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ABSTRACT BOOK

POSTER PRESENTATIONS AND RAPID FIRE SESSIONS

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P001

Copper chelation downregulates YTHDF2 RNA-binding protein causing growth inhibition and decreased MYCN expression

Bell J[1,2], Bertoldo J[1,2], Vittorio O[1,2]

[1]Children's Cancer Institute, Lowy Cancer Research Centre, Sydney, Australia, [2]School of Clinical Medicine, UNSW Medicine & Health, University of New South Wales, Sydney, Australia

Rapid Fire session 1A, May 15, 2023, 11:35 AM - 11:50 AM

Background: RNA-binding proteins such as LIN28B and IGF2BP1 which reduce miRNA attack of oncogenic transcripts were previously reported as oncogenic in animal models of neuroblast-derived cancers. This suggests that post-transcriptional regulation intervention represents a novel strategy against neuroblastoma. We previously reported that FDA-approved copper chelation therapy has in vivo efficacy against neuroblastoma.

Aim: Investigate post-transcriptional effects of copper chelation as a means to elucidate novel combination strategies against neuroblastoma.

Methods/Results: RNA-seq and Mass spectrometry analyses in neuronal cancer cells lines suggested that several methylated RNA (m6A)-binding proteins were deregulated by copper chelator treatment. Indeed, copper chelation treated neuroblastoma cells lines showed a decrease in YTHDF2 protein expression. YTHDF2 knock out (DepMap Chronos dependency score, 0.32) and knock-down studies (siRNA pool, 38% decrease in cell confluence) in neuroblastoma cell lines, indicated that YTHDF2 depletion is selective against neuroblastoma cell growth. Despite YTHDF2 being essential in glioblastoma cancer stem cell population maintenance and tumorigenesis, no specific inhibitor is reported. Thus, to elucidate tool YTHDF2 inhibitors we utilized bioinformatic docking of small molecules against the YTHDF2 RNA-binding domains. Two molecules yielded highly significant virtual binding. In vitro studies showed that both compounds enhanced copper chelation clonogenicity effects in neuroblastoma cells. This suggested that YTHDF2 function has molecular redundancies with copper chelation. YTHDF2 RNA-binding inhibitors downregulated MYCN protein expression. High throughput PAR-CLIP pull downs of YTHDF2 protein and interacting RNAs demonstrated that the MYCN RNA binds at three sequence locations (PARalyzer Peak calling, POSTAR3 online tool). Ongoing work will involve unbiased RNA-sequencing strategies to better define the interplay between YTHDF2 and copper signalling in neuroblastoma.

Conclusion: YTHDF2 is a novel drug target in neuroblastoma and likely contributes to copper signalling in neuronal cancers. YTHDF2 is yet to be pharmaceutically targeted or studied in the neuroblastoma context. Our work therefore represents a significant breakthrough in drugging oncogenic post-transcriptional processes either indirectly through depleting copper, or with our small molecule inhibitors.

P002

Non-canonical PRC1.1 targets BTG2 to maintain cyclin gene mRNAs and cell proliferation in neuroblastoma.

Satoh S[1], Haruta M[1], Ohira M[1], Kamijo T[1]
[1]*Research Institute for Clinical Oncology, Saitama Cancer Center, Saitama, Japan*

Background & Aims
Polycomb repressive complexes (PRCs) are histone modification writers to silence the target genes, especially tumor suppressor genes in oncology. One of the assemblies, PRC1, includes a RING1-PCGF E3 ligase heterodimer and deposits H2AK119 mono-ubiquitination (H2AK119ub1). Recent studies have revealed that PCGF family members (PCGF1-6) in the heterodimer and other unique accessory proteins classify PRC1 assemblies into several sub-complexes. Although targeting PRC1 components is to be promising in cancer therapeutics, the clinical and biological importance of PRC1 subtypes is still elusive in neuroblastoma (NB).

Methods & Results
We found that NB cells exhibited higher dependency against RING1A, one of the two E3 ligases in PRC1 complexes, among various cancer cell lines in Depmap data (https://depmap.org/portal/). In our experiments, the knockdown of RING1A suppressed H2AK119ub1 as well as NB cell viability. Gene expression profiling identified BTG2 gene, which bridges mRNAs to polyA deadenylases, as one of the RING1A targets. ChIP assays uncovered that non-canonical PRC1.1 (ncPRC1.1) accessory proteins (RING1A, PCGF1, KDM2B and BCOR) and H2AK119ub1 occupied the hypomethylated CpG island of BTG2 in NB cells. The PCGF1 knockdown also de-repressed BTG2 expression, suggesting that ncPRC1.1 was responsible for the silencing of BTG2. Besides, ectopic BTG2 impaired NB cell growth in vitro and tumor formation in vivo. The lower expression of BTG2 was associated with poor outcomes in NB clinical samples (HR = 0.7897 [0.6741-0.91233], P = 0.0033 against event-free survival; HR = 0.7474 [0.6057-0.9193], P = 0.0062 against overall survival). Furthermore, BTG2 induction perturbed the cell cycle, decreased the expression of a part of cyclin genes, and enhanced the degradation of the cyclin mRNAs in NB cells. By contrast, a mutant BTG2 that is deficient to interact with the polyA deadenylases did not alter the cyclin gene expression and NB cell proliferation. Finally, the expression of BTG2 was inversely correlated with those of the cyclin genes in NB clinical samples.

Conclusion
ncPRC1.1 epigenetically regulates BTG2 gene to maintain the amount of the cyclin gene mRNAs and NB cell proliferation. This study has first clarified the biological significance and druggability of ncPRC1.1 in NB.

P003

m6A modification of TERRA RNA is required for telomere maintenance and is a therapeutic target for ALT positive Neuroblastoma

Vaid R[1], Thombare K[1], Mendez A[1], Burgos-Panadero R[1], Djos A[1], Jachimowicz D[2], Bartenhagen C[3], Kumar N[4], Sihlbom C[5], Fransson S[1], Johnsen J[6], Kogner P[6], Martinsson T[1], Fischer M[3], Mondal T[1,7]
[1]Department of Laboratory Medicine, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden, [2]Translational Genomics, Discovery Biology, Discovery Sciences, BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden, [3]Department of Pediatric Oncology and Hematology, University of Cologne, Cologne, Germany, [4]Department of Microbiology and Immunology, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden, [5]Proteomics Core Facility, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden, [6]Childhood Cancer Research Unit, Department of Women's and Children's Health, Karolinska Institute, and Pediatric Oncology, Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden, [7]Department of Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg, Sweden

Background: Telomerase-negative tumors can maintain telomere length by alternative lengthening of telomeres (ALT) but the mechanism behind ALT is poorly understood. Aggressive Neuroblastoma (NB), in particular, relapsed tumors are positive for ALT (ALT+) which suggests that better dissection of the ALT mechanism could provide novel therapeutic opportunities. TERRA long non-coding RNA (lncRNA) which is derived from the telomere ends is localized to telomeres in R-loop dependent manner and is essential for telomere maintenance. RNA modification at the N6 position of internal adenosine (m6A) plays a critical role in regulating many key steps in RNA metabolism and METTL3 is the enzyme that deposits m6A in RNA.

Aims: We aim to dissect the role of m6A RNA modification in the TERRA and characterize the significance of such modification in R-loop formation and telomere maintenance in ALT+ NB cells. Further, we wanted to explore the consequence of the removal of this modification in ALT+ NB.

Methods: We performed short and long-read RNA-seq and m6A RIP-seq (m6A RNA immunoprecipitation followed by sequencing). We used the latest Telomere to telomere (T2T) human genome assembly for our analysis. To unravel the critical role of m6A modification in TERRA RNA, we modulated m6A using either METTL3 knock-down or the dCasRx-FTO system or by METTL3 inhibition. We have also standardized a new method DRImR (DRIP followed by m6A RIP) to detect m6A modification present in R-loop structures.

Results: Our results suggest m6A modification in TERRA by METTL3 is essential for telomere maintenance in ALT+ cells and loss of TERRA m6A/METTL3 leads to telomere damage. We observed that R-loop enriched TERRA is abundantly m6A modified and m6A mediated recruitment of hnRNPA2B1 (m6A reader protein) to TERRA RNA is essential for R-loop formation. Our data suggest that m6A drives telomere targeting of TERRA via R-loops. Furthermore, treating ALT+ NB cells with METTL3 inhibitor leads to compromised telomere targeting of TERRA and accumulation of DNA damage over telomere.

Conclusion: In conclusion, we find that m6A modification of TERRA RNA is vital for telomere maintenance and this can be explored as a therapeutic target for ALT+ Neuroblastoma.

P004

The novel long noncoding RNA linc53372 promotes neuroblastoma by inducing protein synthesis and AURKA and Myc over-expression

Wu J[1], Liu P[1], Mondal S[1], Cheung B[1], Mestdagh P[2], Dinger M[3], Zhang X[4], Marshall G[1], **Liu T[1]**

[1]Children's Cancer Institute Australia, Randwick,, Australia, [2]Center for Medical Genetics, Department of Biomolecular Medicine, Ghent University, Ghent,, Belgium, [3]University of New South Wales, Kensington, , Australia, [4]School of Biomedical Sciences and Pharmacy, The University of Newcastle, Newcastle, Australia

Background. Long noncoding RNAs are emerging as novel oncogenic drivers and therapeutic targets.

Aims. To identify novel tumorigenic drivers as therapeutic targets.

Methods & Results. We have re-analysed the publicly available RNA sequencing dataset from 493 human neuroblastoma patients and performed genome-wide Kaplan-Meier survival analysis of the prognostic value of every single transcript, using the median expression as the cut-off point. The long noncoding RNA linc53372 was identified as a transcript, high expression of which in human neuroblastoma tissues significantly correlated with poor overall survival and event-free survival in both MYCN-amplified patients and MYCN-non-amplified patients.

Linc53372 has never been studied in physiology or disease. We found that linc53372 expression was higher in tumour tissues from various organ origins compared to normal tissues in The Cancer Genome Atlas datasets, and the highest expression of linc53372 was observed in neuroblastoma, compared with all other cancers. The linc53372 gene was associated with transcriptional super-enhancers, and treatment with the bromodomain/super-enhancer inhibitor OTX015 significantly reduced linc53372 expression. RNA-binding protein pull-down assay identified the protein translation initiation factor eIF4B as a strong linc53372 RNA-binding protein. Linc53372 or eIF4B knockdown significantly reduced protein synthesis, and ribosome profiling confirmed reduction in mRNA translation after linc53372 knockdown. Immunoblot confirmed that linc53372 or eIF4B knockdown reduced AURKA, N-Myc and c-Myc expression in MYCN-amplified and non-amplified neuroblastoma cells. Conversely, linc53372 over-expression led to enhanced mRNA translation into protein, AURKA and Myc over-expression. In addition, linc53372 or eIF4B knockdown significantly reduced MYCN-amplified and non-amplified neuroblastoma cell proliferation, cell cycle progression and colony formation. In mice xenografted with MYCN-amplified and non-amplified neuroblastoma cell lines, linc53372 knockdown significantly inhibited tumour growth and improved mouse survival. In the publicly available RNA sequencing dataset from 493 neuroblastoma patients, high levels of linc53372 and eIF4B expression in neuroblastoma tissues independently predicted poor patient prognosis.

Conclusions. Linc53372 is over-expressed in neuroblastoma due to transcriptional super-enhancers, and linc53372 RNA interacts with eIF4B protein to promote protein translation, leading to MYCN-amplified and non-amplified neuroblastoma cell proliferation, cell cycle progression and tumour progression. We are currently screening small molecule compounds for inhibitors of linc53372 and eIF4B interaction for neuroblastoma treatment.

P005

Characterization of a novel 3D bone marrow metastatic neuroblastoma model

Corallo D[1], Pantile M[1], Menegazzo S[1,2], Biffi A[2], Aveic S[1,3]

[1]Laboratory of Target Discovery and Biology of Neuroblastoma, Istituto di Ricerca Pediatrica Fondazione Città della Speranza, Padova, Italy, [2]Maternal and Child Health Department, Division of Pediatric Hematology, Oncology and Stem Cell Transplant, University of Padova and Istituto di Ricerca Pediatrica Città della Speranza, Padova, Italy, [3]Department of Dental Materials and Biomaterials Research, RWTH Aachen University Hospital, Aachen, Germany

Background

The bone marrow (BM) infiltration represents an unfavorable prognostic marker for patients with neuroblastoma (NB), whose survival is still below 40%. To improve the chemotherapy effectiveness in patients with BM metastatic disease, it is required to understand the underlying mechanisms that sustain the aggressive behavior of disseminated NB cells.

Aim

Development of an artificial 3D metastatic NB bone/bone marrow model for the elucidation of the key signaling events taking part in the infiltrated BM niche.

Methods

Here, we applied a 3D collagen-β-tricalcium phosphate-based bone scaffold and repopulated it in a sequential mode with the stromal, endothelial, and hematopoietic stem cells. The metastatic niche was then generated by including NB cells. Organization and interaction between cell types in the 3D environment were assessed through immunofluorescence and histological analyses. Also, the alterations in viability and differentiation of tumor cells grown in the engineered 3D model were tested by applying flow cytometry and qPCR approaches.

Results

The osteoinductive properties of the selected biomaterial, as well as the deposition of the stromal cells-derived extracellular matrix (ECM), in adopted 3D growth conditions, were confirmed. Also, a functional elongation of the endothelial cells in the stromal cell meshwork was confirmed, together with a balance between the growth, and survival of cells residing inside the scaffold.

Conclusion

The proposed model system provided a novel 3D in vitro biomimetic scaffold allowing the study of complex microenvironmental conditions of BM infiltrated with NB cells. As a cost-effective in vitro model for the growth of disseminated NB cells, this platform will allow the characterization of the behavior of disseminated tumor cells in a reproduced BM niche, and for related biological and pharmacological surveys.

P006

TARGETING THE MAPK PATHWAY TO ALTER TRK RECEPTOR MEDIATED CELL FATE DECISIONS IN NEUROBLASTOMA

Maher S[1], Wynne K[1], Zhernovkov V[1], Halasz M[1]
[1]*Systems Biology Ireland, School of Medicine, University College Dublin, Dublin, Ireland*

BACKGROUND: Clinical heterogeneity is a hallmark of neuroblastoma, ranging from spontaneous regression to aggressive metastatic progression. Differential expression of the Tropomyosin related kinase receptors (TrkA/TrkB/TrkC) and their respective ligands (NGF/BDNF/NT3) is observed in these opposing phenotypes. TrkA strongly associates with patient survival and is highly expressed in patients with low stage tumours and tumours that spontaneously regress. In contrast, elevated expression of TrkB, is closely associated with 'high-risk' tumours that demonstrate relentless progression and an overall poor prognosis.

AIM: Understanding how Trk-mediated signalling directs cell fate decisions in neuroblastoma is an unsolved problem. Combining quantitative MS-based proteomics with cell fate assays, we aim to link cell signalling dynamics to the divergent cellular phenotypes in TrkA/B/C-overexpressing neuroblastoma cells (with different MYCN status).

METHODS: We generated SH-SY5Y (non-MYCN-amplified), NBLS (MYCN-overexpression) and NLF (MYCN-amplified) neuroblastoma cells stably overexpressing TrkA/NTRK1, TrkB/NTRK2 or TrkC/NTRK3. Then, following ligand stimulation, we observed cell fate decisions by light microscopy and measured proliferation by CyQuant assay. In parallel, mass spectrometry (MS)-based label-free total proteomics and phosphoproteomics was carried out with ligand stimulation (0-10-45min,24h). MS-coupled co-immunoprecipitation of the TrkA/B/C receptors at 0-5-10 min ligand treatment was also investigated to provide a comprehensive overview of the Trk-receptor signalling networks. Mitogen activated protein kinases (MAPKs) p38/ERK/JNK were inhibited pharmacologically in Trk-overexpressing neuroblastoma cells, where impact on phenotype was assessed by light microscopy and CyQuant proliferation assay.

RESULTS: Phenotypic characterisation revealed NGF-TrkA signalling to induce differentiation and BDNF-TrkB to induce proliferation irrespective of MYCN status of neuroblastoma cells. NT3-TrkC signalling showed different phenotypes dependant on the MYCN status. MS-based investigation of the proteome and phosphoproteome identified 5085 proteins and 25099 phosphosites. Subsequent Kinase Substrate Enrichment Analysis (KSEA) of the phosphoproteomics data revealed kinases that align with the divergent cell phenotypes including that of the MAPK pathway. Further pharmacological inhibition of MAPK effectors (ERK/p38/JNK) with phenotype characterisation underlined their significance where inhibition altered Trk-mediated cell fate decisions in neuroblastoma cells.

CONCLUSION: This study provides a comprehensive overview of the Trk-signalling network in neuroblastoma cells with different MYCN status and highlights MAPK effectors as targetable nodes to regulate Trk-receptor mediated cell fate decisions in neuroblastoma.

P007

Targetable genetic alterations in high-risk NB patients. A SIOPEN study

Bellini A[1,2], Bhalshankar J[1,2], Del Castillo Del Rio S[1,2], Saint-Charles A[1,2], Raynal V[3], Masliah Planchon J[4], Beiske K[5], Bernkopf M[6], Betts D[7], Capasso M[8], De Preter K[9], Combaret V[10], Cotteret S[11], Jeison M[12], Mazzocco K[13], Morini M[14,15], Martinsson T[16], Mühlethaler-Mottet A[17], Noguera R[18], Font de Mora J[19], Vicha A[20], George S[21], Chesler L[22], Valteau-Couanet D[23], Delattre O[1,3], Pötschger U[24], Ladenstein R[24,25], Taschner-Mandl S[6], Tweddle D[26], Allinson L[26], Goodman A[27], Schleiermacher G[1,2]

[1]INSERM U830, Laboratoire de Génétique et Biologie des Cancers, Research Center, PSL Research University, Institut Curie, Paris, France, [2]SiRIC RTOP « Recherche Translationelle en Oncologie Pédiatrique », Translational Research Department, Research Center, PSL Research University, Paris, France, [3]Institut Curie Genomics of Excellence (ICGex) Platform, PSL Research University, Institut Curie Research Center, Paris, France, [4]Unit of Somatic Genetics, Institut Curie, Paris, France, [5]Department of Pathology, The Norwegian Radium Hospital, Oslo University Hospital, Oslo, Norway, [6]St. Anna Children's Hospital, Department of Pediatric Hematology and Oncology, Vienna, Austria, [7]Department of Clinical Genetics, Children's Health Ireland at Crumlin, Dublin, Ireland, [8]Università degli Studi Napoli Federico II, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Napoli, Italy, [9]Center for Medical Genetics, Ghent University, Ghent, Belgium, [10]Laboratoire de Recherche Translationnelle, Centre Léon-Bérard, Lyon, France, [11]Department of Pathology and Laboratory Medicine, Translational Research Laboratory and Biobank, AMMICA, INSERM US23/CNRS UMS3655, Gustave Roussy Cancer Campus, Université Paris-Saclay, Villejuif, France, [12]Schneider Children's Medical Center of Israel, Petah Tikva, Israel, [13]Department of Pathology, IRCCS Istituto Giannina Gaslini, 16147, Genova, Italy, [14]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [15]Laboratory of Molecular Biology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [16]Department of Clinical Genetics, University of Göteborg, East Hospital, Gothenburg, Sweden, [17]Pediatric Hematology-Oncology Research Laboratory, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland, [18]Department of Pathology, Medical School, University of Valencia-Incliva Health Research Institute/CIBERONC, Madrid, Spain, [19]Instituto de Investigación Sanitaria La Fe, Valencia, Spain, [20]Department of Paediatric Haematology and Oncology, Second Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czech Republic, [21]Division of Clinical Studies, The Institute of Cancer Research, London, United Kingdom, [22]Paediatric Tumour Biology, Division of Clinical Studies, The Institute of Cancer Research, Sutton, United Kingdom, [23]Département d'Oncologie Pédiatrique, Gustave Roussy, Villejuif, France, [24]Department for Studies and Statistics and Integrated Research, Vienna, Austria, [25]Department of Paediatrics, Medical University of Vienna, Vienna, Austria, [26]Wolfson Childhood Cancer Research Centre, Newcastle Centre for Cancer, Translational & Clinical Research Institute, Newcastle University, Newcastle upon Tyne, United Kingdom, [27]Newcastle Genetics Laboratory, Newcastle upon Tyne Hospitals NHS Trust, Newcastle upon Tyne, United Kingdom

Background and aims:
In high-risk neuroblastoma (NB), new treatment strategies are urgently required to improve outcome. We sought to determine the frequency of genetic alterations (SNVs/Indels) in genes considered to be targetable and/or to play a role in oncogenesis in high-risk NB at diagnosis.

Methods:
721 patients enrolled in the SIOPEN-HR-NBL1 trial were included in this study. Diagnostic NB samples were sequenced at Institut Curie and in nine other European centres. 204 samples (tumour/cell-lines) were sequenced ≥2 times across different centres. 465 tumour samples, 30 NB cell-lines and 11 controls (healthy donor DNA) were sequenced by True-seq custom amplicon (TSCA®) which targets 85 genes relevant in NB oncogenesis. Paired germline-tumour samples (55/721) were sequenced by panel(n=22), WES(n=27) and WGS(n=6). 328 tumours, 26 cell-lines, 2 controls were sequenced by targeted panel and 6 tumours by WES. Panel designs from the European centres target different genes and 200 of them were included in the preliminary analysis. 50/200 genes were considered directly targetable. Mean read depth coverage per sample varied from 30x to 1000x depending on the sequencing technique.
Results:

For tumours sequenced by ≥2 centres, pathogenic ALK/other COSMIC hotspots SNVs/indels (n=26) and high Pearson correlation of 0.97(p-value=12e-15) of mutated allele fraction was observed by comparing TSCA versus other sequencing techniques.
Per NB tumour sample, a mean of 7 SNVs/Indels (95% CI -5.4-8.3; range 0-143) was observed with the most frequent events in known cancer hotspot mutations in the following genes ALK(8.46%), TP53(8.2%), CDKN2A(8.5%), HRAS(1.68%), PTPN11(1.68%), KRAS(1.21%). SNVs/Indels with low MAF (<20%) were observed largely in 8 genes (CDKN2A, TENM4, CHD7, TNEM2, NF1, SMARCB1, PTPRD, PTCH1, ATM).
Detailed variant and copy number analysis of all 721 diagnostic tumour samples is ongoing. Furthermore, these data will enable of (targetable) gene alterations with HR-NBL1 clinical parameters including, treatment response, and their prognostic impact to be determined.
Conclusion:
We observed high reproducibility of variant analysis irrespective of different sequencing techniques. Distinct targetable genetic alterations could be observed in 35% of high-risk NB patients at diagnosis, an important finding to support the introduction of possible targeted therapy approaches in upfront treatment strategies.

P008

CLEAN - Cell Line ExplorAtion in Neuroblastoma: an interactive online tool for exploration of existing high throughput neuroblastoma studies

Gabre J[1,3], Merseburger P[1,3], Siaw J[1,3], Claeys A[1,2], Bekaert S[2,4], Speleman F[2,4], Hallberg B[3], Palmer R[3], Van den Eynden J[1,2]

[1]Ghent University, Department of Human Structure and Repair, Ghent, Belgium, [2]Cancer Research Institute Ghent, Ghent, Belgium, [3]Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [4]Department of Biomolecular Medicine, Ghent, Belgium

Background

Neuroblastoma (NB) is the most frequent cancer in infancy and there's an urgent need for more efficient targeted therapies. The development of novel (combinatorial) treatment strategies often starts with extensive explorations of signaling perturbations in neuroblastoma cell lines, using RNA-Seq or other high throughput technologies (e.g., proteomics, phospho-proteomics). This requires dedicated bioinformatics support, which is not always available. Additionally, while data from past, published studies are highly valuable and raw data (e.g., fastq files) are nowadays released in public repositories, processing them is time-consuming and difficult without bioinformatics support.

Aim

To facilitate NB research, more user-friendly and faster ways to explore newly generated and existing high throughput data are needed. Therefore, we developed a highly interactive data centralization and visualization web application, called CLEAN (Cell Line ExplorAtion in Neuroblastoma).

Method/Result

CLEAN uses normalized counts and differential gene/protein expression input data (i.e., log fold changes and P values generated with state-of-the-art tools such as DESeq2). The output is an extended series of highly interactive plots (e.g., volcano plots, gene set enrichment plots) and tables. These plots and tables are easily downloadable in publication-ready formats (png or pdf format for figures, xlsx format for tables). CLEAN smoothly connects to other complementary platforms that are often used in neuroblastoma research when evaluating the broader biomedical relevance of genes of interest, such as the R2: Genomics Analysis and Visualization Platform, Gene Cards, DepMap or ChEMBL.

The user also has the option to upload their own data for exploration and comparisons to already existing data available in the app. In a similar fashion, the user can also add custom gene sets for the gene set enrichment analysis.

Conclusion

CLEAN represents an ongoing effort to implement all published, past and future neuroblastoma cell line data in a standardized way and making it available to everyone in an interactive environment. The platform constitutes a much needed bridge between disciplines, as well as a great opportunity to revisit data that still holds potential for important discoveries.

P009

High-fat diet promotes metabolic alterations linked to inhibition of anaplastic lymphoma kinase.

Aniszewska A[1], Merseburger P[1,2], Kurhe Y[1], Umapathy G[1], Van den Eynden J[2], Hallberg B[1], Palmer R[1]
[1]Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [2]Department of Human Structure and Repair, Anatomy and Embryology Unit, Ghent University, Ghent, Belgium

Background
Anaplastic lymphoma kinase (ALK) is a therapeutic target in neuroblastoma and ALK inhibitors such as lorlatinib are employed clinically in ALK-positive neuroblastoma patients. ALK was recently linked to thinness, and it was suggested that the use of ALK inhibitors would promote weight loss. However, clinical data in ALK-driven cancer patients, including adult ALK-driven NSCLC patients, as well as pediatric patients treated with ALK inhibitors suggest the opposite. It has been reported that treatment of ALK-driven tumors with lorlatinib leads to metabolic alterations, including increased appetite, weight gain, hyperlipidemia and insulin resistance. The role of ALK in the control of metabolism is currently poorly understood and since the ALK inhibitor lorlatinib is employed in the clinic for ALK-positive NB we decided to investigate the underlying molecular mechanisms.

Aims
Investigation of the role of ALK in metabolism and body weight regulation in preclinical mouse models.

Methods
ALK gain-of-function (ALK-GOF), wild-type (WT) and ALK knock-out (ALK-KO) mice were fed with regular chow or high fat diet and response to treatment with lorlatinib was monitored. Body weight and composition analyses as well as glucose (GTT) and insulin (ITT) tolerance and behavioral evaluations were performed. Plasma samples and organs were harvested upon termination for histological, biochemical, and molecular analyses. Transcriptomic analyses on samples from liver, kidneys, brain and white and brown adipose tissue were performed.

Results
Mice fed with HFD and treated with lorlatinib developed metabolic alterations, including increased body weight and proportion of fat mass, elevated glucose levels and insulin resistance. No abnormalities were observed in mice fed with regular diet. ALK-KO mice showed minor metabolic perturbation in response to lorlatinib, supporting a direct involvement of ALK. We also note an additional metabolic response to lorlatinib in HFD-fed mice that appears to be ALK-independent. Our transcriptome analysis revealed tissue-dependent induction of mitochondrial dysfunction, specifically alterations of key metabolic pathways such as oxidative phosphorylation and fatty acid metabolism. The extent of these transcriptional effects were modulated by the ALK genotype.

Conclusion
ALK inhibition by lorlatinib promotes obesity and insulin resistance in HFD treated mice, mediated in part by dysregulation of mitochondrial energy metabolism.

P010

E2F3 interactome profiling uncovers a new regulatory axis with MYCN to increase oncogenic reprogramming in neuroblastoma

Milazzo G[1], Ciaccio R[1], De Rosa P[1], Cimadom L[1], Santulli M[1], Zadran S[1], Aloisi S[1], Rigamonti A[1], Raught B[2], Penn L[2], Perini G[1]

[1]University of Bologna, Department of Pharmacy and Biotechnology, Bologna, Italy, [2]University of Toronto, Princess Margaret Cancer Centre, Toronto, Canada

BACKGROUND

The most aggressive subtype of neuroblastoma (NB), which carries the worst overall prognosis, occurs where MYCN is amplified. Although recent studies have shown that MYC as well as MYCN factors can act as wide transcriptional amplifiers and participate in super enhancer complexes, many questions remain regarding what distinguishes MYCN-amplified from non-amplified tumors. Several preliminary findings from our lab indicate that high MYCN can establish a strong functional axis with E2F3 in neuroblastoma that is pivotal to the development of a high-risk cancer phenotype.

AIM

Because the action of MYCN on E2F3 can occur despite the RB1 status of the neuroblastoma cell, we speculate that in a high MYCN dosage, MYCN through direct interaction with E2F3 can alter the E2F3 interactome to overcome RB1 function.

METHODOLOGY

To better understand how E2F3 works in different MYCN backgrounds and to specifically assess the contribution of the two nuclear E2F3 isoforms (E2F3a and E2F3b), we determined the complexity of the E3F3a/b interactomes by adopting a proximity-dependent biotin labeling (BioID) screen in both high and low MYCN conditions, using the TET21/N cells as a biological model. Interaction of E2F3a or –b with several critical protein candidates was validated by the Proximity Ligation Assay in both MYCN amplified and non-amplified neuroblastoma cell lines.

RESULTS

LC-MS/MS analyses of pulled-down E2F3a/b biotinylated interactors revealed several candidates belonging to the comprehensive proteomics map of both E2F3a and E2F3b proteins, underlining the fundamental role of MYCN in defining the peculiar interactomic profiles of these two transcription factors in NB cells. More specifically, we identified protein candidates that are uniquely associated with either E2F3a or E2F3b only when MYCN is high. Interestingly, the majority (>90%) of the 80 proteins picked up by the screen belong to transcription and chromatin regulatory complexes like STAGA, TIP60/NuA4, DREAM, and ATAC complexes. Finally, gene expressions of most candidates are independent strong predictors of clinical outcome in Kaplan-Meier analyses.

CONCLUSION

Our findings shed light on the molecular principles through which high MYCN controls E2F3a/b activity to foster the NB oncogenic program and disclose a potentially druggable proteome for the development of innovative therapy.

P011

Survivin over-expression is associated with a good prognosis in neuroblastoma patients

Pinhatti F[1], Sayala S[1], Olandoski M[1], Cardoso de Castro A[2], de Noronha L[1], Elifio-Esposito S[1]
[1]Graduate Program in Health Sciences, School of Medicine and Life Sciences, Pontifícia Universidade Católica do Paraná., Curitiba, Paraná, Brazil, [2]Pequeno Príncipe Pediatric Hospital , Curitiba, Paraná, Brazil

Background: Neuroblastoma (NB) diversity in clinical behavior cannot be explained by prognostic markers currently used in clinical practice and reflects the low survival in high-risk patients. The protein Survivin (SVV) is an apoptosis regulator with high expression related to aggressiveness in different tumors. However, little is known about its potential as an indicator of aggressiveness in neuroblastoma.

Aims: To investigate the prognostic value of the immunohistochemical expression of SVV protein alone or associated with clinical and biological factors in the clinical course of neuroblastoma.

Methods: The clinical and pathological data of 66 patients diagnosed with NB and treated at Pequeno Príncipe and Erasto Gaertner hospitals, Curitiba, PR, Brazil, between 1990 and 2016, were collected and plotted from medical records. Tissue microarray slides were subjected to immunohistochemical staining and analyzed for SVV total expression by color morphometry or score of SVV expression in the nucleus and the extracellular matrix (EM)/cytoplasm through hotspot. The association between SVV and clinical data was analyzed by fitting a ROC curve for SVV expression associated with stage and medullary infiltration. Association analysis was done using Fisher's exact test, the Chi-square test, and Spearman's correlation coefficient. A comparison of two groups was made using Student's t-test for independent samples or the nonparametric Mann-Whitney test.

Results: SVV was expressed in both benign and malignant tumors, with expression in malignant tumors above 2.6% ($p<0.001$). SVV expression was associated with medullary infiltration in the malignant tumor population along the patient's clinical course ($p=0.037$). SVV expression ≤4.4% was associated with more aggressive tumors, and nuclear SVV was associated with age, tumor histology, and metastasis. High SVV EM/cytoplasm was associated with favorable histology tumors. There was no significant association between SVV alone and survival. No statistical difference was found between no SVV expression, nuclei SVV, and ME/Cytoplasmic SVV.

Conclusion: The immunohistochemical expression of SVV analyzed by morphometry showed potential as a factor of good prognosis when associated with a non-metastatic tumor profile for patients diagnosed with malignant NB.

P012

P300 interacted with N-Myc and regulated its protein stability via altering its posttranslational modifications in neuroblastoma.

Wu Y[1], Wu Z[1], Cheng C[1]
[1]*Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Pediatric Surgery, Shanghai, China*

MYCN amplification is an independent risk factor for poor prognosis in neuroblastoma (NB), but its protein product cannot be directly targeted due to protein structure. Thus, this study aimed to explore novel ways to indirectly target N-Myc by regulating its posttranslational modifications (PTMs) and therefore protein stability. N-Myc coimmunoprecipitation combined with HPLC–MS/MS identified 16 PTM residues and 114 potential N-Myc interacting proteins. Notably, both acetylation and ubiquitination were identified on lysine 199 of N-Myc. We then discovered that p300, which can interact with N-Myc, modulated the protein stability of N-Myc in MYCN-amplified NB cell lines, and simultaneously regulated the acetylation level on lysine-199 and ubiquitination level of N-Myc protein in vitro. Furthermore, p300 correlated with poor prognosis in public NB datasets. Taken together, p300 can be considered as a potential therapeutic target to treat MYCN-amplified NB patients, and other identified PTMs and interacting proteins also provide potential targets for further study.

P013

Targeting m6A epitranscriptomic modification as a therapeutic strategy for MYCN amplified Neuroblastoma

Thombare K[1], Vaid R[1], Mendez A[1], Burgos-Panadero R[1], Rehan A[1], Mondal T[1]
[1]*Department of Laboratory Medicine, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden*

Background: Epitranscriptomics refer to covalent modification in the RNA bases. One of the most important epitranscriptomic modifications is the addition of a methyl group to the N6 position of internal adenosine (m6A) in cellular RNA by METTL3/METTL14 enzyme complex. This dynamic RNA modification regulates several steps of RNA metabolism during cellular differentiation and DNA damage response. Role of m6A in Neuroblastoma (NB) tumorigenesis is not known. High-risk NB tumors often do not respond to multimodal therapy. MYCN amplification is associated with high-risk NB with poor prognosis.

Results: We observed that removal of m6A epitranscriptome in MYCN amplified cells by METTL3 knock-down induces DNA damage and reduces the growth of mouse xenografts. We have explored role of m6A epitranscriptome in trunk neural crest cells (tNCC) and found that m6A modification controls expression of HOX genes including HOXC8 and HOXC9. We found MYCN and m6A co-operate with each other to control transition of tNCC to sympathoadrenergic progenitor cells (SAP). MYCN over-expression in tNCC leads to perturbation in the expression of m6A related genes and creates an undifferentiated state. Small molecule (STM2457) mediated METTL3 inhibition sensitizes MYCN NB cells to chemotherapeutic drugs to m6A loss as observed in mouse Xenografts.

Conclusions: Our observation suggests that targeting m6A epitranscriptome could be a novel therapeutic opportunity for MYCN amplified Neuroblastoma

P014

Effect of Casiopeína-IIIia® on the expression of genes involved in intrinsic apoptosis of SK-N-SH neuroblastoma cells.

Hernández Benítez J[1], Martínez-Pacheco M[1], Morales-González A[1], Ruiz-Azuara L[2], Mejía C[1]
[1]*Autonomous University of Queretaro, Laboratory of Interdisciplinary Biomedicine, Queretaro, Queretaro, Mexico,*
[2]*National Autonomous University of Mexico, Laboratory of Medicinal Chemistry, Mexico City, Mexico*

Background
Neuroblastoma (NB) is the most common extracranial solid tumor in childhood. Due to the toxicity of current treatments and the poor response to them, it is urgent to study new therapeutic agents such as Casiopeínas®, which are copper(II) compounds that induce intrinsic apoptosis in cancer cells. This type of cell death involves the release of pro-apoptotic molecules through the mitochondrial permeability transition pore (mPTP). Therefore, changes in the expression of genes of the mPTP may contribute to restoring the balance between apoptosis and proliferation.

Aims
To determine the effect of Casiopeína-IIIia® on the expression of apoptotic and cell proliferation genes and the cell cycle of NB cells.

Methods
Neuroblastoma cells of the SK-N-SH line were cultured in DMEM under standard conditions and treated with 37 µg/mL cisplatin (positive control) or 31 µg/mL Casiopeína-IIIia® for four hours. Control cells received no treatment. We isolated total RNA and evaluated, by RT-qPCR, the expression of VDAC1, VDAC2, VDAC3, N-MYC, TP53, and GADD45A genes in each of the experimental conditions. We assessed the cell cycle stages by flow cytometry and using 50,000 cells. Expression data were analyzed by the comparative Ct method, and significant differences ($p<0.05$) were calculated using the one-way ANOVA and Tukey's post hoc test. Cell cycle data were analyzed by the two-way ANOVA, and significant differences ($p<0.05$) were calculated with Dunnett's post hoc test.

Results
Casiopeína-IIIia® induces the expression of VDAC1 and TP53 genes and the downregulation of VDAC2, VDAC3, and N-MYC but does not influence GADD45A expression. These effects are like those observed with cisplatin. Also, this copper(II) compound causes an increase in the percentage of cells in the G0/G1 phase and a diminishment of the S phase of the cell cycle.

Conclusion
Casiopeína-IIIia® is an efficient antineoplastic that can promote intrinsic apoptosis in NB cells through the induction of over-expression of the pro-apoptotic genes VDAC1 and TP53 and under-expression of the N-MYC oncogene. In addition, it inhibits proliferation by arresting NB cells in the G0/G1 phase of the cell cycle.

P015

Identification and Validation of Tumor-Specific circRNAs as Biomarkers in Neuroblastoma

Kok L[1], Lin Q, Lak N[1], Deleu J[2], van Dinter J[1], Vandesompele J[2], Tytgat L[1], van Heesch S[1]
[1]Princess Máxima Center for Pediatric Oncology, Utrecht, Netherlands, [2]Department of Biomolecular Medicine, Universiteit Ghent, Ghent, Belgium

Background & Aims: Circular RNAs (circRNAs) are stable and abundant non-coding RNA molecules with potential as diagnostic and therapeutic biomarkers. In this project, we set out to identify circRNAs that are specifically associated with neuroblastoma and evaluate their potential as biomarkers for diagnosis and treatment monitoring.

Methods: We conducted a circRNA detection analysis on large datasets of RNAseq data from 69 tumor tissue samples from neuroblastoma patients, and 613 samples from ten other tumor types as a control. Each sample was obtained from a unique pediatric patient at the time of diagnosis or during treatment. We also analysed cfRNA from 24 plasma samples from five neuroblastoma patients.

Results: As preliminary filter strategy, we performed differential expression analyses between the neuroblastoma samples and each of the ten other tumor types. This resulted in a set of 231 circRNAs that were enriched in neuroblastoma samples. These were also detectable in an external neuroblastoma RNAseq dataset from another childhood cancer center. 42 of these circRNAs were also detected in at least one plasma sample.

Conclusion & Outlook: In order to further validate and refine this group of circRNAs, we are conducting additional experiments and analyses. These include negative control datasets to establish neuroblastoma-specificity by including plasma samples from patients with other tumors and non-diseased children, and targeted circRNA detection in plasma using qPCR or ddPCR. If successful, our findings could lead to the development of innovative diagnostic tests and personalised treatment monitoring strategies for neuroblastoma patients.

P017

β3-adrenergic receptor as prognostic marker of neuroblastoma relapse and malignancy

Favre C[1], Boaretto A[1], Tellini M[1], Carra F[1], Tondo A[1], Calvani M[1]
[1]*Department of Pediatric Hematology-Oncology, IRCCS A. Meyer University Children's Hospital, Florence, Italy*

Background
Neuroblastoma (NB) is the most common extracranial solid tumor in childhood with a wide clinical heterogeneity. Risk stratification is pivotal in determining appropriate treatment and new biomarkers are under investigation. The presence of the β3-adrenergic receptor (β3-AR) has been reported in several tumors. We evaluated by flow cytometry analysis (FCM) its expression on circulating tumor cells (CTCs) in Ewing sarcoma patients, showing a role as a possible predictive marker of disease recurrence. In NB, β3-AR has been demonstrated to be involved in the proliferation and in the regulation between stemness and differentiation through mTOR and SK2/S1P2 pathways.

Aims
We aimed to deepen the role of β3-AR expression in NB oncogenesis obtaining NB murine models from ADRB3-/- clones of NB cell lines. We evaluated β3-AR expression by FCM on CTCs and disseminated tumor cells (DTCs) in bone marrow of NB patients to correlate it with disease prognosis.

Methods
Human and murine knock-out NB cell lines (Be(2)-c and N2a) were edited using CRISPR-Cas9 technology to obtain ADRB3-/- clones that were subcutaneously inoculated in NU-Foxn1nu and A/J mice to evaluate tumor growth and to perform RNA-sequencing analysis. Furthermore on NB patients, we evaluated the expression of CD56, GD2 antigen and β3-AR on CTCs, DTCs and bioptic derived cells by FCM.

Results
Knock-out clones mostly failed in developing a tumor mass and when they succeeded, still showed decreased tumor growth with respect to wild type (WT) ones. RNA sequencing of WT and ADRB3-/- Be(2)-c tumor masses resulted in 113 statistically significant altered pathways involved in tumor development and progression. The preliminary data revealed a higher β3-AR expression on BM samples and DTCs derived from metastatic patients, suggesting a role as a possible predictive marker of disease extension.

Conclusion
The in vivo experiment showed that β3-AR is fundamental for tumor formation and the analysis of ADRB3-/- tumor masses' transcriptome enlightened the involvement of this receptor in several typical cancer mechanisms as angiogenesis, differentiation, motility. Moreover, we underlined its prognostic value as a marker of NB aggressiveness, since it is overexpressed in NB patients compared to healthy donors and in high-risk compared to low-risk tumors.

P018

The druggable target KAT2A forms a feedforward loop with MYCN to drive an oncogenic neuroblastoma transcriptional program

Liu Z[1], Hong J[1], Zhang X[1], Xu M[1], Loria S, Carter S, Lei H[1], Shern J[1], Thiele C[1]
[1]*Pediatric Oncology Branch, National Cancer Institute, Bethesda, United States*

Rapid Fire session 1A, May 15, 2023, 11:35 AM - 11:50 AM

Background: MYCN drives malignancy in many types of cancers including neuroblastoma (NB). However, as a transcription factor, MYCN is challenging to target due to its structural flexibility. Since MYCN needs to cooperate with cofactors to regulate gene transcription, these cofactors offer potential for the indirect therapeutic targeting of MYCN. We identified that MYCN interacts with coactivator histone acetyltransferase, KAT2A, but their relationship has not been well-characterized.
Aims: Investigate whether KAT2A functions as a MYCN cofactor, and if it is a druggable target to inhibit MYCN activity in NB.

Methods: We performed RNA-seq, chromatin immunoprecipitation followed by sequencing (ChIP-seq) and loss of KAT2A function studies to investigate the role of KAT2A and its relationship with MYCN.

Results: ChIP-seq results showed that MYCN knockdown in IMR32 cells resulted in a >30% decrease of KAT2A ChIP-seq signals at the summit of MYCN peak centers, whereas MYCN overexpression in SHEP cells increased genome-wide binding of KAT2A. Transcriptional profiling shows that KAT2A silencing results in a significant negative enrichment of MYC target genes. ChIP-seq and RNA-seq results reveals that MYCN binds to KAT2A promoter and activates KAT2A transcription. By overexpressing KAT2A and MYCN in 293T cells, we found that KAT2A acetylates MYCN and increases MYCN protein half-life 7-fold from 34 minutes to 240 minutes. KAT2A knockdown in NB (IMR32, BE(2)C and KCNR) results in a 40-60% decrease in MYCN protein levels and a 20-30% decrease in cell proliferation ($p<0.01$) as indicated by the IncuCyte cell confluence assay. Consistently, the treatment of NB cells with KAT2A PROTAC degrader (GSK983) results in a 50% decrease in MYCN protein levels and a 30% decrease in cell proliferation. Since our previous study identified that MYCN recruits corepressor G9a to repress neuronal differentiation genes in NB, we combined the KAT2A PROTAC degrader with a G9a inhibitor to downregulate canonical MYC targets and upregulate neuronal differentiation genes. This combination synergistically suppressed NB tumor growth.

Conclusion: We identify that KAT2A and MYCN form a feedforward loop to drive an oncogenic transcriptional program in NB. Moreover, our study highlights that MYCN cofactors are viable targets for the development of anti-MYCN therapies.

P019

High-resolution analysis of structural genomic alterations in neuroblastoma by linked-read whole-genome sequencing

Bartenhagen C[1,2], Rosswog C[1,2], Kahlert Y[1,2], Becker K[3], Nürnberg P[2,3], Peifer M[2,4], Fischer M[1,2]
[1]Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Cologne, Germany, [2]Center for Molecular Medicine Cologne (CMMC), Medical Faculty, University of Cologne, Cologne, Germany, [3]Cologne Center for Genomics (CCG), Medical Faculty, University of Cologne, Cologne, Germany, [4]Department of Translational Genomics, Medical Faculty, University of Cologne, Cologne, Germany

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Background: The genetic events driving aggressive clinical courses in most high-risk tumors are still unknown. The paucity of single nucleotide variants in cancer-related genes along with the frequent occurrence of segmental chromosomal copy number alterations suggest that structural genomic variants (SVs) may play a significant role in the pathogenesis of high-risk neuroblastoma. However, detection of SVs by conventional whole-genome sequencing (WGS) is often impaired by false-positive results, incorrect and ambiguous alignments, or alignment gaps within genomic repeats and uneven sequence coverage.

Aims: To identify pathogenetically relevant SVs in primary and relapsed neuroblastoma samples using linked-read WGS, supplemented by information on gene expression profiles and clinical data.

Methods: Linked-read WGS, which has been developed as a library preparation method prior to standard short-read WGS, provides a solution to several issues of conventional WGS by linking reads originating from the same barcoded DNA molecule over ~100 kb regions. Linked-read WGS reduces misalignments and ambiguous alignments, and maps repeat regions with greater resolution and more uniform sequence coverage. The long-range information of linked-reads can significantly contribute to resolving the haplotypes of SVs and the structure of complex, highly rearranged loci.

Results: Linked-read WGS data of 103 neuroblastoma samples were analyzed with the 10x Genomics Long Ranger pipeline. The cohort comprised tumors covering the entire spectrum of the disease, with a specific focus on high-risk neuroblastoma, as well as longitudinal samples obtained at diagnosis and relapse. Between 40 and 400 SVs per sample were detected in total, with an average of 25% of them affecting one or more genes, and <5% leading to gene fusions. Most SVs were deletions and inversions. In addition, we found that SVs were more abundant in relapsed tumors compared to matched tumors at the time of diagnosis. Various genes were associated with SVs, with only few of them being recurrently affected within the subgroups. In-depth analysis of the potential impact of SVs on high-risk neuroblastoma pathogenesis and progression is currently ongoing.

Conclusion: Linked-read WGS detected SVs with higher sensitivity compared to paired-end conventional WGS and revealed a detailed portrait of rearrangement patterns within multiple neuroblastoma subgroups.

P020

Defining the landscape of circular RNAs in neuroblastoma unveils a global suppressive function of MYCN

Fuchs S[1,2,3,4,5,6], Danssmann C[1], Klironomos F[1], Winkler A[1], Fallmann J[11], Kruetzfeldt L[1], Szymansky A[1], Naderi J[1,7], Bernhart S[11], Grunewald L[1], Helmsauer K[8], Rodriguez-Fos E[1,8], Kirchner M[9], Mertins P[9], Astrahantseff K[1], Suenkel C[10], Toedling J[1], Meggetto F[5,6], Stadler P[11], Hundsdoerfer P[12], Deubzer H[1,2,3,8], Kuenkele A[1,2,3], Lang P[13], Fuchs J[14], Henssen A[1,2,3,8], Eggert A[1,2,3,4], Rajewsky N[1,10], Hertwig F[1,2,3], Schulte J[1,2,3,4]

[1]Charité-Universitätsmedizin Berlin, Department of Pediatric Oncology and Hematology, Berlin, Germany, [2]The German Cancer Consortium (DKTK), Partner Site Berlin, Berlin, Germany, [3]The German Cancer Research Center (DKFZ), Heidelberg, Germany, [4]Berlin Institute of Health at Charité – Universitätsmedizin Berlin, Berlin, Germany, [5]CRCT, Inserm, CNRS, Université Toulouse III-Paul Sabatier, Centre de Recherches en Cancérologie de Toulouse, Université de Toulouse, Toulouse, France, [6]Laboratoire d'Excellence Toulouse Cancer-TOUCAN, Toulouse, France, [7]Department of Genome Regulation, Max Planck Institute for Molecular Genetics, Berlin, Germany, [8]Experimental and Clinical Research Center (ECRC) of the Charité and Max-Delbrück-Center for Molecular Medicine (MDC) in the Helmholtz Association, Berlin, Germany, [9]Core Unit Proteomics, Berlin Institute of Health at Charité - Universitätsmedizin Berlin and Max Delbrück Center for Molecular Medicine (MDC), Berlin, Germany, [10]Systems Biology of Gene Regulatory Elements, Berlin Institute for Medical Systems Biology, Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany, [11]Bioinformatics Group, Department of Computer Science, and Interdisciplinary Center for Bioinformatics, University of Leipzig, Leipzig, Germany, [12]Department of Pediatric Oncology, Helios Klinikum Berlin-Buch, Berlin, Germany, [13]Department I - General Pediatrics, Hematology/Oncology, University Children's Hospital, Eberhard Karls University Tuebingen, Tuebingen, Germany, [14]Department of Pediatric Surgery and Pediatric Urology, University Children's Hospital, Eberhard Karls University Tuebingen, Tuebingen, Germany

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Background
Circular RNAs (circRNAs) are a regulatory RNA class and abundant in neural tissues. While cancer-driving functions have been identified for single circRNAs, it is not well understood yet, how they modulate gene expression in cancer. Neuroblastoma is an embryonal cancer and one of the prime diseases responsible for cancer-related death in children.

Aims
Here, we delineate the complex expression patterns of circRNAs in cancer and their functional role for cancer-progression in neuroblastoma, with a focus on the regulation by MYCN, the main oncogenic driver of this disease.

Methods
Deep whole-transcriptome sequencing was performed to analyze samples from 104 primary neuroblastomas covering all risk groups. Cell models with inducible overexpression or knockdown systems of the MYCN oncogene, which defines a subset of high-risk neuroblastoma cases, were employed to assess the specific effects of MYCN on circRNA expression. Knockdown and interaction studies were performed to elucidate the function of a candidate circRNA in vitro.

Results
We identified 5,203 circRNAs transcribed from 2,302 genes. Hierarchical clustering and differential expression analysis revealed that MYCN-amplified neuroblastoma samples showed the lowest expression of circRNAs. Mechanistically we were able to demonstrate that this global negative regulation is dependent on the DHX9 RNA helicase, which is controlled by MYCN. Interestingly, we detected similar mechanisms in

shaping circRNA expression in the pediatric cancer medulloblastoma implying a general MYCN effect. In order to identify neuroblastoma-specific circRNAs, we compared our dataset to other cancers and identified 25 circRNAs, including circARID1A, which are specifically upregulated in neuroblastoma. Transcribed from the ARID1A tumor suppressor gene, circARID1A promotes proliferation and cell viability in neuroblastoma cells. Mechanistically, we identified by RNA pulldown and subsequent mass spectrometry that circARID1A exerts its function by stabilizing the KHSRP RNA-binding protein to control TP53 signaling.

Conclusion
In summary, we here extend knowledge of the neuroblastoma transcriptome to the noncoding RNA class of circRNAs. Our study offers mechanistic insights into the regulation of circRNAs by MYCN in childhood cancer. We emphasize the importance of this class of noncoding RNAs for neuroblastoma cell maintenance and survival, and present new angles for therapy design to tackle this malignancy.

P021

The chromatin reader PHF6 interacts with RRM2 to control replication stress and DNA damage responses in neuroblastoma

Depestel L[1,2], Bekaert S[1,2], Sanders E[1,2], Van Damme C[1,2,3], Eggermont A[1,2], Loontiens S[1,2], Bek J[1,2], Delhaye L[1,2,3], Eyckerman S[1,2,3], Speleman F[1,2], Durinck K[1,2]

[1]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium, [2]Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [3]VIB Center for Medical Biotechnology, Ghent University, Ghent, Belgium

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Background: Neuroblastoma (NB) cells undergo increased replication fork stalling and replicative stress. PHF6 is an essential factor for fork restart and DNA damage repair and is highly expressed in NB versus other entities. RRM2 is part of the ribonucleotide reductase (RNR) enzyme which provides dNTPs for DNA replication and repair, and is tightly controlled throughout the cell cycle in response to replicative stress. It has been suggested that part of the nuclear RNR is associated with replication forks but how it is guided towards chromatin is unknown.

Aims: We aimed to further investigate the role of PHF6 in NB, in particular in relation to the control of replicative stress. To this end, we evaluated (1) the transcriptional effects of PHF6 depletion, (2) the PHF6 interactome and (3) the genome-wide chromatin binding activity of PHF6.

Methods/materials: We performed immunoprecipitation-coupled mass spectrometry (IP-MS) analyses to map the PHF6 interactome and used the NanoBRET technology for validation of PHF6-protein interactions. In addition, we performed RNA-sequencing upon PHF6 depletion and CUT&RUN analyses to gain insight into putative transcriptional effects.

Results: IP-MS analysis revealed top-ranked PHF6 interactors including RRM2, which we recently identified as a MYCN co-dependency and synergistic target with CHK1 in NB (Nunes, et al. Science Advances, 2022). Using NanoBRET assays, we validated this interaction, while deletion mapping revealed a critical role for the first PHD domain of PHF6 in RRM2 interaction. These results suggest that PHF6 acts as a critical component of the so-called replitase, which has been proposed to locally direct RRM2 to sites of stalled/collapsed forks to facilitate fork restart and/or DNA repair. GSEA following PHF6 knockdown in four NB cell lines revealed strong association of PHF6 with DNA repair and G2/M checkpoint. CUT&RUN data showed enrichment of PHF6 binding sites with H3K4me1 and H3K27ac marked (super)enhancer sites and binding sites of NB core regulatory circuitry transcription factors, suggesting a role for suppressing transcription-associated DNA damage.

Conclusion. We propose PHF6 as a crucial component of the replitase to direct RRM2 to damaged replication forks in high-risk NB and a potential role in suppressing transcription-associated DNA damage.

P022

Genetic predisposition to neuroblastoma results from a regulatory polymorphism promoting the adrenergic cell state in zebrafish and human.

Weichert-leahey N[1,2], Shi H[1], Tao T[1], Oldridge D[3], Durbin A[4], Abraham B[4], Zimmerman M[1], Zhu S[5], Wood A[3], Reyon D[6], Joung K[6], Young R[7], Diskin S[3], Maris J[3], Look T[1,2]

[1]Dana-Farber Cancer Institute, Department for Pediatric Oncology, Boston, United States, [2]Boston Children's Hospital, Department for Pediatric Hematology and Oncology, Boston, United States, [3]Children's Hospital of Philadelphia, Division of Oncology and Center for Childhood Cancer Research, Philadelphia, United States, [4]St. Jude Children's Research Hospital, Department of Oncology and Comprehensive Cancer Center, Memphis, United States, [5]Mayo Clinic College of Medicine, Department of Biochemistry and Molecular Biology, Rochester, United States, [6]Massachusetts General Hospital, Molecular Pathology Unit, Center for Computational and Integrative Biology, and Center for Cancer Researc, Boston, United States, [7]Whitehead Institute for Biomedical Research, Cambridge, United States

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Background: Childhood neuroblastomas exhibit plasticity between a neural crest-like "mesenchymal" cell state and a more differentiated sympathetic "adrenergic" cell state. These cell states are governed by autoregulatory transcriptional loops called core regulatory circuitries (CRCs), which drive the early development of sympathetic neuronal progenitors from migratory neural crest cells during embryogenesis. The adrenergic cell identity of neuroblastoma requires LMO1 as a transcriptional co-factor. Both LMO1 expression levels and the risk of developing neuroblastoma in children are associated with the single nucleotide polymorphism rs2168101 G>T that affects a GATA motif in the first intron of LMO1. The permissive TATA allele prevents GATA3 binding at this locus and ablates not only the formation of a tissue-specific enhancer, but also the LMO1 expression in mutant neuroblasts.

Aims: Here, we sought to define the causal mechanism underlying the striking association between rs2168101 and the risk of developing neuroblastoma in vivo.

Methods: We used genetic engineering (TALEN and CRISPR/Cas9) in a zebrafish model of MYCN-driven neuroblastoma to introduce the precise TATA allele into the first intron of lmo1 of the zebrafish. We performed tumor watch experiments and analyzed RNA sequencing data from human and zebrafish tumors with the different genotypes and compared their transcriptional profile.

Results: We found that wild-type fish with the GATA allele have high neuroblastoma penetrance, whereas the germline gene editing to introduce a TATA allele is protective against tumor formation in zebrafish, mirroring the protective effects of the TATA allele in children. In both children and zebrafish with a germline GATA allele, neuroblastomas employ the adrenergic CRC, relying on high levels of LMO1, whereas tumors that develop in the TATA background employ the mesenchymal CRC, independent of LMO1, and are low risk at diagnosis.

Conclusion: Thus, not only the increased risk associated with the regulatory GATA motif in the first intron of LMO1 is conserved over 400 million years of evolution that separate zebrafish and humans, but also the oncogenic regulatory circuitries involved in the initiation of neuroblastoma.

P023

Designing Novel Drug Combinations to Improve Sensitivity of ALK-Driven Neuroblastoma Cells to Lorlatinib.

Matkar S[1], Kalna J[1], O'Driscoll E[2], Santhosh S[1], Balyasny S[1], Gerelus M[1,2], Shalem O[1,2], Mosse Y[1,2]
[1]Children's Hospital of Philadelphia, Division of Oncology, Department of pediatrics, Philadelphia, United States, [2]University of Pennsylvania, Philadelphia, United States

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Background: Gain-of-function mutations in ALK have provided the opportunity to develop targeted therapies for patients with neuroblastoma. Lorlatinib has now completed Phase 1 testing in the NANT Consortium and has moved to frontline therapy in the Children's Oncology Group. While robust and sustained activity has been observed with minimal toxicity, this effect is transient in relapsed patients harboring both MYCN amplification and an activating ALK mutation.

Aim: Elucidate non-mutational mechanisms of Lorlatinib resistance, and design combination treatments that will maximize clinical benefit.

Methods: We utilized CRISPR-CAS9 loss of function screen (LOF) using the Brunello library to probe targets that can synergize with Lorlatinib using Kelly cell line (ALK F1174L, MYCN amplified). sgRNAs were ranked utilizing computational pipeline established in our lab. Cell viability assays using CellTiter-Glo and immunoblotting were performed for orthogonal validation of top hits. We established Felix (ALK F1245C, MYCN non-amplified) and COG-N-453 (ALK F1174L, MYCN amplified) PDXs resistant to Lorlatinib to test efficacy of combination treatments in vivo.

Results: From the ranked list of depleted sgRNAs upon treatment with lorlatinib compared to DMSO, we selected BCL2L1 (BCL-XL) for further analysis since it ranked high and anti-apoptotic BCL2 family proteins are often implicated in resistance to targeted therapy. Moreover, the sgRNAs targeting pro-apoptotic protein Bax were enriched upon Lorlatinib treatment. The utility of small molecules targeting BCL-XL is limited by thrombocytopenia. Recently developed BCL-XL PROTAC, DT2216 (NCT04886622), utilizes VHL E3 ubiquitin ligase for targeted degradation and is not expressed in platelets. Our studies show that DT2216 potently degrades BCL-XL within 24 hours in Kelly, LAN5 (ALK R1275Q, MYCN amplified), and NB1643 (ALK F1174L, MYCN amplified), which was sustained for 5 days. Moreover, DT2216 decreased cell viability significantly in combination with Lorlatinib compared to lorlatinib alone in LAN5 and NB1643 cells ($P < 0.005$). We are currently performing preclinical studies to test the efficacy of lorlatinib and DT2216 combination treatment in ALKI-naïve and resistant models to elucidate mechanism and for clinical testing.

Conclusion: Novel PROTAC targeting BCL-XL can improve the efficacy of lorlatinib to delay or prevent the emergence of resistance in patients with MYCN amplified ALK-driven NB.

P024

Investigating the role of HMMR as a pro-oncogenic protein in neuroblastoma

Karapouliou C[1], Cutillas P[2], Rajeeve V[2], Stoker A[1]
[1]Great Ormond Street Institute of Child Health, University College London, London, United Kingdom, [2]Barts Cancer Institute, Queen Mary University of London, London, United Kingdom

Background
The hyaluronic receptor HMMR (RHAMM) is a multifunctional protein residing both on cell surfaces and in the cytoplasm and nucleus. HMMR is documented as having pro-tumorigenic roles in a number of cancers, with signaling effects through ERK, AKT, FAK and AURKA. Here we report the first study of this protein in Neuroblastoma-derived tumour cell lines.

Aim
Hypothesising that HMMR plays a pro-oncogenic role in neuroblastoma, we aim to characterise the molecular and cellular actions of this protein in neuroblastoma cell lines. Moreover we are investigating whether or not this HMMR action is dependent upon extracellular hyaluronic acid (HA) signals.

Methods
Cox regression model analysis of human patients datasets was used to assess independent prognostic factors. To examine protein directly, we have used CRISPR/Cas9 to generate neuroblastoma cell derivatives that lack HMMR function. The effect of HMMR depletion on KELLY neuroblastoma cells was analysed by in-vitro proliferation, 2D colony formation and scratch assays and the biochemical effectors of HMMR investigated by phosphoproteomics analysis and western blot.

Results
We show that HMMR is an independent prognostic factor that correlates with poor prognosis. This suggests a potential pro-tumorigenic role in this pediatric tumour's aetiology. KELLY cell derivatives lacking HMMR survive, but demonstrated significantly reduced cell proliferation rates and migration. These cells also have reduced ability to form 2D colonies, suggesting that their stemness is hindered as well. These findings support the hypothesis that HMMR can actively support hallmarks of cancer cell biology in neuroblastoma. High molecular weight HA reduced proliferation of neuroblastoma cells, but this was not affected by loss of HMMR. This supports an HA-independent role of HMMR in neuroblastoma cells and the contribution of several signalling pathways is being investigated.

Conclusion
We present the first evidence revealing that HMMR is an independent prognostic indicator in neuroblastoma and that HMMR protein supports the proliferative capacity and motility of neuroblastoma cells. HMMR signaling thus presents a new, candidate therapeutic pathway for investigation in this cancer.

P025

Targeting cholesterol metabolism in neuroblastoma

Ding H[1], Ding J[1], Tran G[1]
[1]*University of Alabama at Birmingham, Birmingham, United States*

Background: High-risk neuroblastoma exhibits transcriptional activation of the mevalonate pathway that produces cholesterol and non-sterol isoprenoids. Cholesterol is a precursor for producing steroid hormones, oxysterols, bile acids, and vitamin D. In addition, it is a structural component of cellular membranes, accounting for approximately 25 mole percent of plasma membrane lipids. Thus, its continuous production is required for cell proliferation. We aimed to investigate the functional significance of cholesterol metabolism in high-risk neuroblastoma and its potential as a therapeutic target.

Methods: Statins are a family of cholesterol-lowering drugs that block the mevalonate pathway by inhibiting the rate-limiting enzyme hydroxymethylglutaryl-CoA reductase. We examined the effect of statins on neuroblastoma cell proliferation and tumorigenic activity using cell lines and animal models. We used specific enzyme inhibitors to dissect the contributions of cholesterol and non-sterol isoprenoids to neuroblastoma cell proliferation. We performed gene expression profiling to identify the cellular and metabolic processes that are affected by statins in neuroblastoma cells. We further tested strategies to enhance the antitumor effect of statins.

Results: Both the cholesterol and non-sterol geranylgeranyl-pyrophosphate branches are critical to sustain neuroblastoma cell proliferation. Blocking the mevalonate pathway by simvastatin impedes neuroblastoma growth in TH-MYCN and PDX mouse models. Transcriptional profiling reveals that the mevalonate pathway is required to maintain the FOXM1-mediated transcriptional program that drives mitosis. High FOXM1 expression contributes to statin resistance and is a therapeutic vulnerability to a combination of statin and FOXM1 inhibition. Furthermore, we show that caffeine synergizes with simvastatin to inhibit neuroblastoma cell proliferation and PDX growth by blocking statin-induced feedback activation of the mevalonate pathway. This action of caffeine depends on its activity as an adenosine receptor antagonist, and the A2A adenosine receptor antagonist istradefylline, an add-on drug for Parkinson's disease, can recapitulate the synergistic effect of caffeine.

Conclusions: Our study reveals that the FOXM1-mediated mitotic program is a molecular target of statin action and identifies new classes of agents for maximizing the therapeutic efficacy of statins with implications in treatment of cancer and metabolic diseases with high cholesterol levels.

P026

Insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) regulates the cargo of extracellular vesicles and promotes neuroblastoma metastasis.

Dhamdhere M[1], Gowda C[1], Singh V[1], Liu Z[2], Carruthers N[4], Grant C[5], Sharma A[1], Sundstrom J[3], Wang H[1], Spiegelman V[1]

[1]Division of Pediatric Hematology and Oncology, Department of Pediatrics, The Pennsylvania State University College of Medicine, Hershey, United States, [2]Department of Public Health Sciences, The Pennsylvania State University College of Medicine, Penn State Cancer Institute, Hershey, United States, [3]Department of Ophthalmology, The Pennsylvania State University College of Medicine, Hershey, United States, [4]Bioinformatics Core, University of Michigan Medical School, Ann Arbor, United States, [5]Division of Pediatric Surgery, Department of Surgery, The Pennsylvania State University College of Medicine, Hershey, United States

Background

Neuroblastoma (NB) is one of the leading causes of cancer-related deaths in pediatric patients. IGF2BP1 is one of the candidate proto-oncogenes at frequently amplified 17q21 locus in NB. IGF2BP1 is upregulated in patients with metastatic NBs, and is associated with poor prognosis, however, its importance in NB progression is poorly understood.

Aims

This study was designed to uncover the role and mechanisms of IGF2BP1 in NB metastasis.

Methods

We utilized several immunocompetent mouse models, including our novel highly metastatic mouse NB cells to understand the role of IGF2BP1 in NB metastasis. IGF2BP1-modulated NB cells were IV-injected, and the development of metastases was monitored using in vivo bioluminescence imaging. Mouse survival was analyzed by Kaplan-Meier method. Importance of small extracellular vesicles (EVs) and EV-mediated role of IGF2BP1 were accessed by examining the effects of NB-EVs on the growth of metastasis, and pre-metastatic niche (PMN) formation. An unbiased proteomic analysis on these EVs was performed to determine the EV-dependent mechanism of IGF2BP1. IGF2BP1-associated regulation of the identified candidates was accessed by RNA stability and binding assays.

Results

Downregulation of IGF2BP1 significantly reduced the metastatic potential of the highly metastatic NB cells, while overexpressing it in less metastatic cells increased the metastatic ability of these cells in mice. NB cell-derived EVs promoted the development of experimental metastasis, and increased the formation of PMN in mouse livers. Importantly, IGF2BP1-downregulated cell-derived EVs failed to accelerate the growth of metastasis, and induce PMN formation in mice. Proteomic analysis of these EVs revealed a differentially regulated cargo. Identified proteins – SEMA3A and SHMT2 phenocopied the EV-mediated effect of IGF2BP1, and their downregulation in IGF2BP1-overexpressing NB cells reduced the EV-mediated effect of IGF2BP1 on recipient cells. IGF2BP1 increased the expression of SEMA3A/SHMT2 in NB cells by directly binding and stabilizing their mRNAs.

Conclusion

Our findings demonstrate:

• Significance of IGF2BP1 in promoting NB metastasis.

• Importance of EVs in NB progression, and EV-dependent pro-metastatic function of IGF2BP1 by governing the EV-protein cargo.
• Novel targets of IGF2BP1 that mediate this pro-metastatic function of IGF2BP1 in NB.
Overall, our study establishes the role of IGF2BP1 in NB metastasis.

P027

Inhibition of the RNA m6A Methyltransferase METTL3 Induces Neuroblastoma Differentiation and Impairs Tumor Growth

Pomaville M[1,2], Jiang Z[2], Wang P[2], Chennakesavalu M[1], Brodnik M[1], Ge R[2], Zhong Y[2], Ye C[2], Moore K[1], Salwen H[1], Chlenski A[1], George R[3], Applebaum M[1], He C[2], Cohn S[1]

[1]Department of Pediatrics, University of Chicago, Chicago, US, [2]Department of Chemistry, Howard Hughes Medical Institute, University of Chicago, Chicago, US, [3]Department of Pediatric Hematology/Oncology, Dana-Farber Cancer Institute, Boston, US

Background

N6-methyladenosine (m^6A) RNA modifications are important regulators of gene expression, and the distribution of m^6A in the epitranscriptome affects tumor progression. m^6A is deposited by a methyltransferase complex that includes methyltransferase-like 3 (METTL3). METTL3 functions as an oncogene in many types of adult cancers, and METTL3 inhibitors are emerging as novel anticancer agents. Our preliminary studies show that METTL3 also promotes the growth of neuroblastoma. Here, we functionally characterized the role METTL3 plays in regulating the m^6A epitranscriptome, gene expression, neuroblastoma phenotype, and in vivo tumor growth. The therapeutic potential of pharmacologic METTL3 inhibition in neuroblastoma was also investigated using the tool compound STM2457.

Aims

1. To elucidate the molecular mechanisms by which METTL3 drives malignant neuroblastoma growth.
2. To investigate the therapeutic potential of pharmacologically targeting the enzymatic activity of METTL3.

Methods

MTT assays were used to assess the effects of siRNA METTL3 knockdown or pharmacologic (STM2457) METTL3 inhibition on neuroblastoma cell viability. METTL3-mediated changes in cell phenotype were evaluated by microscopy, RNA sequencing and Western blot analysis. The anti-tumor effects of STM2457 treatment (50mg/kg daily for 14 days) were investigated in nude mice with subcutaneous neuroblastoma xenografts derived from the adrenergic Kelly cell line. Transcriptome-wide m^6A modifications were mapped in control and STM2457-treated neuroblastoma cells using m^6A methylated RNA immunoprecipitation (MeRIP) with RNA sequencing. Sequencing data were functionally analyzed using ClusterProfiler.

Results

siRNA-mediated METTL3 knockdown decreased neuroblastoma cell viability compared to controls ($p<0.0001$) and induced differentiation with increased neurite outgrowth and up-regulation of axon development transcripts. The IC50 of STM2457 ranged from 8.8μM-25.1μM among five adrenergic cell lines. In three mesenchymal cell lines, IC50 ranged from 70μM to greater than 400μM. STM2457 treatment significantly decreased the growth of neuroblastoma xenografts compared to vehicle control ($p=0.035$). MeRIP with RNA sequencing demonstrated decreased m^6A deposition and increased expression in a network of neuronal differentiation genes in the neuroblastoma cells treated with STM2457.

Conclusion

The anti-neuroblastoma activity of STM2457 demonstrated in this study suggests that METTL3 inhibition may be an effective therapeutic strategy against neuroblastoma.

P028

Activating ALK signaling stabilizes SLC3A2 protein for ALK-driven neuroblastoma tumorigenesis

Lai W[1], Chuang T[1], Borenäs M[1], Palmer R[1], Hallberg B[1]
[1]*Department of Medical Biochemistry and Cell Biology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden*

Background: Neuroblastoma is the most common childhood tumor that grows in the adrenal glands or sympathetic ganglia. Approximately 10% of pediatric neuroblastoma patients harbors mutations on anaplastic lymphoma kinase (ALK). Based on our previous study, we identified SLC3A2 as a potential interacting partner with ALK intracellularly using BioID-based in vivo proximity labeling. SLC3A2 is a multifunctional protein that mediates integrin-dependent signalings, acts as a trafficking chaperone for amino acid transporters, and regulates polyamine transportation. However, the detailed mechanisms and functional consequences behind SLC3A2 and ALK interaction on ALK-driven neuroblastoma are still unclear.

Methods: The interaction of SLC3A2 and ALK was validated with immunoprecipitation. The effect of ALK signaling on SLC3A2 expression and protein stability was evaluated by quantitative PCR and immunoblots. The functional effects of SLC3A2 on cell viability were investigated by siRNAs and inhibitor treatment in both human and mouse ALK-driven neuroblastoma cells.

Results: SLC3A2 was confirmed to interact with ALK in both anti-ALK and anti-SLC3A2 antibodies co-immunoprecipitations from NB1 cells (ALK-wild-type) and CLB-GE (ALK-F1174V). Moreover, this interaction was decreased by ALK inhibitor (lorlatinib) treatment. Upon ALKAL2 ligand treatment, the activated ALK signaling increased the protein level of SLC3A2 and abrogated by lorlatinib in NB1 cells. Furthermore, lorlatinib treatment suppressed SLC3A2 expression and significantly shortened the protein stability in neuroblastoma cells with different ALK mutations including CLB-BAR (ALK exon4-11 deletion), CLB-GAR (ALK-R1275Q), and CLB-GE (ALK-F1174V) under cycloheximide chase analysis. Knockdown of SLC3A2 significantly inhibited cell growth and down-regulated the amino acid transporter SLC7A5 (LAT1) expression in CLB-GE cells (ALK-F1174V). While single treatment of lorlatnib and AMXT-1501 (polyamine transport inhibitor) only showed moderate effect, the combined treatment revealed synergistic effect on cell growth in ALK-driven primary-cultured mouse neuroblastoma #9883 (Th-MYCN; Alk-F1178S) and #111 (Th-MYCN; Rosa26_Alkal2).

Conclusion: SLC3A2 protein stability and its interaction with ALK was dependent on ALK signaling in ALK-driven neuroblastoma. Synergistic effect of combined ALK and polyamine transport inhibitors suggests a potential therapeutic option for ALK-driven neuroblastoma.

P029

Drugging MYCN protein complexes in neuroblastoma

Fleischhauer D[1], Eilers M[1], Büchel G[1,2]
[1]Department of Biochemistry and Molecular Biology, Theodor Boveri Institute, Biocenter, Julius-Maximilians-University, Würzburg, Germany, [2]Mildred Scheel Early Career Center (MSNZ), University Hospital, Würzburg, Germany

Background:
Amplification of MYCN is a driver of pediatric cancers such as neuroblastoma, and its expression correlates with poor overall survival in patients. MYCN is considered “undruggable”, because MYC family members lack enzymatic activity and a defined secondary protein structure. However, MYC proteins have conserved regions (MYC boxes) through which they associate with different co-factors and form functional protein complexes.

Aims:
We aim to identify and characterize the mechanism of oncogenic MYCN-protein interactions that can be targeted for proteasomal degradation using small bifunctional molecules (PROTACs).

Methods:
We carried out a pooled shRNA screen for known MYC/MYCN-interactors [1, 2] in a MYCN (IMR5)- and a MYC (SH-EP)-driven neuroblastoma cell line, to identify crucial MYCN interaction partners as potential drug targets. Candidates that dropped out significantly stronger in the MYCN-amplified condition were further validated and characterized by cell viability and biochemical assays.

Results:
The in vitro screen disclosed known essential MYCN complexes like TF3C, PAF1C, USP7, WDR5 and the nuclear RNA exosome including its co-factor NEXT. In addition, the screen revealed other candidates such as SF3B3, NELFE and TERF1 with potential MYCN-associated vulnerabilities in neuroblastoma.

In cell viability assays using SH-EP cells expressing an inducible MYCN-ER chimeric protein, we could show that there is a potential MYCN-specific therapeutic window for these hits. Thus, for example SF3B3, a component of the SF3B complex usually involved in pre-mRNA splicing appears to have another moonlighting function in MYCN-driven neuroblastoma.

Conclusion:
After target validation and characterization of the newly identified MYCN complexes, our aim is to develop complex-specific small molecule compounds. For this we will perform DEL screenings with a stable recombinant protein complex as scaffold. Finally, all efforts are directed towards the overall goal to establish an efficient MYCN degrader that can be used as a potent drug for neuroblastoma therapy in the future.

Literature:
[1] DOI: 10.1016/j.celrep.2017.11.090
[2] DOI: 10.1016/j.molcel.2019.02.031

P031

Treating the Untreatable by Disrupting the Antioxidant Response in Neuroblastoma

Liano Pons J[1], Fahrig F[1], Esteve M[1], Arsenian-Henriksson M[1,2]
[1]Karolinska Institutet, Department of Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden, [2]Lund University, Division of Translational Cancer Research, Department of Laboratory Medicine, Lund, Sweden

BACKGROUND. High-risk neuroblastomas still have poor outcomes and relapse in 50% of the cases, with metastasis resistant to current therapies. MYCN-amplification is present in 40% of high-risk patients and is a hallmark of poor prognosis. We previously described that MYCN induces a metabolic reprogramming, coupled with increased expression of the antioxidant enzymes (Oliynyk et al., 2019).
AIM. To explore the potential of using enzymes in the antioxidant systems as targets for differentiation-inducing therapies.

METHODS. We analyzed neuroblastoma (NB) patient data from Kocak et al., (2013), SEQC (SEQC/MAQC-III Consortium, 2014), and Versteeg (Molenaar et al., 2012). Experiments were performed in MYCN-amplified (SK-N-BE2, KCN-n69) and non-MYCN-amplified (SH-SY5Y, SK-N-AS) cell lines. We used inhibitors of the antioxidant enzymes and evaluated their effect on cell viability by WST-1 and Incucyte live-cell imaging. Differentiation and apoptosis markers were analyzed by Western blot and immunofluorescence, lipid droplet formation by Oil red O staining, and metabolic changes with an Agilent Extracellular Flux Analyzer. We used a NB xenograft in vivo model and analyzed tumor burden after treatment with our inhibitors.

RESULTS. High expression of several of the genes from the thioredoxin, glutathione, and peroxiredoxin pathways correlate with poor prognosis in NB patients. We treated NB cell lines using inhibitors of these antioxidant pathways, and observed decreased cell viability, enhanced neurite outgrowth, and upregulation of markers associated with neural differentiation. The effects were robust when inhibiting peroxiredoxin 6 (PRDX6), a unique member of the peroxiredoxin family involved in ROS scavenging, lipid metabolism, and ferroptosis. Inhibition of PRDX6 led to an arrest in cell proliferation, cell death, and neural differentiation, especially in MYCN-amplified NB cell lines. These changes were accompanied by decreased levels of MYC/MYCN proteins, an abolishment of oxidative phosphorylation, and an accumulation of lipid droplets. The combined inhibition of the antioxidant enzymes PRDX6 and GSTP-1 potentiated differentiation of NB cells, and reduced tumor burden in vivo.

CONCLUSION. Our results show the potential of using the antioxidant PRDX6 and GSTP-1 enzymes as targets for differentiation therapy in neuroblastoma, providing opportunities for the development of less toxic and more specific strategies for patient treatment.

P032

Germline variation affecting DNA repair genes correlates with chromosomal instability in Neuroblastoma

D'alterio G[1,2], Lasorsa V[2], Bonfiglio F[2,3], Aievola V[4], Fischer M[5], Westermann F[6,7], Diskin S[8,9], Iolascon A[2,4], Capasso M[2,4]

[1]University of Milan, European School of Molecular Medicine, Milan, Italy, [2]CEINGE Biotecnologie Avanzate s.c.ar.l., Naples, Italy, [3]University of Naples Federico II, Department of Chemical, Materials and Production Engineering, Naples, Italy, [4]University of Naples Federico II, Department of Molecular Medicine and Medical Biotechnologies, , Italy, [5]University of Cologne, Children's Hospital and Center for Molecular Medicine, Medical Faculty, Cologne, Germany, [6]Hopp Children's Cancer Center, Heidelberg, Germany, [7]Division of Neuroblastoma Genomics, German Cancer Research Center, , Germany, [8]Children's Hospital of Philadelphia, Philadelphia, USA, [9]University of Pennsylvania, Perelman School of Medicine, Philadelphia, USA

Background

Inherited genetic variants can predispose to cancer onset by altering genes that control cell growth and proliferation. Beyond a simple predisposition, however, recent evidence suggests that inherited (or germline) genetic variation can lead to specific tumor (or somatic) phenotypes, according to a theory known as germline-to-somatic (GxM) correlation.

Aims

In this study we sought to assess the GxM correlation in Neuroblastoma (NBL). More in detail, our hypothesis is that predicted rare pathogenic germline variants in genes that control genome stability can contribute to the development of a Chromosomal Instability (CIN) phenotype at somatic level.

Methods

We leveraged Whole Genome Sequencing (WGS) data of matched control-tumor NBL samples from two publicly available datasets (N=316). We profiled samples for copy number alterations (CNAs) and structural variants (SVs), which allowed us to compute 5 CIN scores (number of segments, fraction of altered bases, aneuploidy score, large state transitions and structural variants burden) according published guidelines. Upon quality control filter application, we selected predicted pathogenic germline variants in a set of 276 genes, divided in 10 pathways, involved in DNA repair processes. Finally, we assessed enrichment of predicted pathogenic germline variants in samples with different CIN scores through a pathway based collapsing test.

Results

CIN scores which indicate whole chromosomes CNAs (aneuploidy score and fraction of altered bases) positively correlated with markers of favorable clinical outcome, while CIN scores reflective of SVs and focal CNAs (number of segments, large state transitions and structural variants burden), correlated with poor prognosis markers. Regarding the GxM correlation, we observed an enrichment of predicted pathogenetic germline variants of Non-Homologous End Joining (NHEJ) pathway in tumors with high number of segments.

Conclusion

Our results, beyond highlighting an association among CIN scores and clinical markers in NBL, suggest that germline variants in NHEJ pathway genes – which are known to be impaired in several tumors characterized by CIN - are associated to the development of a high number of SVs at somatic level.

P033

CHD5 inhibits metastasis of neuroblastoma

Laut A[1,2], Dorneburg C[1,2], Stadler G[1,2], Fürstberger A[3], Barth T[4], Kestler H[3], Debatin K[2], Beltinger C[1,2]
[1]Section Experimental Pediatric Oncology, Dept. of Pediatrics and Adolescent Medicine, University Medical Center Ulm, Ulm, Germany, [2]Dept. of Pediatrics and Adolescent Medicine, University Medical Center Ulm, Ulm, Germany, [3]Institute of Medical Systems Biology, Ulm University, Ulm, Gemany, [4]Dept. of Pathology, University Medical Center Ulm, Ulm, Germany

Background: CHD5, a tumor suppressor at 1p36, is frequently lost or silenced in poor prognosis neuroblastoma (NB) and many adult cancers. The role of CHD5 in metastasis in NB still remains unclear. Aims: Since low CHD5 expression is associated with advanced stage in NB patients, we reasoned that CHD5 may inhibit metastasis in NB and if so, which are the underlying molecular mechanisms.

Methods: After generating CHD5 overexpressing NB cells the metastatic potential was investigated in vitro and in vivo. Genome-wide mRNA sequencing was used to identify potential mechanisms causing effects on the metastatic potential.

Results: Using a large, clinically annotated patient database, first hints could be gathered that CHD5 affects patient outcome by decreasing the metastatic potential of NB cells. We confirm that low expression of CHD5 is associated with stage 4 NB. Forced expression of CHD5 in NB cell lines with 1p loss inhibited key aspects of the metastatic cascade in vitro: anchorage-independent growth, migration and invasion. It was found that forced overexpression of CHD5 inhibited the above described key aspects of the metastatic cascade, most pronounced in 1p deleted p53 wt cells, irrespective of MYCN-amplification. In vivo, formation of bone marrow and liver metastases developing from intravenously injected NB cells was delayed and decreased by forced CHD5 expression. Genome-wide mRNA sequencing revealed reduction of genes and gene sets associated with metastasis when CHD5 was overexpressed. Known metastasis-suppressing genes preferentially up-regulated in CHD5-overexpressing NB cells included PLCL1. In patient NB, low expression of PLCL1 was associated with metastatic disease and poor survival. Knock-down of PLCL1 and p53 in IMR5 NB cells overexpressing CHD5 reversed CHD5-induced inhibition of invasion and migration in vitro.

Conclusion: CHD5 not only holds the potential to suppress tumor growth but was found to be a potent metastasis suppressor in NB, providing further explanations for the observed association of high CHD5 expression with good prognosis.

P034

Expression of the Notch ligand JAGGED1 in neuroblastoma is associated with a better prognosis

Dorneburg C[1,2], Stadler G[1,2], Wei N[1,2], Barth T[3], Debatin K[2], Beltinger C[1,2]

[1]Section Experimental Pediatric Oncology, Dept. of Pediatrics and Adolescent Medicine, University Medical Center Ulm, Ulm, Germany, [2]Dept. of Pediatrics and Adolescent Medicine, University Medical Center Ulm, Ulm, Germany, [3]Dept. of Pathology, University Medical Center Ulm, Ulm, Germany

Background: The Notch ligand JAGGED1 has been described to promote several solid tumors. Little is known about JAGGED1 in neuroblastoma (NB), where a JAGGED1 peptide has been reported to increase proliferation.
Aim: To elucidate the role of the ligand JAGGED1 in the aggressiveness of NB.

Methods: Clinically annotated mRNA microarrays of NB (R2 genomics analysis and visualization platform (http://r2.amc.nl), Kocak-GSE45547, Tumor Neuroblastoma - Kocak - 649 - custom - ag44kcwolf, n=649) were analyzed in silico. JAGGED1 expression was correlated with survival, the risk factors age, stage, MYCN copy number and 1p36 loss, and with risk groups . NB patient samples were assessed by immunohistochemistry for JAGGED1 expression and by histology for tumor differentiation status. JAGGED1 was forcibly overexpressed in representative NB cell lines and cells were analyzed for proliferation, clonogenic growth, adhesion to endothelial cells, activity of Notch signaling and for differentiation.

Results: In patient NB increased JAGGED1 mRNA expression was associated with increased activation of Notch targets, a better prognosis and lower risk factors. Along this line, strong JAGGED1 protein expression, as determined by immunohistochemistry, was associated with a more differentiated tumor phenotype. In concordance, MYCN non-amplified NB cell lines expressed JAGGED1 protein, while MYCN-amplified cell lines did not. In contrast to the tumor-suppressive function of endogenous JAGGED1, its forced overexpression elicited a tumor-promoting or no response. Thus, forced expression of JAGGED1 increased proliferation, clonogenicity, anchorage-independent growth and invasion in MYCN-amplified and JAGGED1-deficient SK-N-BE(2)C NB cells, and forced expression in MYCN non-amplified JAGGED1-expressing SH-SY5Y NB cells did not alter their behavior. Of note, forced expression of JAGGED1 did not increase Notch signaling activity.

Conclusion: As JAGGED1 expression in NB is associated with a better prognosis, its inclusion in expression profiles may improve patient stratification. The contrasting responses to endogenous expression of JAGGED1 or its forced overexpression suggest either differential effects of these expression modes or interactions of NB cells with stroma and immune cells modulating JAGGED1 effects. Further investigations are warranted in this regard.

P035

Noncoding regulatory mutations as driving event for the oncogenic core regulatory circuitries of neuroblastoma

Aievola V[1,2], Lasorsa V[2], Montella A[2,3], Bonfiglio F[2,4], Tirelli M[2,5], D'Alterio G[2,5], Fischer M[6], Westermann F[7,8,9], Iolascon A[2,3], Capasso M[2,3]

[1]University of Naples Federico II, Department of Electrical Engineering and Information Technologies, Naples, Italy, [2]CEINGE Biotecnologie Avanzate s.c.ar.l., Naples, Italy, [3]University of Naples Federico II, Department of Molecular Medicine and Medical Biotechnologies, Naples, Italy, [4]University of Naples Federico II, Department of Chemical, Materials and Production Engineering, Naples, Italy, [5]University of Milan, Milan, Italy, [6]University of Cologne, Experimental Pediatric Oncology, Children's Hospital and Center for Molecular Medicine, Medical Faculty, Cologne, Germany, [7]Hopp-Children's Cancer Center at the NCT Heidelberg (KiTZ), Heidelberg, Germany, [8]Division of Neuroblastoma Genomics, German Cancer Research Center (DKFZ), Heidelberg, Germany, [9]German Cancer Consortium (DKTK), Heidelberg, Germany

Background:
Neuroblastoma is a pediatric tumor composed of two cell types reflecting developmental stages of the adrenergic lineage: mesenchymal-type and adrenergic-type cells. These phenotypes result from the impaired activity of Core Regulatory Circuitries (CRCs), sets of tightly regulated transcription factors (TFs) that control gene expression governing cell state transitions and cellular identity.

Aims:
Our hypothesis is that noncoding somatic single nucleotide variants (SNVs) alter neuroblastoma CRC TF binding sites (TFBSs) affecting cellular differentiation and promoting tumor onset. Our aim is to investigate such pattern of regulatory elements and identify putative driver SNVs, exploring their role and functionally characterize their action mechanisms.

Methods:
Transcriptionally active TFBSs belonging to adrenergic CRC were identified through ChIP-seq and ATAC-seq experiments in neuroblastoma adrenergic-like cell lines (SKNBE2C and Kelly). SNVs from 317 neuroblastomas whole genomes were mapped on them and the enrichment of mutations in core regions respect to flanking ones was tested using Fisher test. SNVs selected by CADD, FuncSeq2 and motifbreakR tools were functionally tested with Luciferase assay. TFBSs target-genes were identified through HiC analysis in SKNBE2C and their expression evaluated using RNA-seq data of 89 patients with matched WGS. Target-genes expression values were correlated with clinical and survival data.

Results:
Preliminary integrating SNVs from a subset of 151 neuroblastoma whole genomes with 6 ChIP-seq and one ATAC-seq in SKNBE2C, we found significantly enriched PHOX2B, TBX2 and ISL1 active binding sites (FDR<0.05). Among the selected variants, chr7:126481446:A>C mutation was predicted to disrupt one PHOX2B motif and showed allele-specific transcriptional activity in Luciferase assay. HiC data highlighted significant PHOX2B motif interactions with GRM8 and PAX4, which result downregulated in mutated samples respect to wild types (P<0.05). Low PAX4 expression correlated with stage 4 and reduced overall survival (P<0.01). Extending the analysis to a larger dataset (SNVs from 317 neuroblastoma whole genomes, 19 ChIP-seq and 6 ATAC-seq in SKNBE2C and Kelly), we observed the mutation enrichment in PHOX2B, TBX2 and ISL1 active TFBSs (FDR<0.05), suggesting a common somatic mutation burden affecting adrenergic CRC TFBSs in adrenergic-like neuroblastoma cells.

Conclusion:
These results demonstrate that combined noncoding somatic mutations affecting the CRC activity can promote neuroblastoma onset.

P036

MYCN cooperates with both WDR5 and G9a to orchestrate global gene transcription and govern a malignant phenotype of neuroblastoma

Liu Z[1], Zhang X[1], Xu M[1], Hong J[1], Ciardiello A[1], Lei H[1], Shern J[1], Thiele C[1]
[1]*Pediatric Oncology Branch, National Cancer Institute, Bethesda, United States*

Background: MYCN is a master transcriptional regulator known to activate canonical MYC target genes involved in ribosome biogenesis, protein synthesis and repress neuronal differentiation genes in neuroblastoma (NB). However, how MYCN orchestrates global gene expression in cancer cells remains incompletely understood.

Aims: We aim to identify druggable MYCN cofactors that mediate MYCN transcriptional activity.

Methods: To investigate how MYCN regulates gene transcription at the genome-wide level, we performed protein interactome assay, RNA-seq and chromatin immunoprecipitation followed by sequencing (ChIP-seq) experiments.

Results: RNA-seq and MYCN ChIP-seq results showed that MYCN activates canonical MYC target genes via binding to promoters whereas it binds enhancers to repress neuronal differentiation genes in NB (IMR32, BE(2)C, LAN5 and KCNR cell lines). MYCN knockdown in IMR32 cells decreased H3K27ac and H3K4me3 ChIP-seq signals at MYCN-bound promoters of canonical MYC target genes ($p<0.0001$) but increased H3K27ac ChIP-seq signals at MYCN-bound enhancers of neuronal differentiation genes ($p<0.0001$). This suggests that MYCN activates canonical MYC targets through increasing promoter activity and represses differentiation genes through inhibiting enhancer activity. MYCN interactome assays revealed that MYCN interacts with WDR5 (a component of histone methylation complex) and G9a (histone methyltransferase). ChIP-seq results showed that WDR5 facilitates MYCN binding to promoters of ribosome biogenesis genes, whereas MYCN recruits G9a to enhancers of neuronal differentiation genes. Transcriptional profiles revealed that WDR5 knockdown antagonized MYCN-mediated activation of ribosomal genes, whereas G9a knockdown antagonized MYCN-mediated repression of neuronal differentiation genes. Genetic silencing of WDR5 or G9a resulted in a 50%-80% reduction of cell proliferation ($p<0.001$). Importantly, combined treatment with a WDR5 inhibitor (OICR-9429) and a G9a inhibitor (UNC0642) antagonized both the activating and repressing actions of MYCN, and synergistically inhibited cell proliferation with an average bliss synergy score greater than 10 in multiple MYCN-amplified NB cell lines (IMR32, IMR5 and KCNR).

Conclusion: We identify that WDR5 facilitates MYCN promoter binding to activate canonical MYC target genes, whereas MYCN recruits G9a to enhancers to repress neuronal differentiation genes. Targeting both WDR5 and G9a simultaneously represents a more effective approach to target MYCN in cancers.

P037

Genome analysis of 95 cases registered in JCCG-JNBSG JN-H-11 and JN-H-07 clinical studies for high-risk neuroblastoma

Ohira M[1], Haruta M[1], Sugawara M[1], Shichino H[2], Hishiki T[3], Yoneda A[4], Matsumoto K[4], Takimoto T[4], Nakazawa A[5], Okita H[6], Higashimoto H[7], Ushijima T[8], Nagase H[9], Ichikawa H[10], Tajiri T[11], Nakagawara A[12], Kamijo T[1]
[1]Research Institute for Clinical Oncology, Saitama Cancer Center, Saitama, Japan, [2]National Center for Global Health and Medicine, Tokyo, Japan, [3]Graduate School of Medicine, Chiba University, Chiba, Japan, [4]National Center for Child Health and Development, Tokyo, Japan, [5]Saitama Children's Medical Center, Saitama, Japan, [6]Keio University School of Medicine, Tokyo, Japan, [7]H.U. Group Research Institute, Tokyo, Japan, [8]Hoshi University, Tokyo, Japan, [9]Chiba Cancer Center Research Institute, Chiba, Japan, [10]National Cancer Center Research Institute, Tokyo, Japan, [11]Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, [12]Saga HIMAT, Saga, Japan, [13]Japan Children's Cancer Group Neuroblastoma Committee (JCCG-JNBSG), , Japan

Background and Aims: Neuroblastoma (NB) is the second most common solid tumor in children and the survival rate of NB patients with high-risk tumors still needs to be improved. International Neuroblastoma Risk Group (INRG), which uses tumor stage, age, histology, MYCN copy number, DNA ploidy, and 11q loss, has been applied for the tumor risk classification and collaborative effort for its update is currently underway. To contribute to the effort, we assessed the clinical impact of additional genomic features such as representative SNVs and CNVs, CpG island methylator phenotype (CIMP) and telomere maintenance mechanisms in 95 high-risk NBs registered in the JCCG-JNBSG clinical studies (JN-H-11, n=50; JN-H-07, n=45; median follow-up:51 months).

Methods: We modified the 'NCC Oncopanel' which is clinically available and approved for national health insurance coverage in Japan to make the customized 'NCC Oncopanel Ped'. It can survey mutations in 211 genes as well as 9 known gene fusions frequently observed in pediatric cancer. Tumor and paired lymphocyte (if available) DNAs were analyzed by using Ion Torrent Comprehensive Cancer Panel (409 genes) or NCC Oncopanel Ped with Ion Torrent Proton (n=45) or Illumina NextSeq sequencers (n=50), respectively (read depth>600). Alternative lengthening of telomere (ALT) and CIMP (methylation level in PCDHB locus) were determined by c-circle assay and pyro-sequencing, respectively.

Results: At least one mutation was detected in 80 tumors (84%). Ten tumors had copy number alterations in cell cycle-related genes (amplifications in CDK4, MDM2, and CCND1, or homozygous deletion in CDKN2A/2B). Hotspot mutations in ALK, FGFR1, MYCN, and HRAS genes were also identified. ALT was found in 23 MYCN-non-amplified cases, and 9 of those (39%) had ATRX alterations. In this cohort, ALT showed limited contribution to the overall survival (p=0.252 in all 91 analyzed cases). On the other hand, 11q loss (-) and CIMP-high (>60%) were independently correlated with poor patient prognosis (p=0.037 and p=0.003, respectively). CIMP-high showed a good correlation with MYCN amplification (p=0.002) and was also involved in 83% of ALT (+) MYCN-non-amplified cases.

Conclusion: Thus, CIMP and these genomic features could be useful for the sub-classification of high-risk tumors as the next version of INRG.

P038

Retrospective analysis of INRG clinical and genomic markers for 605 neuroblastomas in Japan: A report from JCCG-JNBSG

Ohira M[1], Nakamura Y[2], Takimoto T[3], Nakazawa A[4], Hishiki T[5], Matsumoto K[3], Shichino H[6], Iehara T[7], Fukushima T[8], Yoneda A[3], Tajiri T[9], Nakagawara A[10], Kamijo T[1]

[1]Research institute for Clinical Oncology, Saitama Cancer Center, Saitama, Japan, [2]Chiba Cancer Center Research Institute, Chiba, Japan, [3]National Center for Child Health and Development, Tokyo, Japan, [4]Saitama Children's Medical Center, Saitama, Japan, [5]Graduate School of Medicine, Chiba University, Chiba, Japan, [6]National Center for Global Health and Medicine, Tokyo, Japan, [7]Kyoto Prefectural University of Medicine, Kyoto, Japan, [8]Comprehensive Cancer Center, Saitama Medical University International Medical Center, Saitama, Japan, [9]Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, [10]Saga HIMAT, Saga, Japan, [11]Japan Children's Cancer Group Neuroblastoma Committee (JCCG-JNBSG), ,

Background and Aims: Neuroblastomas (NBs) exhibit divergent clinical behaviors and tumor risk classification at diagnosis is crucial for the section of an appropriate therapeutic strategy for each patient. The present study aimed to validate the clinical relevance of International Neuroblastoma Risk Group (INRG) prognostic and genomic markers in a Japanese NB cohort using a retrospective analysis.

Methods: Follow-up data based on 30 common INRG queries in 605 NB cases diagnosed in Japan between 1990 and 2014 were collected and the genome signature (identified by array-CGH analysis) of each tumor sample was integrated.

Results: As previously reported, age, tumor stage, MYCN, DNA ploidy, primary tumor site: adrenal, serum ferritin and lactate dehydrogenase (LDH) levels, segmental chromosome aberrations, and the number of chromosome breakpoints (BP) correlated with lower survival rates, while primary tumor site: thorax and numerical chromosome aberrations correlated with a favorable prognosis. We examined several reported cut-off values for LDH and ferritin levels in survival analysis and confirmed the potential of LDH ≥ 1400 U/L and ferritin ≥ 250 ng/mL as poor prognosis markers in our cohort. In the patient group with stage 4, MYCN non-amplified tumors (n=225), one of the challenging subsets for risk stratification, age ≥18 months, LDH ≥1400 U/L, and BP ≥7 correlated with lower overall and event-free survival rates ($p<0.05$). The genome subgroup GG-P2s (partial chromosome gain/loss type with 1p/11q losses and 17q gain, n=30) was strongly associated with a lower overall survival rate (5-year survival rate: 34%, $p<0.05$). We also examined 'ultra-high-risk' markers (MSI: metastatic site index, LDH, and age > 5 years) and found that MSI>1 and LDH>1250 U/L strongly correlated with a poor prognosis in Japanese patients, however, age > 5 years had no significant clinical impact in high-risk patients in our cohort.

Conclusion: The present study provides 605 Japanese NB data with clinical and genomic factors in the INRGdb. The combination of the tumor genomic pattern (GG-P2s and BP ≥7) with age at diagnosis and LDH will be a promising predictor for high-risk NBs in patient subsets, in accordance with previous findings from the INRG project.

P039

Germline Genetic Contribution to Tumorigenesis and Progression in Neuroblastoma

Seo E[1,2], Lee J[1], Lim J[2], Cho H[1], Ju H[1], Yoo K[1], Park W[2], Sung K[1]
[1]Department of Pediatrics, Samsung Medical Center, Gangnam-gu, South Korea, [2]Samsung Advanced Institute for Health Sciences & Technology, Sungkyunkwan University, Gangnam-gu, South Korea

Background: As cancer is primarily a disease of alterations in the DNA sequence, the germline genetic context where a somatic mutation occurs may impact tumor development and progression and the impact of germline genetics could be more prominent in neuroblastoma than in adult-onset cancers.

Aim: We hypothesized that putative damaging rare variants (pDRV) that were predicted to alter the structure or function of protein-coding genes may have a biologic impact on neuroblastoma, even if these variants are not sufficiently elevating neuroblastoma risk alone.

Methods: We performed whole-exome sequencing of germline and tumor DNA in 125 neuroblastoma patients. Validation cohort of 160 neuroblastoma patients from the TARGET database and comparison cohorts of 10,389 adult-onset cancer patients in the TCGA as well as 916 controls from the Korean general population database (KOREA1K) were analyzed and compared to study cohort.

Results:
Our analysis showed that neroblastoma patients carried an average of 41 pDRVs and we observed a weak but a positive correlation between the number of pDRVs and somatic mutations in neuroblastoma patients ($r = 0.18$, $P = 0.046$). Additionally, we found patients with a higher number of pDRVs had an inferior outcome than those with a lower number of pDRVs ($P = 0.034$), and patients with pDRVs in cancer biology-related genes also had a poorer outcome ($P < 0.001$).
In comparison to neuroblastoma, the TCGA cohort did not show a positive correlation between the number of pDRVs and somatic mutations. However, several cancer types or late-onset disease showed significant negative correlation. In addition, survival difference according to the pDRVs in cancer biology-related genes was not significant in the TCGA cohort. When examining the Korea1K, the total number of pDRVs and prevalence of pDRVs in cancer biology-related genes were significantly lower than those in neuroblastoma patients ($P < 0.001$ and $P = 0.025$ respectively). These findings suggest germline variants may have a substantial role in the development and progression of neuroblastoma and may have potential clinical implications for pediatric cancer patients.

P040

DIFFERENTIAL IMPACTS OF ALK-WT, ALK-F1174L AND ALK-R1275Q ON NEUROBLASTOMA TUMOR HETEROGENEITY AND METASTATIC DISSEMINATION

El Natour M[1], Vivancos Stalin L[1], Praz V[2], Balmas Bourloud K[1], Riggi N[3], Renella R[1], Mühlethaler-Mottet A[1]
[1]Pediatric Hematology-Oncology Research Laboratory (LHOP), Woman-Mother-Child Department, University Hospital CHUV, Lausanne, Suisse, [2]Lausanne Genomic Technologies Facility, UNIL, Lausanne, Suisse, [3]Genentech Inc, Department of Cell and Tissue Genomics (CTG), South San Francisco, CA, USA, San Francisco, États-Unis

Background: Neuroblastoma (NB) displays a high intratumoral heterogeneity with interconvertible noradrenergic (NOR) and drug resistant mesenchymal (MES) cell identities. Alterations of the anaplastic lymphoma Kinase (ALK) receptor are frequent in NB and correlated to poor outcome, with ALK-F1174L and ALK-R1275Q being the most potent activating mutations.
Aims: Identify the specific impact of ALK-wt, ALK-F1174L and ALK-R1275Q-mediated signaling on NB tumorigenesis and progression.

Methods: SH-EP cells (MES) expressing ALK-wt, ALK-F1174L and ALK-R1275Q isoforms and controls (SH-EP-pLIV) were injected subcutaneously into NOD-scid-gamma mice. Tumors were analyzed by RNA-seq and immunohistochemistry (IHC). Metastases were detected by human-Alu in situ hybridization.

Results: While SH-EP-ALK-R1275Q tumors conserved the spindle/MES-like stroma-rich phenotype of SH-EP-pLIV controls, SH-EP-ALK-wt and SH-EP-ALK-F1174L tumors displayed a phenotypic change with mainly differentiating or poorly differentiated neuroblasts. ALK-wt and ALK-F1174L-expressing tumors displayed an increase in the NOR signature score relative to ALK-R1275Q and control tumors and a heterogeneous reduction of CD44 protein expression, suggesting a role for ALK-wt and ALK-F1174L in mediating a MES to NOR phenotypic switch. Furthermore, ALK expression conferred metastatic properties to SH-EP cells, mainly to the lung, with an ALK-status specific dissemination pattern. ALK-R1275Q expression lead to numerous isolated or small clusters of disseminated tumor cells (DTC) in the lung parenchyma, whereas ALK-F1174L resulted in few but large intravascular tumor emboli (ITE) and rare DTC, and ALK-wt resulted in DTC, ITE and established metastases. An ALK-status differential signaling was also detected in the tumor transcriptome. Gene ontology analyses revealed that the major pathways deregulated by ALK, involved in extracellular matrix organization, hemidesmosome assembly, cell-cell signaling and cell migration, were predominantly upregulated in ALK-R1275Q tumors and downregulated in ALK-wt/ALK-F1174L compared to pLIV tumors. The upregulation of specific genes associated with migration and metastasis were validated by qPCR in ALK-R1275Q tumors. Finally, analyses of the differentially expressed murine genes in the tumor transcriptomes revealed a pronounced alteration in the stroma of ALK-R1275Q–expressing tumors compared to other groups.

Conclusion: Our in vivo data revealed an ALK-status dependent differential signaling. ALK-F1174L and ALK-wt may promote the transition toward the NOR cell identity. Further analyses by single-cell RNAseq are needed to validate these findings.

P041

Mutations of 1p genes do not consistently abrogate tumor suppressor functions in 1p-intact neuroblastoma

Kuick C[1], Tan J[2,3], Jasmine D[2,3], Tohari S[4], Ng A[4], Venkatesh B[4], Chen H[1], Loh E[1], Jain S[1], Seow W[1], Ng E[1], Lian D[1], Soh S[5,6,7], Chang K[1,5,7], Chen Z[1,2,5,8], Loh A[5,7,9]

*[1] Department of Pathology and Laboratory Medicine, KK Women's and Children's Hospital, Singapore, Singapore, [2]Neurodevelopment and Cancer Laboratory, NUS Center for Cancer Research, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, [3]Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, [4]Comparative and Medical Genomics Laboratory, Institute of Molecular and Cell Biology, A*STAR, Singapore, Singapore, [5]VIVA-KKH Paediatric Brain and Solid Tumour Programme, KK Women's and Children's Hospital, Singapore, Singapore, [6]Department of Paediatric Subspecialties Haematology Oncology Service, KK Women's and Children's Hospital, Singapore, Singapore, [7]Duke NUS Medical School, Singapore, Singapore, [8]National University Cancer Institute, Singapore, National University Health System, Singapore, Singapore, [9]Department of Paediatric Surgery, KK Women's and Children's Hospital, Singapore, Singapore*

Background:
Deletion of 1p is associated with poor prognosis in neuroblastoma, however selected 1p-intact patients still experience poor outcomes. Mutations resulting in functional impairment of key 1p genes may mimic the deleterious functional effects of 1p deletion in neuroblastoma

Aims:
To understand the incidence, spectrum and effects of mutational variants in 1p-intact neuroblastoma using a custom targeted sequencing panel of 21 candidate genes that were commonly mutated and prognostically significant in neuroblastoma, to profile the mutational variants in a retrospective cohort of 1p-intact neuroblastoma tumors. Then, to correlate the identified variants with clinical, cytogenetic and pathological characteristics, and functionally validate selected novel variants of 1p36 genes identified from our patient cohort.

Methods:
We characterized the 1p status of 325 neuroblastoma patients, and correlated the mutational status of 1p tumor suppressors and neuroblastoma candidate genes with survival outcomes among 100 1p-intact cases, then performed functional validation of selected novel variants of 1p36 genes identified from our patient cohort.

Results:
Among patients with adverse disease characteristics, those who additionally had 1p deletion had significantly worse overall survival. Among 100 tumor-normal pairs sequenced, somatic mutations of 1p tumor suppressors KIF1Bβ and CHD5 were most frequent (2%) after ALK and ATRX (8%), and BARD1 (3%). Mutations of neuroblastoma candidate genes were associated with other synchronous mutations and concurrent 11q deletion (P = 0.045). In total, 24 of 38 variants identified were novel and predicted to be deleterious or pathogenic. Functional validation identified novel KIF1Bβ I1355M variant as a gain-of-function mutation with increased expression and tumor suppressive activity, correlating with indolent clinical behavior; another novel variant CHD5 E43Q was a loss-of-function mutation with decreased expression and increased long-term cell viability, corresponding with aggressive disease characteristics.

Conclusions:

Our study showed that chromosome 1 gene mutations occurred frequently in 1p-intact neuroblastoma, but may not consistently abrogate the function of bonafide 1p tumor suppressors. These findings may augment the evolving model of compounding contributions of 1p gene aberrations toward tumor suppressor inactivation in neuroblastoma.

P042

DIAPH3 regulates proliferation and cytoskeletal modulation in neuroblastoma

Yuan Y[1], Liano Pons J[1], Bedoya-Reina O[1], Rodriguez Garcia A[1], Arsenian Henriksson M[1]
[1]*Department of Microbiology, Tumor and Cell Biology (MTC), Biomedicum, Karolinska Institutet, SE-17165 Stockholm, Sweden*

Background: Cell differentiation and adhesion are associated with better outcome in neuroblastoma (NB). DIAPH3 formin governs actin dynamics in proliferating cells, supports cell migration, and is a key mediator of mesenchymal-ameboid transition. In addition, up-regulation of DIAPH3 enhances migration and invasion of liver and lung cancer cells.
Aims: In the present study, we investigated the role of DIAPH3 in NB prognosis, proliferation, cell cycle, and in mouse and human sympathoadrenal development.

Methods: We used two NB single-cell/nuclei datasets: i) Kildisiute et al. 2021 and ii) Bedoya-Reina et al. 2021 and identified cell populations in which DIAPH3 was significantly up-regulated, together with known diagnostic markers. Additionally, we investigated the SEQC bulk-seq RNA dataset (GSE62564). Further, DIAPH3 was knocked-down using shRNA in NB cell lines, and its role in proliferation was assessed by EdU staining and Incucyte analysis, while cell cycle arrest was evaluated by FACS.

Results: We identified a significant upregulation of DIAPH3 in high-risk patients with significantly lower survival in SEQC cohort. Consistently, using single-cell analysis, a significant upregulation of DIAPH3 was identified in undifferentiated cell populations, specifically in a cluster of NB cells enriched in high-risk patients with MYCN overexpression. During development, the gene is significantly upregulated in both cycling Schwann cell precursors (SCPs) and neuroblasts in humans, and in SCPs in mice. DIAPH3 is also significantly expressed in post-natal progenitors of chromaffin cells and in peripheral dividing mesenchymal cells of neural crest origin. Further analysis indicates that DIAPH3 is expressed in cells of undifferentiated aggressive NB with embryonic stem cell identity. EdU staining showed that knocking down DIAPH3 reduced the proliferation rate in NB cell lines as well as G2-M phase arrest using FACS. Importantly, we observed pronounced changes in cell morphology.

Conclusions: We found that DIAPH3 is significantly expressed in poorly differentiated cells in high-risk NB patients and is important in NB cell proliferation, cell cycle, and morphology. Further studies will give insights into its role in actin dynamics, neural differentiation, and metabolism.

P043

Identifying unique genomic gains associated with chromosome 17q gain translocation breakpoint in neuroblastoma

Chandrasekaran S[1], Rodriguez-Fos E[1,7], Szymansky A[1], Peifer M[3], Henssen A[1,4,5], Kehr B[2], Schulte J[1,4,5,6]
[1]Department of Pediatric Oncology and Hematology, Charité – Universitätsmedizin Berlin, Berlin, Germany, [2]Algorithmic Bioinformatics, RCI Regensburg Center for Interventional Immunology,Franz-Josef-Straußß-Allee 11,93053, Regensburg, Germany, [3]Department of Translational Genomics, Center of Integrated Oncology Cologne- Bonn, Medical Faculty, University of Cologne, Robert-Koch-Str. 21, 50931, , Cologne, Germany, [4]The German Cancer Consortium (DKTK), Partner Site Berlin, Germany, Berlin, Germany, [5]The German Cancer Research Center (DKFZ), Heidelberg, Germany, Heidelberg, Germany, [6]Department of General Pediatrics, Hematology/Oncology, University Children's Hospital Tuebingen, , Tuebingen, Germany, [7]Berlin Institute of Health (BIH) at Charité - Universitätsmedizin Berlin, Berlin, , Berlin, Germany

Background:
Neuroblastoma is associated with strong clinical heterogeneity and are stratified as high- and low-risk based on clinical and molecular features. In high-risk neuroblastomas, the MYCN oncogene locus is amplified. The copy number signature most frequently associated with high-risk neuroblastoma is a partial gain on the long q arm of chromosome 17 occurring in
approximately 80% of cases. However, its contribution to neuroblastoma disease pathogenesis and molecular risk is incompletely understood.

Aim:
Chromosome 17q gain is often accompanied with other chromosomal aberrations such as 3p or 11q loss. The aim of this study was to classify the chromosome 17q gains based on the breakpoint position and structural variation, and to understand the transcriptomic changes that occur in neuroblastoma as a result of chromosome 17q gain rearrangements.

Methods:
We analyzed whole genome sequencing data of 120 neuroblastoma samples with structural variant and copy number results from novoBreak tool and ASCAT R
package respectively, for the validation of the chromosome 17q21 gain breakpoint genomic position. Further differential expression analysis was performed using DESeq between the most significant translocation and the rest of the samples to account for a unique gene set.

Results:
Around 60% of the samples with chromosome 17q gain had a translocation to chromosome 11q14, leading to a loss around 60mbp at the breakpoint region. This subtype encompassing 17q21gains and 11q14 losses was observed in 3 low-risk and in 12 high-risk non-MYCN amplified neuroblastoma samples. This subtype (17:11) had 1463 genes differentially expressed genes. In addition, we were able to identify a copy number gain observed in the 11q13 region which is unique only to the high-risk samples of the translocation (17:11) subtype. This region of 5mbp gain hosts oncogenes such as
SYT7, that are seen in prostate cancer and DPF2 and NAA40 that are known in neuroblastoma.

Conclusion:
This unique region of chromosome 11q gain observed in high-risk non-MYCN

amplified neuroblastoma samples paves the way to understanding the mechanism that is associated with this translocation (17:11). In addition, it could enable identifying genes that could be associated with neuroblastoma progression and therefore possibly could be used in disease prognosis.

P044

In vivo structure-function analysis supports a role for FANCJ DNA helicase G4-unwinding activity in MYCN-driven neuroblastoma formation

Hilgert E[1,2], Leys S[1,2], Vanhauwaert S[1,2], Depestel L[1,2], Look AT[3,4], Schulte JH[5,6,7], Goossens S[1,2,8], Fischer M[9,10], De Wilde B[2,11], Vermeirssen V[1,2,12], Bek JW[1,2], Durinck K[1,2], Speleman F[1,2]

[1]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium, [2]Cancer Research Institute Ghent (CRIG), Ghent University, Ghent, Belgium, [3]Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, USA, [4]Division of Pediatric Hematology, Oncology Boston Children's Hospital, Boston, USA, [5]Department of Pediatric Oncology and Hematology, Charité University Medical Center Berlin, Berlin, Germany, [6]German Cancer Consortium (DKTK), Berlin, Germany, [7]German Cancer Research Center (DKFZ), Heidelberg, Germany, [8]Department of Diagnostic Sciences, Ghent University, Ghent, Belgium, [9]Center for Molecular Medicine Cologne, Cologne (CMMC), Medical Faculty, University of Cologne, Cologne, Germany, [10]Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Cologne, Germany, [11]Department of Internal Medicine and Pediatrics, Faculty of Medicine & Health Sciences, Ghent University, Ghent, Belgium, [12]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

Background: MYC(N) overexpression in neuroblastoma (NB) causes enhanced replication stress marked by elevated ATR-CHK1 signalling. Gene regulatory network analysis in NB identified the BRIP1 gene (encoding the Fanconia anemia complex protein FANCJ) as driver of a large module encompassing genes implicated in CHK1 addiction. FANCJ has been proposed as master regulator at the replication fork acting upstream of ATR-CHK1 activation and has a well-studied DNA helicase function implicated in unwinding stable G-quadruplexes (G4) which can form impediments to progressing replication forks.

Aims: We aimed to further unravel BRIP1/FANCJ dependency in NB.

Methods: An established zebrafish model for MYCN-driven NB, in which tumors develop from 9 weeks on with a penetrance of 20% (Zhu, et al., Cancer Cell 2012) was used to investigate the role of wildtype and mutant BRIP1 overexpression in development, maintenance and/or aggressiveness of the NB tumors.

Results: We evaluated conditional BRIP1 overexpression in the sympathetic neuronal lineage using a zebrafish model for dβh-MYCN-driven NB formation and noted significantly increased tumor penetrance ($p < 0.0001$). Next, we tested the impact of a mutated BRIP1 allele which is known to be DNA helicase deficient (BRIP1(K52R)). We compared MYCN-only versus MYCN/BRIP1 and MYCN/BRIP1(K52R) co-overexpression in a total of 120 animals confirming the tumor-accelerating effect of wildtype BRIP1 while observing complete loss of this effect in the MYCN/BRIP1(K52R) zebrafish which showed the normal tumor penetrance as observed in MYCN-only overexpressing zebrafish. We also generated a Th-BRIP1 conditional overexpressing mouse line which will now be crossed with the Th-MYCN line in order to test the impact of BRIP1 overexpression on tumor penetrance in mice. Additional experiments to further explore the role of G4-unwinding in NB formation in vivo and the impact on cellular phenotype in vitro are ongoing.

Conclusion: We demonstrated the importance of the FANCJ DNA helicase function on NB tumor formation in vivo using a dβh-MYCN-driven zebrafish model. These results will fuel further experiments to better understand the role and impact of factors controlling DNA replication and replicative stress and boost efforts toward the identification of FANCJ-specific helicase inhibitors for therapeutic application.

P045

SH-SY5Y cells overexpressing LIN28B gene release exosomal-microRNA that promote cell migration

Ardito M[1,2,6], Segalerba D[1,2,6], Rombolà L[1], Cocchi S[3], Corallo D[4], Aveic S[4], Quattrone A[3], Capasso M[5], Eva A[1], Morini M[1,2]

[1]Laboratory of Molecular Biology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [2]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [3]Laboratory of Translational Genomics, CIBIO-Department of Cellular, Computational and Integrative Biology, University of Trento, Trento, Italy, [4]Laboratory of Target Discovery and Biology of Neuroblastoma, Istituto di Ricerca Pediatrica (IRP), Fondazione Città della Speranza, Padova, Italy, [5]Department of Molecular Medicine and Medical Biotechnologies, University of Napoli Federico II, Napoli, Italy and CEINGE Biotecnologie Avanzate s.c.ar.l., Napoli, Italy, [6]Authors contributed equally to this work, ,

Background

Overexpression of LIN28B has been extensively reported in high-risk neuroblastoma (HR-NB). It is known that LIN28B downregulates the expression of the let-7 miRNA family, leading to a worse outcome for NB patients. We hypothesized that LIN28B could further promote tumor progression by affecting the miRNA content of exosomes (exo-miRs).

Aims

We aimed at (i) characterizing the exo-miR expression of SH-SY5Y cells overexpressing LIN28B (SH-SY5Y/LIN28B) compared to SH-SY5Y control cells (SH-SY5Y/CTR) to highlight differentially expressed exo-miRs and (ii) assessing whether the identified exo-miRs are able to induce SH-SY5Y/CTR cells shift toward the aggressive tumor phenotype of SH-SY5Y/LIN28B cells.

Methods

We measured a panel of 384 exo-miRs in SH-SY5Y/LIN28B and SH-SY5Y/CTR cells by quantitative Real Time PCR (qPCR). We focused on the most significantly upregulated exo-miRs for functional studies by miRNA mimics transfection in SH-SY5Y/CTR cells. qPCR was used to assess transfection efficiency by measuring specific miRNA expression. Next, we evaluated miRNA mimics effects on cell migration and viability.

Results

We found that the overexpression of LIN28B was associated with a specific exo-miR profile. We identified 20 downregulated and 145 up-regulated exo-miRs. So far, we focused on the upregulated exo-miR-501, -92a and -539, which have been associated with cell motility. We performed functional studies by assessing cell migratory capacity upon transfection of SH-SY5Y/CTR cells with miRNA mimics. We observed that the expression of miR-501, -92a and -539 mimics increased the migratory rate of SH-SY5Y/CTR cells to levels comparable to those of SH-SY5Y/LIN28B cells. We also evaluated the expression of a miR-501-predicted target gene, LPAR1, which inhibits cell migration. Transfected SH-SY5Y/CTR cells showed a reduction of LPAR1 expression to levels comparable to those of SH-SY5Y/LIN28B cells, suggesting the involvement of a miR-501/LPAR1 axis in the regulation of cell migration in NB.

Conclusion

Our results indicate that LIN28B overexpression modulates the exo-miRs cargo of SH-SY5Y/LIN28B cells. The higher migration rate observed in SH-SY5Y/CTR cells upon miRNA mimic transfection demonstrates that miR-501 (via LPAR1 modulation), -92a and -539 are actively involved in shaping cell motility and in promoting more aggressive tumor traits.

P046

The prognostic impact of chromosome 11q loss in stage 4S neuroblastoma

Theißen J[1], Lorenz W[1], Hemstedt N[1], Gecht J[2], Berthold F[2], Simon T[2], Hero B[2], Fischer M[1]
[1]Department of Experimental Pediatric Oncology, University Children´s Hospital, and Center for Molecular Medicine Cologne (CMMC), Medical Faculty, University of Cologne, Cologne, Germany, [2]Department of Pediatric Oncology and Hematology, University Children´s Hospital of Cologne, Cologne, Germany

Background:
Genomic loss of chromosome 11q is associated with poor outcome in neuroblastoma. In the current INRG risk classification, stage 4S patients harboring 11q loss are considered to be at high risk, however, this categorization was developed on few patients only.

Aims:
We aimed to assess the prognostic impact of 11q loss in a large cohort of stage 4S neuroblastoma patients.

Methods:
Patients with stage 4S neuroblastoma lacking MYCN amplification, who had been registered in GPOH trials between 1990 and 2016, were included in this study. The copy number status of chromosome 11q at diagnosis and, where available, at relapse or progression was investigated by interphase fluorescence in situ hybridization (FISH), with probes covering 11q22.3 and centromere as control. Loss of 11q (deletion/imbalance) was defined according to the international consensus criteria.

Results:
Tumor samples of 208 stage 4S patients were analyzed for 11q copy number status. The median follow-up of patients was 11 years (0-26 years). Loss of chromosome 11q was found in 10/208 tumors (4.8%). Median age at diagnosis was 5.9 months and 2.9 months for patients with and without 11q aberration, respectively (range, 0–11 versus 0-12 months; p=0.01). Tumor progression was observed in 4/10 patients with 11q loss, and in 35/198 patients with normal 11q (p=0.10). Acquisition of novel 11q loss at relapse/progression was found in one of 16 patients. Five-year event-free survival was 0.70±0.15 in patients with 11q loss and 0.82±0.03 in patients with normal 11q (p=0.07), with favorable overall survival in both groups (5-year-OS 11q loss, 0.90±0.10; 11q normal, 0.93±0.02; p=0.21). Of note, metastatic progression to stage 4 disease was observed in all four patients with 11q loss who had an event. By contrast, only 15 of the 198 patients with normal 11q developed metastatic progression into stage 4 disease. Accordingly, metastasis-free survival differed significantly between the groups (0.70±0.15 versus 0.92±0.02; p<0.001).

Conclusion:
Loss of chromosome 11q is rare in stage 4S neuroblastoma and does not define high-risk disease per se. The relatively high frequency of metastatic progression into stage 4 disease in this subgroup, however, suggests that close monitoring is required in these patients.

P047

Circadian clock disruption in neuroblastoma, role of metabolic programming and effects on clinical outcomes

Sjoeberg Bexelius T[1,2], Bedoya-Reina O[3]

[1]Paediatric oncology unit, Astrid Lindgren's Children Hospital, and Karolinska University Hospital, Solna, Sweden, [2]Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden, [3]Karolinska Institutet, Department of Microbiology, Tumor- and Cell Biology, Solna, Sweden

Background

Circadian clock genes and their output constitute the molecular basis of transcriptional-translational feedback loop in tissues, and may clinically play a role in neuroblastoma outcome. Circadian controlled biomarkers include metabolic pathways, such as lactate, glucose and glutamine. The oncoprotein MYC's activity may disrupt the circadian rhythm in neuroblastoma, but its its role in the clinical outcome of patients and the mechamisms mediating this disruption is unclear. Mechanistically, MYC induces the expression of the circadian gene repressor Nr1d1, whose product in turn suppresses the core clock gene Bmal1. BMAL1 and CLOCK form a transcription factor, binding to same E-boxes as MYC.

Aims

To investigate if core clock gene dysregulation is correlated with clinical outcomes of neuroblastoma patients, and to determine if this is linked to a metabolic reprogramming.

Methods

Clock gene disruption was assessed in more than 1,000 patients beloging to the SEQC, Kocak, and TARGET cohorts by calculating a metric: Clock Correlation Distance (CCD). This metric compares the co-expression of 12 core clock genes with that of a synchronized wild-type reference, so that a zero value indicates an identical co-expression-, and a high score a larger difference to that of the reference. The significance of the CCD is assessed by using the same method with 12 random genes. Subsequently, CCD was analysed in relation to known risk factors and for prognostication. To study the effect of CCD on metabolic reprogramming, the expression of genes relevant to glycolysis, and oxidative phosphorylation were analysed and clustered into groups.

Results

In the provisional analysis, CCD is significantly associated with MYCN status, age at diagnosis, and INSS stage. In particular, 1) low-stage-, 2) low-risk-, and 3) non-MYCN amplified tumours, have a significantly lower CCD ($p<0.05$). Oppositely, 1) high-stage, 2) high-risk, and 3) MYCN amplified tumours, have higher non-significant CCD scores, suggesting that a larger clock disruption is linked with worse prognosis.

Conclusion

Clock disruption in neuroblastoma is linked to prognosis. This occurs in connection with established prognostic risk factors such as MYCN amplification. We plan to further analyse the metabolic pathways and their reprogramming to understand mechanism of clock disruption in neuroblastoma.

P048

Conditional knock-out of the TERT gene in neuroblastoma cell lines

Riedel J[1,2], Ackermann S[1,2], Brummel L[1,2], Kahlert Y[1,2], Fischer M[1,2]
[1]Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Cologne, Germany, [2]Center for Molecular Medicine Cologne, Medical Faculty, University of Cologne, Cologne, Germany

Background:
Telomere maintenance mechanisms characterise high-risk neuroblastoma, which is conferred by telomerase activation in most cases. While telomerase is known to maintain telomere lengths in cancer cells in general, the mechanistic consequences of its absence and its potential value as therapeutic target in neuroblastoma have remained unclear.

Aim:
We aimed to create a conditional knock-out of the TERT gene in telomerase dependent neuroblastoma cell lines to investigate the mechanistic relevance and potential therapeutic utility of TERT expression and telomerase activity in this malignancy.

Methods:
CRISPR/Cas9 gene editing was used to generate conditional TERT knock-out cell lines by inserting an invertible intronic cassette in exon 2 of the TERT locus on one allele and induce an inactivating frameshift mutation on the other allele. Polyclonal and monoclonal cell lines were validated by nested PCR and dideoxy-sequencing. Cre recombinase was used to induce inversion of the cassette into a mutagenic configuration to generate a conditional TERT knock-out. Antibiotic resistance genes and fluorescent markers enabled identification of successfully modified cells, validation of integration and visualisation of cassette inversion by fluorescence microscopy.

Result:
We successfully integrated the cassette into five high-risk neuroblastoma cell lines. Antibiotic selection produced polyclonal cell lines, from which monoclonal cell lines were established. Using nested PCR and dideoxy-sequencing, we identified 124 clones with specific integration of the cassette into the TERT gene locus. Furthermore, we successfully inverted the cassette using Cre recombinase in these clones to induce a conditional TERT knock-out on the allele containing the cassette. Integration and inversion of the cassette were validated by fluorescence microscopic detection of ZsGreen and mCherry, respectively. Ongoing experiments aim at identification of clones with an inactivating frameshift mutation on the second allele, and at determining the functional consequences of conditional TERT inactivation.

Conclusion:
We created a conditional knock-out of the TERT gene on one allele in five neuroblastoma cell lines using a CRISPR-Cre system. This approach may facilitate to investigate the mechanistic role of telomerase in high-risk neuroblastoma development and progression, as well as its potential value as a therapeutic target.

P049

Intercellular extrachromosomal DNA copy number heterogeneity drives cancer cell state diversity

Stöber M[1], Chamorro González R[3], Brückner L[3], Conrad T[1], Wittstruck N[1,3], Szymansky A[3], Eggert A[3], Schulte J[3], Koche R[7], Henssen A[1,3,4,6,8], Schwarz R[2,4,1], Haase K[3,5]

[1]Berlin Institute for Medical Systems Biology at the Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany, [2]Institute for Computational Cancer Biology, Center for Integrated Oncology (CIO), Cancer Research Center Cologne Essen (CCCE), Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany, [3]Department of Pediatric Oncology/Hematology, Charité-Universitätsmedizin Berlin, Berlin, Germany, [4]BIFOLD - Berlin Institute for the Foundations of Learning and Data, Berlin, Germany, [5]German Cancer Consortium (DKTK), partner site Berlin, and German Cancer Research Center (DKFZ), Heidelberg, Germany, [6]Berlin Institute of Health, Berlin, Germany, [7]Center for Epigenetics Research, Memorial Sloan Kettering Cancer Center, New York, USA, [8]Experimental and Clinical Research Center (ECRC) of the MDC and Charité Berlin, Berlin, Germany

Neuroblastoma is characterised by extensive inter- and intra-tumor genetic heterogeneity and varying clinical outcomes. One possible driver for this heterogeneity are extrachromosomal DNAs (ecDNA), which segregate independently to the daughter cells during cell division and can lead to rapid amplification of oncogenes. While ecDNA-mediated oncogene amplification has been shown to be associated with poor prognosis in many cancer entities, the effects of ecDNA copy number heterogeneity on intermediate phenotypes are still poorly understood.

Here, we leverage DNA and RNA sequencing data from the same single cells in cell lines and neuroblastoma patients to investigate these effects. We leverage ecDNA amplicon structures to determine precise ecDNA copy numbers and reveal extensive intercellular ecDNA copy number heterogeneity. We further provide direct evidence for the effects of this heterogeneity on gene expression of cargo genes, including MYCN and its downstream targets, and the overall transcriptional state of neuroblastoma cells.

These results highlight the potential for rapid adaptability of cellular states within a tumour cell population mediated by ecDNA copy number, emphasising the need for ecDNA-specific treatment strategies to tackle tumour formation and adaptation.

P050

MYCN mRNA is a post-transcriptional regulator of RNA transcripts via microRNAs in High-Risk Neuroblastoma

Gómez-González S[1], García López M[1], Lemos I[2], Rodríguez E[3], Muñoz Aznar O[2], Suñol M[3], Carcaboso Á[2], Mora J[2], Lavarino C[1]

[1]Translational Genomics Group, Developmental Tumor Biology Laboratory, SJD Pediatric Cancer Center (PCCB), Institut de Recerca Sant Joan de Déu, Barcelona, Spain, [2]SJD Pediatric Cancer Center Barcelona (PCCB), Hospital Sant Joan de Déu, Barcelona, Spain, [3]Department of Pathology, Hospital Sant Joan de Déu, Barcelona, Spain

Background

MicroRNAs (miRNAs) are small RNA sequences which downregulate gene expression through targeting complementary sequences in mRNA 3'-untranslated region (UTR), known as miRNA response elements (MRE). Competing endogenous RNA (ceRNA) act as miRNA sponges for shared miRNAs.

In NB, the MYCN amplification (MNA) is associated with high-risk disease. The highly conserved MYCN 3'UTR in MNA tumors can act as a miRNA sponge that regulate other ceRNA via shared miRNAs. The effect of concomitant deregulated levels of multiple miRNAs on gene regulatory processes in NB has not been studied.

Aim

This study aimed to explore MYCN mRNA as a post-transcriptional regulator of ceRNA transcripts via shared miRNAs pool, and characterize the MNA ceRNA associated network.

Methods

We generated and analyzed genome-wide miRNA and gene expression microarray data from twenty primary NB tumors. MYCN status was characterized using qPCR or/and SNP arrays. Clinical and biological data was available for all NB included in the study. Bioinformatic tools and publicly available databases were used to examine mRNA/miRNA interactions. NB cell lines, patient-derived xenografts (PDXs) and primary NB tumors were used to validate the miRNA expression and ceRNA network by real-time qPCR, luciferase assays and MYCN transfection in non-amplified samples.

Results

Unsupervised analysis of the miRNA microarray data revealed differential profiles among NB clinical risk groups. miRNA expression profiles were significantly associated with MNA. We developed and applied an R-based bioinformatic tool that performs correlation expression analyses between miRNA and RNA transcripts. The analysis also identified a potential ceRNA network associated with MNA. After data filtering, the most promising MYCN targets and their associated miRNAs were validated in NB cell lines, PDX and an independent NB cohort. The results enabled us to identify a ceRNAs network that affect biological pathways that contribute to the aggressive phenotype of the disease.

Conclusion

High levels of MYCN mRNA act as a miRNA sponge deregulating the expression of several ceRNAs that compete for common miRNAs. These findings could provide a functional explanation of how genes with no previously known direct interactions are potentially interlinked and identify interactions between relevant signaling pathways.

P051

LSD1/CoREST complexes contribute to neuroblastoma aggressiveness by modulating core regulatory TFs' transcriptional programs

Aloisi S[1], Santulli M[1], Russo M[1], Zadran S[1], Cimadom L[1], Rigamonti A[1], Capranico G[1], Perini G[1], Milazzo G[1]
[1]*University of Bologna, Department of Pharmacy and Biotechnology, Bologna, Italy*

Background:
The HDAC-containing complexes have been defined as promising drugguble targets for tumour treatment, since the transcriptional programs they govern are characteristically dysregulated in tumour development. The CoREST complex possesses an additional histone demethylating activity, carried out by the LSD1 enzyme. CoREST protein family (CoREST1,2,3) plays an important role during neurodevelopment and analyses of different cohorts of neuroblastoma (NB) patients revealed a negative correlation between CoRESTs expression and survival rates.

Aims:
To investigate the functional and clinical relevance of the three LSD1/CoREST1,2,3 complexes in NB.

Methods:
CoREST1,2,3 complexes were purified from NB cells overexpressing single tagged CoREST factors. Biological effects (growth, clonogenicity and cell cycle) were tested after both chemical CoREST complex inhibition (corin, CoREST_inhibitor) and conditional CoREST1,2,3 Knock-Down (KD). CoREST1,2,3 and LSD1 ChIP-sequencing were performed and the resulting genomic distribution data were integrated with global transcriptomic analyses obtained in NB cells conditionally KD for each CoREST1,2,3.

Results:
We investigated the trimeric composition of CoREST1,2,3-complexes, highlighting differences in how each CoREST protein interact with LSD1 and HDAC1/2. Moreover, our data revealed a strong dependency of NB cell lines on CoREST factors, the downregulation of which led to a reduction of cell growth, dysregulation of the cell cycle and induced massive apoptosis. Consistently, corin treatment induced a drastic reduction of NB cell proliferation. Genome-wide occupancy of CoRESTs and transcriptomic analyses revealed a non-functionally redundant role of CoRESTs. Indeed, they seem to regulate the expression of unique sets of target genes, and, interestingly, they co-bind on overlapping genomic regions. Surprisingly, CoRESTs occupancy was also associated with open chromatin regions (H3K27Ac) also bound by TFs that are members of the NB Core Regulatory Circuitry (CRC): an auto-regulatory TFs loop essential for maintaining neuroblastoma cell identity.

Conclusions:
Our findings support a model in which CoREST complexes, so far known only for their repressive activity, might have a non-canonical role acting as auxiliary co-regulatory complexes of the NB CRC and thus halting the "active state" of chromatin. Hence, LSD1/CoREST complexes represent major vulnerabilities for neuroblastoma and interesting therapeutic targets, not only for blocking LSD1 tumorigenic activity, but also for disrupting CRC-mediated transcriptional networks.

P052

Aryl Hydrocarbon Receptor (AhR) is a Tumor Promoter in MYCN-Amplified Neuroblastoma Via Suppression of Differentiation

Chaudhry K[1], Jacobi J[1], Gillard B[2], Long M[3], Twist C[4], Smiraglia D[1], Bianchi-Smiraglia A[1]
[1]Department of Cell Stress Biology, Roswell Park Comprehensive Cancer Center, Buffalo, United States, [2]Department of Pharmacology & Therapeutics, Roswell Park Comprehensive Cancer Center, Buffalo, United States, [3]Department of Biostatistics & Bioinformatics, Roswell Park Comprehensive Cancer Center, Buffalo, United States, [4]Department of Pediatrics, Roswell Park Comprehensive Cancer Center, Buffalo, United States

Background
Neuroblastoma's current standard-of-care differentiation therapies, including retinoic acid, eventually fail in ~50% of high-risk neuroblastoma patients due to resistance, necessitating novel treatment options. Amplification of MYCN, a driver of neuroblastoma disease progression, correlates with unfavorable patient prognosis and poor response to retinoid therapies. Thus, targeting MycN expression is an attractive strategy to suppress neuroblastoma and improve retinoic acid treatment response.

Aims
We hypothesized that the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, is a tumor promoter that positively regulates MycN and alters retinoic acid treatment efficacy in MYCN-amplified neuroblastoma.

Methods
Human neuroblastoma cell lines were interrogated for AhR and MycN levels. MYCN-amplified cells were genetically depleted of AhR by shRNA or treated with clofazimine (CLF), a safe and orally bioavailable FDA-approved drug and novel AhR antagonist. Proliferation, invasion, colony formation, and in vivo tumor growth were used to assess AhR tumorigenicity. RNA-, ATAC-, and CUT&RUN-sequencing were performed to interrogate regulation of MycN and differentiation. Synergy analysis was utilized to determine interaction of CLF and all-trans retinoic acid (ATRA).

Results
AhR protein expression is positively associated with MycN in human neuroblastoma cell lines. Genetic depletion of AHR or its inhibition with CLF decreases clonogenicity, invasion, and proliferation of MYCN-amplified neuroblastoma cells. AhR positively regulates MycN, as AHR knockdown or CLF treatment decreases MycN protein levels. RNA-sequencing reveals negative enrichment of "Hallmark Myc targets" in AHR-depleted cells. CUT&RUN sequencing verified AhR binding to the MYCN promoter. Moreover, AhR suppresses differentiation. AHR depletion or inhibition induces neurite outgrowth and upregulates neuronal differentiation gene signatures. Mechanistically, ATAC-sequencing results suggest AhR mediates chromatin remodeling at distal enhancer regions. Upon AHR knockdown, lost differentially accessible regions (DARs) overlapped strongly with MYCN-related cistromes, while gained DARs aligned with drivers of neuroblastoma differentiation, including the retinoic acid receptor, RARA. Finally, CLF synergizes with retinoic acid therapy. CLF augments retinoic acid-induced differentiation, decreases MycN, and synergistically suppresses growth in vitro and in vivo.

Conclusion
Our work uncovers AhR as a novel tumor promoter in MYCN-amplified neuroblastoma and could lead to an already FDA-approved drug, CLF, as a promising new therapy that improves efficacy of retinoic acid-based treatments.

P053

Inhibition of TENEURIN-4 promotes differentiation and suppresses neuroblastoma growth

Abu Ajamieh S[1], Andonova T[1], Pepich A[1], Olsen T[1], Tümmler C[1], Thankaswamy-Kosala S[2], Kanduri C[2], Fransson S[3], Martinsson T[3], Baryawno N[1], Näsman A[4], Kogner P[1], Johnsen J[1], Wickström M[1]
[1]Department of Women and Children's Health, Karolinska Institutet, Stockholm, Sweden, [2]Department of Medical Genetics and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [3]Department of Laboratory Medicine, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [4]Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden

Background: High-risk neuroblastoma presents significant clinical challenges, and further therapeutic options are needed. Neuroblastoma originates from neural crest cells resulting in impaired neural differentiation. Teneurins (TENM1-4) are cell adhesion molecules highly expressed during embryonal development and function in differentiation. Somatic mutations and structural aberrations of TENM genes have been identified in neuroblastoma.

Aims: To identify the significance of TENM4 in neuroblastoma growth, tumorigenicity, and differentiation.

Methods: TENM4 immunohistostaining was performed in tumors from neuroblastoma patients. Genetic inhibition was mediated by siRNA and CRISPR-Cas9 in neuroblastoma cells to analyze the effects on morphology, proliferation, tumorigenicity, and molecular signaling through transcriptomics, proteomics, and quantification of gene expression.

Results: We examined primary neuroblastomas and detected a significantly higher protein and mRNA expression level of TENM4 in high-risk vs. non-high-risk and MYCN-amplified vs. non-MYCN amplified tumors. Moreover, tumors positive for TENM4 protein were associated with poor patient outcome. siRNA-mediated knockdown of TENM4 significantly decreased proliferation in all investigated neuroblastoma cell lines. Two TENM4-/- clones from the CRISPR-Cas9 gene-edited SK-N-BE(2) were uncovered; both clones demonstrated neuronal differentiation-like morphology with impaired clonogenic capacity and reduced proliferation compared to wild-type cells. Using RNA-Seq, qPCR, and proteomics, we characterized neuroblastoma cell responses of inhibited TENM4, identifying key components as induced differentiation, inhibited cell cycle progression, and mTOR signaling together with metabolic changes as TENM4 targets in neuroblastoma cells. More specifically, genes associated with neuronal differentiation, such as SCG2 and NGFR, were upregulated after the siRNA knockdown of TENM4. Similar changes were observed in one of the TENM4-/- clones, while the second clone exhibited upregulation of SCG2. Also, protein levels of SCG2 were upregulated in all TENM4-inhibited cells. Furthermore, both MYCN and the Schwann cell precursor marker ERBB3 were downregulated in the TENM4-inhibited cells compared to their controls. Finally, TENM4-/- cells did not lead to tumor formation when grafted into nude mice as opposed to wild-type SK-N-BE(2) cells that formed tumors.

Conclusion: Our data suggest that TENM4 is expressed in a subpopulation of neuroblastomas with MYCN-amplification and plays an essential role in neuroblastoma growth and differentiation. TENM4 could be a potential target in neuroblastoma therapy.

P054

Can overexpression of genes located on chromosome 17q in Schwann cell precursors cause neuroblastoma?

Ihrmark Lundberg K[1], Tsea I[1], Zhang S[2], Otte J[1], Uhlén P[2], Kogner P[1], Baryawno N[1], Johnsen J[1]
[1]Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden, [2]Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

Background
Neuroblastoma originates from cells within the neural crest, but the cell of origin is still not known. Recently, our research group analyzed primary neuroblastoma tumors using a combination of single-cell RNA/DNA sequencing and genotyping SNP arrays. Bioinformatic analysis showed that a few clones of SOX10-expressing pre-malignant Schwann cell precursors (SCPs) contained only one cytogenetic aberration, gain of chromosome 17, that also appeared in all other tumor clones in addition to other chromosomal aberrations. These data together with evolutionary trajectories of neuroblastoma samples suggest this genomic aberration to be an early genomic event and SCP to be the cell of origin for neuroblastoma.

Aims
We aim to investigate candidate genes on chromosome 17q, with or without MYCN overexpression, using zebrafish as a model system in order to discover oncogenic drivers involved in the development of high-risk neuroblastoma.

Methods
Zebrafish were of AB background or a transgenic line; Tg(sox10:mCherry). DNA constructs were cloned with the genes of interest driven by the sox10 promoter. The constructs were injected into the zebrafish at the one-cell stage followed by a long-term screening for tumor development. Analysis was done through live fluorescence imaging and IHC/IF.

Results
The DNA constructs have been successfully cloned. One group of Tg(sox10:mCherry) zebrafish have been injected with sox10:MYCN-GFP constructs and one group of AB zebrafish have been co-injected with sox10:MYCN-GFP and sox10:PPM1D. Both groups are being screened for tumor development. The MYCN-GFP+ group started to show signs of increased GFP signal in the interrenal gland region after 3 wpf. IF stainings of these fish show GFP expression close to the interrenal gland. Ongoing experiments aim to conclude whether these findings are neuroblastoma tumors.

Conclusion
Zebrafish are useful models to study early events in neuroblastoma. We hope to find out if overexpression of genes from 17q, with or without MYCN overexpression, in SCPs can initiate the development of neuroblastoma.

P055

Comparison of telomere maintenance and gene expression-based classification for risk assessment of neuroblastoma patients

Rosswog C[1,2,3], Hemstedt N[1,2], Kahlert Y[1,2], Kurilov R[4], Brors B[4], Bartenhagen C[1,2], Ackermann S[1,2], Berthold F[3], Simon T[3], Hero B[3], Fischer M[1,2,3]

[1]Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Faculty of Medicine and University Hospital of Cologne, Cologne, Germany, [2]Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany, [3]Department of Pediatric Oncology and Hematology, University Children's Hospital of Cologne, Medical Faculty, Cologne, Germany, [4]Division of Applied Bioinformatics, DKFZ, Heidelberg, Germany

Background

Previous studies have demonstrated that gene expression-based classification improved outcome prediction of neuroblastoma patients when integrated into current risk stratification systems (Oberthuer A et al., Clin Cancer Res 2015). Another study suggested accurate risk assessment by determining telomere maintenance (TM) mechanisms, including activation of telomerase and alternative lengthening of telomeres (ALT; Ackermann S et al., Science 2018).

Aims

We aimed to evaluate and compare the performance of gene expression-based and TM-based risk assessment for outcome prediction of neuroblastoma patients.

Methods

TM and microarray-based gene expression profiles were determined in tumor samples of 346 neuroblastoma patients covering the entire spectrum of the disease. Tumor samples were considered as TM-positive if they harbored TERT rearrangements, MYCN amplification (both determined by FISH), elevated TERT expression, or ALT-associated PML nuclear bodies (as determined by combined immunofluorescence/FISH). Patients were classified into favorable or unfavorable subgroups using the previously described SVM-th10 gene expression classifier (NB-Profiler).

Results

TM was found in 165/346 (47.7%) and unfavorable NB-Profiler prediction in 144/346 tumors (41.6%). TM was associated with unfavorable NB-Profiler prediction (P<0.001). TM and unfavorable NB-Profiler prediction were associated with stage 4, MYCN amplification, age ≥18 months, high-risk disease (P<0.001 each) and poor outcome. Five-year event-free survival (5-y-EFS) of patients with TM-positive versus TM-negative tumors was 0.36±0.04 versus 0.79±0.03 (P<0.001), and 5-year-overall survival (5-y-OS) was 0.63±0.04 versus 0.95±0.02 (P<0.001). 5-y-EFS of patients with unfavorable versus favorable NB-Profiler prediction was 0.37±0.04 versus 0.73±0.03 (P<0.001), and 5-y-OS was 0.62±0.04 versus 0.93±0.02 (P<0.001). Similar performances of both classifiers were observed in non-high-risk patients (P<0.001 each). Only TM was an independent prognostic marker in multivariable analysis based on EFS for the entire cohort (hazard ratio, HR=4.2, P<0.001), whereas both TM and NB-Profiler were retained in the model based on OS (HR=4.7, P<0.001, and HR=2.3, P=0.014, respectively). In multivariable analyses for the non-high-risk subgroup, only TM remained in the model based on EFS (HR=6.3; P<0.001), whereas only the NB-Profiler remained in the model based on OS (HR=20; P<0.001).

Conclusion

The prognostic accuracy of TM appears to be slightly better than that of the NB-Profiler, however, prospective comparison of the two classification systems is warranted.

P056

Assessment of telomere maintenance mechanisms in neuroblastoma

Rosswog C[1,2,3], Meeser A[1,2], Bartenhagen C[1,2], Werr L[1,2], Hellmann A[1,2], Kahlert Y[1,2], Hemstedt N[1,2], Nürnberg P[2,4], Altmüller J[4,5,6], Ackermann S[1,2], Hero B[3], Simon T[3], Peifer M[2,7], Fischer M[1,2,3]

[1]Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Cologne, Germany, [2]Center for Molecular Medicine Cologne, Medical Faculty, University of Cologne, Cologne, Germany, [3]Department of Pediatric Oncology and Hematology, University of Cologne, Cologne, Germany, [4]Cologne Center for Genomics (CCG), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany, [5]Berlin Institute of Health at Charité – Universitätsmedizin Berlin, Core Facility Genomics, Berlin, Germany, [6]Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin, Germany, [7]Department of Translational Genomics, Center of Integrated Oncology Cologne-Bonn, Medical Faculty, University of Cologne, Cologne, Germany

Background

Telomere maintenance (TM), conferred by telomerase or alternative lengthening of telomeres (ALT), is a hallmark of high-risk neuroblastoma. Previous studies suggested that the individual risk of neuroblastoma patients might be precisely assessed by a mechanistic classification that takes TM into account. Detection of TM, however, is not yet part of clinical routine, and consensus on TM detection, especially on ALT assessment, remains to be achieved.

Aims

We here aimed to develop a workflow to reliably assess TM in neuroblastoma and thus allow evaluation of the prognostic significance of TM in clinical settings.

Methods

Sixty-eight primary neuroblastoma samples were analyzed by whole genome sequencing (WGS). Fluorescence in situ hybridization (FISH) was performed to assess MYCN amplification (n=68) and TERT rearrangements (n=23). RNA sequencing (n=64) was used to determine expression of TERT and telomeric long non-coding RNA (TERRA). Telomerase activity was examined by telomerase repeat amplification protocol assay (n=15). ALT was assessed by C-Circle assay (CCA, n=67) and detection of ALT-associated PML nuclear bodies (APB, n=68). Telomere length was calculated from WGS data (n=68) or by telomere restriction fragment analysis (n=39).

Results

Tumors were considered as telomerase positive (TEL+) if they had TERT rearrangements, MYCN amplification or high TERT RNA expression (45.6%, 31/68), and ALT-positive (ALT+) if they were positive for both APB and CCA (19.1%,13/68). If all these markers were absent, tumors were considered TM negative (TM-, 25.0%, 17/68). Based on these criteria, 61/68 tumors were classified unambiguously (89.7%). Although CCA and APB results were largely concordant (94%, 63/67), we found that uncertain TM classification was mainly due to ambiguous ALT assessment. Determination of additional ALT-associated parameters, such as high telomere content, elevated TERRA expression, and presence of ATRX mutations, enabled unequivocal classification of all seven ambiguous cases.

Conclusion

We show that unambiguous classification of TM in neuroblastoma is feasible following a stepwise approach that considers activation markers of telomerase and ALT-associated parameters. We propose a workflow that can be used to determine the prognostic value of TM in future risk stratification systems.

P057

Genomic ALK alterations in primary and relapsed neuroblastoma

Rosswog C[1,2,3,4], Fassunke J[5], Ernst A[4], Schömig-Markiefka B[5], Merkelbach-Bruse S[5], Bartenhagen C[1,2], Cartolano M[2], Ackermann S[1,2], Theissen J[1,4], Blattner-Johnson M[6,7], Jones B[6,7,8], Schramm K[6,7], Altmüller J[9,10,11], Nürnberg P[2,9], Ortmann M[5], Berthold F[4], Peifer M[2,12], Büttner R[5], Westermann F[13], Schulte J[14], Simon T[4], Hero B[4], Fischer M[1,2,4]

[1]Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Medical Faculty, University of Cologne, Cologne, Germany, [2]Center for Molecular Medicine Cologne (CMMC), Medical Faculty, University of Cologne, , Cologne, Germany, [3]Else Kröner Forschungskolleg Clonal Evolution in Cancer, University Hospital of Cologne, Cologne, Germany, [4]Department of Pediatric Oncology and Hematology, University Children's Hospital of Cologne, Medical Faculty, University of Cologne, Cologne, Germany, [5]Institute of Pathology, University Hospital of Cologne, Cologne, Germany, [6]Hopp Children's Cancer Center Heidelberg (KiTZ) , Heidelberg, Germany, [7]Division of Pediatric Glioma Research, German Cancer Research Center (DKFZ) and German Cancer Consortium (DKTK), Heidelberg, Germany, [8]Department of Pediatric Oncology, Hematology, Immunology and Pulmonology, Heidelberg University Hospital, Heidelberg, Germany, [9]Cologne Center for Genomics (CCG), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany, [10]Berlin Institute of Health at Charité – Universitätsmedizin Berlin, Core Facility Genomics, Berlin, Germany, [11]Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin, Germany, [12]Department of Translational Genomics, Medical Faculty, University of Cologne, Cologne, Germany, [13]Division Neuroblastoma Genomics, B087, German Cancer Research Center and Hopp Children´s Cancer Center at the NCT (KiTZ), Heidelberg, Germany, [14]Department of Paediatric Oncology and Haematology, Charité University Medical Centre Berlin, Berlin, Germany

Background

Genomic alterations of the anaplastic lymphoma kinase gene (ALK) are among the most frequent somatic mutations in neuroblastoma. However, their prevalence and prognostic relevance in relapsed disease has remained unclear.

Aims

We aimed to assess ALK alteration frequencies in primary and relapsed neuroblastoma, to determine their impact on patient outcome and their development over the course of disease.

Methods

We determined ALK alterations in a cohort of 943 neuroblastoma patients by dideoxy-sequencing, next-generation-sequencing approaches, and fluorescence in situ hybridization. ALK mutations and amplification were assessed in tumors at diagnosis (n=717 and n=298, respectively) and relapse/progression (n=198 and n=172, respectively). Information on diagnostic and relapsed samples from the same patients was available in 101 cases for mutation and 102 cases for amplification status.

Results

We found ALK mutations in 10.5% (75/717) of neuroblastomas obtained at diagnosis and in 17.7% (35/198) at relapse. While ALK mutations were not associated with risk group in general, we found that mutation frequencies were highest in the subgroup of patients <18 months with stage 4 disease (21.7% at diagnosis, 28.6% at relapse). ALK mutations were associated with worse outcome in high-risk patients if occurring at allelic fractions >20% (allelic fractions >20% versus ≤20% versus ALK wild-type, 5-year-OS, 28% versus 57% versus 54%; P=0.028 for allelic fractions >20% versus ALK wild-type). ALK amplification was detected in 4.7% (14/298) of tumors at diagnosis and in 3.5% (6/172) at relapse (P=0.639) and was associated with poor outcome at diagnosis and relapse (ALK-amplified versus ALK non-amplified, 5-year-OS, 22% versus 60%, P=0.016; and 0% versus 57%, P<0.001, respectively). The ALK mutation frequency increased at relapse by

70% (P=0.009), corresponding to the occurrence of de novo mutations in 7/101 cases with information on longitudinal samples. The increased ALK mutation frequency at relapse was accompanied by significant enrichment of R1275Q mutations (34.7% versus 54.3%, P=0.001).

Conclusion
The high prevalence of ALK mutations in stage 4 tumors of patients <18 months suggests evaluating ALK-targeted treatment in this patient subgroup. The considerably increased frequency of ALK mutations at relapse/progression underscores the importance of surveying the genomic ALK status regularly in relapsed and progressive patients.

P058

Inhibition of OCT4 binding at the MYCN locus induces neuroblastoma cell death accompanied by alteration of protein-coding potential of RNAs

Nakatani K[1,2], Kogashi H[1,2], Sakuma T[3], Yamamoto T[3], Hippo Y[1,2], Suenaga Y[2]
[1]Graduate School of Medical and Pharmaceutical Sciences, Chiba University, Chiba, Japan, [2]Department of Molecular Carcinogenesis, Chiba Cancer Center Research Institute, Chiba, Japan, [3]Graduate School of Integrated Sciences for Life, Hiroshima University, Hiroshima, Japan

Background
Amplification of MYCN is found in high-risk neuroblastomas (NBs) and correlates with poor prognosis. MYCN is regulated by multiple transcription factors, including OCT4, MYCN, and p53. Inhibition of p53 binding at the MYCN locus induces NB cell death; however, it remains unclear whether inhibition of other transcription factors contributes to NB cell death.

Aims
We aim to inhibit a transcription factor that binds to the MYCN locus and evaluate its effects on MYCN-amplified NB cell survival.

Methods
We used CRISPR/dCas9 technology to specifically inhibit transcription factors from binding to the MYCN locus in the MYCN-amplified NB cell lines, CHP134 and IMR32. Total RNA extracted from dCas9-transfected NB cell was analyzed by combining short-read and long-read RNA-seq. Total protein extracted from dCas9-transfected NB cell was analyzed by western blotting.

Results
In both cell lines, the inhibition of OCT4 binding at the MYCN locus induced cell death accompanied by induction of p53, cleaved MDM2, and activation of caspase 2. Differentially expressed genes identified using short-read RNA-seq were enriched in MYCN-target genes. In addition, GO analysis revealed that the splicing pathway was inactivated in both cell lines. Recently, we developed an RNA coding-potential index, open reading frame dominance (ORF-D), which correlates with translational efficiency for both coding and noncoding transcripts. Because global splicing changes can dramatically alter RNA sequences and ORF-D, we calculated ORF-D of transcripts from long-read RNA-seq analysis. After the inhibition of OCT4 binding at the MYCN locus, the expression levels of high-ORF-D transcripts were decreased in both cell lines. Furthermore, database analysis revealed that high-ORF-D transcripts were significantly associated with the genes whose high expression is associated with poor prognosis in neuroblastoma.

Conclusion
These results suggest that OCT4-mediated MYCN gene regulation maintains the expression of the transcripts with high translational efficiency and contributes to NB cell survival.

P059

The adrenergic-specific lncRNA NESPR regulates PHOX2B expression and neuroblastoma cell viability

Delhaye L[1,2,3], de Bony E[1,2], Lim F[4,5], Rombaut D[1,2], Decaesteker B[1,2], Louwagie A[1,2], D'haene E[1,2], Volders P[1,2,3], Yigit N[1,2], De Sutter D[1,3], Anckaert J[1,2], Tan T[4,5], Bekaert S[1,2], Everaert C[1,2], Menten B[1,2], Vandesompele J[1,2], Lefever S[1,2], Speleman F[1,2], Sanda T[4,5], Eyckerman S[1,2,3], Mestdagh P[1,2]

[1]Ghent University, Department of Biomolecular Medicine, Ghent, Belgium, [2]Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [3]VIB-Ghent University, Center for Medical Biotechnology, Ghent, Belgium, [4]National University of Singapore, Cancer Science Institute of Singapore, Singapore, Singapore, [5]National University of Singapore, Yin Loo Lin School of Medicine, Department of Medicine, Singapore, Singapore

Neuroblastoma is a childhood cancer of the sympathetic nervous system. Recent studies have shown that neuroblastoma tumors are composed of two cell identities, i.e. an adrenergic and mesenchymal identity. Both identities are driven by a core regulatory transcriptional circuitry, which acts as an autoregulatory positive feedforward loop, to delineate the cell identity through regulation of its target genes. We identified the long non-coding RNA NESPR to be specifically expressed in neuroblastoma cells of the adrenergic cell identity. NESPR expression correlates with several parameters of high-risk neuroblastoma and poor patient survival. Knockdown of NESPR decreased neuroblastoma cell proliferation and induced cell death, highlighting NESPR's importance in the survival of the adrenergic neuroblastoma cells. We show that NESPR is contained within an insulated gene neighborhood with the adrenergic core regulatory transcription factor PHOX2B, and that NESPR regulates PHOX2B expression in cis. ChIRP-sequencing demonstrated that NESPR does not bind the PHOX2B locus, suggesting that PHOX2B regulation is not driven by in cis NESPR DNA-binding. Localization experiments showed that NESPR concentrates in distinct nuclear foci, presumably at its own locus. Together, his puts forth the hypothesis that NESPR creates or maintains transcriptional condensates at the PHOX2B-NESPR insulated gene neighborhood to regulate PHOX2B expression in cis.

P060

Discovering vulnerabilities of MYCN-independent neuroblastoma

Bozatzi P[1], Perepelkina P[1], Fetahu I[1], Sturtzel C[1], Ramirez S[1,2], Dnyansagar R[2], Scheuringer E[1], Tarlungeanu D[1], Gehl V[1], Esmaeiligoudarzi D[1], Distel M[1], Fortelny N[2], Taschner-Mandl S[1]
[1]CCRI, Children's Cancer Research Institute, Vienna, Austria, [2]Computational Systems Biology Group, Department of Biosciences and Medical Biology, Paris Lodron University of Salzburg, Salzburg, Austria

Background
High-risk neuroblastoma (NB) is characterized by poor prognosis despite intensive multimodal treatment. Current research has focused extensively on MYCN oncogene-driven neuroblastoma, whereas the understanding of neuroblastoma without MYCN amplification (MNA), harboring e.g., ATRX exon-spanning mutations are not well elucidated. Synthetic lethality is an exploitable phenomenon in cancer therapy when the co-occurrence of disrupted genes results in cell death. Genome-wide knock-out and drug screens have recently identified major vulnerabilities of pediatric cancers, but have not focused on genetic subtypes of NB.

Aims
This research aims to identify genetic and therapeutic vulnerabilities of high-risk MYCN-dependent vs -independent neuroblastoma.

Methods
A panel of three NB patient-derived cultures and four cell lines with distinct MYCN status was screened using the CLOUD library of 308 FDA-approved compounds, covering a broad spectrum of chemical structures and molecular targets. Drug candidates were validated in a panel of eight NB cell lines. In addition, we performed synthetic lethality genome-wide CRISPR-Cas9 knock-out screens in two non-MNA NB cell lines and bioinformatically integrated the results of the screenings with public data from the DepMap database to identify specific genes for NB survival with non-MNA (including ATRX mutated) versus MNA genetic backgrounds and other cancers.

Results
We successfully established a semi-automated workflow for genome-wide CRISPR-Cas9 knock-out screening and high-throughput compound screening in vitro. Our bioinformatic analysis has revealed 964 genes conferring dependency (adj. $p<0.05$) in ATRX mutated NB and in 80 genes differential dependency (adj. $p<0.2$) was found in MNA versus non-MNA NB. In parallel, we identified three drugs, which showed significant sensitivity associated with the MYCN status and nineteen drugs effective across all subtypes. Cell viability and migration assays showed high efficacy of drugs already implicated in the treatment of NB, e.g., Auranofin, as proof-of-principle and new drug candidates effective across and unique to genetic subtypes.

Conclusion
The combined information on the genomic dependency and drug screen may enable us to address the challenge of lacking directly targetable mutations in high-risk NB. Currently, we are exploring the molecular pathways involved in the survival of non-MNA NB.

P061

Oncogene amplification identifies a subgroup of intermediate-risk neuroblastoma patients with inferior outcome: A SIOPEN study

Fong F[1], Stevenson L[1], Gabriel A[1], Beckett E[1], Hartley H[1], Allinson L[1], Nath S[1], Goodman A[2], Harding F[2], Cuthbert G[2], Watts K[2], Pearce K[2], Potts A[2], Whittle E[2], Batet-Matinez A[2], Herd F[3], Mazzocco K[4], Pezzolo A[4], Morini M[4], Ardito M[4,5], Eva A[4], Ognibene M[4], Fischer M[6], Ackermann S[6], Rosswog C[6], Hero B[7], Molenaar J[8], Matser Y[8], Langenberg K[8], Tytgat L[8], Noguera R[9], Berbegall A[9], Font de Mora J[10], Combaret V[11], Mühlethaler-Mottet A[12], Schoumans J[13], Tchinda J[14], Banzola I[14], Jeison M[15], Hameiri-Grossman M[15], Beiske K[16], Auger N[17], Van Roy N[18], Basta N[19], McNally R[19], Papadakis V[20], Di Cataldo A[21], Wheeler K[22], Beck-Popovic M[23], Segura V[24], Canete A[25], Schleiermacher G[26], Tweddle D[1,27]

[1]Wolfson Childhood Cancer Research Centre, Institute of Translational & Clinical Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom, [2]Newcastle Genetics Laboratory, Newcastle upon Tyne Hospitals NHS Trust, Newcastle upon Tyne, United Kingdom, [3]Department of Paediatric Oncology, Royal Aberdeen Children's Hospital, Aberdeen, Scotland, United Kingdom, [4]Laboratory of Molecular Biology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [5]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [6]Department of Experimental Pediatric Oncology, University Children's Hospital, Medical Faculty, University of Cologne, Cologne, Germany, [7]Children's Hospital, Department of Pediatric Oncology and Hematology, University of Cologne, Cologne, Germany, [8]Princess Máxima Center for Pediatric Oncology, Utrecht University, 3584 CS Utrecht, The Netherlands, [9]Department of Pathology, Medical School, University of Valencia-Incliva Health Research Institute/CIBERONC, Madrid, Spain, [10]Clinical and Translational Research in Cancer, Instituto de Investigación Sanitaria La Fe, Valencia, Spain, [11]Laboratoire de Recherche Translationnelle, Centre Léon-Bérard, Lyon, Auvergne-Rhone-Alpes, France, [12]Pediatric Hematology-Oncology Research Laboratory, Woman-Mother-Child Department, Lausanne University Hospital, Lausanne, Switzerland, [13]Oncogenomics Laboratory, Hematology Service, Department of Laboratory Medicine & Pathology, University Hospital CHUV, Lausanne, Switzerland, [14]University Children's Hospital Zurich, Oncology Laboratory, Zurich, Switzerland, [15]Schneider Children's Medical Center of Israel, Petah Tikva, Israel, [16]Department of Pathology, Oslo University Hospital, and Medical Faculty, University of Oslo, Oslo, Norway, [17]Laboratoire de cytogénétique, Gustave Roussy, 144 rue Edouard Vaillant, 94805 Villejuif, France, [18]Center for Medical Genetics, Ghent University, Ghent, Belgium, [19]Population Health Sciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom, [20]Department of Paediatric Haematology and Oncology, Agia Sofia Children's Hospital, Athens, Greece, [21]Department of Paediatric Haematology & Oncology, Azienda Policlinico-OVE, Catania, Italy, [22]Department of Paediatric Oncology, Oxford Children's Hospital, Oxford, United Kingdom, [23]Unit of Pediatric Hematology-Oncology, University Hospital CHUV, Lausanne, Switzerland, [24]Instituto de Investigación Sanitaria La Fe , Valencia, Spain, [25]Paediatric Oncology Unit, Hospital Universitario y Politecnico La Fe, Valencia, Spain, [26]SIREDO Integrated Pediatric Oncology Center and Translational Research in Pediatric Oncology, U830 INSEM, Institut Curie, Paris, France, [27]Great North Children's Hospital, Newcastle upon Tyne, United Kingdom

Background:
Intermediate-risk neuroblastoma (NB) patients >18 months of age with non-MYCN amplified, localised, unresectable disease (L2) and unfavourable histology according to the International Neuroblastoma Pathology Classification have inferior outcomes compared with other intermediate-risk cases. Within the current SIOPEN low and intermediate neuroblastoma (LINES) trial, these patients are treated with chemotherapy, surgery, local radiotherapy, and 13-cis-retinoic acid.

Aims:
To undertake a detailed genetic study of this intermediate-risk subgroup to identify prognostic markers.

Materials/Method:
Tumour/data were collected from 10 European countries, including 7 in the LINES trial. Numerical and segmental chromosomal aberrations (SCAs) were assessed using SNP arrays, whole exome sequencing

(WES), array CGH, MLPA, and/or FISH. Mutational data was studied by WES, targeted sequencing, Sanger sequencing, or Agena MassARRAY. RNA sequencing was performed on 17 cases.

Results:
101 patients were included (median age at diagnosis 49.0 months; range: 18.1–204.5). 5-year EFS was 56.4% (95% CI: 47.1%-67.6%), and 5-year OS 74.7% (95% CI: 65.8%-84.8%). 16/101 (15.8%) had numerical chromosomal aberrations (NCAs) only, 67/101 (66.3%) typical (t) SCAs, 10/101 (9.9%) atypical SCAs only, and 8/101 (7.9%) oncogene amplifications (3x MDM2-CDK4, 2x CDK4, 1x MDM2 (unknown CDK4 status), 1x MYC and 1x TERT). The most frequent tSCAs were: 17q gain 49/96 (51.0%), 11q loss 39/95 (41.1%), 2p gain 31/98 (31.6%) and 1q gain 27/93 (29.3%). RAS/MAPK/p53 pathway alterations were detected in 26/82 (32.9%) cases, 12 (14.6%) involving ALK.

Oncogene-amplified cases were all near-triploid and had one to four SCAs, including 9p loss (3/8) and 17q gain (3/8). One CDK4-MDM2 co-amplified case demonstrated high MDM2 expression but not CDK4. None had known RAS/MAPK/p53 pathway mutations.

Cases with tSCA had a significantly worse EFS than NCA (5-year EFS: 51.7% vs 93.3%, $p<0.01$) but not OS ($p=0.4$). Oncogene-amplified cases had the worst EFS ($p<0.0001$) and OS ($p<0.001$), with 7 patients relapsing within 5 years. 5-year OS was 37.5% (95% CI: 15.3–91.7%). RAS/MAPK/p53 altered cases showed worse EFS ($p=0.03$) but not OS ($p=0.2$).

Conclusion:
This intermediate-risk subgroup of patients with oncogene-amplified NB shows a distinct genetic profile and markedly worse outcomes. These patients may benefit from high-risk strategies and/or CDK4 or MDM2 targeted treatments.

P062

Identifying a Synthetic Lethal Signature for MYCN-Amplified Neuroblastoma

Nagy M[1], Schischlik F[3], Wang K[1], Sahni S[1], Nguyen R[2], Thiele C[2], Ruppin E[1]
[1]Cancer Data Science Laboratory, National Cancer Institute, National Institutes of Health, Bethesda, United States, [2]Pediatric Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, United States, [3]Boehringer Ingelheim RCV Gmbh & Co KG, Vienna, Austria

Background & Aims
MYCN amplified (MYCN-amp) neuroblastoma (NB) is associated with poor outcomes and is particularly challenging to target, calling for novel therapeutic approaches. Here we aim to identify candidate synthetic lethal (SL) target genes of MYCN-amp NB whose alteration is predicted to form new therapeutic avenues to specifically target MYCN-amp NB while sparing MYCN non-amplified (non-amp) tumors.

Methods
Three NB transcriptomic datasets were used to infer SL genes (GSE49711, GSE120572, and GSE85047) via a modified version of the published SELECT algorithm (Lee et al., 2021 Cell), employing the following four analysis steps: if they were 1) upregulated in MYCN-amp relative to non-amp samples, 2) phylogenetically similar to MYCN, 3) found to be rarely depleted in the MYCN-amp tumor transcriptome, and 4) associated with improved survival in patients with MYCN-amp but not non-amp NB. Subsequently, an SL signature score was assigned for each tumor sample by the fraction of downregulated SL genes, where a larger score predicts better survival. The predictive power of this signature was validated in three independent patient datasets (GSE16476, TARGET RNA, and TARGET CNV) and by comparing survival of MYCN-amp NB cell lines with non-amp NB and pediatric cancer cell lines (Pediatric DepMap: Broad Institute).

Results
One hundred top candidate genes were selected to form the SL score, which accurately predicts five-year survival in all three training (AUCs: 0.72-0.99) and 2/3 patient validation datasets (AUCs: 0.79-0.8) for patients with MYCN-amp but, reassuringly, not non-amp NB (AUCs 0.34-0.55). Cell survival following knock-out of candidate genes was lower for MYCN-amp compared to non-amp NB cell lines (p=0.0095, AUC 0.77) and all six other pediatric cancers (p's 0.016 – <0.0001, AUC 0.85). Using the top 9 identified genes, MYCN-amp NB was accurately discriminated from non-amp NB (p=0.0001, AUC 0.87) and other tested pediatric cancers (p's 0.007 – <0.0001, AUC 0.90).

Conclusions
We identified a SL signature that predicts 1) survival of patients with MYCN-amp NB via expression and copy number profile and 2) cell fitness following the knockout of SL genes. This lays the basis for SL approaches for prognostication and identification of new therapeutic targets in NB.

P063

Telomere maintenance mechanisms are associated with inferior survival and segmental chromosomal abnormalities in an intermediate-risk neuroblastoma subgroup: A SIOPEN study

Stephenson L[1], Fong F[1], Gabriel A[1], Beckett E[1], Hartley H[1], Allinson L[1], Nath S[1], Goodman A[2], Harding F[2], Cuthbert G[2], Watts K[2], Pearce K[2], Potts A[2], Whittle E[2], Batet-Martinez A[2], Herd F[3], Mazzocco K[4], Pezzolo A[4], Morini M[4], Ardito M[4,5], Eva A[4], Ognibene M[4], Fischer M[6], Ackermann S[6], Rosswog C[6], Hero B[7], Molenaar J[8], Matser Y[8], Langenberg K[8], Tytgat L[8], Noguera R[9], Berbegall A[9], Font de Mora J[10], Combaret V[11], Mühlethaler-Mottet A[12], Schoumans J[13], Tchinda J[14], Banzola I[14], Jeison M[15], Hameiri-Grossman M[15], Beiske K[16], Auger N[17], Van Roy N[18], Basta N[19], McNally R[19], Papadakis V[20], Di Cataldo A[21], Wheeler K[22], Beck-Popovic M[23], Segura V[24], Canete A[25], Schleiermacher G[26], Tweddle D[1,27]

[1]Wolfson Childhood Cancer Research Centre, Institute of Translational & Clinical Medicine, Newcastle University, Newcastle Upon Tyne, United Kingdom, , , [2]Newcastle Genetics Laboratory, Newcastle upon Tyne Hospitals NHS Trust, Newcastle Upon Tyne, United Kingdom, , , [3]Department of Paediatric Oncology, Royal Aberdeen Children's Hospital, Scotland, United Kingdom, , , [4]Laboratory of Molecular Biology, IRCCS Istituto Giannina Gaslini, Genova, Italy, , , [5]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy, , , [6]Department of Experimental Pediatric Oncology, University Children's Hospital, Medical Faculty, University of Cologne, Cologne, Germany, , , [7]Children's Hospital, Department of Pediatric Oncology and Hematology, University of Cologne, Cologne, Germany, , , [8]Princess Máxima Center for Pediatric Oncology, Utrecht University, 3584 CS Utrecht, The Netherlands, , , [9]Department of Pathology, Medical School, University of Valencia-Incliva Health Research Institute/CIBERONC, Madrid, Spain, , , [10]Clinical and Translational Research in Cancer, Instituto de Investigación Sanitaria La Fe, Valencia, Spain, , , [11]Laboratoire de Recherche Translationnelle, Centre Léon-Bérard, Lyon, Auvergne-Rhone-Alpes, France, , , [12]Pediatric Hematology-Oncology Research Laboratory, Woman-Mother-Child Department, Lausanne University Hospital, Lausanne, Switzerland, , , [13]Oncogenomics Laboratory, Hematology Service, Department of Laboratory Medicine & Pathology, University Hospital CHUV, Lausanne, Switzerland, , , [14]University Children's Hospital Zurich, Oncology Laboratory, Zurich, Switzerland, , , [15]Schneider Children's Medical Center of Israel, Petah Tikva, Israel, , , [16]Department of Pathology, Oslo University Hospital, and Medical Faculty, University of Oslo, Oslo, Norway , , , [17]Laboratoire de cytogénétique, Gustave Roussy, 144 rue Edouard Vaillant, 94805 Villejuif, France, , , [18]Center for Medical Genetics, Ghent University, Ghent, Belgium, , , [19]Population Health Sciences Institute, Newcastle University, Newcastle Upon Tyne, United Kingdom, , , [20]Department of Paediatric Haematology and Oncology, Agia Sofia Children's Hospital, Athens, Greece, , , [21]Department of Paediatric Haematology & Oncology, Azienda Policlinico-OVE, Catania, Italy, , , [22]Department of Paediatric Oncology, Oxford Children's Hospital, Oxford, United Kingdom, , , [23]Unit of Pediatric Hematology-Oncology, University Hospital CHUV, Lausanne, Switzerland, , , [24]Instituto de Investigación Sanitaria La Fe, Valencia, Spain, , , [25]Paediatric Oncology Unit, Hospital Universitario y Politecnico La Fe, Valencia, Spain, , , [26]SIREDO Integrated Pediatric Oncology Center and Translational Research in Pediatric Oncology, U830 INSEM, Institut Curie, Paris, France, , , [27]Great North Children's Hospital, Newcastle Upon Tyne, United Kingdom, ,

Background: Intermediate-risk neuroblastoma (NB) patients >18 months old with non-MYCN amplified, unfavourable histology, localised, unresectable (L2) tumours, have a worse prognosis compared with other intermediate-risk patients. Segmental chromosomal abnormalities (SCAs) in these cases correlate with poor outcome. The presence of telomere maintenance mechanisms (TMM) has prognostic significance in NB, but the frequency of TMM in this subgroup is unknown.

Aims: To test this intermediate-risk NB subgroup for TMM then compare with SCA and clinical outcome.

Methods: Copy number analysis of NB tumours (n=101), mutational analysis (n=81), and RNAseq analysis (n=17) was undertaken. TMM were assessed by TERT FISH (n=25) or TERT expression for telomerase activity, and immunofluorescence fluorescence in situ hybridization (immuno-FISH) for telomerase-independent

alternative lengthening of telomeres (ALT) (n=29). Cases with ATRX intragenic deletions or truncating mutations were also considered ALT-positive. Only samples testing negative for both TERT structural alterations and immuno-FISH were classified as TMM-negative.

Results: 30 NB tumours were classified for TMM status, 21/30 (70%) possessed TMM, with 6/30 (20%) inferred telomerase activity and 15/30 (50%) ALT. For ALT-positive cases, five had ATRX intragenic deletion, while one had an ATRX mutation (p.R781*). TMM presence correlated with typical (t) SCAs (-1p, +1q, +2p, -3p, -4p, -11q, +17q; Fisher-exact test, OR: 16.35, 95% CI: 1.90–242.68; p<0.01). TMM-positive NB showed significantly worse EFS than TMM-negative (5-year EFS: 19.0% vs 77.8%, p= 0.002), but no difference in OS (p=0.4). One case without SCAs who died of disease (DOD) had high TERT expression and a HRAS mutation. Of two patients who DOD with TMM-negative status, one had CDK4-MDM2-co-amplification, the other TP53 loss secondary to chromosome 17 hyper-rearrangement. An ALT-negative case with undetectable TERT expression, no ATRX alteration, and TP53 loss of heterozygosity with a TP53 mutation (p.C141Y), also DOD. These cases suggest a TP53-related mechanism may be prognostic outside of known TMM.

Conclusions: TMM are associated with inferior EFS and tSCAs in this intermediate-risk NB subgroup. Since TMM classification can be technically challenging, the presence of tSCAs may predict TMM, but validation in a larger cohort is required. A prognostically relevant TP53-dependent mechanism may exist outside of TERT activation and ALT.

P064

TERC AND MYNN VARIANTS ASSOCIATED WITH MYCN NON-AMPLIFIED NEUROBLASTOMA : A PRELIMINARY STUDY.

Gimenez T[1,2], Peralta V[1,2], Dias A[1,2], Lerario A[3], Benedetti A[4], Halley Neves N[1,5], Camargo Vince C[1,5], Victorino Krepischi A[6], Cristofani L[1], Odone Filho V[1,5], Novak E[7,8]

[1]Instituto de Tratamento de Câncer Infantil(ITACI), Instituto da Criança, HC/FMUSP, São Paulo, Brazil, [2]bLaboratório de Investigação Médica (LIM36),Instituto da Criança,HC/FMUSP, São Paulo, Brazil, [3]Hammer Laboratory. Endocrine Oncology Program.University of Michigan, Michigan, USA, [4]Núcleo de Sequenciamento em Larga Escala (SELA). Faculdade de Medicina da Universidade de São Paulo, São Paulo , Brazil, [5]Hospital Israelita Albert Einstein (HIAE), São Paulo , Brazil, [6]Department of Genetics and Evolutionary Biology, Institute of Biosciences, São Paulo, Brazil, [7]Fundação Pró-Sangue Hemocentro de São Paulo, São Paulo, Brazil, [8]Laboratório de Investigação Médica (LIM31),Departamento de Hematologia HC/FMUSP, São Paulo , Brazil

BACKGROUND

Neuroblastoma, a neoplasm of the sympathetic nervous system, is the most common solid tumor in infancy. Polymorphisms in telomere maintenance genes are known to affect telomere length and may contribute to neuroblastoma susceptibility because can affect a critical apoptotic checkpoint of the cells. In previous studies, germline SNPs MYNN (rs10936599) and TERC (rs2293607) and longer telomeres were associated with the risk to colorectal adenomas and bladder cancer.

AIMS

In this preliminary study, we speculate if MYNN (rs10936599) and TERC (rs2293607) and telomere length could be related with risk for MYCN non-amplified neuroblastoma.

METHODS

Peripheral blood leukocytes from 52 Brazilian patients with MYCN non-amplified neuroblastomas stages IV (according International Neuroblastoma Staging System) and unfavorable histology were analyzed using a customized targeted panel sequenced on MiSeq (Illumina. USA) . The patient's median age at diagnosis was 13 months (range 3-20 months) and 57% female. Furthermore, a group of 12 health children's matched by age and sex were used as control. Relative telomere length quantification was performed by Real-Time PCR as described by Cawthon 2002. GraphPad Prism 8.0 software (GraphPad Inc, USA)was used to statistical analysis. Written informed consent approved by the Ethics Committee of São Paulo's University were obtained from parents.

RESULTS AND DISCUSSION

Our results shown in 12/52 patients the MYNN variant (rs10936599) and in 10/52 patients the TERC variant (rs2293607) with C → T substitution at nucleotide 18). Although treatment regimens, our results also demonstrated that 15% of patients with both variants(MYNN and TERC) died.

Relative telomere length of patients carrying the SNPs were significantly longer compared to the age-matched healthy controls (T/S ratio:3.0 in patients vs 1 in controls (p□0.05), suggesting the existence an association between longer telomeres and SNPs rs2293 607 and rs10936599 variants.

CONCLUSION

Despite the small casuistry of this study, our findings suggest that rs10936599 and rs2293607 variants and longer telomeres may be associated with poor prognostic of MYCN non amplified neuroblastomas. However, further studies in larger cohorts of MYCN non amplified neuroblastoma patients are required in order to explore the impact of these variants in neuroblastoma risk in Brazilian population.

P065

Haddad Syndrome and Neuroblastoma: a case report

Carra F[1], Peri M[2], Trevisan F[1], Sieni E[1], Perrone A[3], Olianti C[4], Tondo A[1]
[1]Department of Pediatric Oncology and Hematology, Meyer Children's Hospital IRCCS, Florence, Italy, [2]Meyer Children's Hospital IRCCS, University of Florence, Florence, Italy, [3]Department of Radiology, Meyer Children's Hospital IRCCS, Florence, Italy, [4]Nuclear Medicine Unit, Careggi University Hospital, Florence, Italy

Background: Haddad Syndrome (HS) is a very rare disease including Congenital Central Hypoventilation Syndrome (CCHS) and Hirschsprung disease (HSCR). We report a case of HS complicated by neuroblastoma.
Case description: Second child born at full-term with perinatal asphyxia. During hospitalization, she presented respiratory failure, needing assisted ventilation and tracheostomy. Genetic studies showed a de novo heterozygous mutation (C.780dupT) of PHOX2B gene, confirming CCHS. At 1 month of age HSCR was diagnosed, with subsequent segment resection and coloanal anastomosis. The presence of CCHS and HSCR led to the diagnosis of Haddad Syndrome. As CCHS and HSCR may be associated with Peripheral Neuroblastic Tumor, a close follow up was performed. At 13 months of age a right surrenalic mass (mm 14x10x17) was found, associated with an increment of urinary VanillylMandelic Acid (25.94 mg/gr urinary creatinine). Magnetic Resonance Imaging showed a voluminous thoraco-abdominal mass, from D5 to L2, with predominant left paravertebral thoracic development, inhomogeneously hyperintense in TR sequences and positive in DWIBS (mm 108 x 36 x 37); IDRF were present. MIBG-Scintigraphy confirmed the presence of the tumor in the thoraco-abdominal region. The ultrasound-guided needle-biopsy showed fragments with proliferation of neuronal cellular elements with maturation phenomena and calcifications, low MKI and MYCN-non-amplified. Findings were compatible with neuroblastic tumors not further classifiable. Bone marrow biopsy were negative.
During hospitalization, the patient presented recurrent life-threatening event with hypoglycemia, hyperglycemia and metabolic acidosis. Therefore, because of the complex clinical presentation of the baby and her metabolic instability, in agreement with the family, we chose a wait and see approach.

Conclusion: We highlight the importance of a multidisciplinary follow up for CCHS patients in order to early detect associated illnesses such as HSCR and tumors derived from the neural crest and to plan the best treatment for the patient.

P066

Altered arginine metabolism is an exploitable vulnerability in neuroblastoma

Hanssen K[1,2], Pandher R[1,2], Murray J[1], Alfred S[1], Gamble L[1], Kamili A[1,2], Mussai F[3], Cheng P[4], Ziegler D[1,5], Fletcher J[1,2], Norris M[1,6], Haber M[1,2]

[1]Children's Cancer Institute, Sydney, Australia, [2]School of Clinical Medicine, UNSW Medicine & Health, UNSW Sydney, Sydney, Australia, [3]Birmingham Children's Hospital and the University of Birmingham, , United Kingdom, [4]Bio-cancer Treatment International Ltd, Shatin, New Territories, Hong Kong, [5]Kids Cancer Centre, Sydney Children's Hospital, Randwick, Australia, [6]UNSW Centre for Childhood Cancer Research, UNSW Sydney, Sydney, Australia

Background: Cancers frequently exhibit dysregulated amino acid metabolism, causing cancer-specific dependencies that may be exploited therapeutically. Neuroblastomas have an increased demand for arginine but, unlike normal tissues, frequently lack the enzymes needed for its de novo synthesis, making them reliant on systemic arginine for proliferation and survival. We previously demonstrated that pegylated arginase (BCT-100), which depletes plasma arginine via metabolism to ornithine, delays tumour growth in Th-MYCN mice, suggesting arginine depletion as a novel neuroblastoma therapy. Determinants of response and optimal combinations with BCT-100 in neuroblastoma, however, have yet to be identified.

Aims: To investigate the molecular mechanism of response and clinical potential of BCT-100 combination treatment in pre-clinical models of high-risk neuroblastoma.

Methods: Human neuroblastoma cell lines were treated with BCT-100 and effects characterised by LC-MS/MS metabolite profiling, puromycin incorporation, Western blotting, clonogenic, and viability assays. Efficacy of BCT-100 (60mg/kg) treatment was assessed in Th-MYCN mice and in aggressive neuroblastoma PDX models.

Results: In vitro, BCT-100 depleted up to 90% of intracellular arginine, reduced mTORC1 signalling, decreased global protein translation, and arrested cell proliferation independent of MYCN amplification. BCT-100 also caused dose-dependent reduction in viability and clonogenic capacity in neuroblastoma cell lines and PDX cells; an effect enhanced by combination with irinotecan/temozolomide or mafosfamide/topotecan. In Th-MYCN mice, BCT-100 significantly depleted arginine levels in both plasma and tumour while liver levels were unaffected. Pre-emptive BCT-100 treatment from time of weaning significantly delayed tumour development, whilst treatment of established tumours delayed tumour progression. BCT-100 was particularly effective when combined with irinotecan/temozolomide, with 60% of Th-MYCN mice tumour-free 100 days after beginning treatment compared to <60 days median survival for mice treated with BCT-100 or chemotherapy alone. Significant delay of tumour progression following BCT-100/irinotecan/temozolomide treatment was also observed in two PDX models.

Conclusion: BCT-100 profoundly inhibits neuroblastoma growth in vitro and in vivo and these effects are enhanced in combination with standard-of-care chemotherapy. A phase II trial of BCT-100 monotherapy in children with relapsed/refractory solid, CNS, and liquid tumours has recently completed, and our data support further trials of BCT-100 combined with chemotherapy as a promising new treatment strategy for high-risk neuroblastoma.

P068

Integrative network analysis characterizes regulatory programs for dosage sensitive genes in high-risk neuroblastoma

Vermeirssen V[1,2,3], Bekaert S[3,4], Decaestecker B[3,4], Hilgert E[3,4], Depestel L[3,4], Bek J[3,4], Durinck K[3,4], Speleman F[3,4]
[1]Laboratory for Computational Biology, Integromics and Gene Regulation (CBIGR), Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [2]Department of Biomedical Molecular Biology, Ghent Universit, Ghent, Belgium, [3]Department of Biomolecular Medicine, Ghent, Belgium, [4]Pediatric Precision Oncology Laboratory (PPOL), Cancer Research Institute Ghent (CRIG), Ghent, Belgium

Background: Neuroblastoma (NB) tumors with segmental chromosomal aberrations are associated with poor survival. Although we previously identified oncogenic (co-)drivers located on 2p and 17q, many (co)-drivers remain to be found in NB copy number aberrations. Gene regulatory networks (GRNs) provide systems-level understanding of key pathways and drivers in tumor biology. Network inference, the prediction of GRNs from omics data, is a useful hypothesis generator of the molecular interactions between regulatory factors, such as transcription factors and chromatin modifiers, or other oncogenic and tumor suppressor (co-)drivers, and their target genes.

Aims: We hypothesize that dosage sensitive genes located on regions affected by segmental aberrations act as cooperative drivers during NB development and could represent novel druggable targets. We aim to identify causal (co-)drivers and their regulatory programs using integrative computational analysis and network inference.

Methods: Regulatory network inference was performed through the multi-omics module network inference software Lemon-Tree. First, based on the SEQC transcriptome dataset for 497 primary NBs, 11,089 highly variable protein-coding genes were selected and clustered in consensus coexpression modules. Next, based on a reported meta-analysis of copy number alterations in 556 high risk NBs (Depuydt et al., 2018), dosage sensitive genes were chosen and assigned as a prioritized list of drivers to each of the modules using an ensemble of decision trees. All modules and drivers in the GRNs were characterized through network analysis, functional enrichment analysis, clinical data correspondence and further integration with other data.

Results: Module network inference resulted in over 100 coexpression modules containing over 9,000 genes. Over 90% of the modules showed significant functional enrichment, often related to the hallmarks of cancer. Retaining the 1% top predictions, we identified novel drivers on segmental chromosomal aberrations, including the previously in vivo validated regulatory factors RRM2 and BRIP1. We will present these prioritized candidate drivers and their regulatory programs and provide further evidence for their biological relevance, amongst others based on correlation to NB specific transcriptomic signatures and survival data.

Conclusion: We report on the relevance of network inference for identifying novel key (co-)driver genes in segmental chromosomal aberrations and their regulatory programs in neuroblastoma.

P069

Actionable mechanisms and biomarkers to predict selective Bcl2-family inhibitor responses in neuroblastoma

Saforo D[1], Zapata C[2], Liu K[2], Goutnik M[2], Bodducherla R[2], Coku J[2], Vemu R[2], Nance J[3], Jonas H[1], Reynolds C[3], Goldsmith K[1], Hogarty M[2]

[1]Children's Healthcare of Atlanta/Emory University, Atlanta, United States, [2]Children's Hospital of Philadelphia/University of Pennsylvania, Philadelphia, United States, [3]Texas Tech University Health Sciences Center, Lubbock, United States

Background: Apoptosis is governed by protein-protein interactions (PPIs) among Bcl2 family members. Cancer therapies activate Bim to engage Bak/Bax and trigger apoptosis and we've shown Bim is instead sequestered by either Bcl2 or Mcl1 in neuroblastomas, maintaining viability.

Aims: We seek a mechanistic understanding of neuroblastoma survival dependencies to leverage Bcl2-family inhibitors for clinical use.

Methods: Bcl2-PPIs in diverse cell lines, PDXs and tumors were identified by co-immunoprecipitation, with or without inhibitor exposure [ventoclax (BCL2i), navitoclax (BCL2/BCLXi), A1155463 (BCLXi), S63845 (MCL1i)], and compared with inhibitor responses in vitro and in vivo, including PDX tissue slice cytotoxicity. Oncogenes were knocked-down via shRNA and Bim-PPI and drug sensitivity measured.

Results: We studied >50 neuroblastoma models and defined two dominant Bim-PPI patterns, "Bim:Bcl2" (Bim preferentially bound to Bcl2) or "Bim:Mcl1". Bim:Bcl2 tumors are sensitive to BLC2i's in vitro and in vivo through Bim displacement that activates Bak/Bax. Surprisingly, Bim:Mcl1 tumors are insensitive to MCL1i's alone, as Bim is displaced from Mcl1 but sequestered by Bclx. Such tumors are sensitive to dual MCL1/BCLX inhibition. MYCN- and ALK-activated tumors occur in both classes, but all MAPK mutated tumors (excluding receptor-tyrosine kinase mutants) were Bim:Mcl1. Confirming this, EGFR knock-down in a Bim:Mcl1 tumor rendered it Bim:Bcl2 and newly BCL2i sensitive, while NF1 knock-down in a Bim:Bcl2 tumor rendered it Bim:Mcl1 and MCL1i/BCL2i sensitive. Approved MEK inhibitors also displaced Bim from Mcl1, phenocopying MCL1i activity. Since Bim-PPI heterogeneity within a tumor would hinder the clinical efficacy of selective inhibitors, we assessed 11 tumor-model pairs established from the same patient at differing times or anatomic locations. All 11 were Bim-PPI concordant (<0.2% probability if heterogeneous). PDXs showed a similar pattern of dominant PPI at diagnosis, but post-therapy tumors more frequently developed multiple Bim-PPIs concurrently (Bim:Bcl2>Bim:Mcl1>Bim:Bclx).

Conclusions: Neuroblastomas sequester Bim via a singular Bcl2-homolog, and drugs that displace Bim induce apoptosis. This Bim-PPI biomarker is temporally and spatially stable with nominal intra-cellular heterogeneity, although therapeutic pressure in situ may engage additional survival proteins (inter-cellular heterogeneity). We also define a hierarchical Mcl1-Bclx dependency linked to MAPK activation. Our findings robustly inform clinical integration strategies for Bcl2-family inhibitors.

P070

Clinically relevant treatment of PDX models reveals patterns of neuroblastoma chemoresistance

Mañas A[1], Aaltonen K[1], Andersson N[2], Hansson K[1,3], Adamska A[1], Seger A[1], Yasui H[2,4], van den Bos H[5], Radke K[1], Esfandyari J[1], Bhave M[1], Karlsson J[2], Spierings D[5], Foijer F[5], Gisselsson D[2,6], Bexell D[1]

[1]Division of Translational Cancer Research, Department of Laboratory Medicine, Lund University, Lund, Sweden, [2]Division of Clinical Genetics, Department of Laboratory Medicine, Lund University, Lund, Sweden, [3]Cancer Stem Cell Laboratory, The Breast Cancer Now Toby Robins Research Centre, The Institute of Cancer Research, London, United Kingdom, [4]Department of Gynecologic Oncology, Aichi Cancer Center Hospital, Nagoya, Japan, [5]European Research Institute for the Biology of Ageing (ERIBA), University of Groningen, University Medical Center Groningen, , The Netherlands, [6]Department of Pathology, Laboratory Medicine, Skane University Hospital, Lund, Sweden

Rapid Fire session 1A, May 15, 2023, 11:35 AM - 11:50 AM

Background and Aim

Chemoresistance and relapse are common in high-risk neuroblastoma (HR-NB) and are often associated to high intratumor heterogeneity and certain genetic aberrations, but more specific mechanisms have remained elusive. Recently, transcriptional and epigenetic analyses identified at least two phenotypic cell states in NB: noradrenergic (ADR) and mesenchymal (MES). However, their relevance for treatment resistance had not been confirmed beyond cell culture before. Our goal was to explore the mechanisms of HR-NB chemoresistance in a clinically relevant setting.

Methods

One of the main limitations of preclinical studies of chemotherapy resistance is the use of single agents, rather than the combination of multiple drugs used for HR-NB patients' treatment. We developed a clinically relevant in vivo protocol mimicking the first line five-chemotherapy treatment regimen of HR-NB (COJEC) and applied this protocol to mice bearing MYCN-amplified HR-NB patient-derived xenografts (PDXs). Genomic and transcriptomic analyses were used to reveal the mechanisms involved in NB chemoresistance.

Results and Conclusions

The NB PDX response to chemotherapy resembled the response of the corresponding patients. We identified convergent and parallel evolution of key genetic aberrations in NB progression. Intrinsic chemoresistance was associated with high genetic diversity and an embryonic phenotype. NB-PDX tumors that initially responded to treatment but eventually relapsed with acquired resistance showed a lower ADR phenotype and an enhanced immature MES-like phenotype resembling Schwann cell precursors found in the developing adrenal gland. In contrast, tumors with a favorable response presented a lineage-committed ADR phenotype similar to normal neuroblasts and negative regulation of the MAPK/ERK cascade. We compiled a set of integrated phenotypic gene signatures that (i) summarize multiple ADR/MES signatures, (ii) represent multiple NB models, (iii) represent different sequencing methods, (iv) reflect COJEC response in PDXs, (v) and have prognostic power in NB patients. Additionally, we established NB organoids from relapsed PDX tumors that retained COJEC resistance, tumorigenicity, and transcriptional cell states. This work sheds light on mechanisms involved in NB chemoresistance using a clinically relevant treatment protocol and emphasizes the importance of phenotypic plasticity and transcriptional cell states in chemotherapy response.

P072

High Content imaging screen identifies lysosomal activity and MAPK signaling as specific vulnerabilities of mesenchymal neuroblastomas

Herter S[1,2,3], Ridinger J[1,2], Kreth S[4,5], Peterziel H[1,2], Westermann F[4,5], Hamacher-Brady A[6,7], Witt O[1,8,2], Oehme I[1,2]

[1]Hopp Children's Cancer Center Heidelberg KiTZ, Clinical Cooperation Unit CCU Pediatric Oncology, Heidelberg, , Germany, [2]German Cancer Research Center DKFZ and German Cancer Consortium DKTK, Clinical Cooperation Unit Pediatric Oncology CCU, Heidelberg, , Germany, [3]Heidelberg University, Faculty of Bioscience, Heidelberg, , Germany, [4]Hopp Children's Cancer Center Heidelberg KiTZ, Division of Neuroblastoma Genomics, Heidelberg, , Germany, [5]German Cancer Research Center DKFZ and German Cancer Consortium DKTK, Division of Neuroblastoma Genomics, Heidelberg, , Germany, [6]Johns Hopkins University, Bloomberg School of Public Health- W. Harry Feinstone Department of Molecular Microbiology and Immunology, Baltimore, , United States, [7]Heinrich-Heine University Düsseldorf, Institute of Biochemistry and Molecular Biology I- University Hospital Düsseldorf, Düsseldorf, , Germany, [8]Heidelberg University Hospital, Department of Pediatric Oncology- Hematology and Immunology, Heidelberg, , Germany

Background:
Neuroblastoma tumors include adrenal linage committed and more undifferentiated mesenchymal cancer cells that differ also in their response to drug treatment. Lysosomes are involved in many cellular processes that contribute to drug resistance mechanisms.

Aim:
This project aims to unravel how lysosomes contribute to the specific resistance of mesenchymal neuroblastomas and how they are connected to MAPK pathway. This enables the identification of a treatment specifically targeting the vulnerabilities of mesenchymal neuroblastoma.

Methods:
High throughput drug screening: To analyze the drug resistance of a panel of 24 neuroblastoma cell lines are seeded in 384-well plates with 75 anti-cancer drugs in 5 concentrations covering the clinically relevant concentration range of the drugs. Cells grow as spheroids for 72h. The viability of the cells is measured with an ATP- based luminescence readout and compared to negative and positive controls. Therapy response is described with a drug sensitivity score (DSS) to make drug response comparable among different cell lines.

High content imaging: To characterize the lysosomal compartment of the tumor cell lines at basal levels and in response to drug treatment, lysosomes are stained w ith functional and immunofluorescence stainings and analyzed with confocal high content imaging. The measurements
are summarized in a lysosomal score.

Results: We generated drug sensitivity profiles for each cell line and correlated them with basal lysosomal scores. This revealed that MEK inhibitors were specifically effective in mesenchymal tumor models with a high lysosomal score. Functional analysis of lysosomal features as a response to MAPK pathway inhibition showed an increase of lysosome numbers in mesenchymal neuroblastoma models.

Conclusion: Understanding the mechanisms of underlying resistance mechanisms in high-risk pediatric tumors is essential to develop novel treatment approaches. Natural variations in lysosome numbers of cancer cell lines emerge as a potential indicator for intrinsic drug resistance and create new vulnerabilities to break resistance by targeting lysosomes.

P073

MYCN drives neuroblastoma oncogenesis via molecular clock disruption

Li L[1], Wolf A[1], Hernandez B[1], Badachhape A[2], Patel T[3], Ghaghada K[2], Coarfa C[3], Barbieri E[1]
[1]Department of Pediatrics, Dan L Duncan Comprehensive Cancer Center, Baylor College of Medicine, Houston, United States, [2]Department of Radiology, Texas Children's Hospital, Baylor College of Medicine, Houston, United States, [3]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, United States

Rapid Fire session 1A, May 15, 2023, 11:35 AM - 11:50 AM

Background: Amplification of the MYCN oncogene drives high-risk neuroblastoma (NB) and poor patient clinical outcomes. MYCN has been deemed "undruggable", thus a more precise elucidation of targetable MYCN-induced pathways is needed. Increasing evidence suggests a correlation between the molecular clock and cancer development, however the mechanisms underlying this association remain elusive. Recently, we have found that pharmacological restoration of BMAL1, a core component of the molecular clock, inhibits MYCN-induced de novo lipogenesis and tumor growth. These data suggest that MYCN promotes oncogenic metabolic reprogramming through disruption of the molecular clock. Aim: We aim to unveil the molecular mechanisms underlying MYCN-induced oncogenesis through the inhibition of BMAL1 expression and oscillatory behavior.

Methods: In vitro, MYCN-amplified (MNA) and non-amplified, as well as genetically engineered (SK-N-AS MYCN-ER™ and Sh-MYCN) NB models were employed to assess consequences on metabolic rhythmicity. Sh-BMAL1 cells were synchronized to characterize the metabolic and biological phenotypes associated with genetic depletion of BMAL1. We employed ChIP-seq to study the BMAL1 interactions with chromatin in NB cells under low and high MYCN expression. In vivo, the oscillatory behavior and expression of BMAL1 and lipogenic genes were evaluated in MNA and non-MNA tumors.

Results: Activation of MYCN profoundly represses the expression and oscillation of BMAL1, while promotes lipid and cholesterol biosynthetic genes. BMAL1 ChIP-seq revealed the most downregulated pathways upon MYCN activation are steroid biosynthesis, and cell differentiation and morphogenesis (known MYCN functions) suggesting that MYCN and BMAL1 may compete at specific loci controlling these processes. BMAL1 knock-down provides NB cells a proliferative advantage and perturbs the circadian oscillation of metabolic genes. MNA tumors are characterized by a lower BMAL1 and a higher lipogenic rhythmic expression compared with non-MNA tumors. The anti-phase cycles between BMAL1 and lipogenic genes suggests that BMAL1 rhythmically regulates the metabolic state of these tumors.

Conclusion: Collectively, our data indicate that MYCN dysregulates the oscillation and expression of metabolic genes through disruption of the molecular clock. On-going efforts will elucidate the epigenetic remodeling occurring during this rhythmic perturbation. These study provide a novel chronologic therapeutic strategy targeting time-specific metabolic states to block NB growth.

P074

Study of spatial and temporal epigenetic heterogeneity in high-risk neuroblastoma suggests role of H3K27me3-mediated repression

Butterworth C[1,2], Saint-Charles A[1,2], Bhalshankar J[1,2], Iddir Y[1,2], Bellini A[1,2], Bobin C[1,2], Wecht E[7], Park Y[7], Plantaz D[4], Sartelet H[5], Combaret V[6], Pierron G[3], Janoueix-Lerosey I[2], Henrich K[7], Delattre O[2], Westermann F[7], Schleiermacher G[1,2,8]

[1]SiRIC RTOP (Recherche Translationelle en Oncologie Pédiatrique); Translational Research Department, Institut Curie Research Center, PSL Research University, Institut Curie, Paris, France, [2]INSERM U830, Equipe Labellisée Ligue contre le Cancer, PSL Research University, Institut Curie Research Center, Paris, France, [3]Somatic Genetics Unit, Institut Curie, Paris, France, [4]Centre Hospitalier Universitaire Grenoble-Alpes, Grenoble, France, [5]Centre Hospitalier Régional Universitaire de Nancy, Nancy, France, [6]Laboratoire de Recherche Translationnelle, Centre Léon-Bérard, Lyon, France, [7]German Cancer Research Center (DKFZ), Heidelberg, Germany, [8]SIREDO Integrated Pediatric Oncology Center, Institut Curie, Paris, France

Rapid Fire session 1A, May 15, 2023, 11:35 AM - 11:50 AM

Background
Neuroblastoma (NB) has a relatively silent mutational landscape, but changes in epigenetic regulation might explain its wide clinical and biological heterogeneity. However, recent epigenetic studies have predominantly focused on the previously identified adrenergic and mesenchymal cell states found in NB tumours, defined by distinct super enhancer histone marks and their core regulatory circuits. Less is understood about changes in repressive control.

Aims
The aim of our work is to study the heterogeneity of epigenetic mechanisms in neuroblastoma; specifically focusing on differences in H3K27me3-repressed genes between MYCN-amplified and non-amplified tumors, and changes induced by chemotherapy treatment or associated with disease relapse.

Methods
We performed extensive data analysis on a large cohort of > 40 diagnostic NB tumor samples studies for which different histone marks were studied by histone ChIP-Seq and our findings further validated in both MYCN knockdown and overexpressing cell line models.
Additionally, we optimized the histone ChIP-Seq protocol for low input DNA levels and performed ChIP-Seq on high-risk neuroblastoma patient blood plasma samples at either diagnosis or relapse timepoints, to follow histone marks over time.

Results
In NB tumor samples, clinically similar patient samples cluster into their MYCN status subgroups based on H3K27me3 patterns. We see differential suppression of many gene signatures including the mesenchymal signature and various developing adrenal medullary cell states, between MYCN-amplified and non-amplified high-risk tumours, highlighting a potential role of H3K27me3 in regulating cell lineage commitment and differentiation ability.
Secondly, when comparing bulk sequencing of tumours collected pre- and post-chemotherapy, we identify modifications at the epigenetic level, with bivalently controlled changes observed via H3K27me3 and H3K4me3, seen more significantly in high-risk cases - further validation that these changes are tumor cell specific is required.

To further validate these identified H3K27me3 changes, incorporation of additional transcriptomic, proteomic, and ChIP-Seq datasets from the same patients as well as ongoing analysis of histone ChIP-Seq performed on plasma samples at multiple timepoints.

Conclusions

Our study expands the understanding of the differential role of H3K27me3 in neuroblastoma, both at different disease stages and between MYCN status subgroups. This knowledge will be important when considering future epigenetic treatment options.

P075

Palbociclib releases the latent differentiation capacity of neuroblastoma cells

Ferguson K[1], Gillen S[1], Chaytor L[1,2], Poon E[3], Marcos D[1,2], Woods L[1,2], Mykhaylechko L[1,2], Elfari L[4], Chesler L[3], Ali F[5], Carroll J[6], Philpott A[1,2]

[1]Wellcome-MRC Cambridge Stem Cell Institute, Jeffrey Cheah Biomedical Centre, Cambridge Biomedical Campus, Cambridge, United Kingdom, [2]Department of Oncology, University of Cambridge, Cambridge, United Kingdom, [3]Division of Clinical Studies and Cancer Therapeutics, Institute of Cancer Research (ICR), London and Royal Marsden NHS Trust, Sutton, United Kingdom, [4]Wellcome-MRC Cambridge Stem Cell Institute Advanced Imaging Facility, Cambridge, United Kingdom, [5]College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai Healthcare City, Dubai, United Arab Emirates, [6]Cancer Research UK Cambridge Institute, University of Cambridge, Cambridge, United Kingdom

Rapid Fire session 1A, May 15, 2023, 11:35 AM - 11:50 AM

Background

In normal development, sympathoadrenal precursor cells differentiate into cell types including sympathetic neurons; however, in neuroblastoma these precursors are locked into an immature state that drives tumour growth. Based on anti-proliferative effects, preclinical studies suggest CDK inhibitors as promising treatments, with cyclin D-CDK4/6 particularly attractive targets owing to their extensive overexpression. Here, we investigate the CDK4/6 inhibitor palbociclib, a drug already approved for front-line combination treatment in breast cancer, as a clinically relevant differentiation therapy for neuroblastoma.

Results

We find that palbociclib not only drives cell cycle exit but induces extensive neuronal differentiation of adrenergic-type neuroblastoma cells. RNA-seq analysis shows a robust reprogramming of the transcriptome towards reduced proliferation and induction of neuronal differentiation across three adrenergic-type cell lines. This altered gene expression pattern significantly correlates with improved patient survival. Interrogation of enhancer and super-enhancer regions using H3K27ac ChIP-seq demonstrates that palbociclib treatment restructures the epigenetic landscape of neuroblastoma cells to favour neuronal differentiation and maturation, as well as to restrict alternative developmental pathways. Furthermore, ultrastructural features consistent with maturing neurons were observed upon palbociclib treatment, including neuritic processes containing filaments and microtubules, as well as dense-core neurosecretory granules.

Conclusion

Palbociclib-mediated differentiation of neuroblastoma cells is manifested by extensive phenotypic and transcriptional changes, accompanied by the establishment of a new epigenetic programme driving expression of mature neuronal features. Novel, kinder therapies are needed to treat this devastating childhood cancer and reactivating a latent ability to undergo differentiation is a promising approach. Here, we identify CDK4/6 inhibition as a novel strategy to dramatically enhance neuroblastoma differentiation efficacy.

P076

Atypical neuroblastoma: screening of urinary catecholamine excretion and mIBG avidity at diagnosis, a retrospective study.

BOROVKOV A[1], Assy J[1], Aerts I[1], Bourdeaut F[1,2], Orbach D[1], Michon J[1], Cyrta J[3], Mosseri V[4], Doz F[1,5], Gauthier A[3], Cardoen L[6,7], Luporsi M[7,8], Schleiermacher G[1,2]

[1]SIREDO Integrated pediatric, adolescents and young adults oncology center, Institut Curie, Paris, France, [2]SIRIC RTOP Laboratory of translational research in pediatric oncology, INSERMU830, Institut Curie, Paris, France, [3]Department of pathology, Institut Curie, Paris, France, [4]Department of research, Institut Curie, Paris, France, [5]Université Paris Cité, Paris, France, [6]Department of radiology, Institut Curie, Paris, France, [7]LITO Laboratory of translational radiology in oncology, INSERMU1288, Institut Curie, Orsay, France, [8]Department of nuclear medicine, Institut Curie, Paris, France

Rapid Fire session 1B, May 16, 2023, 11:30 AM - 11:45 AM

Background

In neuroblastoma (NB), urinary catecholamine excretion and mIBG avidity, proportional to norepinephrine transporter's (NET) expression, have been used for decades as diagnostic tools.

Recently, cellular plasticity has been described, with a mesenchymal phenotype related to an absence of enzymes involved in catecholamine synthesis and an absence of NET. The frequency and prognostic impact of atypical NB, without catecholamine excretion and/or without mIBG avidity, remains to be determined.

Aims

To determine the frequency and prognosis of atypical NB.

To investigate the urinary catecholamine profile and to evaluate its correlation with mIBG avidity.

Methods

From 2000 to 2020, 253 children treated for NB at Institut Curie, France, were analyzed for catecholamine excretion and mIBG avidity.

Results

62 of 253 NB had atypical features: 16 without mIBG avidity with positive catecholamine excretion, 35 without catecholamine excretion with mIBG avidity, and 11 with neither mIBG avidity nor catecholamine excretion. Concerning atypical NB, 63% had INRG stage L1/2 (n=37), 27% had stage M (n=16), 10% had stage MS (n=6), versus typical NB (n=191) which 28% had stage L1/2 (n=52), 61% had stage M (n=114) and 11% had stage MS (n=21). NMYC amplification/segmental and numeric genomic profile were found in 12%/48% and 52% in atypical NB, versus 31%/74% and 26% in typical ones.

OS and EFS were better in atypical than typical NB with 5-year OS of 87% vs 64% ($p<0.0007$) and EFS of 77% vs 51% ($p<0.0009$). Catecholamine excretion in overall NB was dominated by elevated vanillylmandelic acid/homovanillic acid (VMA/HVA) in 53% (n=103), dopamine in 41% (n=80), with only 2% epinephrine and 4% norepinephrine excretion predominance respectively. mIBG non-avid tumors had catecholamine excretion in 59% (n=16), with elevation of VMA/HVA in 69% (n=9). Elevated dopamine excretion had worse 5-year OS comparatively to VMA/HVA positivity, 43% and 80% respectively ($p<0.0001$).

Conclusion

Atypical NB are observed in 25% of patients, have more favorable tumor features and are associated with a better prognosis. Catecholamine excretion profiles reveal clinical and biological subgroups which might be of clinical relevance.

Complementary biological studies including single cell RNAseq analysis will provide insight into disease associated features for prognostic and therapeutic value.

P077

Cause and consequence of intra- and inter-tumor heterogeneity in zebrafish models of neuroblastoma.

Fresmann N[1], Spanjaard B[1], Gauert A[2], Bramè L[2], Heeren-Hagemann A[2], Junker J[1]
[1]*Berlin Institute for Medical Systems Biology, Max Delbrück Center for Molecular Medicine, Berlin, Germany,*
[2]*Department of Paediatrics, Division of Oncology and Haematology, Charité, Berlin, Germany*

Rapid Fire session 1B, May 16, 2023, 11:30 AM - 11:45 AM

Neuroblastoma is a childhood cancer originating from multipotent sympathoadrenal progenitor cells. Survival rates for high-risk patients remain below 50 %, as most experience metastatic relapse after initially responding to therapy. High-risk disease is often associated with MYCN-amplification, but there is an overall paucity of high-frequency recurring somatic mutations and druggable targets. Therefore, it is crucial to better understand non-genetic mechanisms of neuroblastoma plasticity in relation to disease progression and to ultimately identify ways to target specific cellular programs.

We aim at elucidating the role of transcriptional heterogeneity and plasticity in neuroblastoma tumorigenesis and progression by employing single-cell RNA-sequencing (scRNA-seq) and high-throughput lineage tracing on MYCN-driven tumors from several zebrafish models of neuroblastoma with differing penetrance, primary tumor site and metastatic potential.

We performed multi-region scRNA-seq on 36 full-blown primary tumors from transgenic zebrafish expressing either MYCN alone or in combination with LMO1 under the control of the sympathoadrenal lineage-specific dbh-promoter. By employing CRISPR/Cas9-based lineage tracing, we simultaneously gathered clonal information for the cells. We then profiled neuroblastoma cell transcriptomes before and after transplantation into zebrafish larvae to measure state plasticity in response to a new niche.

ScRNA-seq revealed diverse neuroblastoma cell states as well as microenvironmental and immune cell types. In line with findings in human neuroblastoma, zebrafish neuroblastoma cells show a largely adrenergic transcriptomic profile. We further observe multiple sub-states, whose occurrence and frequency differs between individual tumors, despite the controlled genetic background.
Single-cell lineage tracing showed that several progenitor cells transform and contribute to tumor growth in these models. In some cases, neuroblastoma cell subgroups from different progenitors and tumor regions were transcriptionally divergent, highlighting the impact of the cell of origin and early events in tumorigenesis on the tumor cell state. Using transplantation assays, we show that a cell's transcriptional state greatly influences its response to and survival in a new niche.

Our data provides an in-depth look into intra- and inter-tumor heterogeneity of zebrafish neuroblastoma models on a single-cell level. We are actively working on linking our findings on state plasticity and pre-disposition to survival to human neuroblastoma to ultimately gain insights into new treatment options.

P078

Hypoxia Promotes a Transition from Adrenergic to Mesenchymal Cell State in Neuroblastoma

Chaves G[1], Moore K[1], Wilkinson E[1], Gaynes J[1], Vayani O[1], Chennakesavalu M[1], Chlenski A[1], He C[2], Cohn S[1], Applebaum M[1]

[1]University of Chicago, Department of Pediatrics, Chicago, United States, [2]University of Chicago, Department of Chemistry, Chicago, United States

Background: We previously demonstrated that in adrenergic (ADRN) neuroblastoma cells, hypoxia induces TET1, an enzyme that catalyzes the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), a modification known to facilitate active gene expression. Hypoxia also induces global increases and accumulation of 5-hmC at canonical hypoxia response genes in ADRN cells. Here we investigated the role these 5-hmC changes play in regulating the hypoxic response and cell state in ADRN and MES neuroblastoma cells.

Aims: Identify mechanisms of the hypoxia response by characterizing the genome-wide epigenetic and transcriptional response to hypoxia in a panel of ADRN and MES cells.

Methods: Three pairs of isogenic ADRN and MES cells were assessed (NBLW-N/NBLW-S, LA1-55n/LA1-5s, and SH-SY5Y/SHEP). Changes in the levels of global and whole genome 5-hmC in cells cultured in normoxia and hypoxia were accessed using HPLC-MS and Nano-hmC-Seal. Growth was assessed by MTT assay. Gene expression was evaluated by RNA-Seq and qPCR. Differentially expressed genes were identified using DESeq2. ClusterProfiler assessed pathway enrichment using MSigDB gene sets. Gene Set Variation Analysis (GSVA) quantified ADRN and MES phenotypes.

Results: While TET1 expression increased in hypoxia in both ADRN (log2 fold change=6.4; p=0.005) and MES cells (log2 fold change=1.4; p=3.4x10-5), global 5-hmC deposition only increased in ADRN but not MES cells (p=0.01 and 0.43, respectively). Compared to normoxic conditions, ADRN cells cultured in hypoxia had significantly reduced proliferation rates (p=5.4x10-5) whereas MES did not (p=0.15). In normoxic conditions, genes in neuronal pathways were enriched for 5-hmC deposition and expression in ADRN cells compared to MES cells. Conversely, MES cells were enriched for pathways of extracellular matrix, inflammatory signaling and the hypoxia response. Hypoxia increased expression and 5-hmC deposition of genes enriched for pathways in ADRN cells that are detected in MES cells cultured in normoxia including the hypoxia response, collagen extracellular matrix deposition and inflammatory signaling. GSVA identified increase in MES scores induced by hypoxia in ADRN (p=0.007) but not MES cells (p=0.92), while ADRN scores did not change for ADRN or MES cells (p=0.43 and p=0.48).

Conclusion: Hypoxia induces epigenomic modifications that promote a transition from ADRN to MES cell state.

P079

Adaptive phenotypic cell plasticity is a cancer evolutionary driver in paediatric solid cancers: “plasticity-first” evolvavility

Bruna A[1]
[1]*The ICR, London, United Kingdom*

BACKGROUND
We hypothesize that in response to treatment, adaptive phenotypic changes (“plasticity-first”) drive evolution. This is an important new concept because it predicts identifying the molecular mechanisms enabling plasticity-first evolution may present a recipe for innovative anticipatory biomarkers of drug responses, including those to immunotherapy, and therapeutic strategies including plasticity-inducing compounds which could turn into superior therapeutic responses. To identify phenotypic plasticity in mediating neuroblastoma malignant phenotype, we need innovative technology that will simultaneously determine lineage relationships (single cell ancestries) and phenotypes. To this end, we aim:

i/ To develop and benchmark a new Molecular Tape (herein referred to as Plasticity Molecular Recorder or PMRec) to temporally dissect plasticity at single-cell resolution and delineate its contribution to adaptive evolutionary behaviours.
ii/ To identify the interplay between plasticity and genetic diversity and how this influences the outcome of evolution.
iii/ To translate these evolutionary findings into the discovery of improved treatment strategies that will alter the mechanisms enabling plasticity to avoid and prevent relapse.

METHODS
A new molecular tape (PMRec) will be created and used to identify phenotypic switching (RNA dynamics) whilst simultaneously recording lineage relationships (DNA dynamics) at single-cell resolution in evolving tumours prototypical of low and high genetic diversity (patient-derived ex vivo and in vivo children and adult cancer models, respectively).
The plasticity capability of PMRec will be engineered into live imaging and FLOW approaches and coupled to high-throughput functional screens to identify novel plasticity compounds. Innovative treatment strategies will be tested in a dynamic patient-derived ex vivo preclinical setting and validated in vivo. Single-molecule FISH and RNA sequencing will be used to identify transcriptional noise as a source of plasticity.

HOW THE RESULTS OF THIS RESEARCH WILL BE USED
This study will provide the identification of druggable targets, their validation and preclinical investigation paving the way towards the derivation of innovative preclinical testing platforms to refine/innovate rationally targeted treatment strategies for cancer patients that have relapsed or are at high-risk of relapses.

P080

Cellular and molecular mechanisms involved in neuroblastoma plasticity

Gautier M[1,2], Pierre-Eugène C[1,2], Louis-Brennetot C[1,2], Kramdi A[1,2], Peltier A[1,2], Delattre O[1,2], Thirant C[1,2], Janoueix-Lerosey I[1,2]

[1]Institut Curie, PSL Research University, Inserm U830, Equipe Labellisée Ligue contre le Cancer, Paris, FRANCE, [2]SIREDO: Care, Innovation and Research for Children, Adolescents and Young Adults with Cancer, Institut Curie, Paris, FRANCE

BACKGROUND: Half of neuroblastoma patients present with high-risk metastatic cancers usually responding to initial chemotherapy but frequently becoming refractory even to multimodal therapies. Tumor cells are defined by a main sympathetic noradrenergic (NOR) identity, but epigenetic analyzes of cell lines highlighted a second tumor identity associated with mesenchymal (MES) properties. Indeed, in vitro, MES cells are more invasive and migratory than their NOR counterparts and more resistant to standard chemotherapies. While "pure" MES tumor cells have not been found in vivo yet, our data revealed a population of NOR cells expressing MES features in patients and PDX models. Using cellular models, we described a plasticity potential defining a "Noradrenergic to Mesenchymal Transition" (NMT) and its reverse mechanism (MNT), associated with epigenetic and transcriptional reprogramming.

AIMS: We hypothesize that the acquisition of mesenchymal properties through tumor cell plasticity could support metastasis and chemoresistance, two hallmarks of high-risk neuroblastoma. We aim to characterize intrinsic and extrinsic factors controlling plasticity between tumor identities.

METHODS: We have developed dynamic models of NMT/MNT relying on the CD44 cell surface marker that allow to discriminate MES from NOR cells in several heterogeneous cell lines. We document potential transition states during NMT/MNT by scRNAseq experiments. Pharmacological and genetic tools (siRNAs, shRNAs and CRISPR KO) allow to investigate the role of several factors, including members of the HIPPO pathway, in the acquisition and maintenance of mesenchymal properties.

RESULTS: Bulk and scRNAseq expression data show that several regulators such as YAP, TAZ and some of their target genes are specifically induced during NMT and maintained in MES cells. Different approaches show that YAP inactivation induces a decreased expression of some targets genes and affects moderately the proliferation of MES cells. Nevertheless, an overexpression of TAZ protein is observed suggesting a compensation mechanism. The co- inactivation of the two factors induces the mortality of MES cells. ChIPseq experiments are ongoing to characterize their molecular role on chromatin in NMT and the MES identity.

CONCLUSION: In conclusion, we are currently investigating the roles of key intrinsic factors that influence the acquisition and/or the maintenance of mesenchymal properties in neuroblastoma tumor cells.

P081

Targeting lineage heterogeneity via cGAS-STING

Dessau J[1], Narch A[2], Shapiro M[2], Jung P[2], Dong M[1], Dang C[3,4,5,6], Wolpaw A[2,7]
[1]Wistar Institute, Philadelphia, USA, [2]Children's Hospital of Philadelphia, Philadelphia, USA, [3]Ludwig Institute for Cancer Research, New York, USA, [4]Johns Hopkins University, Bloomberg-Kimmel Institute for Cancer Immunotherapy, Baltimore, USA, [5]Johns Hopkins University School of Medicine, Department of Oncology, Baltimore, USA, [6]Johns Hopkins Bloomberg School of Public Health, Department of Biochemistry and Molecular Biology, Baltimore, USA, [7]Perelman School of Medicine at the University of Pennsylvania, Departmen of Pediatrics, Philadelphia, USA

Background:
Lineage heterogeneity is an important mechanism of cancer therapy resistance. In neuroblastoma two states co-exist, a chemoresponsive adrenergic state and a chemoresistant mesenchymal state. We recently demonstrated that the mesenchymal state has elevated expression of inflammatory sensors (Wolpaw et al., PNAS 2022), including STING, which can promote anti-tumor immunity when activated by cGAS binding to cytosolic DNA. Cytosolic DNA can be present in untreated cancer cells or produced by therapy, including agents used in neuroblastoma.

Aim:
Define mechanisms and consequences of cGAS-STING suppression in neuroblastoma to identify approaches to enhance the immunologic response to therapy

Methods:
We examined cGAS and STING mRNA expression, H3K27 trimethylation, and CpG island methylation in genome-wide datasets. Using immunoblot and qPCR, we assessed expression in 20 neuroblastoma cell lines and changes after treatment with epigenetic inhibitors. To probe functional pathway activity, we measured changes in pSTAT1 and pIRF3 after DNA transfection or irradiation, with or without exogenous cGAS expression.

Results:
We found that STING protein is detectable in mesenchymal but not adrenergic lines while cGAS is largely undetectable. After expression of cGAS, adrenergic lines lacking STING remained unresponsive while STING-expressing mesenchymal lines responded to both transfected DNA and irradiation. In some cases, cGAS expression led to signaling in the absence of a pathway agonist, implying the presence of endogenous cytosolic DNA. ChIP-seq data revealed more repressive H3K27me3 marks at the promoter of cGAS in adrenergic than in mesenchymal lines. Consistently, EZH2 inhibition increased cGAS in adrenergic but not mesenchymal lines. The CpG island in the cGAS promoter is heavily methylated. Treatment with decitabine increased cGAS transcript in all lines tested and, in a subset of mesenchymal cell lines, was sufficient to restore a functional response to transfected DNA.

Conclusions:
cGAS-STING is inactivated in neuroblastoma, which may be selected for due to endogenous cytosolic DNA. cGAS expression restores signaling only in mesenchymal lines as STING expression is limited to the mesenchymal state. cGAS is repressed by both histone and DNA methylation and expression restored by epigenetic inhibitors. These data support the therapeutic potential of cGAS reactivation to enhance immunotherapeutic targeting of the chemoresistant mesenchymal state.

P082

Uncovering Initiation Mechanisms of Neuroblastoma via Transcriptional Profiling of Neural Crest Development

Hsin J[1,2], Thiele C[3], Kerosuo L[1]

[1]National Institute of Dental and Craniofacial Research, Neural Crest Development and Disease Unit, Bethesda, MD, United States, [2]University of Cambridge, Department of Engineering, Cambridge, England, [3]National Cancer Institute, Pediatric Oncology Branch, Bethesda, MD, United States

Background: Neuroblastoma, the most common extracranial pediatric solid tumor, is a neural crest cell (NCC) derived cancer of the peripheral nervous system with a widely varying clinical course. NCCs are an early transient population of stem cells that emerge in young embryos, in the third week of gestation in humans. After detaching from the neural epithelium, NCCs migrate to various destinations in the vertebrate body to form the peripheral nervous system, craniofacial skeleton, pigmentation, and several cell types of the hormonal regulatory system. The exact stage of NCC development in which neuroblastoma occurs remains unclear. While most research has focused on studying neuroblastoma initiation in sympathoadrenal progenitors, chromatin cells, and sympathetic neurons, recent studies by us and others suggest the initiation may in fact already occur earlier during NCC development.

Aims: We hypothesize we can pinpoint the initiation mechanism of neuroblastoma by searching for matching transcriptional profiles between stages of normal neural crest development and neuroblastoma samples with different prognosis and stage of aggressive behavior.

Methods: We have optimized protocols for differentiating human embryonic stem cells to early pre- and post-migratory NCC and their respective differentiating sympathetic neuron and chromaffin cell lineage specific derivatives in vitro. From these, we have generated single cell and bulk RNA sequencing samples. We compare developmental gene modules unique to each stage of NCC development to the published single cell RNA sequencing data of neuroblastoma samples to identify stage specific resemblance of NCC development and neuroblastoma.

Results: Our preliminary results show that patient-derived neuroblastoma samples are not equal in regard to their transcriptional profile reflecting embryonic developmental status. Even though all neuroblastomas are NCC derived, only some tumors demonstrate a NCC profile while other samples are more similar to neural stem cells, a finding not directly correlating with the MYCN amplification status. The differences in these cancer profiles may explain the differences seen in the progression of neuroblastoma.

Conclusion: Based on our initial results we suggest our approach may lead to insights behind the heterogeneity within neuroblastoma and provide information on the molecular tumor initiation and metastasis mechanisms necessary for designing novel targeted neuroblastoma treatments.

P083

The DDR2 (Discoid Domain Receptor 2) defines SCP/bridge subpopulations and is a novel therapeutic target in Neuroblastoma

Rozen E[1], Frantz W[1], Wigglesworth K[1], Shohet J[1]
[1]*University of Massachusetts Chan Medical School, Department of Pediatrics, Worcester, United States*

Introduction: DDR2 (Discoid Domain Receptor-2) is a collagen-binding Receptor Tyrosine Kinase (RTK) expressed in mesenchymal stem cells and dedifferentiated mesenchymal tumors. This RTK drives drug resistance and angiogenesis in ovarian cancer, and critically regulates tumor/stroma interactions and metastasis in breast cancer. Importantly, DDR2 has the strongest correlation with high-risk disease and overall poor survival among all known RTKs kinases across multiple neuroblastoma patient cohorts.

Aims: We sought to characterize the role of DDR2 in neuroblastoma pathogenesis
Results: To address the transcriptional functions of DDR2-mediated signaling in NB cells we performed RNA-seq analysis of SH-SY5Y NB cells expressing a control shRNA vs. shDRR2. Initial analysis of the results showed 1017 genes that were statistically up-regulated upon DDR2 knock-down (Qvalue < 0.05; log2 FC > 0.59), whereas 611 genes were significantly down-regulated (Qvalue < 0.05; log2 FC < -0.59). Remarkably, 34 of the top 100 genes repressed by DDR2 knockdown are adrenergic signature genes (Groningen et al. 2017) including HAND1, DBH, PHOX2B, TFAP2B, SOX11, RET, and TH suggesting DDR2 functions to support adrenergic phenotype. However, DDR2 knockdown also repressed TWIST1, ZEB1 and PDGFRA and over 25 other mesenchymal signature genes including MAGED4B, RORB, and PCOLCE. DDR2 ShRNA also inhibits neuroblastoma metastasis, and further efforts to define pathogenic functions of DDR2 are ongoing. Remarkably, analysis of single cell-seq data reveals high expression of DDR2 restricted to Schwann cell precursor (SCP) and 'bridge' cell populations co-expressing MPZ, SOX10, CDH19 and other SCP signature genes. These findings were consistently demonstrated using multiple independent data sets and patient samples (Jansky et al. 2021, Kildisiute et al. 2021, Dong et al. 2020, and https://scpca.alexslemonade.org).

Conclusions: These data contribute to our understanding of neuroblastoma tumor heterogeneity and suggest DDR2 activity in SCPs promotes proliferation of adrenergic and mesenchymal subpopulations. Additional oncogenic functions of DDR2 include activation of MAPK, integrin, and Notch signaling. DDR2 inhibition sensitizes multiple tumor types to checkpoint inhibition altering T-cell effector ratios within the tumor microenvironment. We define DDR2 as a marker of SCPs in neuroblastoma. Specifically targeting SCPs via DDR2 may limit drug resistance and metastasis while improving immune recognition and killing.

P084

Micro-environmental regulation of cancer cell fate plasticity

Andersson S[1,2,3], Chan A[4], van Nes J[4], Mohlin S[1,2,3]

[1]Division of Pediatrics, Department of Clinical Sciences, Lund University, Lund, Sweden, [2]Lund University Cancer Center, Lund University, Lund, Sweden, [3]Lund Stem Cell Center, Lund University, Lund, Sweden, [4]Department of Oncogenomics, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

Although the cause of neuroblastoma (NB) remains elusive, it is well recognized that two distinct cellular phenotypes underpin its development and progression: adrenergic (ADRN) and mesenchymal (MES). The committed ADRN phenotype is more proliferative but less motile and more sensitive to therapy, while the undifferentiated MES phenotype is less proliferative but resistant to therapy. Evidence suggests that cells are capable of interconversion between these phenotypes, i.e., a cell with a MES phenotype can adopt an ADRN phenotype and vice versa. We aim to understand how this interconversion is controlled, which contextual in vivo signals that are important, and how malignancy progress. To study this we use ADRN and MES NB cell line pairs of isogenic origin and implant them to the chick embryo at the time of neural crest cell migration. Our chick embryo model reflects human nervous system development, and provides an endogenous in vivo environmental niche allowing for cell tracing throughout terminal fetal development. We study how implanted NB cells behave and respond to environmental cues by cell tracing using fluorescent tags and species-specific probes. Our preliminary data show that NB cells respond to chick embryo-derived endogenous cues and migrate along healthy neural crest cells. Our next step is to apply single-cell spatial omics analyses to pinpoint environment-driven phenotype plasticity during in situ NB development.

P085

Deciphering the transcriptomic landscape of a neuroblastoma transgenic mouse model

Embaie B[1], Sarkar H[2], Alchahin A[1], Olsen T[1], Otte J[1], Mei S[2], Johnsen J[1], Kharchenko P[2], Baryawno N[1]
[1]Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sverige, [2]Harvard Medical School, Department of Biomedical Informatics, Boston, USA

Background
MYCN amplification is found in approximately 20% of neuroblastoma (NB), representing a critical stratifying prognostic marker. Given that MYCN plays a key role in NB tumorigenesis and aggressiveness, the TH-MYCN transgenic mouse model is widely used. This model is characterized by the overexpression of MYCN, driven by tyrosine hydroxylase (TH) promoter, giving rise to tumors exclusively in the sympathoadrenal system, reflecting human NB.

Aims
Here we aim to explore the transcriptional landscape of TH-MYCN tumors and bone marrow samples by scRNA-seq, and provide comparative analysis with murine fetal adrenal gland and human NB, to decipher the cellular identity, heterogeneity, interactive network, and clinical relevance. Harnessing the bioinformatically predicted essential interactions, we establish organoids (tumoroids).

Methods
Tumors and bone marrow from three homozygous and two hemizygous (and bone marrow from three WT) TH-MYCN mice were dissociated into single cells, profiled by scRNA-seq (10x Genomics), sequenced with Illumina NextSeq, and analyzed using Seurat. TH-MYCN transcriptomes were further aligned with previously published transcriptomes from three murine fetal adrenal glands and 19 NB patient tumors. Intercellular communication networks were inferred by CellChat. Tumoroids were established and expanded for immunofluorescence staining.

Results
scRNA-seq of TH-MYCN tumors revealed 16 clusters, spanning stromal, immune and tumor compartments. MYCN+ tumor cells predominantly resembled sympathoblasts, while MYCN+ chromaffin cells were rare. Alignment with fetal adrenal samples confirmed that TH-MYCN tumor cells resembled normal embryonic chromaffin cells and sympathoblasts. Comprehensive comparison of tumors from NB patients and TH-MYCN mice showed resemblance in adrenergic tumor cell composition. CellChat analysis showed diverse cellular communication networks. Twelve signaling pathways were common to human and mouse tumors and receptor-ligand analysis of conserved pathways unraveled targetable vulnerabilities. Notably, high expression of ITGB2-ICAM2 and NCAM1-FGFR1 were significantly correlated with poor survival in human NB. Subsequent analysis of bone marrow from matched tumor-bearing and wild type (WT) mice, revealed compositional and transcriptional shifts in the bone marrow microenvironment. Moreover, ex vivo tumoroid cultures were robust, highly proliferative and exhibited histological resemblance with the originating tumor.

Conclusion
We provide a comprehensive tumor cell atlas, which is fundamental for the therapeutic application of NB models in preclinical research.

P087

Characterization of neural crest stem-like cells and their contribution to aggressiveness

Amador-Álvarez A[1,2], Gómez-Muñoz M[1,3], Bravo-Cordero J[4], Pardal R[1,3], Vega F[1,2]

[1]Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Seville, Spain, [2]Departamento de Biología Celular, Universidad de Sevilla, Seville, Spain, [3]Departamento de Fisiología Médica y Biofísica, Universidad de Sevilla, Seville, Spain, [4]Department of Medicine, Division of Hematology and Oncology, The Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York , United States

Background/introduction:
Neuroblastomas (NBs) are heterogeneous, metastatic tumours originating from the neural crest of the sympathetic nervous system. Intratumor cellular heterogeneity has been observed, including at least two cell populations: undifferentiated stem-like mesenchymal cells and compromised adrenergic cells. The undifferentiated cell population seems to exhibit stem cell properties and has been suggested to contribute to neuroblastoma malignization by evading intensive multimodal therapy and being responsible for tumour relapses and metastasis formation, although the exact mechanism is not clear. The formation of aggressive metastases takes place thanks to a multi-stage process in which cell migration and invasion are essential. Migratory and invasive cancer cells undergo dramatic molecular and cellular changes by reshaping their cytoskeleton. These changes in cell shape can modulate cell phenotype and biological properties and are indicative of the behaviour and evolution of the cells, influencing biological processes, such as proliferation, differentiation and stem cell fate.

Aims:
To study the specific adhesive, morphological, migratory and invasive properties of neural crest stem-like cells (NCSLC), as well as their transcriptomic profiling.
Methods/Materials:
Cultures are enriched in NCSLC by tumorespheres assay in defined culture conditions and FACS sorting from NB cell lines and patient derived cells. Adhesion and spreading assay, cell morphology assay, migration analysis, membrane dynamics analysis, matrix degradation assays and RNA-seq were performed.

Results:
The results obtained show that NCSLC present defined, persistent adhesive and morphological features. These characteristics are concordant with a greater cellular plasticity. In addition, NCSLC migrate at a slower speed, but with greater directionality, exhibit increased blebs-mediated membrane actin dynamics, and degrade the extracellular matrix to a greater extent than the rest of tumour cells. NCSLC also present differentially expressed genes.

Conclusions:
These preliminary results show that NCSLC are characterized by a particular morphological, migratory and invasive behaviour, suggesting that they possess significant metastatic features calling for further analysis of their contribution to aggressiveness in vivo.

P088

DISSECTING THE ROLE OF AUTOPHAGY IN METASTATIC NEUROBLASTOMA

Galardi A[1], Lampis S[1], Di Paolo V[1], Colletti M[1], Carinci M[2], Ferraina C[1], Locatelli F[1], Nazio F[1], Di Giannatale A[1]
[1]Bambino Gesù Children's Hospital IRCCS – Department of Onco-Haematology, Gene and Cell Therapy, Rome, Italy, [2]Department of Medical Sciences, Section of Experimental Medicine, Laboratory for Technologies of Advanced Therapies, University of Ferrara, Ferrara, Italy

Background and aims: Around 70% of patients with metastatic NB at diagnosis present bone marrow (BM) infiltration, which is considered a marker of poor outcome. Although several prognostic factors have been identified in patients with NB, the mechanisms underlying this specific tropism to the BM have not been completely elucidated. Autophagy is a self-degradative process that plays a homeostatic role in normal cells by eliminating organelles, pathogens and protein aggregates. In cancer cells, autophagy plays a different role based on the context: suppresses tumorigenesis by inhibiting cancer-cell survival and inducing cell death, but it also facilitates tumorigenesis by promoting cancer-cell proliferation and tumor growth. Emerging evidences suggest that, bone metastasis can be supported by MSCs through the creation of metastatic niches and that the dialogue between tumor cells and the surrounding tumor microenvironment may be mediated by extracellular vesicles such as exosomes. The aim of this work is to evaluate the role of autophagy in the BM metastatic niche formation through ii) the analysis of autophagy-related proteins into NB cells-derived exosomes; iii) the study of the autophagic pathway in MSCs cells.

Methods: Autophagic flux has been evaluated by western blot analysis in 8 NB cell lines (2 derived from primary tumor and 5 derived from BM metastasis) and MSCs isolated from BM of NB patients with/without BM involvement and healthy control (HC-MSCs). Exosomes of NB cell lines has been isolated, characterized and analyzed at proteomic level for the presence of proteins implicated in autophagy. Modification in autophagy flux in MSCs has been evaluated after co-culture with NB-derived exosomes.

Results: Analysis of basal levels of autophagy revealed that: i) primary tumor-derived cell lines have higher basal levels of autophagy than the lines derived from BM metastases; ii) HC-MSCs have higher basal level of autophagy than MSCs derived from NB patients; iii) NB-derived exosomes contain proteins implicated in autophagy pathway regulation.

Conclusions: Our study give new insights to the characterization of the autophagy pathway in NB and in the BM niche. The understanding of this molecular process could help us to develop new therapeutic approaches in patients affected by metastatic NB.

P089

Modelling neuroblastoma using human induced pluripotent stem cells

Santopolo G[1], Hafkesbrink C[1,2], Manca E[1], Liu M[1], Wilhelm M[1], Arsenian-Henriksson M[1,3]
[1]Karolinska Institutet, Department of Microbiology, Tumor and Cell Biology, Stockholm, Sweden, [2]University of Cologne, Cologne, Germany, [3]Lund University, Department of Laboratory Medicine, Lund, Sweden

Background:
Neuroblastoma (NB) cell of origin is still debated, but it is known to arise from the neural crest cells of the trunk (tNCC) during development of the peripheric nervous system. Mutations of few genes have been associated with development of NB, where ALK and PHOX2B have been shown to be mutated in familial forms of NB.
Human induced pluripotent stem cells (hiPSCs) are somatic human cells that have been reprogrammed into pluri-potent stem cells, with the potential to generate cells from all the three germ layers. By controlling the composition of culturing medium, it is possible to direct hiPSC differentiation into a desired cell type, including tNCC and cells from the sympathoadrenal lineage. Because of these properties, hiPSCs represent a reliable model to study and characterize development of different organs and tissues, and to understand the role of gene mutations in disease development.

Aim:
We aim to generate a novel model of NB development based on hiPSCs. While most of the present models are focused on studying events associated with the late stages of disease, we aim to characterize initiation of NB development.

Methods:
Differentiation of wild type hiPSCs to sympathoadrenal progenitors. Differentiation is assessed by expression of neural crest markers by immunofluorescence and RT-qPCR. Positive cells will be sorted by FACS and analysed for neural crest markers as well as for metabolic profile.

Results:
We optimized the protocol to differentiate wild type hiPSC lines into tNCC and then further to sympathoadrenal progenitors. This has been verified by analysis of marker expression. While hiPSCs are positive for pluripotency markers NANOG, OCT4 and SSEA4, the tNCC express the trunk identity marker HOXC9 and the neural crest marker SOX10.

Conclusions:
Our approach has the advantage of identifying key steps and processes involved in NB formation. This knowledge could be used to test therapeutic targets to induce regression of NB cells or promote their differentiation. By further characterizing the differences between PHOX2B wild type and knockout hiPSCs during differentiation into sympathoadrenal cells, we intend to elucidate how deregulation of normal developmental pathways may lead to disease development.

P090

Targeted DamID identifies novel transcriptional targets of Alk signalling in Drosophila neuroendocrine cells.

Sukumar S[1], Antonydhason V[1], Molander L[1], Sandakly J[2], Umapathy G[1], Masudi T[1], Mendoza-Garcia P[1], Shirinian M[2], Palmer R[1]

[1]Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden, [2]Department of Experimental Pathology, Immunology and Microbiology, Faculty of Medicine, American University of Beirut, Beirut, Lebanon

Background:
Anaplastic lymphoma Kinase (ALK) is a receptor tyrosine kinase that is implicated in various human cancers including neuroblastoma and lung cancer. In Drosophila, Alk is expressed in the developing embryonic visceral mesoderm, CNS and at neuromuscular junctions. Numerous roles for Alk have been described in the CNS, but the molecular details are poorly understood.

Aim:
The aim of the study is to identify novel transcriptional targets of Alk signalling in CNS.

Methods:
To identify transcriptional targets of Alk signalling in the Drosophila larval CNS using the transcriptional profiling technique known as Targeted DamID (TaDa). TaDa was employed in larval CNS tissues, while simultaneously genetically manipulating Alk signalling output. We also employed immunohistochemistry and CRISPR/Cas9 technology to characterise the candidate target genes.

Results:
Analysis of TaDa datasets identified transcriptional regulation of genes expressed in neuroendocrine cell clusters by Alk. Further integration with bulk/scRNA-seq and proteomics datasets from larval brains in which Alk signalling was manipulated, identified a previously uncharacterized Drosophila neuropeptide encoded by CG4577, which we named Sparkly (Spar). To further characterize Spar, we generated anti-Spar antibodies showing that Spar is expressed in a subset of Alk-positive neuroendocrine cells in the developing larval CNS. In agreement with our TaDa analysis, inhibition of Alk signalling with a dominant-negative Alk transgene led to decreased Spar protein levels, while overexpression of the Drosophila Alk ligand Jeb resulted in increased levels of Spar protein in the larval CNS. Further in-depth analysis of spar mutant flies showed a significant reduction in life-span, and behavioral phenotypes including defects in activity, sleep and circadian rhythm. The molecular function of Spar in the context of Alk signalling in Drosophila larval CNS is being studied in detail and our recent findings will be presented.

Conclusion:
Our study suggests a novel role for Alk in regulation of endocrine function in Drosophila. These results agree with the previously reported role of Alk in the hypothalamic-pituitary-gonadal axis in mice (Witek et al., 2015) and identify spar as the first molecular target of Alk to be described in the regulation of fly activity and circadian clock.

P093

Verteporfin-induced proteotoxicity impairs neuroblastoma cell homeostasis and survival independent of YAP/TAZ expression

Taub N[1], Condurat A[2], Ferlemann F[2], Menon V[2], Thomas R[2], Malnar M[1], Opitz L[1], Aminzadeh-Gohari S[3], Kofler B[3], Pruszak J[1]

[1]Institute of Anatomy and Cell Biology, Paracelsus Medical University (PMU), Salzburg, Austria, [2]Former association: Institute of Anatomy & Cell Biology, University of Freiburg, Freiburg, Germany , [3]Research Program for Receptor Biochemistry and Tumor Metabolism, Department of Pediatrics, University Hospital of the Paracelsus Medical University, Salzburg, Austria

Background/Introduction

Neuroblastoma (NB) is the most common extra-cranial solid tumor in children. Originating from the embryonic neural crest, this highly-aggressive tumor remains a therapeutic challenge.

Aims

Our research exploits differential expression patterns of cluster-of-differentiation (CD) antigens to better resolve NB heterogeneity for phenotypic study and pharmacological in vitro assays (see Ferlemann et al., 2017). The Hippo effectors YAP/TAZ are among the oncogenic factors associated with NB tumorigenesis. Verteporfin (VPF), a clinically approved ophthalmologic drug, was shown to directly inhibit YAP/TAZ activity. Our present study aims to investigate VPF's potential as a therapeutic agent in NB.

Methods/Materials

Using a range of established NB cell lines (GI-ME-N, SK-N-AS, BE(2)-M17, IMR-32) and normal fetal fibroblasts, we determined the cell viability of YAP-positive versus YAP-negative NB subtypes by crystal violet staining after VPF treatment (5 and 10 µM; 3DIV). VPF's impact (5 µM, 24h) on the phosphorylation profiles in GI-ME-N cells was identified by using phospho-kinase arrays (R&D Systems) and immunoblots. Accumulation of proteotoxic high molecular weight (HMW) complexes was determined after VPF treatment (5 µM; 30', 1h). In vivo effects of VPF (100 mg/kg) were analyzed by using a well-established NB xenograft model (n=7).

Results

We demonstrate the utility of flow cytometric readout for small molecule screens towards identifying differential responsiveness of NB subpopulations to pharmacological candidates. We show that VPF selectively and efficiently impairs the cell viability of YAP/TAZ-expressing NB cells, such as GI-ME-N and SK-N-AS cells, but not of normal fibroblasts. Interestingly, VPF-mediated NB cell killing is independent of YAP expression confirmed by using CRISPR-mediated YAP/TAZ knock-out GI-ME-N cells, as well as YAP-negative/-low BE(2)-M17 NB cells. Additionally, VPF exerts a suppressive effect on tumor growth in NB in vivo. Furthermore, we demonstrate that the formation of HMW oligomeric complexes involving STAT3, GM130 and COX IV proteins is an early mechanism of VPF-induced cell death, triggering cellular stress and impairing homeostasis.

Summary/Conclusions

Our study shows that VPF acts independently of YAP/TAZ as a potent suppressor of NB growth in vitro and in vivo, making VPF a potential therapeutic candidate. Moreover, our work further elucidates NB surface antigen expression dynamics and subpopulation profiles.

P094

Single-cell Multi-omics Identifies Dynamic Cancer Cell State Reprogramming and Immune Environment Dysfunction in Human Metastatic Neuroblastoma

Wu N[1], Weiss B[2], von Allmen D[3], Belcher K[1], Ferrell J[1], Rao R[1], Adam M[4]

[1]Division of Experimental Hematology and Cancer Biology, CBDI, Brain Tumor Center, Cincinnati Children's Hospital Medical Center , Cincinnati, United States, [2]Division of Oncology, CBDI, Neuroblastoma Program, Cincinnati Children's Hospital Medical Center , Cincinnati, United States, [3]Department of Surgery, Cincinnati Children's Hospital Medical Center, CINCINNATI, United States, [4]Department of Developmental Biology, Cincinnati Children's Hospital Medical Center, CINCINNATI, United States

Background: Neuroblastoma (NB) – originating from the sympathetic nervous system derived from the neural crest - is the most common, deadly extracranial childhood tumor and accounts for 15% of all pediatric cancer deaths. Despite aggressive multimodal therapy, patients with high-risk NB only have about a 50-60% survival rate. Novel effective treatments for NB patients are urgently needed.

Aims: Nearly half of neuroblastoma patients have metastatic (stage M) disease at diagnosis. Metastasis at diagnosis and relapse remains a major challenge in NB therapy and results in poor patient outcome. Molecular events driving NB metastasis are incompletely understood. We hypothesize that a better understanding of the cellular heterogeneity, tumor-initiating cells and the tumor ecosystem complexity in primary and metastatic NB at a single-cell level will facilitate identifying molecular and cellular determinants underlying metastatic triggers and defining potential therapeutic opportunities, including immune-based therapies against this deadly disease.

Methods and Results: By single-cell transcriptome and chromatin accessibility profiling comparing cellular diversity in a cohort of high-risk primary and metastatic NB, we uncover a dynamic acquisition of discrete metastatic cancer cell states. Integrative multi-omic profiling further identifies unique transcriptional regulatory networks relevant to embryogenic or epithelial-to-mesenchymal transition (EMT) programs governing transition to metastatic cell states. In addition, our metastatic high-risk NB cohort displayed enriched metabolism-related and migratory mesenchymal gene signatures. Furthermore, we identify that the immune milieu of high-risk metastatic NB is predominately characterized by accumulation of immunosuppressive myeloid subsets and exhausted progenitor T cells, whereas primary NB harbor both anti-tumor and pro-tumor immune cells post therapy. We identify features of immune cell dysfunction in metastatic NB.

Conclusions: In summary, our studies using single-cell multi-omics profiling of metastatic NB identifies tumor cellular heterogeneity and immunoregulatory interactions, which may inform potential targets for immunotherapy in high-risk NB.

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Cohesin-mediated 3D genome structure defines Neuroblastoma cell subtypes and their plasticity.

Kang J[1], Liu Z[1], Sun M, Thiele C[1]
[1]*Pediatric Oncology Branch, National Cancer Institute, Bethesda, United States*

BACKGROUND
Neuroblastoma is highly heterogenous with different clinical and molecular cell subtypes, which are marked by distinct epigenetic profiles with distinct 3D genome organization defined by the interaction of their super-enhancers and promoters of their core regulatory circuitry (CRC) transcription factors. In addition to its role in DNA replication and damage, Cohesin (subunits; SMC1a, SMC3, RAD21, STAG1/STAG2) mediates the looping structure between promoters and distal enhancers to regulate target gene expression.

AIM
Our study aims to evaluate the role of the cohesin in the control of cell-type specific super-enhancer networks in neuroblastoma and their contribution to differentiation and neuroblastoma subtype plasticity.

METHODS AND RESULTS
Data mining using R2 database (SEQC-498 data), all cohesin subunits were significantly over-expressed in NB tumors compared to neural crest cells or adrenal gland and were significantly associated with poor event-free/overall survival. Studies evaluating H3K27Ac have shown distinct super-enhancer landscapes between isogenic MES/ADRN cell types. We performed ChIP-seq for Cohesin components Rad21, SMC1a and H3K27Ac in NB MES/ADRN isogenic pairs. Our RAD21, SMC1a ChIP-seq data show the cohesin binding pattern between two cell subtypes is very distinct, with cell type specific enhancers and promoters bound by cohesin subunits. Homer de novo motif scan of cohesin binding site in MES cell line identified well known MES specific CRC TF, RUNX2 and FOSL2, as highest ranked motif, whereas Homer de novo motif scan of cohesin binding site in ADRN cell line identified well known ADRN specific CRC TF, TWIST, ISL1 and GATA3, as highest ranked motif. We used two different siRNAs to assess whether cohesin regulates cell type specific gene expression and identified that MES specific genes were differentially expressed after RAD21 knockdown in MES cells. These results indicate the possibility of cohesin bound at cell type specific enhancer-promoter regions regulating its target genes through enhancer-promoter looping.

CONCLUSION
In this study we are defining the molecular mechanisms underlying regulation of 3D genome structure in phenotypically distinct isogenic NB cells. This is crucial for understanding how neuroblastoma cell types acquire and stabilize their cellular identity and the basic mechanisms involved in cell state changes during therapeutic interventions and differentiation.

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Adrenal medulla development markers as a tool for improved patient risk stratification: elucidation of the neuroblastoma clinical heterogeneity

Sourada L[1,2], Neradil J[1,2,3], Polaskova K[3], Sterba J[2,3], Veselska R[1,2], Skoda J[1,2]

[1]Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic, [2]International Clinical Research Center, St. Anne's University Hospital, Brno, Czech Republic, [3]Department of Pediatric Oncology, University Hospital Brno and School of Medicine, Masaryk University, Brno, Czech Republic

Background:
Although the exact cellular origin of neuroblastoma has not been elucidated yet, recent single-cell transcriptomics studies have suggested a possible role of particular cell populations in the differentiation pathways from neural crest to adrenal medulla (i.e., bridge cells or Schwann cell precursors, SCPs). Identification of novel biomarkers that would better reflect the different etiology of neuroblastoma among individuals may improve patient risk stratification.

Aims and Methods:
Here, we aimed to investigate whether neuroblastoma cellular heterogeneity could be pinpointed with the use of markers of different cell states identified in the developing adrenal medulla. In a panel of 14 patient-derived and 6 established neuroblastoma cell lines, we analyzed protein expression of >20 selected markers, including several receptors (HTR3A, ERBB3, NTRK2), stemness markers (SOX2, c-MYC, CD133, NES) and differentiation markers (ASCL1, TH, CHGA, ISL1, PRPH). Expression profiles were correlated with cancer stem-like traits assessed by sphere formation assay and with the response to the HTR3 receptor agonist, N-methylquipazine.

Results:
Expression screening revealed marked differences in the expression of HTR3A and ASCL1 (markers of bridge cells), ERBB3 (SCP marker), and PRPH (marker of sympathoblasts). High levels of ASCL1, HTR3A and PRPH were characteristic for high-risk patients, while ERBB3 was predominantly detected in cell lines derived from low-risk neuroblastomas and negatively correlated with patient age. Moreover, ERBB3-high cells showed reduced cancer stem-like traits, as demonstrated by impaired sphere formation capacity. Identified expression signatures also corresponded with sensitivity to modulation of the serotonin signaling using N-methylquipazine, which significantly suppressed growth solely in the HTR3A-overexpressing cell lines.

Conclusion:
Together, our results pointed to a presence of two distinct expression signatures associated with different aggressiveness of neuroblastoma cell lines, which may correspond with their cellular origin and potentially reflect different neuroblastoma subtypes. Our research suggests ERBB3, ASCL1, HTR3A and PRPH as candidates for a large-scale study to validate their potential in patient risk stratification that is crucial for improving neuroblastoma therapy.

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Subtyping and diagnostic classification of neuroblastic tumours using multi-scale transcriptomics

Cohen-gogo S[1,2], Comitani F[2], Nash J[2], Chang Schwertschkow A[2], Wen T[2], Maheshwari A[2], Brunga L[2], Malkin D[1,2], Villani A[1], Irwin M[1], Shlien A[2,3]

[1]Division of Hematology/Oncology, The Hospital for Sick Children, Toronto, Canada, [2]Genetics and Genome Biology, The Hospital for Sick Children Research Institute, Toronto, Canada, [3]Department of Pediatric Laboratory Medicine, The Hospital for Sick Children, Toronto, Canada

Background: The transcriptional diversity between and within pediatric tumour types can be leveraged to improve diagnostic and prognostic tools and potentially identifying subgroups of tumours

Aims/Methods: To develop transcriptional definitions of cancers with the potential to subclassify tumour types, we designed a scale-adaptive unsupervised method (RACCOON - Resolution-Adaptive Coarse-to-fine Clusters OptimizatiON), which groups samples into hierarchically organized clusters. We used this approach on a dataset of 2,178 pediatric tumours (including 180 neuroblastomas), 9,400 adult tumours, and 1,735 non-neoplastic samples. We then trained an ensemble of convolutional neural networks to classify tumours to these transcriptional clusters with the OTTER tool (Oncologic TranscripTome Expression Recognition).

Results: Applying this method to 13,313 transcriptomes, RACCOON yielded a hierarchy of 455 tumour and normal classes. RACCOON identified clusters for most major types of pediatric cancers including 4 subtypes of neuroblastoma. These subtypes, which overlap with previously reported clusters, have significant differences in immune activity, differentiation level and patient survival. The prognostic impact of these subtypes is independent of COG risk group and stage. Named based on the expression of previously established marker genes, ERBB2 (T062), NTRK1 (T063), MYCN (T064) and TERT (T065), these subtypes may be rooted in the tumour's lineage. The ERBB2-overexpressing subtype is highly differentiated, with high immune activity, and reflects a neural crest cell/mesenchymal identity. Conversely, the TERT subtype is associated with a sympathoadrenal identity and has the highest level of stemness. Notably, 25% of cases in T064 were not previously identified as MYCN amplified by standard testing, but still maintained significant enrichment of downstream MYCN-dependent pathways. Temporal analyses demonstrated that unlike most pediatric tumours, the majority of neuroblastoma tumours were comprised of more than one transcriptionally identified subtype, suggesting that our algorithms can identify plasticity and heterogeneity.

Conclusion: RNA-seq is a promising tool for both subtype discovery and subclassifying neuroblastoma in patients based on expression. Further studies will be aimed at determining the prognostic impact in well characterized homogeneously treated patient cohorts.

P098

Tumor-associated macrophages and mesenchymal stromal cells modulate adrenergic to mesenchymal state transition in neuroblastoma

Lee M[1], Louault K[1], Giannikou K[1], Ding X[2], Yanovsky A[1], Park J[1,3], DeClerck Y[1,3], Asgharzadeh S[1,3]
[1]Children's Hospital Los Angeles, Cancer and Blood Diseases Institute, Department of Pediatrics , Los Angeles, United States, [2]Children's Hospital Los Angeles, The Saban Research Institute, Los Angeles, United States, [3]University of Southern California, Keck School of Medicine , Los Angeles, United States

Background
Neuroblastoma (NB), a common solid tumor of children, can adopt a lineage-committed adrenergic (ADRN) or an immature mesenchymal (MES) tumor cell type, which differs in phenotype, core regulatory circuitries, and differential responses to therapy. These cell types can spontaneously interconvert, but the contribution of tumor microenvironment (TME) to this mechanism remains largely unknown. In this study, we focused on the role of tumor-associated macrophages (TAMs) and mesenchymal stromal cells that convert to cancer-associated fibroblasts (CAF) in NB TME to ADRN-MES lineage transition (AMT).
Aims
To evaluate whether TAMs and CAF contribute to pro-tumorigenic factors production and drive AMT in neuroblastoma and pathways involved in this transition.

Methods
The ADRN and MES lineage identity of NB cell lines were assessed by either RNA sequencing (RNAseq) or single cell RNAseq (scRNAseq), Western blot, and immunocytochemistry comparing NB cell lines co-cultured with and without TAM+CAF or monocytes (MO)+CAF in transwell culture system. TAMs were generated by transwell co-culture of MO with NB cell lines for five days before using in the experiments.

Results
Initial experiments demonstrated morphologic changes in TAM-CAF co-cultures NB cell lines with increased spreading area and spindle shape compared to controls. As compared to control cells, MO+CAF or TAM+CAF co-culture induced the MES lineage markers SOX9 and NOTCH1 of NB cell lines and reduced ADRN lineage markers PHOX2B, and GATA3. scRNAseq studies conducted on two cell lines exposed to MO+CAF or TAM+CAF showed enrichment of MES signature over time in both conditions providing clear evidence AMT of NB cells. Enrichment of pathways involving TGFβ, extracellular matrix deposition, epithelial to mesenchymal transition (EMT), and JAK pathway were observed in all experimental conditions. Treatment of Galunisertib (TGFβR1 inhibitor) and Ruxolitinib (JAK inhibitor) inhibited SOX9 and NOTCH1 expression respectively and enhanced ADRN lineage markers expression in NB-TAM-CAF co-culture experiment.

Conclusions
The presence of TAM and CAF in the TME drives neuroblastoma AMT through at least two independent signaling pathways involving TGFβ and JAK-STAT3, and it is akin to that observed in EMT in adult tumors. Our studies point to a therapeutic vulnerability by targeting cells and pathways activated in the NB-TAM-CAF axis.

P099

Single-cell multiomic investigation of chromosomal alterations in normal adrenal development, neuroblastoma, and pheochromocytoma

Schloo C[1,4], Wecht E[1], Habibolahi F[2], Herrmann C[2], Bechmann N[3], Westermann F[1]

[1]Division of Neuroblastoma Genomics, German Cancer Research Center (DKFZ) and Hopp-Children's Comprehensive Cancer Center at the NCT (KiTZ), Heidelberg, Germany, [2]Biomedical Genomics Group - Health Data Science Unit, Medical Faculty Heidelberg University, Heidelberg, Germany, [3]Department of Internal Medicine III & Institute for Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus Dresden, Technische Universität Dresden, Dresden, Germany, [4]Faculty of Biosciences, Heidelberg University, Heidelberg , Germany

Background

Many chromosomal alterations are involved in neuroblastoma tumourigenesis, such as amplifications and translocations, but how 3D chromatin conformation contributes to these phenomena is not fully understood. Neuroblasts, a putative cell-of-origin for neuroblastoma, are a transient cell population derived from the neural crest that may be uniquely vulnerable to such events. Moreover, the timing of these events across development may be an explanatory factor underlying the heterogeneity that neuroblastomas present in clinical outcome.

Aims

This project aims to address questions regarding neuroblastoma tumourigenesis, such as which chromatin architectures may exhibit increased vulnerability to enhancer hijacking and other oncogenic events. In addition, we intend to describe the changes that occur in the chromatin landscape across normal adrenal development, and investigate how these aspects differ among neuroblastoma, pheochromocytoma, and normal developing adrenal tissue.

Methods

This study utilized snRNA-seq of normal developing adrenal glands (n = 17), sympathetic ganglia (n = 5), and adult adrenal glands (n = 3), as well as neuroblastomas (n = 36) and pheochromocytomas and paragangliomas (n = 17). Additionally, multiomic analysis (snRNA/ATAC-seq) will be used for developing adrenal (n = 2) and neuroblastoma (n = 2) samples, with plans to expand the cohort. In addition to the snATAC-seq, scHiC will be utilized to explore chromatin conformational changes. The tumour microenvironment interactions will also be investigated through receptor-ligand pair predictions and spatial transcriptomics.

Results

snRNA-seq analyses have revealed that neuroblastoma transcriptomically resembles neuroblasts, and that its severity correlates with an immature phenotype. In contrast, pheochromocytomas demonstrate transcriptional similarity to chromaffin cells, with the more aggressive pseudohypoxic subtype resembling more immature cells. While fetal neuroblasts and Schwann cell precursors are similar regardless of location in the adrenal medulla or sympathetic ganglia, the sympathetic ganglia show a higher percentage of neuroblasts at 10-11 pcw, which declines thereafter.

Conclusion

Chromosomal alterations are key drivers in neuroblastoma, and characterizing how chromatin conformation allows these events to occur will shape our understanding of tumourigenesis. Furthermore, the description of the chromatin landscape throughout normal adrenal development may be particularly insightful for identifying key developmental timepoints and cell lineages that display increased vulnerability to oncogenic events.

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The Role of ASCL1 and Its Interactors in Neuroblastoma

Mykhaylechko L[1], Woods L[1], Gomez R[2,3], Ramachandran R[2,3], Ali F[2,3], Carroll J[4], Philpott A[1]

[1]Wellcome-MRC Cambridge Stem Cell Institute, University of Cambridge , Cambridge, United Kingdom, [2]Centre for Genomic Discovery, Mohammed Bin Rashid University of Medicine and Health Sciences, Dubai, United Arab Emirates, [3]Al Jalila Genomics Centre, Al Jalila Children's Hospital, Dubai, United Arab Emirates, [4]Cancer Research UK Cambridge Institute, University of Cambridge, Cambridge, United Kingdom

Background

Neuroblastoma (NB) arises due to incomplete differentiation of sympathoadrenal cells during development, and is maintained by either an adrenergic or mesenchymal core transcriptional regulatory circuitry (CRC). Differentiation therapies are an attractive alternative therapeutic approach to the current first-line treatments. However, we still do not fully understand how to modulate NB cell behaviour to limit proliferation and potentiate differentiation.

Achaete-scute complex-like 1 (ASCL1) is a transcription factor required for both progenitor maintenance and neuronal differentiation in normal sympathoadrenal development. In NB, ASCL1 can also play a dual role. As a component of the adrenergic CRC, ASCL1 supports cell proliferation, while promoting differentiation and cell-cycle exit on ASCL1 over-expression. The mechanism behind these two functions is not fully understood but is likely to involve ASCL1 interactors regulating its transcriptional activity on chromatin.

Aims

Our goal was to, for the first time, identify proteins associated with ASCL1 in two adrenergic NB cell lines. Further, we aimed to investigate the role of ASCL1 interactors in driving NB differentiation.

Methods

IMR-32 and SK-N-BE(2)C NB cell lines were lentivirally transduced to overexpress ASCL1 in a doxycycline-inducible manner. After ASCL1 induction, RNA and ASCL1 ChIP sequencing was performed to investigate the ASCL1-driven transcriptional regulation. In addition, analysis of the ASCL1 interactors across these cell lines was performed using quantitative multiplexed rapid immunoprecipitation mass spectrometry of endogenous proteins (qPLEX-RIME).

Results

We found that ASCL1 overexpression inhibited proliferation and induced morphological and transcriptional features of neuronal differentiation to a differing extent in each cell line, with IMR-32 cells showing more responsiveness. In IMR-32 cells, ASCL1 associated more with proteins known to positively impact neuronal differentiation during development, such as BCL11B, MEIS1, PBX1, PHOX2B, SOX11, and others, whereas in SK-N-BE(2)C, ASCL1 associated more with proteins involved in cell-cycle regulation, such as CDKs and cyclins, in particular CDK2 and Cyclin A2.

Conclusion

This work suggests ASCL1 interactors could help determine whether this key transcriptional regulator promotes cell proliferation or differentiation of NB cells. We are currently exploring specific interactors from this large data-set, allowing us to target modulation of ASCL1-mediated differentiation to improve therapeutic options for this devastating disease.

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Chromaffin-to-neuroblast cell state transitions drive tumor plasticity in NF1 and KIF1Bb deficient neuroblastoma, pheochromocytoma and composite tumors.

Cui P[1], Li W[1], Plescher M[1], Bullova P[1], Dalarun B[1], Fell S[1], Kogner P[4], Larsson C[2], Tischler A[3], Juhlin C[2], Bedoya Reina O[1], Schlisio S[1]

[1]Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden, [2]Department of Oncology-Pathology, Karolinska Institutet, Karolinska University Hospital-Solna, Stockholm, Sweden, [3]Department of Pathology and Laboratory Medicine, Tufts Medical Center, Tufts University School of Medicine, Boston, United States, [4]Childhood Cancer Research Unit, Department of Women's and Children's Health, Karolinska Institutet, , Stockholm, Sweden

Background:
Intratumor heterogeneity and high-plasticity accounting for therapy resistance and poor clinical outcome in neuroblastoma (NB) and paraganglioma (PPGL). Causes of plasticity during tumor progression remain poorly understood.

Aims:
Here, we profile tumor cell state transitions of genetically engineered mouse sympatho-adrenal tumors at several stages, from embryonic, pre-neoplastic hyperplasia to pheochromocytoma, neuroblastoma and composite tumors.

Methods:
We have generated conditional gene targeting of KIF1B tumor suppressor gene in the mouse sympatho-adrenal system and generated crosses with mice expressing Cre-recombinase under the control of the gene for dopamine beta hydroxylase (DBH-Cre). The resulting mice we crossed with NF1fl/fl mice to generate double knockout (referred as DKO). We combined H&E staining, immunofluorescence, in situ hybridization (RNAscope) and single-cell transcriptome analysis combined with RNA velocity to explore Chromaffin-to-neuroblast tumor state transitions.

Results:
Loss of KIF1Bβ potentiated the oncogenic activity of NF1 loss causing the development of large, bulky and locally invasive masses that arose in the adrenals. Histopathology revealed pheochromocytoma, neuroblastoma and composite tumours in aged mice. We observed abundant embryonic neuroblast hyperplasia in mutant embryonic medulla that transit to a neuroplastic chromaffin state postnatally. We found chromaffin tumor cells obtain a neuroblastic feature postnatally (3 month or older) and continue to form NB, PCC and composite tumors of both types. Meanwhile, these tumors have a remarkable heterogeneity. In early tumor development at 3-month-old, a distinctive three segment structure has been found breaking through the cortex from medulla, which shows the transitional state of chromaffin cells to neuroblasts. We further validate these transitions in human PPGL and NB. The transitions are consistent with the single-cell RNA velocity prediction.

Conclusions:
Deep single cell RNA sequencing combined with immunohistochemistry and RNA scope revealed chromaffin-neuroblast cell state transitions at embryonic and postnatal stages driving tumour plasticity. Cancer cells progressively adopt neuroblast lineage identity, computationally predicted to be mediated

through a common chromaffin- neuroblast transitional, high-plasticity cell state. Such newly discovered lineage transitions suggest important implications for understanding neuroblastoma and pheochromocytoma heterogeneity.

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A key role for HIF2α in determining differentiation potential and a noradrenergic cellular state in neuroblastom

Yuan J[1,2], Bedoya-Reina O[2], Liu M[2], Toskas K[1], Şafak Demirel İ[1], Li W[2], Shi Y[1], Konger P[3], Schlisio S[2], Holmberg J[1]
[1]Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden, [2]Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden, [3]Department of Women's and Children's Health, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden

Background
Neuroblastoma arises within the sympathetic nervous system and is the most frequent extra cranial solid childhood cancer. It exhibits a high degree of clinical heterogeneity also presents itself with considerable intratumor heterogeneity of which sympathetic noradrenergic (NORAD) and mesenchymal (MES) cells are two identified entities. The hypoxia inducible factor, EPAS1/HIF2a, has been proposed to be NB oncogene. However, several studies challenge this concept and high expression levels of EPAS1 is associated with increased survival of neuroblastoma patients.

Aims
We aim to, in an unbiased manner, determine the functional role of HIF2α in NB, which is essential prior to target HIF2α in the clinic which has been suggested.
Methods
We have performed analysis of single cell sequenced neuroblastoma and initiated gain-and loss-of-function experiments in neuroblastoma cells.

Results
Our analysis of single cell sequenced human neuroblastoma tumors revealed that EPAS1 is significantly enriched in cells from low-risk tumors, but lower in neuroblastoma cells expressing markers defining mesenchymal cellular state. Crispr/Cas9 depletion of EPAS1 in SK-N-SH cells which harbor both MES and NORAD populations, resulted in rapid loss of PHOX2B and PHOX2A. Besides, depletion of EPAS1 in SH-SY5Y cells, which is NORAD population of SK-N-SH cells, leaded to a significant loss of key NORAD transcription factors such as PHOX2B, NEFL, but an enrichment of MES factors such as VIM, DESMIN and DACH1 upon RA treatment. Conversely, overexpression of EPAS1 in SH-EP2 neuroblastoma cells that only harbor the MES population,resulted in reduced YAP1 expressionand altered morphology. More interesting, overexpression of EPAS1 in NORAD state of LAN-1 cells resulted in the induction of both TH and DBH, a significant reduced in vivo xenograft tumor sizes and weights. In both SH-EP2 and LAN-1 cells, overexpression of EPAS1 caused reduction of proliferation, a down regulation of MYCN, and a less migration and invasion capacity in vitro. This implies that high EPAS1/HIF2a levels are required to maintain the NORAD cellular state and that transition to a MES cellular states is associated with reduced EPAS1/HIF2a levels.

Conclusion
Our preliminary data suggested a novel role for EPAS1/HIF2a as a determining factor of neuroblastoma differentiation potential and heterogeneity.

P103

Chromaffin-to-neuroblast cell state transitions drive tumor plasticity in NF1 and KIF1Bb deficient neuroblastoma, pheochromocytoma and composite tumors

Cui P[1], Li W[1], Plescher M[1], Bullova P[1], Fell S[1], Dalerun B[1], Kogner P[2], Larsson C[2], Tischler A[3], Juhlin C[2], Reina O[1], Schlisio S[1]

[1]Karolinska Institutet, Department of Microbiology, Tumor and Cell Biology, Stockholm, Sweden, [2]Karolinska Institutet, Karolinska University Hospital-Solna, Department of Oncology-Pathology, Stockholm, Sweden, [3]Tufts University School of Medicine, Tufts Medical Center, Department of Pathology and Laboratory Medicine, Boston, United States

Introduction:
Intratumor heterogeneity and high-plasticity accounting for therapy resistance and poor clinical outcome in neuroblastoma (NB) and paraganglioma (PPGL). Causes of plasticity during tumor progression remain poorly understood.

Aims:
Here, we profile tumor cell state transitions of genetically engineered mouse sympatho-adrenal tumors at several stages, from embryonic, pre-neoplastic hyperplasia to pheochromocytoma (PCC), neuroblastoma and composite tumors.

Methods:
We have generated conditional gene target of KIF1Bb (tumor suppressor gene) in the mouse sympatho-adrenal system, and crossed this mouse line with mice expressing Cre-recombinase under the control of the gene for dopamine beta hydroxylase (DBH-Cre). The offspring we crossed with NF1fl/fl mice to generate double knockout (referred as DKO) mice. H&E staining, immunofluorescence, in situ hybridization (RNAscope) and single-cell transcriptome analysis have been combined with RNA velocity to explore Chromaffin-to-neuroblast tumor state transitions.

Results:
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Conclusions:
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transitions suggest important implications for understanding neuroblastoma and pheochromocytoma heterogeneity.

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NTRK1/TrkA-induced nuclear reorganisation and abrogation of checkpoint responses are critical determinants of differentiation and proliferation in neuroblastoma cells

Janetzko J[1], Soni A[2], Funke L[3], Bracht T[3], Sitek B[3], Hassiepen C[1], Oeck S[1], Iliakis G[2], Schramm A[1]
[1]University of Duisburg-Essen, University Hospital Essen, Dept. of Medical Oncology, Essen, Germany, [2]University of Duisburg-Essen, Institute of Medical Radiation Biology, Essen, Germany, [3]Ruhr-University Bochum, Medical Faculty, Medizinisches Proteom-Center, Bochum, Germany

The physiological functions of NTRKs include regulating migration of developing neurons and facilitating their survival and differentiation. These capabilities can be hijacked by neuroblastoma to enable sustained proliferation and therapy resistance of tumor cells. While the canonical pathways fueled by NTRK1/TrkA activation are known for decades, the critical targets that convert signals downstream of NTRK1/TrkA into cellular responses of neuroblastoma cells are less well understood. We have previously shown that activation of NTRK1 by its ligand, NGF, upregulates differentiation markers and increases the interaction of neuroblastoma cells with immune cells and Schwann cells. Using data from mRNA profiling and combined proteomics / phosphoproteomics screens of preclinical models of neuroblastoma harboring inducible NTRK1 expression, we here report that the cell-autonomous effects induced upon NTRK1-activation by NGF involve nuclear reorganization processes guided by lamin A/C and stathmin 1 phosphorylation. These structural changes were accompanied by induction of CDC25B upon NTRK1 activation in both, MYCN-normal and MYCN-amplified cells. CDC25B expression and nuclear re-organisation affecting tubulin polymerization could contribute to the quite paradoxical enhanced proliferation that is preceding differentiation e.g. in SK-N-AS cells with inducible NTRK1 expression. Mechanistically, NTRK1 activation was able to overcome the G2/M checkpoint even in the presence of genotoxic stress such as ionizing radiation (IR). Abrogation of IR-induced G2 block by NTRK1/TrkA was epistatic to ATM or ATR inhibition, while small molecule-mediated inhibition of NTRK1/TrkA by Loxo-101 rescued the IR-induced G2 checkpoint. These effects were NTRK1-specific and not a general phenomenon of receptor tyrosine kinase-mediated IR response, as EGFR activation in lung cancer cell lines did not affect cell cycle distribution upon IR. Moreover, MYCN-normal cells did not induce or activate CHK1 upon combined NTRK1 activation and IR. This is in sharp contrast to MYCN-amplified cells, which strictly rely on CHK1 activation to prevent mitotic catastrophe even in the absence of IR. Our results help to better understand the multifaceted effects of NTRK1 activation on nuclear reorganization preceding proliferation and differentiation of neuroblastoma cells.

P105

Hypoxia induces expression of periostin and chemotherapy-specific resistance in SH-SY-5Y cells.

Karapiperi K[1], Bayliss R[2], Elliott M[3], Burchill S[1]

[1]Children's Cancer Research Group, Leeds Institute of Medical Research, St. James's University Hospital, Leeds, United Kingdom, [2]Astbury Center for Structural Molecular Biology, School of Molecular and Cellular Biology, University of Leeds, Leeds, United Kingdom, [3]Leeds Teaching Hospitals, NHS Trust, Leeds, United Kingdom

Background

The presence of neuroblastoma cells in the bone marrow (BM) is a poor prognostic indicator for some children, the BM providing a haven for drug resistant metastatic cells. We have recently identified neuroblastoma cells with an immature mesenchymal phenotype in the BM. Since mesenchymal neuroblastoma cells are reported to be resistant to chemotherapy we have examined the hypothesis that hypoxia in the BM may induce a mesenchymal phenotype and resistance to treatment.

Aims

To examine the effects of hypoxia on response to chemotherapies and expression of the mesenchymal marker periostin in SH-SY5Y and SHEP-1 cells.

Methods

SH-SY5Y and SHEP-1 cells were cultured in normoxia (O2 95%;CO2 5%) and hypoxia (O2 1%;CO2 5%) for 24h. Cells were treated with common chemotherapeutics used in the management of children for 48-96h. Cells were harvested and cell viability measured using the trypan blue exclusion assay. Cytospins were prepared and cells immunostained for the mesenchymal marker periostin (Biotech 66491-1-Ig).

Results

The effect of hypoxia on response to chemotherapeutics was drug specific. Hypoxia significantly decreased the sensitivity of both cell lines to doxorubicin (EC50_normoxia_SH-SY5Y 0.038uM vs EC50_hypoxia_SHSY5Y 0.046uM; EC50_normoxia_SHEP-1 0.058uM vs EC50_hypoxia_SHEP-1 0.25uM; $p<0.005$), etoposide (EC50_normoxia_SH-SY5Y 0.65uM vs EC50_hypoxia_SH-SY5Y 1.15uM, $p<0.0001$; EC50_normoxia_SHEP-1 4.67uM vs EC50_hypoxia_SHEP-1 unable to calculate, $p<0.0001$) and vincristine (EC50_normoxia_SH-SY5Y 0.046uM vs EC50_hypoxia_SH-SY5Y 0.075uM, $p=0.03$; EC50_normoxia_SHEP-1 0.79uM vs EC50_hypoxia_SHEP-1 unable to calculate, $p<0.05$). Hypoxia had no effect on the response of SH-SY5Y and SHEP-1 cells to cisplatin or carboplatin ($p >0.05$). Expression of periostin was lower in SY-SY5Y cells than in SHEP-1 cells (Hscore_normoxia_SH-SY5Y 45 vs Hscore_normoxia_SHEP-1 280; $p=0.001$). Hypoxia had no effect on periostin expression in the SHEP-1 cells (mean Hscore_normoxia 280 vs mean Hscore_hypoxia 275; $p=0.6$), although this was significantly increased in SH-SY5Y cells following 92h in hypoxia (mean Hscore_normoxia 45 vs mean Hscore_hypoxia 276; $p=0.0007$).

Conclusion

Hypoxia induced resistance to doxorubicin, etoposide and vincristine in both SH-SY5Y and SHEP-1 cells. This was associated with an increased expression of periostin in adrenergic SH-SY5Y cells, but not in the SHEP-1 cells. Hypoxic areas of the BM microenvironment may play a role in the induction of a mesenchymal phenotype and resistance to treatment.

P106

Optimization of Bulk RNA-seq Deconvolution of Serial Samples from Patients with High Risk Neuroblastoma (HR-NBL) Using Single Cell RNA-seq (scRNAseq)

Pastor S[1], Berko E[2], Mosse Y[2], Maris J[2]

[1]Children's Hopsital of Philadelphia, Department of Biomedical and Health Informatics, Philadelphia, United States, [2]Children's Hopsital of Philadelphia, Cancer Center, Philadelphia, United States

Background

Neuroblastomas exhibit evolving genetic heterogeneity during therapy and at relapse. To study transcriptional changes that occur from diagnosis throughout treatment, we performed RNA-seq on serial patient samples from the Next Generation Personalized Neuroblastoma Therapy (NEPENTHE) clinical trial cohort (NCT02780128). Given the heterogeneity of biopsy sites, sample quality, and often low tumor purity, we sought to utilize a recently published single cell dataset from neuroblastoma to deconvolve bulk RNA-Seq data and provide superior assessment of transcriptional changes.

Aims

To optimize the performance of two single cell deconvolution algorithms, MuSiC and CIBERSORTx, and apply these optimized algorithms to identify cell types associated with clinical features in the NEPENTHE cohort.

Methods

Simulated bulk RNA-seq data were generated from the scRNA-seq dataset from Jansky and colleagues (Nature Genetics, 2021) consisting of 17 HR adrenal NBL samples. Per sample, cells were merged producing counts with known cell type proportions. The remaining 16 samples were retained to deconvolve left-out samples. Cell type estimations were evaluated by comparing the spread (mean absolute deviation, mAD) and correlation (R) of predicted to actual cell types. Parameters reducing spread and increasing correlation were used in MuSiC and CIBERSORTx to deconvolve bulk RNA-seq data from 63 NEPENTHE samples. Results were validated based on recapitulating known tissue compositions in marrow samples and published cell type-specific marker genes.

Results

We identified optimal parameters for both algorithms to achieve highest correlation of cell type predictions with lowest deviation (MuSiC: R=0.9699, mAD=0.0069; CIBERSORTx: R=0.9631, mAD=0.0261). Using these parameters, we deconvolved the NEPENTHE bulk RNA-seq data into cell types. Since MuSiC predicted marrow sample proportions with 100% accuracy, we used these proportions to compare to CIBERSORTx. CIBERSORTx corroborated MuSiC's results (R=0.8835, mAD=0.0081). Using estimated cell type proportions, we identified decreased proportions of bridge cells, chromaffin cells, and Schwann cell precursors in treated vs diagnostic patients and we are currently analyzing cell type-specific differentially expressed genes to mechanistically explain these differences.

Conclusion

This deconvolution optimization protocol precisely infers cell types in a highly heterogeneous sample cohort, identifying serial changes in cell type proportions, enabling inference of mechanisms of cellular plasticity at a pseudo-single cell level.

P107

Syngeneic TH-MYCN tumors convert to a mesenchymal phenotype, lose GD2, and increase inflammatory signaling: implications for preclinical immunotherapy

Jung P[1], McNerney K[3], Malka O[1], Dessau J[2], Vu A[1], Burudpakdee C[1], Bassiri H[1], Hogarty M[1], Wolpaw A[1]
[1]The Children's Hospital of Philadelphia, Philadelphia, United States, [2]The Wistar Institute, Philadelphia, United States, [3]Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, United States

Background:
Tumor heterogeneity impacts treatment response and progression. In neuroblastoma, two lineage states have been described: an adrenergic state that comprises the majority of tumor cells and a smaller mesenchymal population that is chemo-resistant and enriched in relapse. We showed that human mesenchymal neuroblastoma cells have higher inflammatory signaling than adrenergic neuroblastoma cells (Wolpaw et al, PNAS 2022). Further, we found that murine neuroblastoma cell lines derived from autochthonous TH-MYCN tumors rapidly lose expression of the adrenergic marker Phox2b and surface GD2 and gain expression of the mesenchymal marker Yap1 (McNerney et al., OncoImmunology 2022). Here we further characterize the MES/ADRN-GD2 axis of primary and syngeneic murine tumors and how they differ in inflammatory signaling.

Methods:
We analyzed cell lines and tumors derived from TH-MYCN mice. Tumors included primary tumors, tumors derived from the dissociation and immediate subcutaneous reimplantation of primary tumor cells, and tumors derived from the subcutaneous implantation of cell lines. Samples were analyzed for adrenergic and mesenchymal markers by immunoblotting and IHC, surface GD2 by FACS, and transcriptional differences by RNA-seq.

Results:
All TH-MYCN tumor cells lost expression of GD2 and adrenergic markers and gained expression of mesenchymal markers during outgrowth as cell lines. This pattern was maintained in tumors derived from cell lines, but not in primary tumors or subcutaneous tumors derived from immediate reimplantation of dissociated primary tumors, both of which showed an adrenergic profile and GD2 expression. Transcriptionally, cell lines and cell line-derived tumors had strong mesenchymal and weak adrenergic signatures compared to primary tumors. Consistent with our findings in human neuroblastoma, compared to adrenergic-like primary tumors, mesenchymal-like murine neuroblastoma has higher expression of inflammatory signatures and antigen presentation genes.

Conclusions:
TH-MYCN-derived tumors cells can assume mesenchymal and adrenergic states reflective of the human tumor states that recapitulate differences in inflammatory signaling and GD2 expression. These models will be essential in determining the impact of lineage and surface antigen heterogeneity on response to current and preclinical immunotherapies in an immunocompetent context. Ongoing studies into the impact of 3-dimensional culture and genetic manipulation on lineage state in these models will be reported.

P108

Exploring neuroblast-chromaffin state transitions in human neuroblastoma and paraganglioma

Arceo M[1], Zhu J[1], Bullova P[1], Kogner P[2], Larsson C[3], Juhlin C[3], Bedoya-Reina O[1], Schlisio S[1]
[1]Karolinska Institutet, Department of Microbiology, Tumor and Cell Biology, Stockholm, Sweden, [2]Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden, [3]Karolinska Institutet, Department of Oncology-Pathology, Stockholm, Sweden

Introduction:
Neuroblastoma (NB) and paraganglioma (PPGL) arise from sympatho-adrenal lineage and mostly occur at the adrenal medulla. These tumors are characterized by high inter- and intrapatient heterogeneity. The diversity of progenitors and dynamics of developing medulla might be recapitulated in these tumors. Embryonic intra-adrenal neuroblasts are born within the developing gland and can transition into chromaffin cells, but they also can transition from chromaffin-to-neuroblast. Postnatally, however, we previously described a subtype of TRKB+ chromaffin progenitor population in human post-natal gland that resembles the transcriptome enriched in high-risk NB.

Aims:
To evaluate NB and PPGL cell heterogeneity and developmental status for a cohort of tumor samples covering all NB stages and PPGL standard molecular groups. Building a reference single-nuclei-transcriptome adrenal gland (AG) atlas allows comparative analysis, including embryonic to postnatal AG.

Methods:
We collected six human post-natal and one embryonic AG, 24 NB, and 9 PPGL samples, and processed them with SMART-seq2-Nuc-Seq protocol. We used two peer-reviewed datasets to infer clusters obtained. We integrated our AG with developing AG from Jansky et al. to generate a reference adrenal tissue atlas.

Results:
We found different cell clusters enriched in high- vs low-risk NB. We found PPGL samples belonging to the kinase-signaling group drove differences in clusters, while the genotype mostly drove clusters comprising PPGL cells from the pseudohypoxia group. We identified a cluster of PPGL pseudohypoxia-group cells whose transcriptional profile also resembled an NB noradrenergic cluster (BH-FDR < 1e-85), an NB noradrenergic cluster resembled pseudohypoxia PPGL group (BH-FDR < 4.57e-5). We built a reference healthy AG atlas where embryonic shared cell states grouped mostly together while still retaining meaningful biological heterogeneity.

Conclusion:
Whereas a chromaffin progenitor population identified in human postnatal adrenal medulla resembled the transcriptome of the undifferentiated cluster enriched in high-risk NB, we further characterized two noradrenergic clusters NOR1 and NOR2. NOR1 cluster was enriched in low-risk and 4S NB, characterized by neuroblast transcriptional signature. However, NOR2 (CHGB+) cluster transcriptionally resembled a chromaffin signature enriched also in high-risk NB that shared a transcriptional program observed in the PPGL pseudohypoxia group, suggesting that unfavorable neuroblastomas perhaps arise during postnatal chromaffin differentiation.

P109

Transcriptional-noise-driven phenotypic plasticity as a source of non-genetic evolution in neuroblastoma

Allo Anido A[1], Hamer S[1], Roux C[1], Shea A[1], Butterworth C[3], Bellini A[3], Chen X[4], Weinberger L[4], Scheleiermacher G[3], Chesler L[2], Bruna A[1]

[1]Centre for Evolution and Cancer, Division of Molecular Pathology, The Institute of Cancer Research, London, United Kingdom, [2]Division of Clinical Studies, The Institute of Cancer Research, London, United Kingdom, [3]Inserm U830, Institut Curie, Paris, France, [4]Gladstone/UCSF Center for Cell Circuitry, Gladstone Institutes, San Francisco, USA

Neuroblastoma is one of the most common and deadliest types of paediatric cancer, characterised by a high frequency of treatment resistance and relapse. The low mutational burden and the existence of plasticity between the differentiated adrenergic and the resistant mesenchymal identities, point towards an involvement of non-genetic mechanisms in the evolution of resistance in neuroblastoma. Noise in expression of cell state regulators appears as an attractive mechanism to explain the spontaneous switching between these two identities in genetically homogeneous populations. Here, we used transcriptional, phenotypic and functional assays to study the involvement of transcriptional noise in triggering cell plasticity in neuroblastoma cell lines and patient samples. We showed that induction of noise with the pyrimidine analogue 5-Iodo-2'-deoxyuridine (IdU) promotes a migratory phenotype and expression of CD44 in SK-N-SH, compatible with an enrichment of mesenchymal cells. In patient-derived organoids, IdU potentiates the effect of TGF-ß signalling and promotes a switch towards the mesenchymal identity through activation of epithelial-to-mesenchymal transition, upregulation of mesenchymal transcription factors and downregulation of adrenergic regulators. We hypothesise that phenotypic plasticity is an early driver of neuroblastoma evolution. The identification of transcriptional noise as a source of plasticity would allow us to decipher the mechanisms enabling adrenergic-to-mesenchymal transitions and, ultimately, design therapeutic strategies to prevent treatment resistance and relapse in this devastating cancer.

P110

A single-cell meta-analysis on gene regulatory programs and signaling in neuroblastoma

Zanzani V[1,2,4,5], Bonine N[2,3,4,5], Speleman F[3,5], De Preter K[2,4,5], Vermeirssen V[1,4,5]

[1]Laboratory for Computational Biology, Integromics and Gene Regulation (CBIGR), Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [2]Laboratory for Translational Oncogenomics and Bioinformatics (TOBI), Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [3]Pediatric Precision Oncology Laboratory (PPOL), Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [4]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium, [5]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium

Background:
Single-cell transcriptomics allow to separate heterogeneous neuroblastoma (NB) tissues into specific cell states with their regulatory programs. These can be modelled in gene regulatory networks (GRNs) using single-cell network inference methods. GRNs highlight active pathways and key regulators of tumor biology for a specific patient or cell state, leading to the identification of novel potential drug targets and mechanisms. Several single-cell RNA-seq (scRNA-seq) studies on human NB have been reported. However, regulatory programs and signaling have only preliminarily been explored.

Aims:
In a meta-analysis of published scRNA-seq data of NB tumors, we aim to map the underlying GRNs with a resolution ranging from the single patient to the whole atlas, to robustly derive master regulators, their putative targets, and the pathways they are operating in. Additionally, we want to explore regulatory and signaling interactions between different malignant cell states and the tumor microenvironment (TME).

Methods:
We gathered public 10x Genomics scRNA-seq data for over 50 NB tumors with different biological and clinical characteristics. We accounted for the intrinsic technical variability of the different datasets through the best-performing integration methods from a recent benchmark. By applying scalable GRN inference methods, we characterized the regulatory programs active in the malignant cell states. In addition, we investigated the crosstalk between cell states and the TME through multicellular programs and cell-cell communication methods.

Results:
We generated a comprehensive spectrum of cell-state-specific GRNs from a reference cellular atlas of human NB. For high-risk NB, we identified cell states with regulatory programs driven by adrenergic transcription factors, such as PHOX2A, GATA2, and ISL1. We also retrieved specific cell states governed by mesenchymal transcription factors, such as STAT1 and ELF1. Other cell states were regulated by E2F transcription factors. We also predicted cell state transitioning through simulated transcription factor perturbations in the GRNs, suggesting how GRN rewiring establishes a more mesenchymal-like state. Analysis is ongoing to connect GRNs across different malignant cell states and the TME.

Conclusion:
Our work provides comprehensive insights into the regulatory heterogeneity of NB, which might aid the development of more effective therapies.

P111

Exploring the technology frontiers in the treatment of neuroblastoma from the perspective of patent citations

Zhang T[1], Yang X, Xu D, Chen J, Ouyang Z
[1]*Institute of Medical Information & Library, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China*

Objectives: This paper aimed to explore the technology frontiers in the treatment of neuroblastoma from the perspective of patent citations.

Methods: Patents related to the treatment of neuroblastoma were searched and collected from the Derwent Innovation Index (DII), which were imported into Derwent Data Analyzer (DDA, Clarivate Derwent, New York, NY, USA) for authority control. The patent citations were extracted from the collected patent data in order to select the highly cited patents and highly cited publications, and corresponding citation patents were extracted, respectively. Text clustering is an important method in text mining, where similar documents are grouped into clusters. The similarity between documents are determined by calculating the distance between them, and the two documents with the closest distance are combined. The method of text clustering was used to identify the technology frontiers based on the extracted citation patents, which was according to co-word analysis of the title of the citation patents in this field.

Results: 6240 patents were obtained in the field of the treatment of neuroblastoma, with 71304 cited patents and 88698 cited publications. There were 731 citation patents citing the 158 high cited patents, with the more than 20 cited frequencies, and 500 citation patents were citing the 149 cited publications, with the more than 10 cited frequencies. There were four technology frontiers based on the 731 citation patents, namely: drug target, drug design, tumor indication expansion, and gene expression regulation, and three technology frontiers based on the 500 citation patents, namely: drug design, drug target, and tumor indication expansion.

Conclusions: The treatment of neuroblastoma was currently in a rapid development period. The results of this study identified the technology frontiers in the treatment of neuroblastoma, which could help readers broaden innovative ideas and discover new technological opportunities, and also served as important indicators for government policymaking.

P112

Growth inhibition of neuroblastoma cells by anti-NLRR1 monoclonal antibodies

Takatori A[1], Hossain M[1], Ogura A[1], Akter J[1], Nakamura Y[1], Nakagawara A[2]
[1]Division of Innovative Cancer Therapeutics, Chiba Cancer Center Research Institute, Chiba, Japan, [2]Saga International Carbon Particle Beam Radiation Cancer Therapy Center, Tosu, Japan

Background:
Receptor tyrosine kinases receive different modulation before transmitting proliferative signals. We previously identified neuronal leucine-rich repeat 1 (NLRR1) as a positive regulator of EGF and IGF-1 signals in high-risk neuroblastoma cells. Our previous study has also demonstrated that NLRR1 negatively regulates ALK in neuroblastoma cells, although the contribution of NLRR1 to other types of cancers is not understood.

Aims:
In this study, we examined NLRR1 expression in various adult cancers and non-neuroblastoma cell lines. Screening assays were carried out to obtain monoclonal antibodies against extracellular part of NLRR1 with growth inhibitory effect in neuroblastoma cells.

Methods:
NLRR1 expression was examined by immunohistochemistry, immunoblotting and quantitative real-time PCR. Purified proteins of NLRR1 extracellular domain were used to immunize mice to obtain hybridomas producing monoclonal antibody against NLRR1.

Results:
High expression of NLRR1 was detected in neuroblastoma cell lines and non-neuroblastoma cell lines. Immunohistochemistry showed strong staining of NLRR1 in cancer tissues from skin, lung and breast as compared with the corresponding normal tissues, suggesting that NLRR1 may contribute to the malignant status and serve as a biomarker in not only neuroblastoma, but also adult cancers. NLRR1 with the deletions in extracellular domains demonstrated that the deletion of fibronectin type III (FNIII) domain significantly reduced the cell growth and phosphorylation of ERK upon EGF treatment compared to wild-type, indicating that FNIII domain is a responsible domain for NLRR1 function. Among monoclonal antibodies against the extracellular domains of NLRR1 (N1mAb), we obtained N1mAbs that suppressed cell proliferation in neuroblastoma cells. The N1mAb treatment significantly reduced the tumor growth in SCID mice bearing NLRR1 expressing neuroblastoma xenograft tumors. examined We further examined the binding property of N1mAbs by immunoprecipitation, indirect flow cytometry and peptide microarray and found that the growth inhibitory effect of N1mAb was exerted through binding to FNIII domain of NLRR1.

Conclusion:
NLRR1 is a novel molecular target for treating particular cancers including neuroblastoma and its function to regulate growth signals is dependent on the extracellular domain which can be a target for antibody-based therapy of NLRR1-expressing cancers.

P113

Evaluating the RIST molecular-targeted regimen in a neuroblastoma spheroid cell culture model

Kaess C[1], Matthes M[1], Gross J[1], Waetzig R[2], Heise T[1], Corbacioglu S[1], Sommer G[1]
[1]Department of Pediatric Hematology, Oncology and Stem Cell Transplantation, University Hospital of Regensburg, 93053 Regensburg, Germany, [2]Department of Internal Medicine 5 – Hematology and Clinical Oncology, Friedrich Alexander University (FAU), 91054 Erlangen, Germany

Background: Neuroblastoma is the most common extracranial solid tumor in childhood. Despite intensive treatment regimens the outcome for patients with high-risk, relapsed and refractory neuroblastoma remains dismal and novel treatment strategies are urgently needed. Irinotecan, a topoisomerase inhibitor and the alkylating agent Temozolomide (I/T) are the current backbone to develop novel treatment options for several tumor entities. RIST represents a metronomic molecular targeted treatment strategy, combining I/T with the multikinase inhibitor Dasatinib (Srycel, S) and mTOR inhibitor Rapamycin (R). A prospective randomized phase II clinical trial testing the RIST protocol in relapsed and refractory neuroblastoma patients (NCT01467986) completed recruitment and the results are currently under evaluation. For preclinical drug testing the growth of cancer cells in form of spheroids compared to monolayer cultures is of advantage since it reproduces a wide range of avascular solid tumor characteristics, including the three-dimensional architecture and cancer stem cell (CSC) properties. Aim: Rigorous assessment of the RIST treatment regimen in a neuroblastoma spheroid model characterized by an augmented neoplastic phenotype. Methods: Establishment of growth conditions for spheroids in a 96-well format for five neuroblastoma cell lines. Evaluation of CSC marker expression by mRNA and protein analysis. Testing the viability of spheroids after drug- and control-treatment by applying luminescence-based assays. Assessment of RNA-binding protein La, a well-known factor promoting cancer cell plasticity, in neuroblastoma by tissue microarray analysis and patients' data mining. Results: Compared to monolayer cultures, spheroid cultures not only showed increased expression of CSC marker CXCR4, NANOG, and BMI1, but also augmented phosphorylation of the neuroblastoma-associated La protein at threonine 389. Molecular targeted 'pre-treatment' reduced neoplastic signaling, CSC marker expression and the viability of spheroids. The RIST treatment significantly decreased the viability of spheroids in all neuroblastoma cell lines tested. Conclusion: The RIST protocol efficiently reduced the viability of neuroblastoma cells in a preclinical spheroid model despite the presence of increased CSC properties. Furthermore, the results underscore the importance of the neuroblastoma spheroid model for preclinical drug testing in a rigorous, robust, and efficient high-throughput format which can be valuable to identify more effective treatments for children with high-risk neuroblastoma in the future.

P114

Evaluating the RIST molecular-targeted regimen in an in vitro cell culture model of Ewing sarcoma

Yildirim E[1], Matthes M[1], Heise T[1], Corbacioglu S[1], Sommer G[1]
[1]*University Hospital of Regensburg, Department of Pediatric Hematology, Oncology and Stem Cell Transplantation, Regensburg, Germany*

Background: Ewing sarcoma is a rapidly metastasizing bone and soft-tissue cancer most common in adolescents and young adults. Despite intensified chemotherapeutic treatment concepts the prognosis remains poor, in particular for patients with metastatic presentation and refractory/relapsed diseases. Irinotecan, a topoisomerase inhibitor and the alkylating agent Temozolomide (I/T) are the current backbone to develop novel treatment options for several tumor entities. RIST represents a metronomic molecular targeted treatment strategy, combining I/T with the multikinase inhibitor Dasatinib (Sprycel, S) and mTOR inhibitor Rapamycin (R). A prospective randomized phase II clinical trial testing the RIST protocol in relapsed and refractory neuroblastoma patients (NCT01467986) completed recruitment and the results are currently under evaluation. Recent preclinical studies demonstrated that RIST synergistically targets key cancer promoting pathways and thereby enhances the efficacy of neuroblastoma treatment. Aims: This study tested the efficacy of RIST in an in vitro cell culture model of Ewing sarcoma. Methods: Determination of effective drug concentrations (half-maximal inhibitory concentration (IC50)) and evaluation of potential synergistic effects of drug combinations in Ewing sarcoma cell lines by applying colorimetric cell viability assays. Analysis of the phosphorylation status of key cancer promoting pathways and the expression level of fusion oncogene EWS-FLI after drug- and control- (vehicle) treatment by immunoblotting and RT-qPCR. Results: The molecular targeted 'pre-treatment' inhibited the mTOR pathway as indicated by reduced phosphorylation of translation initiation factor 4E-BP at serine 65 (Ser65) and ribosomal protein S6 kinase at threonine 389 (Thr389) and reduced phosphorylation of Src kinase at tyrosine 416 (Tyr416). The increase of AKT phosphorylation at serine 473 (Ser473) suggests that further improvement of neoplastic inhibition can be achieved by employing an ATP competitive mTOR inhibitor instead of the allosteric mTOR inhibitor Rapamycin. Taken together, compared to the conventional chemotherapeutic treatment the multimodal RIST protocol significantly reduced the cell viability of Ewing sarcoma cells. Conclusion: The results imply that the RIST treatment protocol represents a therapeutic option not only for patients with neuroblastoma but also for advanced stage, relapsed and refractory Ewing sarcoma. The validation is pending an evaluation in extended preclinical and clinical studies.

P115

Identification of TIMP-3 as a metastatic regulator of neuroblastoma

Wu P[1], Kargren M[2], Hsu W[3]
[1]Department of Life Sciences, National Central University, Taoyuan, Taiwan, [2]Department of Life Science, National Taiwan University, Taipei, Taiwan, [3]Department of Surgery, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan

Background: Neuroblastoma (NB) is the most common extra-cranial solid tumor in children. Despite improvements in available treatment options, the prognosis for high-risk neuroblastoma patients remains unfavorable, with the metastatic spread being one of the reasons behind it. Therefore, new therapeutics for patients with advanced NB are urgently needed. MYCN is an oncogene whose amplification is associated with high-risk NB. Additionally, EGFR and ErbB2 were both shown to be highly expressed in NB cells and have been implicated in the metastasis of various tumors. Targeting MYCN or EGFR and ErbB2 may have therapeutic potential for treating NB metastasis.

Aims: In this study, we tested the ability of JQ1, a Bromodomain and Extra-Terminal (BET) inhibitor, and CL-387,785, an EGFR/ErbB2 dual inhibitor, to block metastatic properties of NB cells. In addition, the underlying mechanism was verified.
Methods: The tumor metastasis-related cell behaviors were analyzed by wound healing, trans-well assays, and colony formation assays. A QPCR-based tumor metastasis array was performed to identify the differentially expressed genes (DEGs) in the NB cells treated with JQ1 and CL-387,785. The clinical significance of selected DEG was further analyzed by the web-based R2 platform and a Taiwanese cohort of neuroblastoma patients.

Results: Both JQ1 and CL-387,785 significantly reduce migration and invasion of NB cells. Consistently, both compounds can inhibit the anchorage-independent growth of NB cells. We further identified tissue inhibitor of metalloproteinases 3 (TIMP3) as one of the significantly upregulated genes in NB cells treated with JQ1 or CL-387,785, indicating that TIMP3 may involve in the mechanism of NB cell metastasis. Lastly, both clinical datasets demonstrate that high expression of TIMP3 positively correlated with favorable prognosis of patients and the differentiation histology of the NB tumors.

Conclusion: Our findings provide proof-of-principle evidence that JQ1 and CL-387,785 could be further developed to become the target therapies for high-risk neuroblastoma and verify the prognostic merit of TIMP3 in neuroblastoma.

P116

The multikinase inhibitor Sitravatinb blocks neuroblastoma tumorigenesis in vitro and in vivo.

Rozen E[1], Wigglesworth K[1], Shohet J[1]
[1]*University of Massachusetts Chan Medical School, Department of Pediatrics, Worcester, United States*

Background: Clinical trials are now testing multi-Receptor Tyrosine Kinase inhibitors (mRTKi) that target both tumor intrinsic pathways and immune supressive cells with the tumor microenvironment (TME). The oral drug Sitravatinib (MGCD516) is currently in phase-II and phase-III clinical trials (e.g., as single agent in stage 4 breast cancer (NCT04123704) and in combination with nivolumab for NSCLC (NCT03906071)). This mRTKi targets 4 of the top 5 RTKs recently found to strongly correlate with high stage and worse overall survival in neuroblastoma (DDR2, RET, KIT, and PDGFRA) (Rozen and Shohet, 2022). It also inhibits the immune suppressive TAM receptors (Tyro3/Axl/MERTK) and to a lesser extent the ABCG2 and ABCB1 multidrug resistance efflux pumps.

Aims: We hypothesized Sitravatinib could be a novel therapeutic for relapsed refractory neuroblastoma and sought to determine the efficacy of Sitravatinib against neuroblastoma cell lines, multiple orthotopic xenografts, and the TH-MYCN transgenic murine model of neuroblastoma.

Results: This mRTKi blocked cell proliferation and migration at low micromolar levels and uniformly induced apoptosis at higher doses across a panel of neuroblastoma cell lines. This was independent of MYCN, ALK, or p53 status. We confirmed on-target functional inhibition of GDNF-mediated activation of RET and collagen-mediated activation of DDR2. In vivo, Sitravatinib effectively limited metastasis and blocked proliferation of orthotopic xenografts (KELLY, SHY5Y and NGP). Surprisingly, we maintained TH-MYCN+/+ mice past 90 days of life with oral Sitravatinib starting at 28 days of life with no evidence of tumor related morbidity (control cohort median time to sacrifice 57 days).

Conclusions: As a readily available oral drug with limited human toxicity and promising efficacy in preclinical models, we propose Sitravatinib is an excellent candidate for additional preclinical and clinical testing. This mRTKi may both enhance immune reactivity of the TME while blocking neuroblastoma proliferation and metastasis. Additional work is required to define synergistic combinations and its impact on drug resistance and immune recognition.

P117

Unraveling the polypharmacology of beta-blockers in neuroblastoma using chemoproteomics

Mouysset B[1], Le Grand M[1], Ariey-Bonnet J[1], Brémond P[1], Camoin L[1], Audebert S[1], Gerault M[1], Ghesquière B[2], André N[1,3], Pasquier E[1]

[1]Centre de Recherche en CancérUniveologie de Marseille Inserm U1068, CNRS UMR7258, Aix-Marseille university, Marseille, France, [2]Metabolomics Expertise Center, VIB Center for Cancer Biology, VIB, Leuven, Belgium, [3]Pediatric Hematology and Oncology Department, Hôpital pour Enfant de La Timone, AP-HM, Marseille, France

Background & Aim

Our lab has previously shown that anti-hypertensive drugs, β-blockers, can increase the efficacy of chemotherapy in neuroblastoma. The mechanism(s) involved remains however poorly understood. First, we showed that the enantiomers of propranolol and carvedilol (R-S) were equipotent at increasing the cytotoxic activity of vincristine in neuroblastoma cells. Since the (R)-enantiomers have low affinity for the β-adrenergic receptors, we concluded that their canonical targets are not involved in this effect. Therefore, this project aims at developing an innovative approach to identify the non-canonical targets of beta-blockers and better understand their mechanism of action.

Methods

We developed an integrative method that combines unsupervised and candidate strategies. Using two complementary and independent chemoproteomic approaches, our first goal was to uncover the interactome of β-blockers in neuroblastoma. Based on our results, we then performed pharmaco-metabolomic analyses.

Results

We used click chemistry-based proteomics, a biocompatible chemical reaction coupled with mass spectrometry, and found an enrichment in proteins involved in cell metabolism within the 77 interactors shared by the three tested β-blockers. In parallel, we exploited a biophysical assay called “cellular thermal shift assay” coupled with quantitative mass spectrometry (MS-CETSA) to evaluate the impact of beta-blockers (propranolol and carvedilol) and vincristine alone or in combination in cellulo. Our results highlighted well-known targets of vincristine such as tubulins, but also an enrichment in proteins involved in cell metabolism and mitochondrial respiration in both monotherapies and combination treatment. We then performed 13C glucose and glutamine tracer experiments and showed an alteration of the glucosamine and pyrimidine synthesis pathways under the combination treatment. Using functional genomics and coupling our clickable drug derivatives with an azide-fluorophore to perform co-localization experiments, we will next validate and characterize the identified metabolic targets of β-blockers impacting neuroblastoma biology and drug response. Overall, our results show that β-blockers increase the efficacy of chemotherapy agents in neuroblastoma by interfering with cancer cell metabolism, independently of beta-adrenergic receptors.

Conclusion

This project could help better understand neuroblastoma biology and reveal novel targetable pathways, that could be further therapeutically exploited to develop new treatments and unveil biomarkers for patient selection, thus facilitating future clinical trials.

P119

Investigating Molecular Glues as a New Therapeutic Approach for High-Risk Neuroblastoma

Delahunty I[1], Nishiguchi G[2], Zhang Y[3], Fish A[2], Mayasundari A[2], Qi J[4], Abraham B[3], Shelat A[2], Rankovic Z[2], Durbin A[1]

[1]St. Jude Children's Research Hospital, Department of Molecular Oncology, Memphis, United States, [2]St. Jude Children's Research Hospital, Department of Chemical Biology and Therapeutics, Memphis, United States, [3]St. Jude Children's Research Hospital, Computational Biology, Memphis, United States, [4]Dana Farber Cancer Institute, Boston, United States

Despite extensive study, treatments for high-risk neuroblastoma (NB) remain highly toxic. Thus, there is an urgent need to develop more effective and less toxic treatments. NB cells rely on a small cohort of transcription factors (TFs) and epigenetic proteins to drive cell growth, however, many of these targets are inaccessible with conventional small molecules. In recent years, new modalities such as targeted protein degradation (TPD) have emerged. TPD may permit small molecule degradation of traditionally "undruggable" targets, such as TFs, making this modality of exceptional interest in TF-dependent malignancies. One specific TPD category of interest are the molecular glues (MGs), which are bioavailable monovalent small molecules capable of forming a ternary complex between an E3 ligase receptor and a target protein of interest, resulting in target proteasomal degradation.

Thus, here, we sought to explore the sensitivity of NB cells to MGs. We demonstrate that NB patient-derived xenografts and cell lines display exceptionally high levels of the E3 receptor CRBN, as compared with other tumors. We capitalized on this finding by performing high-throughput screening of a proprietary library of >4000 unique CRBN-based MGs in two MYCN-amplified NB cell lines, Kelly and MHHNB11. The response of NB cells to this panel of MGs was strikingly correlated (Pearson's R2 0.72), as compared with medulloblastoma, lymphoid or myeloid leukemia cells (R2 0.0-0.52). Further, these NB cells are critically sensitive to a cluster of 112 previously uncharacterized MGs. Using CRISPR-cas9 gene editing, we derived NB cells with stable knockout of the E3 ligase receptor CRBN, and coupled these with screening to confirm that the majority of these MGs (84%; 94/112) require CRBN expression. To rule out effects on the common off-target protein GSPT1, candidates were also tested in NB cells expressing wild-type or degradation-resistant GSPT1 (G575N), revealing a subset of MGs retaining cytotoxicity without affecting GSPT1. Ongoing studies are focused on determining the targets of these MGs, as a foundation for preclinical in vivo studies. These studies will identify new compounds with defined mechanisms of action, with an ultimate goal of developing new treatments that are more effective and less toxic for children with NB.

P120

Fenretinide-loaded mesenchymal stromal cell-derived extracellular vesicles as novel drug against human neuroblastoma

Zorzoli A[1], Zuccari G[3], Brignole C[2], Cocco C[1], Morandi F[1], Sabatini F[1], Airoldi I[1], Podestà M[1], Marimpietri D[1]
[1]IRCSS Istituto Giannina Gaslini, Cell Factory, Genoa, Italy, [2]IRCSS istituto Giannina Gaslini, Laboratory of Experimental Therapies in Oncology, Genoa, Italy, [3]University of Genoa, Department of Pharmacy, Genoa, Italy

Background
The synthetic retinoid Fenretinide (4-HPR) displays cytotoxic activity against cancer cells and is well tolerated in clinical trials on neuroblastoma (NB) patients. This molecule presents poor bioavailability, resulting in limited therapeutic efficacy.
Aims
We propose a new strategy based on the use of mesenchymal stem cells (MSC)-derived extracellular vesicles (EV) as endogenous carriers for drug delivery in human NB.
Methods & Results
In order to produce drug-laden EV by passive loading, we treated MSC isolated by human umbilical cord (hUC) with or without 20 µM 4-HPR for 48h. The particles were isolated by ultracentrifugation. Nanoparticle Tracking Analysis analysis revealed that Mesenchymal Stem Cells-derived Extracellular Vesicles (MSC-EV) and Fenretinide-loaded Mesenchymal Stem Cells-derived Extracellular Vesicles (4-HPR-MSC-EV) showed a concentration of 1.25x1011 and 1.53 x1011 particles/ml, respectively, and a size ranging from 70 to 150 µm. Similar results were obtained by DLS. EVs measurements showed a negative Zeta Potential between -10 and -15 mV.
High-performance liquid chromatograph (HPLC) revealed that the concentration of 4-HPR-MSC-EV, was 1,77 µM.
By CYQUANT Direct Cell Proliferation assay kit, we compared the antitumor activity of free 4-HPR at different concentrations (0, 1, 2.5, 5, 10, 20 µM), and 4-HPR-MSC-EV on a panel of human NB cell lines (IMR-32, HTLA-230, SK-N-AS, SH-SY5Y) at 48, 72 and 96h.
We founded that 4-HPR-MSC-EV induced a higher dose and time dependent inhibition of human NB cell proliferation with respect to free 4-HPR. This results were confirmed also on spheroids of SH-SY5Y and IMR-32 NB cells. Furthermore, microscope images revealed the uptake of MSC-EV by NB cells, showing the localization of fluorescence at cell membrane level.
Conclusion
These findings support that MSC-EV acts as drug carrier, and that 4-HPR loaded in EVs has a greater effect, suggesting the increased bioavailability of this drug. This promising results contributed to believe how this approach may improve the therapeutic effect to treat neuroblastoma.

P121

NANOSENSO: a Trojan horse reconditioning high-risk neuroblastoma to a ferroptosis sensitive state

Koeken I[1], Hassannia B[1,2,3], Golba B[4], Walravens M[1], Pellis B[1], Talebi A[5], Deshair J[5], Speleman F[6], de Geest B[4], Vanden Berghe T[1,2,3,7]

[1]University of Antwerp, Department of Biomedical Sciences, Antwerp, Belgium, [2]VIB-UGent Center for Inflammation Research, Ghent, Belgium, [3]Ghent University, Department of Biomedical Molecular Biology, Ghent, Belgium, [4]Ghent University, Department of Parmaceutics, Ghent, Belgium, [5]Catholic University of Leuven, Department of Oncology, Leuven, Belgium, [6]Gent University, Department of Biomolecular Medicine, Ghent, Belgium, [7]University of Antwerp, Infla-Med Centre of Excellence, Ghent, Belgium

Neuroblastoma is the most common solid tumor outside the brain of infants and very young children. A substantial part of neuroblastoma patients presents with high-risk neuroblastoma disease. In fact, these children have a poor prognosis, do not respond to therapy or even relapse. Therefore, there is an urgent need to find novel treatment strategies. The Vanden Berghe lab discovered a new approach to kill aggressive therapy-resistant neuroblastoma cells in mice by inducing a sort of biological rusting in cancer cells, called ferroptosis. By using nanoparticles, the lab was able to minimize side effects of treatment and enhance tumor targeting. However, to get full tumor regression without relapse, it is needed to further improve the efficacy of targeting ferroptosis as therapeutic strategy in neuroblastoma. Therefore, we aim to recondition high-risk neuroblastoma cells to a ferroptosis sensitive state, by acting on anti-ferroptosis mechanisms in cancer cells. As first strategy, we successfully developed a sensitizing strategy targeting the lipid composition of the cancer cells using poly-unsaturated fatty acid containing lipid nanoparticles, similar to current COVID-19 RNA vaccine formulations. In addition, we identified ferroptosis-sensitizing compounds that we are currently encapsulating in these lipid nanoparticles. These ferroptosis-sensitizing nanomedicines will be tested and validated in cell- and patient-derived high-risk neuroblastoma mouse models as non-toxic pre-treatment to ferroptosis induction. As such, this would provide a steppingstone to clinical investigation of ferroptosis targeting as anti-cancer therapy.

P122

Casiopeína-IIIia® treatment inhibits the migration of xenografted neuroblastoma cells in zebrafish (Danio rerio).

Mejía C[1], Zamudio-Hernández J[1], Ruiz Azuara L[2]
[1]Universidad Autónoma de Querétaro, QUERÉTARO, México, [2]Universidad Nacional Autónoma de México, Ciudad de México, México

Background. Neuroblastoma (NB) is a neural crest cell tumor representing childhood's most common extracranial solid tumor. Because patients with high-risk NB present with amplification of the N-MYC oncogene and metastasis, the usual treatments are not effective, giving rise to Casiopeínas®, a family of coordinated copper(II) compounds that have shown an antineoplastic effect in vivo and in vitro. Its mechanism of action includes the induction of mitochondrial apoptosis through the overproduction of reactive oxygen species (ROS).
Aim. To advance in investigating the mechanism of action of these compounds, we proposed to analyze their effect on a model of xenotransplantation of NB cells in zebrafish (Danio rerio).
Methods. 2dpf (days post-fertilization) D. rerio specimens were anesthetized with tricaine to xenograft 1,000 NB cells from the SK-N-SH line, previously stained with 20 µg/ml Dil marker. Subsequently, 1 or 10 µg/ml of Casiopeína-IIIia® was applied to the fish tank water. The cell migration was observed by confocal microscopy at 400X, and by immunohistochemistry, we searched for CD31.
Results. We found that the ideal concentration of tricaine to anesthetize the fish was 250 µg/ml, with a survival rate of 59.4%. In specimens of 7 dpt (days post-transplantation) without treatment, the number of cells that migrated towards the tail of the fish was higher than with the 1 µg/ml treatment ($p<0.05$) and with the 10 µg/ml treatment ($p<0.0001$). At the same time, the cell marker CD31 was not observed under any condition in the organs observed.
Conclusion. We found that the treatment with 10 µg/ml of Casiopeína-IIIia® had a more significant anti-migratory effect on transplanted SK-N-SH cells in Danio rerio, which is interesting in the treatment of high-risk NB.

P123

Evaluation of the activity of the mPTP components in neuroblastoma cells treated with Casiopeína-IIIia®.

Martínez-Pacheco M[1], Jaime-Martínez L[1], Ruiz-Azuara L[2], Mejía C[1]
[1]Autonomous University of Queretaro, Laboratory of Interdisciplinary Biomedicine, Queretaro, Queretaro, Mexico,
[2]National Autonomous University of Mexico, Laboratory of Medicinal Chemistry, Mexico City, Mexico

Background
Neuroblastoma (NB) is childhood´s most common extracranial solid tumor, originating from cells of the neural crest. For high-risk NB patients, the survival rate is low despite multimodal therapy. This scenario has led to the research of copper(II) compounds called Casiopeínas®, which have shown an antineoplastic effect in vivo and in vitro. Its mechanism of action implicates the induction of mitochondrial apoptosis through the overproduction of reactive oxygen species (ROS). Mitochondrial apoptosis is regulated by the formation of the mitochondrial permeability transition pore (mPTP); however, to date, it is unknown whether Casiopeína-IIIia® can activate it.
Aims
Inhibit the activity of the most crucial mPTP proteins in neuroblastoma cells of the SK-N-SH line treated with Casiopeína-IIIia® or cisplatin.
Methods
Using the CRISPR/Cas9 technique, we silenced the expression of BAX and BCL2 in SK-N-SH (NB) and 3T3-L1 (fibroblasts) cells. In NB cells, the structural proteins hexokinase-II (HKII), voltage-dependent anion channel (VDAC), and cyclophilin-D (CypD) were inhibited with 3-bromopyruvate (BrPyr), 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and cyclosporine A (CsA), respectively. Cells were treated with their respective LC50 of Casiopeína-IIIia® or cisplatin in all conditions.
Results
The levels of mitochondrial Ca2+ retention allowed us to determine that the inactivity of BAX, VDAC, and CypD prevented the depolarization of the mitochondrial membranes in the presence of the treatments. In contrast, the opposite occurred in the absence of BCL2 and HKII. We also observed that in cells treated with Casiopeína-IIIia® and cisplatin with BAX silencing, caspase-3 expression was lower than in cells with BCL2 silencing. Annexin-V assays revealed that apoptosis was lower in VDAC- and CypD-inactivated cells treated with Casiopeína-IIIia® and cisplatin than in cells with HKII inhibition.
Conclusion
We conclude that BAX, VDAC, and CypD are the essential components in mPTP formation after Cassiopeína-IIIia® treatment.

P125

Drug repurposing for high-risk neuroblastoma

Radke K[1], Aaltonen K[1], Hansson K[1,2], Mason D[3], Roberts I[3], Lock A[3,5], Brennan J[3], Davies E[3], Brown D[3], Thompson N[3], Spadoni C[4], Bexell D[1]

[1]Translational Cancer Research, Lund University, Lund, Sweden, [2]Cancer Stem Cell Laboratory, The Breast Cancer Now Toby Robins Research Centre, The Institute of Cancer Research, London, UK, [3]Healx Ltd., Charter House, 66-68 Hills Road, Cambridge, UK, [4]aPODD Foundation, , UK, [5]Current address: EMBL-EBI, Wellcome Genome Campus, Hinxton, UK

Rapid Fire session 1B, May 16, 2023, 11:30 AM - 11:45 AM

Background: Chemotherapy resistance and relapse in high-risk neuroblastoma (HR-NB) can be associated with an immature mesenchymal (MES)-like phenotype. The need for new therapeutic agents that can target cells in different states and circumvent the development of resistance is important. Drug repurposing of already approved drugs can allow for efficient and rapid implementation of drug candidates in the clinical setting.

Aims: To identify and test drugs that can be repurposed from other indications to target HR-NB.

Methods: Four publicly available gene expression signatures were analysed in silico by Disease-Gene Expression Matching (DGEM), part of the Healnet platform developed by Healx, to find drugs that may be therapeutic for a given disease. Drugs with the highest scores were subject to pharmacological review, and a subset was then tested in NB patient-derived xenograft (PDX)-derived organoid models. Synergy testing in vitro was performed and assessed using SynergyFinder (FIMM). RNA sequencing of the treated samples was analyzed with the R2 platform and Metascape.

Results: We identified 12 already approved drugs that were further tested in HR-NB organoids. Four substances decreased viability in three NB organoid models (IC50 in the range of 0.15 to 3.57uM at 7 days). Three of these four are classified as dopamine receptor 2 (D2) antagonists while the fourth is a cholesterol synthesis inhibitor. We screened for combinatory effects of the two classes of drugs and observed high synergistic effects (10.29 - 36.73 ZIP synergy scores) with a substantial decrease in cell viability and increased cell death. Combination treatment resulted in the upregulation of pathways linked to neuron projection development, metabolism of lipids, and vesicle-mediated transport. Additionally, the combination led to an increased adrenergic (ADR) profile and decreased immature MES-like profile. In vitro pretreatment with the combinations resulted in the sensitization of HR-NB organoids to cisplatin.

Conclusion: We identified D2 antagonists and a cholesterol synthesis inhibitor to be highly synergistic in HR-NB organoids in vitro. Combination treatment resulted in transcriptional upregulation of an ADR signature, sensitization to chemotherapy, and eventually to cell death.

P126

CDK12 as a target for combinatorial drugging of high-risk neuroblastoma

Goulding J[1,2], Couwenbergh S[3], Bekaert S[1,2], Eggermont A[1,2], Amo-Addae V[3], Koopmans B[3], Dewyn J[1,2], Eising S[3], Molenaar J[3,4], Speleman F[1,2], Durinck K[1,2]

[1]Department of Biomolecular Medicine, Center for Medical Genetics, Ghent University, Ghent, Belgium, [2]Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [3]Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands, [4]Department of Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

Rapid Fire session 1B, May 16, 2023, 11:30 AM - 11:45 AM

Goulding J and Couwenbergh S contributed equally to this work as co–first authors.

Background: High-risk NB patients currently undergo intensive multi-modal treatment, with over half succumbing to their disease. High-risk NB cells exhibit chronic replicative stress, partly through MYCN amplification, and targeting DNA damage response pathways provides a viable therapeutic option. Loci encoding BRCA1 and CDK12 are both located on the recurrently gained 17q region. BRCA1 and CDK12 are both important for maintenance of genomic stability. In addition, MYCN recruits BRCA1 to promoter-proximal regions, stabilises MYCN, and combines with MYCN to suppress R-loop formation. CDK12 loss or selective inhibition leads to a gene-length dependent downregulation of DNA damage response genes, including BRCA1. Subsequently, CDK12 has been proposed as a potential drug target in cancer.

Aims: As a transcriptional regulator of DNA damage response genes, we hypothesised that pharmacological inhibition or degradation of CDK12 could induce a BRCAness phenotype, rendering NB cells vulnerable to the drugging of synthetic lethal targets.
Methods/materials: Single compound efficacy of CDK12 small molecule inhibitors and degraders was evaluated in a panel of NB cell lines and organoids with diverse genomic backgrounds. Molecular effects of CDK12 inhibition and degradation were investigated through immunoblotting and RNA sequencing. We then combined CDK12-targeting agents with a high-throughput compound screen featuring approximately 200 compounds that are clinically approved or used in paediatric oncology research in MYCN-amplified and non-amplified NB cells.

Results: Single compound treatment with a CDK12 inhibitor (SR-4835) and CDK12 degraders (dCeMM2, BSJ-4-116) potently induced cell death in vitro (low nanomolar GI50 values, except dCeMM2). We confirmed induction of the BRCAness signature with decreased BRCA1 and RAD51 levels accompanied by increased DNA damage (elevated γH2AX levels) and downregulated DNA repair gene sets following RNA-sequencing analysis. Combination drug screening revealed extensive synergy with DNA damaging agents, PARP, Ras/MAPK and other inhibitors.

Conclusion: We confirmed CDK12 as a novel target and demonstrated that pharmacological inactivation of CDK12 induced a BRCAness phenotype in NB, which can be exploited through combination treatments. Next steps involve phenotypically testing the most synergistic combinations in zebrafish and mouse xenograft models to further evaluate efficacy and toxicity in vivo as well as potential functional differences of CDK12 inhibition versus degradation.

P127

Exploring the role of MYCN in the mitochondria to discover MYCN-Amplified tumour vulnerabilities

Epp S[1], O'Connell F[2], O'Sullivan J[2], Chalkley A[3], Simpson J[3], Halasz M[1], Kolch W[1]
[1]Systems Biology Ireland, University College Dublin, Dublin, Ireland, [2]Trinity Translational Medicine Institute, Department of Surgery, Trinity College Dublin, St James's Hospital, Dublin, Ireland, [3]School of Biology and Environmental Science Science Centre West, University College Dublin, Dublin, Ireland

Rapid Fire session 1B, May 16, 2023, 11:30 AM - 11:45 AM

Neuroblastoma (NB) is the most common extracranial solid childhood tumour, with high-risk patients still having low survival rates of 40%. Amplification of the MYCN gene (MNA) is strongly correlated with advanced stage disease and treatment failure. Pharmacologic inhibition of MYCN has proved to be very challenging as MYCN cannot be targeted directly, due to the absence of drug-binding pockets. In that context, it is known that MYCN drives metabolic modifications essential for oncogenesis in the tumorigenic cell, thus making mitochondria promising targets for the development of antineoplastic agents in NB.
We aim to inhibit MNA NB growth by targeting mitochondrial function, thereby finding new drug targets that eventually can limit the use of genotoxic treatments on young cancer patients.
To explore the role of MYCN in the mitochondria, we measured changes in mitochondrially-(RT-qPCR) and nuclearly-(database analysis)-encoded gene expression induced by different MYCN status, using doxycycline-inducible cell lines systems. To correlate these gene transcription changes with mitochondrial function and MYCN level, we measured various mitochondrial parameters (membrane potential, ROS, mass, fusion/fission), metabolic capacity (Seahorse Analyzer) and apoptosis. MtDNA-IP-Sequencing was subsequently performed to decipher whether the observed effects were due to MYCN binding directly to mtDNA, or indirectly by another interaction. In parallel, we tested OxPhos inhibitors for efficacy against MNA NB cells that can exploit the vulnerability mentioned above, by targeting mitochondrial function.
Gene expression measurements revealed that Complex I genes of the electron-transport-chain (ETC), but also POLRMT and TFAM, are the most broadly affected by MYCN amplification. Moreover, the ETC complexes are being regulated differently when MYCN is overexpressed, suggesting various metabolic perturbation upon MYCN level in the tumorigenic cell. MNA cells have shown to be more metabolically active than non-MNA cells, which correlates with Complex I genes being upregulated. Furthermore, treatments with OxPhos inhibitors decrease MYCN level, disrupt mitochondrial biogenesis, and increase ROS production and cell death in MNA NB cells. However, MYCN was not found binding to mtDNA, suggesting another indirect regulatory mechanism.
This study implies that altering MYCN level affects mitochondrial function and related gene expression, thereby highlighting therapeutic vulnerability for targeting the ETC in MNA NB.

P128

Unbiased high-throughput screening and preclinical testing in PDX models identifies synergistic combinations with the BCL2 inhibitor venetoclax

Fletcher J[1,2], Kamili A[1,2], Wheatley M[1], Bui C[1,3], Atkinson C[1,2], Elhassadi L[1,3], Qureshi S[1,3], Solomon J[1], Cadiz R[1], Failes T[1,4], Arndt G[1,2,4], Haber M[1,2], Norris M[1], Dolman E[1,2,5], Trahair T[1,2,6]

[1]Children's Cancer Institute, Lowy Cancer Research Centre, UNSW Sydney, Sydney, Australia, [2]School of Clinical Medicine, UNSW Sydney, Sydney, Australia, [3]School of Medical Sciences, UNSW Sydney, Sydney, Australia, [4]ACRF Drug Discovery Centre for Childhood Cancer, Children's Cancer Institute, Lowy Cancer Research Centre, UNSW Sydney, Sydney, Australia, [5]Princess Maxima Center for Pediatric Oncology, Utrecht, Netherlands, [6]Kids Cancer Centre, Sydney Children's Hospital Randwick, Sydney, Australia

Rapid Fire session 2A, May 17, 2023, 12:25 PM - 12:40 PM

Background

Survival for children diagnosed with high-risk neuroblastoma (HR-NB) remains <50% and survivors experience substantial long-term toxicities. The selective BCL2 inhibitor venetoclax, in combination with cyclophosphamide/topotecan, has shown promising activity in HR-NB patients, although limited by myelosuppression with continuous dosing. Venetoclax is also a recurrent recommendation from the Australian child cancer personalised medicine trial, ZERO Childhood Cancer.

Aims

To identify more effective and better tolerated venetoclax combinations using unbiased high-throughput screening in early passage patient derived xenograft (PDX) models, followed by preclinical testing using clinical trial-like experimental designs.

Methods

Co-immunoprecipitation was used to identify PDX models with low, intermediate, and high levels of Bim-BCL2 protein complex, a putative biomarker of venetoclax sensitivity. Combination drug screening was conducted ex vivo in three freshly dissociated PDX models using the IC30 concentration of venetoclax with a library of >120 approved oncology drugs (0.5nM–5μM) enriched for agents with paediatric safety data. Synergistic combinations were tested for tolerability in NSG mice, and then for efficacy in the PDX models in vivo, using schedules approximating those achievable in patients.

Results

Drugs synergistic with venetoclax in combination screens included both conventional chemotherapies (DNA topoisomerase inhibitors and microtubule inhibitors) and targeted agents (PARP, HDAC and AURKA inhibitors). To date, three combinations have been assessed in the PDX models in vivo. Vincristine-venetoclax prolonged survival in all three models tested, while vorinostat-venetoclax had limited efficacy. The most effective combination, alisertib-venetoclax, elicited objective responses in two models tested so far, including one maintained complete response and one complete response, and outperformed venetoclax-cyclophosphamide-topotecan. Furthermore, Bim-BCL2 protein complex levels predicted single agent venetoclax response in PDX models in vivo. Additional combinations are currently undergoing testing. We will further assess promising combinations using less myelosuppressive discontinuous dosing schedules that are more clinically relevant and will test efficacy across of panel of HR-NB PDX models using clinical trial like (n=1) experimental designs.

Conclusion

Unbiased high-throughput screening of PDX models can be utilised to identify more effective venetoclax combinations. Alisertib-venetoclax is a promising combination based on preclinical studies. This study may inform the design of future clinical trials, where venetoclax combinations may improve patient outcomes.

P129

Pseudometastatic allografting of TH-MYCN neuroblastoma cells with loss of p53 function provides an efficient syngeneic model of advanced-stage, high-risk disease

Nguyen F[1], Guan P[1], Gillen S[1], Bratinov G[2], Alferiev I[2], Chorny M[2], Brodeur G[1]
[1]*Children's Hospital of Philadelphia and the University of Pennsylvania, Division of Oncology, Philadelphia, United States,*
[2]*Children's Hospital of Philadelphia and the University of Pennsylvania, Division of Cardiology, ,*

Rapid Fire session 2A, May 17, 2023, 12:25 PM - 12:40 PM

Background. Despite improvements in the treatment of high-risk neuroblastoma (NB), there are no established rescue treatment options effective against recurrent high-risk tumors. More effective new therapies are desperately needed for these patients often present with metastatic disease and acquired drug resistance. Existing preclinical models do not capture the combination of essential biological and pathophysiological characteristics of relapsed aggressive disease. Therefore, developing effective and clinically viable therapies for this patient group requires establishing validated models integrating these features.

Aims. To characterize a pseudometastatic allograft model developed as a tool to evaluate experimental therapeutics in a setting simulating recurrent/refractory MYCN-driven disseminated NB.
Methods. Pseudometastatic allografts were established in heterozygous TH-MYCN mice by intravenous injection of a syngeneic NB cell line that integrates ABCG2 overexpression and loss of p53 function. This syngeneic model was characterized in comparison to a parent, genetically engineered TH-MYCN mouse model of NB with spontaneously arising localized tumors that overexpress ABCG2 but exhibit no loss of p53 function. In vitro antiproliferative effects of topoisomerase I inhibitors, SN38 and SN22, were compared by analyzing cell growth curves. Therapeutic responses to liposomal irinotecan and PEG-[SN22]4, injectable prodrug forms of these chemotherapeutic agents, were compared between the two models using the log-rank test.

Results. Both the established murine NB cell line and primary cells directly obtained from TH-MYCN NB tumors exhibited strongly reduced sensitivity toward SN38 vs. SN22, consistent with a marked difference in affinities of the two compounds for ABCG2. However, sensitivity toward both agents was further reduced in the p53-dysfunctional cell line in accordance with the presence of an additional resistance mechanism characteristic of recurrent disease. PEG-[SN22]4 was markedly more effective than liposomal irinotecan at extending animal survival in both models (median survival: 84 vs 40 days and 180+ vs 54 days in pseudometastatic allograft and spontaneous models, respectively). However, consistent with its more aggressive phenotype, a significant negative shift in response toward either agent was seen in the pseudometastatic allograft model.

Conclusion. Our syngeneic allograft model offers a reliable approach for comparative evaluation of new therapies in the setting of recurrent high-risk disease with intrinsic and acquired drug resistance.

P130

A zebrafish larvae xenograft model for high-throughput drug testing and precision medicine in neuroblastoma

Goulding J[1,2], Loontiens S[1,2], Depestel L[1,2], Hilgert E[1,2], De Vloed F[1,2], Eggermont A[1,2], Tapaneeyaphan P[1,2], Sanders E[1,2], Fieuws C[1,2], Claes K[1,2], De Preter K[1,2], Durinck K[1,2], Bek J[1,2], Speleman F[1,2]
[1]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium, [2]Cancer Research Institute Ghent (CRIG), Ghent, Belgium

Rapid Fire session 2A, May 17, 2023, 12:25 PM - 12:40 PM

Background: Patient-derived neuroblastoma (NB) xenograft mouse models are the current gold standard for preclinical testing of novel drugs or drug combinations. However, this approach has several limitations including the large number of tumour cells required for engrafting, high cost, and relatively slow turn-around time; limiting their use in personalised medicine.
Zebrafish hatch at 2 days post-fertilisation (dpf) with all organs, major cancer signalling pathways, and innate immune system present. With the adaptive immune system not established until 12-14 dpf, engraftment rates are high and their small size dramatically reduces the number of cells required for engrafting. Compound testing requires small volumes and assay-time is reduced to one week, facilitating high-throughput testing.

Aims: Our goal is to optimise this system as a personalised medicine platform in NB. The metastatic potential and innate immune cell invasion of engrafted patient-derived material will be determined. Preclinical drug testing will be performed by testing efficacy and toxicity of compounds with high-throughput.

Methods/materials: A panel of fluorescently stained neuroblastoma cell lines with diverse genomic backgrounds are engrafted into the perivitelline space of 2 dpf zebrafish larvae which are maintained at a hybrid temperature (34°C). Zebrafish larvae are exposed to compounds via waterborne exposure, between 24 and 72 hours post-injection (hpi). Tumour volume of engrafted cells is determined using spinning-disk confocal microscopy pre- and post-compound exposure (24 and 72 hpi). We will utilise transgenic kdrl and mpeg1 fluorescent reporters to assess metastatic potential and macrophage invasion respectively in engrafted patient material.

Results: We successfully engrafted a panel of NB cell lines into the perivitelline space of 2 dpf wild-type zebrafish. We observed increased toxicity in zebrafish larvae when exposed to specific compounds at the hybrid temperature of 34°C compared with 28°C (homeostatic temperature of zebrafish). NB cells exhibited local and distal metastasis 48 hpi. We will further evaluate a panel of compounds targeting replicative stress pathways in their ability to reduce engrafted tumour volume.

Conclusion: This platform will enable fast and cost-effective drug testing using a minimal amount of patient material and can therefore be used as a complementary approach to guide treatment decisions for NB.

P131

Joint inhibition of oncogenic and epigenetic targets PPM1D and KDM6B synergistically suppresses neuroblastoma growth

Treis D[1], Tümmler C[1], Åkerlund E[2], Pepich A[1], Seashore-Ludlow B[2], Sakaguchi K[3], Kogner P[1], Johnsen J[1], Wickström M[1]

[1]Childhood Cancer Research Unit, Dept. of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden, [2]Science for Life Laboratory, Dept. of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden, [3]Laboratory of Biological Chemistry, Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo, Japan

Rapid Fire session 1B, May 16, 2023, 11:30 AM - 11:45 AM

Background

High-risk neuroblastoma frequently contains segmental gain of chromosome 17q, including gain of oncogenic PPM1D which encodes the phosphatase WIP1, a regulator of p53 activity, DNA repair and apoptosis. High expression of PPM1D correlates to poor prognosis, and genetical or pharmacological inhibition of WIP1 suppresses neuroblastoma growth. KDM6B encodes the H3K27 demethylase JMJD3, which has also been implicated as a therapeutic target in neuroblastoma.

Aims

We aimed to identify synergistic drug combinations which potentiate the effect of PPM1D inhibition in high-risk neuroblastoma.

Methods

We screened 527 different cancer compounds in concentrations spanning the dose-response curve together with fixed concentrations of PPM1D inhibitors or vehicle. Potential synergy was assessed in five neuroblastoma cell lines using viability assays and dose-response matrices analyzed with the SynergyFinder tool. We also treated neuroblastoma multi-cellular tumor spheroids, observing size and viability and subsequently collecting them for immunohistochemistry. Further, we performed immunoblot, qPCR and RNA-seq on neuroblastoma cells exposed to vehicle, SL-176 (3–5μM), GSK-J4 (0.3–0.5μM), or the combination, extracting protein or RNA after 1-144 or 6-72 hours, respectively. Hallmark gene sets were used for gene set enrichment analysis (GSEA).

Results

In the screening with PPM1D inhibitor SL-176, we identified the epigenetic modifier GSK-J4 as the most promising combination candidate, a specific inhibitor of histone H3K27 demethylase KDM6B previously implicated in neuroblastoma and other malignancies. Viability assays demonstrated different degrees of synergistic cytotoxicity in all tested neuroblastoma cell lines. Tumor spheroids treated with the drug combination showed smaller size and decreased viability after six days compared to single drug treatment. Immunoblot experiments exhibited a marked effect on WIP1 downstream targets and apoptosis markers, while qPCR confirmed the synergistic upregulation of p53 downstream targets PUMA and p21. RNA-seq showed a vast number of differentially expressed genes, suggesting a pervasive effect on transcription. GSEA revealed an enrichment of the p53 pathway, TNFα signaling via NFκB, and TGFβ signaling, while gene sets for E2F and MYC targets were downregulated.

Conclusion

The combination of the PPM1D inhibitor SL-176 and the epigenetic modifier GSK-J4 induces synergistic cytotoxicity and growth inhibition in neuroblastoma cells and spheroids by potentiating p53 downstream effects.

P132

Splicing dependency of neuroblastoma

YANG J
[1]*Department of Surgery, ST JUDE CHILDREN'S RESEARCH HOSPITAL, Memphis, United States*

Background
Oncogenic transcription factors drive a variety of types of cancer including neuroblastoma, which is responsible for 15% of cancer related deaths in childhood cancers. Neuroblastoma originates from neural crest lineage cells that are transformed by dysregulated MYC oncogenes. The “undruggability” of MYC transcription factors and few recurrent somatic mutations of neuroblastoma lead to enormous challenges for developing more effective targeted therapies. Pre-mRNA splicing is a process coupled with active gene transcription and catalyzed by spliceosome for production of mature mRNAs. Genome-wide studies of transcriptomics in thousands of human tumors revealed dysregulated pre-mRNA splicing existing across all types of cancer, which act as a critical pathway in tumorigenesis and may consequently create vulnerabilities for cancer cells.

Aims
The objective of this study is to explore the feasibility of targeting the vulnerable splicing machinery in MYC-driven neuroblastoma.
Methods
RNA-seq analysis, shRNA knockdown, CRISPR knockout and knockin, in vitro viability assay and in vivo efficacy, transgenic MYC models and PDXs, molecular glue "indisulam".

Results
The splicing factor RBM39 is exquisitely essential to neuroblastoma. Indisulam, a “molecular glue” that selectively recruits RBM39 to the CRL4-DCAF15 E3 ubiquitin ligase for proteasomal degradation, is highly efficacious against neuroblastoma, leading to significant responses in multiple high-risk disease models, without overt toxicity. The high efficacy of indisulam against neuroblastoma is determined by high-levels of DCAF15 expression.

Conclusion
Targeting the dysregulated spliceosome by targeted degradation of RBM39 is an extremely promising precision therapeutic strategy against neuroblastoma.

P133

Neuroblastoma resistant to lorlatinib upregulates FGFR2 and acquires sensitivity to FGFR inhibition

Pucci P[1], Barrett C[1], Trigg R[1], Jahangiri L[1], Matthews J[1], Mologni L[2], Burke G[3], Turner S[1,4,5]
[1]University of Cambridge, Cambridge, United Kingdom, [2]Dept. of Medicine and Surgery, University of Milano-Bicocca, Monza, Italy, [3]Department of Paediatric Oncology, Addenbrooke's Hospital, Cambridge, United Kingdom, [4]European Research Initiative for ALK related malignancies (ERIA), , , [5]CEITEC, Masaryk University, Brno, Czech Republic

Background
High-risk neuroblastoma (NB) has a poor prognosis despite multimodal treatment. To improve survival and minimise treatment side-effects, research has focused on developing more effective therapeutics. Anaplastic lymphoma kinase (ALK) is a promising druggable target as its expression rapidly decreases in healthy tissues postnatally, and ~14% of high-risk NB express ALK hyperactivated mutant forms. The ALK tyrosine kinase inhibitor lorlatinib is a promising treatment, but resistance has been reported, hindering long-term benefits.

Aims
We aim to identify genes whose expression is associated with decreased sensitivity to lorlatinib creating novel therapeutic vulnerabilities in high-risk NB.

Methods
We used a trimodal approach, consisting of genome-wide CRISPR-Cas9 overexpression (CRISPRa), RNA-sequencing and high-throughput automated drug screens.

Results
We validated the CRISPRa screen hit FGFR2 as a bypass signalling mechanism desensitising mutant ALK-expressing NB to lorlatinib; overexpression of FGFR2 increased, while silencing decreased resistance of NB cells to lorlatinib. Furthermore, RNA-sequencing of lorlatinib resistant (LR) NB cells developed in our lab, compared to parental cell lines, showed FGFR2 to be expressed to a higher level in the LR cells. High-throughput drug screens exposing LR and parental NB cell lines to an FDA-approved drug library of 1430 compounds showed that drugs targeting receptor tyrosine kinases, including FGFR2, were amongst the compounds most significantly effective in reducing the viability of LR NB cells compared to parental cells. The multi-kinase inhibitor ponatinib and the selective FGFR inhibitor erdafitinib acted synergistically with lorlatinib in treating both parental and LR NB cells although both inhibitors were more effective in inhibiting the growth of LR compared to parental cells, suggesting that FGFR2 may represent a novel vulnerability to treat lorlatinib resistant NB. In vivo studies using patient-derived xenograft (PDX) models of high-risk NB (MYCN-amplified and ALKF1174L mutant) showed that combinations of either ponatinib or erdafitinib with lorlatinib decreased tumour growth and increased survival compared to PDXs treated with vehicle or either agent alone.

Conclusion
Overall, these findings suggest that FGFR2 alters NB sensitivity to lorlatinib and modulation of this pathway in combination with ALK inhibition is a promising approach to improve NB treatment response and ultimately patient survival.

P134

Ketogenic diet and metformin enhance the effect of cyclophosphamide on neuroblastoma

Catalano L[1], Aminzadeh-Gohari S[1], Stefan V[1], Weber D[1], Feichtinger R[1], Smiles W[1], Bereswill S[2], Heimesaat M[2], Kofler B[1]

[1]Research Program for Receptor Biochemistry and Tumor Metabolism, Department of Pediatrics, University Hospital of the Paracelsus Medical University, Salzburg, Austria, [2]Institute of Microbiology, Infectious Diseases and Immunology, Gastrointestinal Microbiology Research Group, Charité - University Medicine Berlin, Berlin, Germany

Background

Previously, we reported that a ketogenic diet (KD) successfully targets neuroblastoma (NB), when combined with cyclophosphamide (CP). Recent findings have suggested that the inhibition of residual oxidative phosphorylation (OXPHOS) activity with metformin (MET), an inhibitor of complex I of OXPHOS, could be beneficial for cancer treatment.

Aim

The aim of this study was to elucidate whether MET can enhance the anti-tumorigenic effects of a KD in NB.

Methods

To evaluate the effect of MET on mitochondrial function in NB cells in vitro the oxygen consumption rate (OCR) was measured by the Agilent Seahorse XF Analyzer. NB xenografts were established in CD-1 nude mice with the MYCN amplified cell lines SKNBE(2) and KELLY. The NB-bearing mice were fed with a control diet (CTRL) or a KD (ketogenic ratio 8:1) with/without MET (oral gavage, 100 mg/kg body weight) and with/without low-dose cyclophosphamide (CP) (13 mg/kg in SKNBE(2), 20 mg/kg for KELLY), in drinking water. Microbiome analysis of feces was performed by qRT-PCR. 20 different biomarkers potentially involved in tumor progression were measured in plasma by the Mesoscale Discovery Technology.

Results

Maximal mitochondrial respiration was inhibited dose dependently by MET in the MYCN amplified NB cells. MET enhanced the anti-proliferative effect of the KD when combined with CP on both xenograft models. Furthermore, the triple therapy (MET+CP+KD) led to an increase of survival when compared to CP+CTRL diet. The KD-induced elevation of ketone bodies was not influenced by co-treatment with MET, while blood glucose levels were reduced in the KD+CP+MET groups. Fibroblast growth factor 21 (FGF-21) was highest in MET+CP+KD compared to all other treatment groups. KD+CP+MET reduced the expression of Acetyl-CoA-Carboxylase (ACC), a rate-limiting enzyme in fatty acid metabolism, compared to CTRL+CP+MET. Whereas, the phosphorylation status of ACC, the central regulator of energy homeostasis AMP-activated protein kinase (AMPK), as well as the expression of MYCN was unaffected. Lactobacilli levels were reduced in all KD groups. Clostridium cluster IV levels were only reduced in groups treated with MET+KD.

Conclusion

Our data suggest that MET and KD can enhance the anti-tumor efficacy of chemotherapy and therefore be used potentially as novel adjuvant therapy.

P135

PREME: AN ITALIAN PERSONALIZED MEDICINE PROGRAM FOR HIGH-RISK NEUROBLASTOMA

Capasso M[1], Brignole C[2], Bensa V[2], Lasorsa V[3], Sebastiani E[4], Cantalupo S[1], Avitabile M[1], Sementa A[5], Mazzocco K[5], Cafferata B[5], Vellone V[5], Cilli M[6], Calarco E[2], Giusto E[2], Ciampi E[2], Perri P[2], Corrias M[2], Aveic S[7], Fruci D[8], Quattrone A[4], Tondo A[9], Luksch R[10], Mura R[11], Rabusin M[12], De Leonardis F[13], Cellini M[14], Coccia P[15], Iolascon A[1], Conte M[16], Amoroso L[16], Garaventa A[16], Ponzoni M[2], Pastorino F[2]

[1]Università di Napoli Federico II, Dipartimento di Biotecnologie Mediche e Medicina Molecolare, Naopli, Italy, [2]Laboratorio di Terapie Sperimentali in Oncologia, IRCCS Istituto Giannina Gaslini, Genova, Italy, [3]CEINGE Biotecnologie Avanzate, Naopli, Italy, [4]CIBIO, Università di Trento, Trento, Italy, [5]UOC Anatomia Patologica, IRCCS Istituto Giannina Gaslini, Genova, Italy, [6]Stabulario, IRCCS Policlinico San Martino, Genova, Italy, [7]Fondazione Istituto di Ricerca Pediatrica Città della Speranza, Padova, Italy, [8]Dipartimento di Ematologia-Oncologia Pediatrica e Terapia Cellulare e Genica, IRCCS Bambino Gesù Children's Hospital, Roma, Italy, [9]Dipartimento di Ematologia-Oncologia, Anna Meyer Children's Hospital, Firenze, Italy, [10]Unità di Oncologia Pediatrica, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy, [11]Oncoematologia Pediatrica, Ospedale Pediatrico Microcitemico "Antonio Cao" Azienda Ospedaliera Brotzu, Cagliari, Italy, [12]Dipartmento di Pediatria, Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Trieste, Italy, [13]Dipartmento di of Oncologia Pediatrica, Azienda Ospedale Policlinico di Bari, Bari, Italy, [14]U.O.C Oncoematologia Pediatrica Azienda Ospedaliero Universitaria Modena, Modena, Italy, [15]Azienda Ospedaliero Universitaria delle Marche, Presidio Ospedaliero "G. Salesi", Ancona, Italy, [16]UOC Oncologia, IRCCS Istituto Giannina Gaslini, Genova, Italy

Background: In the era of precision medicine, the need for high-risk neuroblastoma (NB) patient-specific therapies is crucial.

Methods: From November 2018 to February 2021, the Italian PeRsonalizEd MEdicine (PREME) program has enrolled 18 NB affected patients. After either histological or flow cytometry immunophenotyping, tumors and bone marrow-infiltrating NB cells were used for: 1) DNA extraction for subsequent exome sequencing, 100X mean coverage, or sequencing of 498 cancer genes, 1000x mean coverage; 2) RNA extraction for subsequent RNAseq (30 millions of reads per sample); 3) Development of Patient-Derived Xenografts (PDX) models in mice and primary NB cell culture (3D/tumor-spheres).

Results: In fourteen out of 18 (77.7%) patients, the Molecular Tumor Board identified one or more targetable somatic alterations (including point mutations, copy number variations and mRNA over-expression). Nine tumors showed alterations, graded as "very high priority", that are validated to be directly targetable by an approved drug or an investigational agent, while 5 tumors displayed alterations, graded as "high priority", that are predicted to be pathogenetic in genes or pathways targetable by approved drug or an investigational agent. A molecular targeted therapy was applied for 4 patients. A genetic counseling was suggested to 4 patients having one pathogenic germline variant in known cancer predisposition genes. Out of 11 tumor samples implanted in mice, 5 gave rise to PDX. Comparing all genomic variants of the 5 tumors with developed PDX samples up to second generation, a high grade of similarity among primary tumors and PDX models (Pearson coefficients>0.8) was observed. The validity and reproducibility of our PDX models was also demonstrated from the high rates of conserved somatic variants (mean average: 81.3%) and of the identification of all the potentially actionable genetic alterations among primary tumors and PDX generations. A high grade of similarity was confirmed when the histological, the immunophenotypic and the transcriptomic profiles among primary tumors and PDX generations were compared. NB cells grown as 3D demonstrated good rates of conserved somatic variants.

Conclusions: PREME program has reported NB patient samples, which harbor targetable genomic alterations and has allowed the development of a Bio-banks to be used for translational research.

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SHP2 and ALK inhibitor combinations are synergistic in ALK-driven neuroblastoma models

Valencia-Sama I[1], Kee L[1], Christopher G[1], Ohh M[2,3], Hayes M[1,4], Irwin M[1,2,5,6]

[1]The Hospital for Sick Children, Cell Biology Program, Toronto, Canada, [2]University of Toronto, Department of Laboratory Medicine and Pathobiology, Toronto, Canada, [3]University of Toronto, Department of Biochemistry, Toronto, Canada, [4]The Hospital for Sick Children, Developmental and Stem Cell Biology Program, Toronto, Canada, [5]University of Toronto, Department of Medical Biophysics, Toronto, Canada, [6]The Hospital for Sick Children, Department of Paediatrics, Toronto, Canada

Background: Combination therapies that target alterations detected in recurrent high-risk neuroblastoma (NB) are needed. RAS/MAPK-activating events are common in NB, with ALK being the most mutated gene in both primary and relapsed tumors. Although ALK tyrosine kinase inhibitors (TKIs) have shown promise for ALK-driven NB, innate or adaptive resistance to single agent ALK-TKIs remains a challenge. Recently, SHP2 inhibitors have been shown to overcome ALK-TKI resistance in ALK-rearranged lung tumors, and we previously reported that SHP2 inhibitor combinations target certain NBs based on their RAS/MAPK pathway status. Thus, we hypothesized that dual SHP2/ALK inhibition may be effective in subsets of NB.

Aims: In this study we evaluated the pre-clinical efficacy of the novel SHP2 inhibitor TNO155 alone and in combination with the ALK inhibitors crizotinib, ceritinib, or lorlatinib for the treatment of ALK-driven NB.

Methods: Drug efficacy and combinations were assessed in vitro on a panel of human NB cell lines, and in vivo using diverse ALK-mutant larval zebrafish and murine xenograft models.

Results: In comparison to wild-type, ALK-mutant NB cell lines were more sensitive to SHP2 inhibition with TNO155. Treatment with TNO155 and ALK-TKIs synergistically reduced cell growth and promoted inactivation of ALK and MAPK signaling in ALK-mutant cell lines. Moreover, ALK-mutant larval zebrafish xenografts treated with single agent inhibitors showed reduced growth and invasion, which was further enhanced upon dual SHP2/ALK inhibition. Likewise, in murine ALK-mutant xenografts, tumor burden was reduced or delayed, and survival was prolonged with TNO155 and lorlatinib combination treatment. Finally, we show that ALK-F1174L mutant NB cells and tumors that have become resistant to lorlatinib can be re-sensitized with the addition of TNO155 in vitro and in vivo.

Conclusions: Overall, our studies provide the first in vitro and in vivo assessment of single agent TNO155 in NB, and importantly demonstrate the efficacy and tolerability of TNO155 combinations with ALK-TKIs in several ALK-mutant NB models. These results highlight the translatability between zebrafish and murine xenografts, and the clinical potential of concurrent SHP2/ALK inhibition as a novel approach for the treatment of ALK-aberrant NB, including those with innate or acquired resistance to ALK-TKIs.

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Large scale drug response comparison across different neuroblastoma cell model systems

Park SW[1], Seashore-Ludlow B[1], Östling P[1], Molenaar JJ[2], Stenman J[3], Karlsson K[1]

[1]Department of Oncology-Pathology, Karolinska Institute, Stockholm, Sweden, [2]Paediatric Oncology, Princess Maxima Centre, Utrecht, The Netherlands, [3]Department of Women's and Children's Health, Karolinska Institute, Stockholm, Sweden

Background

Traditionally, 2D models have been used for drug response studies due to their ease of handling and the lack of better models. Today, various 3D models, including patient-derived tumour organoids (PDO) and patient-derived xenografts (PDX), are gaining preference due to their ability to predict patient treatment responses. These tumour models are thought to better recapitulate the tumour architecture than conventional 2D models and therefore provide better drug response predictions. Here, we present drug screening results comparing neuroblastoma 2D and 3D cell culture models.

Aims

To compare neuroblastoma 2D and 3D models for drug screening purposes.

Methods

Drug screening with 528 existing and emerging cancer drugs was performed on seven neuroblastoma cell lines and three PDOs. Drug responses was measured using the cell viability assay CellTiter-Glo(R) and analyzed with Breeze software. Drug responses were compared to a recent study by Hansson et al (1) that performed similar drug screens on neuroblastoma organoids derived from PDX models.

Results

We identified 10 drugs that were effective across all cell culture models, including targeted therapies like Aurora, IGF1R and CHEK1 inhibitors. There was a clear difference in drug response between 2D and 3D cultures, where for example chemotherapy compounds were generally more effective in PDO and PDX models. Notably, one PDO model that preferred to grow in 2D monolayer clustered together with 2D cell cultures on overall drug response, despite being cultured in organoid media.

Conclusions

Large scale drug screening comparing drug response between neuroblastoma 2D and 3D cell culture models showed a marked difference in response between topologies, but only a subtle difference between PDO and PDX derived organoids lines, despite that fact the PDO and PDX lines were drug treated at different centra and grown using different media compositions. The study suggests that the choice of cell culture topology will have a large effect on drug response, but that PDO and PDX derived lines to a large extent can be used interchangeably for drug screening experiments.

References

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Screening of NB PDTC models with a large compound drug library. A COMPASS study.

Bellini A[1,2], Del Nery E[3], Gestraud P[4], Anthony E[3], Tessier S[3], Surdez D[5], Zaidi S[6], Saint-Charles A[1,2], Iddir Y[1,2], Bobin C[1,2], Bhalshankar J[1,2], Butterworth C[1,2], Geoerger B[7], Delattre O[1], Schleiermacher G[1,2]

[1]INSERM U830, Laboratoire de Génétique et Biologie des Cancers, Institut Curie, PSL Research University, Paris, France, [2]SiRIC RTOP « Recherche Translationelle en Oncologie Pédiatrique », Translational Research Department, Institut Curie, PSL Research University, Paris, France, [3]Biophenics High-Content Screening Laboratory, Translational Research Department, Cell and Tissue Imaging Facility (PICT-IBiSA), Institut Curie, PSL Research University, Paris, France, Paris, France, [4]INSERM U900, Bioinformatics and Computational Systems Biology of Cancer, Institut Curie - Mines Paris Tech, PSL Research University, Paris, France, [5]Balgrist University Hospital, University of Zurich, Zurich, Switzerland, [6]INSERM U830, Équipe Labellisée La Ligue Nationale Contre Le Cancer, SIREDO Oncology Center, Institut Curie, PSL Research University, Paris, France, [7]Gustave Roussy Cancer Center, Department of Pediatric and Adolescent Oncology, Université Paris-Saclay, INSERM U1015, Villejuif, France

Background and aims:
New treatment strategies are urgently required for children with high risk neuroblastome (HR-NB). Here we sought to establish a standardized ex vivo high throughput drug response profiling platform to establish drug efficacies in patient-derived tumor cells (PDTC) established from patient-derived tumor xenografts (PDTX) and to correlate these with potential biomarkers.

Methods:
The drug library consisted of 75 clinically approved cancer drugs including those used in standard of care for HR-NB and small molecule targeted therapies. Drugs were pre-printed in 384 well plates (wp) in five concentrations each. Fourteen PDX models, with distinct genetic profiles (such as MYCN amplification, ALK amplification or mutations, ATRX mutations) were screened in this study.
PDX tumors were dissociated and the cell suspension was seeded into the 384-wp (day 0). After 24 hours of incubation at 37°C, drugs were added in the wells and drug-treated cells were incubated for additional 72 hours. Cell viability was then measured at day 4 by using CellTiter-Glo luminescent cell viability assay (CTGa). Luminescence reading was normalized to and expressed as a relative percentage of DMSO controls. Dose-response curves were fitted for each drug and HR-NB model with corresponding drug sensitivities represented by a single quantitative drug sensitivity score (DSS).

Results: HR-NB models showed broad resistance to most drugs included in the library.

The most commonly used broad-spectrum chemotherapeutics were the most active compounds, with Actinomycine D showing high cytotoxicity.
A high cytotoxicity across all models was observed for the proteasome inhibitor bortezomib, and 3 HDACi (Panobinostat, Vorinostat and Entinostat). The ALK inhibitor ceritinib also showed wide cytotoxicity across all PDX models.
One PDX model (GR-NB5, harboring an ALK amplification) showed a higher drug sensitivity compared to other models, whereas 2 PDX models (IC-pPDX183 and IC-pPDX109, both harboring ALK wt) were globally more resistant.

Conclusions:

This study showed that ex-vivo high throughput drug response profiling platform can be used to find drug efficacies in HR-NB and revealed the high cytotoxic effect of chemotherapeutics and HDACi compounds on the majority of HR-NB models. This platform now enables the exploration of specific drug combinations in PDTC.

P139

Quatsomes are novel platform to deliver RNA-based therapies for Neuroblastoma

Boloix A[1], Köber M[2,3], Molero-Valenzuela A[1], Merlo J[4], Pérez-García M[1], Avilés-Domínguez L[2], Piqué-Ponti J[2], Soriano A[1], Roma J[1], Córdoba A[4], Sala S[4], Moreno L[1], Ventosa N[2], Segura M[1]

[1]Group of Childhood Cancer and Blood Disorders. Vall d'Hebron Research Institute (VHIR), Barcelona, Spain, [2]Institut de Ciència de Materials de Barcelona (ICMAB-CSIC), Cerdanyola del Vallès, Spain, [3]CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Madrid, Spain, [4]Nanomol Technologies SA, Mòdul de Recerca B, Campus Universitari de Bellaterra, Cerdanyola del Vallès, Spain

Background:
Neuroblastoma (NB) is the most common solid tumor in childhood which causes ~15% of pediatric cancers deaths. Despite current therapies have improved patient survival rates, most of high-risk NB patients still present poor survival and need new therapies.
MicroRNAs (miRNAs) are endogenous non-coding small RNAs that are key regulators of gene expression and deregulated in multiple tumors including NB. Mounting evidence showed that restoration of miRNA expression result in anti-tumoral effects in NB preclinical models. However, the lack of a consensus formulation for their clinical delivery is blocking their way into clinical trials.

Aim:
Our aim is to develop a platform for the clinical delivery of miRNAs and/or other nucleic acids for the treatment of high-risk NB.

Methods:
Quatsomes are lipid-based nanovesicles that conjugate miRNAs through electrostatic interactions. MiRNA levels and their target genes expression were monitored by RT-qPCR and western blot. In vitro antitumoral effects were measured by crystal violet staining. Neuroblastoma xenografts were established by injecting SK-N-BE(2) cells into the flank of athymic nude-Foxn1nu mice. The QS-miRNA complexes were injected intravenously or intratumorally and their molecular effects and biodistribution were monitored by RT-qPCR and in vivo imaging (IVIS).

Results:
Quatsomes (QS) are prepared by a green, GMP compliant and scalable procedure, essential requirements for the entrance of new drugs into clinical trials. With this procedure, QS have small size (80-100nm), high homogeneity and positive charge. These properties grant a long-term colloidal stability, scalability, efficient entrapment of miRNAs and the protection of miRNAs from nucleases degradation. In addition, QS were capable of internalizing and releasing miRNAs in the cytoplasm of NB cells. From the functional point of view, treatment of neuroblastoma cells with QS-miR-323a-5p nanoconjugates induced downregulation of bona fide miR-323a-5p targets, such as CCND1 or CHAF1A, at mRNA and protein level and halted the proliferation of neuroblastoma (NB) cells.
In vivo, QS-miRNA injected intravenously reached partially NB tumors and when were injected intratumorally, QS-miR-323a-5p complexes showed a reduction of miR-323a-5p target genes expression, thereby confirming its efficacy in vivo.

Conclusion:
We have developed a novel platform to deliver tumour-suppressive miRNAs for the treatment of high-risk NB.

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Targeting SWI/SNF ATPase SMARCA4 in core transcription factors-addicted neuroblastoma suppresses cell plasticity and invasiveness

Xu M[1], Hong J[1], Zhang X[1], Sun M[1], Jung R[1], Lei H[1], Shern J[1], Thiele C[1], **Liu Z[1]**
[1]*Pediatric Oncology Branch, National Cancer Institute, Bethesda, United States*

Background: Mutations of SWI/SNF subunits SMARCA4, ADRID1A and ADRID1B occur in approximately 10% of neuroblastoma (NB). Although ARID1A functions as a tumor suppressor, the silencing of both ARID1A and ARID1B inhibits NB growth and metastasis. SMARCA4 knockdown suppresses NB cell proliferation, but a recent study showed that PROTAC degradation of SWI/SNF ATPases SMARCA2/4 had no such effect. As a chromatin remodeler, SMARCA4 cooperates with pioneer factor OCT4 to establish an accessible chromatin state. Core transcription factors HAND2 and MYCN cooperate to compete with nucleosomes in NB, but the mechanisms remain unclear. Our recent MYCN interactome assay identified that MYCN interacts with SMARCA4.

Aim: Investigate whether SMARCA4 cooperates with HAND2/MYCN to regulate chromatin accessibility and if it is druggable in NB.

Methods: We performed RNA-seq, assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) and chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq) to investigate SMARCA4's role in NB.

Results: Co-immunoprecipitation confirmed interaction between SMARCA4 and MYCN in NB cells. ChIP-seq results revealed genome-wide co-localization of SMARCA4, HAND2 and MYCN. SMARCA4 silencing in IMR32 resulted in a 50% decrease in MYCN ChIP-seq signals ($p<0.0001$) and also decreased chromatin accessibility shown by a >40% decrease in ATAC-seq signals ($p<0.0001$). Depmap portal gene expression and cancer dependency analyses indicated that SMARCA4 is both highly expressed ($p<0.0001$) and selectively essential ($p<0.0001$) in NB compared to ~1000 other cancer cell lines. Consistently, SMARCA4 depletion using siRNAs or PROTAC degrader, ACBI1, resulted in 20-50% decrease ($p<0.05$) of NB (IMR32, IMR5, KCNR and BE(2)C) cell proliferation shown by CellTiter-Glo assay. Transcriptomic profiling showed that SMARCA4 depletion in IMR5 cells using two different siRNAs (48h) or ACBI1 (8h and 24h) resulted in a negative enrichment of epithelial-mesenchymal transition and collagen containing extracellular genes, genes which are also regulated by HAND2. IncuCyte scratch and single spheroid invasion assays revealed decreased cell migration (>2-fold, $p<0.01$) and matrigel invasion (>10-fold, $p<0.01$) for ACBI1-treated NB cells.

Conclusion: Our study demonstrates that SMARCA4 cooperates with HAND2/MYCN to open chromatin enabling a plastic and invasive transcriptional program in NB. Importantly, we find that SMARCA4 is a druggable target in highly metastatic, core transcription factors-addicted NB.

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Novel combination therapy for MYCN-driven neuroblastoma targeting the deubiquitinase USP5

Cheung B[1,2], Gadde S[1], Chung N[1], Mittra R[1], Kleynhans A[1], Ng S[1], Doughty L[3], Nero T[3], Kim D[4], Zhu S[4], Carter D[1,5], Kumar N[6], Parker M[3], Marshall G[1,7]

[1]Children's Cancer Institute Australia, Lowy Cancer Research Centre, UNSW Sydney, Australia, , , [2]School of Clinical Medicine, Faculty of Medicine & Health, UNSW Sydney, Australia , , , [3]Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Victoria 3010, Australia, , , [4]Department of Biochemistry and Molecular Biology, Cancer Center and Center for Individualized Medicine, Mayo Clinic, Rochester, MN, 55902, USA, , , [5]School of Biomedical Engineering, University of Technology Sydney, Australia, , , [6]School of Chemistry, UNSW Sydney, NSW 2052, Australia , , , [7]Kids Cancer Centre, Sydney Children's Hospital, Sydney, Australia, ,

Background: Following a screen for drugs which synergised with histone deacetylase inhibitors (HDACi), we identified a novel compound (SE-486-11) which repressed levels of the deubiquitinase USP5 in MYCN amplified neuroblastoma (NB) cells. We recently showed that ubiquitin-specific protease 5 (USP5) is a novel MYCN binding protein and co-factor for the MYCN oncogenic signal in NB. Mechanistic studies revealed that MYCN bound the USP5 promoter and induced USP5 gene expression, while overexpression of USP5 increased MYCN protein stability. The two proteins formed a positive forward feedback loop, driving MYCN levels ever higher. Thus, disruption of the USP5/MYCN protein complex is a potential new therapeutic avenue for high-risk NB patients.

Aims: To improve efficacy and reduce toxicity of compounds which synergise with HDACi and inhibit the USP5/MYCN complex.

Methods: We used Surface Plasmon Resonance (SPR) and Cellular Thermal Shift Assay (CETSA) to determine the direct binding interactions between USP5 and newly synthesised USP5 inhibitors. We also performed cycloheximide assay to determine MYCN protein half-life. For in vivo efficacy studies, we used NB TH-MYCN mice, MYCN zebrafish and NB xenograft models.

Results: We designed and synthesised 64 new analogues based on the parental compound, SE486-11, using structure-activity relationship data. A total of 15 UNSW-SC compounds displayed sub-micromolar IC50 values (26-1000 nM) against NB cell lines, and significantly less toxic to normal human fibroblasts. UNSW-SC-22 bound the USP5 protein in a dose-dependent manner demonstrated by SPR and CETSA. UNSW-SC-22 decreased USP5 and MYCN protein expression in NB cells, but not mRNA levels. UNSW-SC-22 reduced MYCN protein half-life and displayed MYCN and USP5 protein dependency for its cytotoxicity in doxycycline inducible MYCN and USP5 shRNA knockdown in NB cell lines. In combination with a histone deacetylase inhibitor SAHA, or chemotherapy drugs (irinotecan and temozolomide), UNSW-SC-22 combinations significantly reduced tumour growth and enhanced the survival of TH-MYCN homozygous mice in comparison with SAHA, UNSW-SC-22, or chemotherapy drugs alone. Experiments are undergoing to determine the in vivo efficacies of the novel combination therapies in multiply NB animal models, and to identify specific biomarkers for predicting which patients are likely to benefit from the novel combination treatment.

P142

Fast, in vivo drug-response evaluation with single cell resolution of human-zebrafish xenografts (ZefiX)

Gauert A[1], Brame L[1], Köppke J[1], Proba J[1], Eggert A[1], Hagemann A[1]
[1]*Charité-Universitätsmedizin Berlin, Dept. of Pediatric Oncology/Hematology, Berlin, Germany*

Background
Children with relapsed neuroblastoma face a long-term survival rate < 10%. New therapy strategies are urgently needed. Currently, potentially suitable molecular targeted or immunotherapeutic drugs are chosen based on molecular profiles in single tumor biopsies, which has proven to be of limited value for reliable prediction of treatment response in patients. The preferred model organism for patient-derived xenografts remains the immunodeficient mouse. However, xenografting neuroblastoma into mice also remains challenging to date, with success rates of only 30% and an engraftment time of several weeks. Zebrafish are an excellent alternative as transplant host organism because of their cost and space efficiency, external embryonic development, and their rapid rate of growth. Embryonic engraftment requires only a few days. However, reliable drug response evaluation, which is mostly imaging based, is limited by manual effort and resolution.

Aims
To optimize pre-clinical drug selection, we established a new model for individualized drug testing on patient-derived xenografts in immunosuppressed zebrafish with single cell evaluation of viability and cell division rate (ZefiX).

Methods
Human neuroblastoma cells are transplanted into several hundred zebrafish embryos with a transiently suppressed, innate immune response. Groups of 25 embryos are subsequently bathed in various doses of selected drugs. After a 3-day incubation period in vivo at 35°C, the cells are isolated and examined for their vitality and the proliferation rate of viable cells by flow cytometry.

Results
We previously established xenotransplantation of primary human B-cell precursor acute lymphoblastic leukemia cells into zebrafish embryos with a 60% success rate for use as testing avatars and have adapted our protocol now for neuroblastoma/solid cancer cells. We find that suppression of the innate host immune system and proper choice of injection site increase graft cell survival substantially. With flowcytometric analysis of graft cells after incubation, the number of cell divisions and the state of vitality can be assessed for each cell individually. Inhibitory drug effects require strongly increased doses compared to conventional cell culture and, therefore, experimental success can be hampered by host toxicity.

Conclusion:
ZefiX is a reliable in vivo assay for drug response prediction with limitations by pharmacokinetics.

P143

Dual AURKA (LY3295668) and BCL2 (venetoclax) inhibition is potently effective in preclinical models of neuroblastoma (NB)

Groff D[1], Casey C[1], Krytska K[1], Tsang M[1], Acholla C[1], Maris J[1], Mossé Y[1]
[1]*Children's Hospital of Philadelphia, Philadelphia, United States*

Background:
Deregulated expression of the MYCN oncogene is associated with the development of many neural-derived pediatric cancers including NB, medulloblastoma and atypical teratoid rhabdoid tumors. While direct therapeutic targeting of MYCN protein remains challenging, we previously demonstrated synergistic activity of dual targeting of BCL-2 (venetoclax) and AURKA (MLN8237) in MYCN-amplified NB (Ham, Cancer Cell, 2016). With MLN8237 no longer being developed due to toxicities related to collateral AURKB inhibition, testing of a highly selective AURKA inhibitor, LY3295668, is warranted.

Aim: To determine the optimal dosing, toxicity, and anti-tumor efficacy of venetoclax and LY3295668 in preclinical models of NB.

Methods:
LY3295668 (Chemie Tek) and venetoclax (Selleck Chem) were tested alone and in combination in nine NB PDX models using 2-3 mice/arm. Six of nine PDX models tested (COG-N-424x, COG-N-453x, NB-1643, Obelix-Rx, Obelix-Fx and Obelix-2x) were MYCN-amplified and three (Felix-PDX, COG-N-619x, SK-N-AS) were MYCN-non-amplified. First, a comprehensive toxicity study, testing LY3295668 at 30mg/kg with venetoclax at 100mg/kg, 75mg/kg, 50mg/kg, and 25mkg/kg, was performed. Based on tolerability of the combination, these were the maximum tolerated doses when tested in combination, that did not result in weight loss greater than 15% or other overt signs of toxicity.

Results:
Toxicity evaluation established 30mg/kg for LY3295668 and 50mg/kg for venetoclax administered once daily, seven days a week, via oral gavage for 60 days as optimal. The combination was well tolerated as monotherapy and when administered in combination with a maximum weight loss of 13%. Single agent therapy resulted in minimal if any growth delay. Four of six MYCN-amplified models demonstrated maintained complete responses and two of six showed significant tumor growth delay ($p<0.001$). Two of three MYCN-non-amplified model demonstrated maintained complete response while one showed no significant growth delay compared to vehicle.

Conclusions: Dual targeting of AURKA and BCL2 with LY3295668 and venetoclax is tolerable and demonstrate compelling anti-tumor activity in MYCN-amplified NB PDX models, validating our earlier findings, but now with a more selective AURKA inhibitor. These preclinical results support clinical development of this combination for NB patients with and without MYCN amplification.

P144

In vitro drug screens as a mirror of in vivo drug sensitivity, a next step in neuroblastoma precision medicine

Schoonbeek M[1], Eising S[1], van Hooff S[1], Koopmans B[1], Vernooij L[1], Amo-Addae V[1], Molenaar J[1]
[1]*Prinses Maxima Center, Utrecht, Netherlands*

Background
Identifying valid drug sensitivities with currently available model systems remains a challenge for precision medicine. Drug screening in vivo or in vitro organoids take too long to be beneficial for the patient. Additionally, in vivo studies are costly and subjected to ethical considerations. Short-term ex vivo drug screening of tumor material might be a solution to timely and efficiently determine tumor drug sensitivity for precision medicine.

Aims
This study aims to compare the drug sensitivities of several neuroblastoma models: the in vivo PDX model, the corresponding in vitro dissociated PDX material and the subsequent established organoids, to determine the best model for precision medicine.

Methods
As part of the pan-cancer ITCC-P4 project, neuroblastoma PDX models were established and in vivo compound sensitivities were determined for eight compounds. We used freshly dissociated PDX tumors to perform short-term ex vivo screens with a drug library of 140 compounds. Subsequently, we aimed to establish organoid cultures and screen those with an extended library of 224 compounds. Moreover, we performed extensive molecular charachterization of the patient tumors, PDX models and organoid cultures (whole exome, low coverage whole genome and RNA sequencing as well as methylation profiling).

Results
So far, the drug sensitivities of six freshly dissociated neuroblastoma PDX models and four organoids are established.We aim to expand this to a cohort of 10 models. Interestingly, for the two models where we performed both a short-term ex vivo and organoid drug screen, we see differences in the correlation of the drug sensitivities (R = 0.96 vs. 0.72). In addition, we successfully performed 16 direct in other pediatric cancer models, which will be used as reference cohort.

Conclusions and future perspectives
We successfully performed direct screens of PDX tumors, obtaining results within two weeks after harvesting the material. This rapid turnaround combined with the concordance with matched organoid screens suggests that direct screens of human tumor material offer great potential for integration with current clinical decision making. We will continue our comparison between the three model systems using techniques such as correlation, principal component and enrichment analysis.

P145

The EZH2 inhibitor PF-06821497 Demonstrates High Specificity and Potency Against ALT Neuroblastoma

Daniels A[1], Matkar S[1], Jostes S[2,3,4], Cooke A[2,3,4,5], Rosemann F[2,3,4,5], Cook A[2,4], Balyasny S[1], Bernstein E[2,3,4,5], Reynolds C[6,7,8], Maris J[1,9], Mosse Y[1,9]

[1]Division of Oncology and Center for Childhood Cancer Research, Children's Hospital of Philadelphia, Philadelphia, United States, [2]Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, New York, United States, [3]Department of Dermatology, Icahn School of Medicine at Mount Sinai, New York, United States, [4]Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, United States, [5]Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, United States, [6]Cancer Center, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, United States, [7]Department of Cell Biology & Biochemistry, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, United States, [8]Cancer Center and Department of Pediatrics, School of Medicine, Texas Tech University Health Sciences Center, Lubbock, United States, [9]Department of Pediatrics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, United States

Background: Indolent neuroblastoma with alternative lengthening of telomeres (ALT) is associated with poor survival and minimal effective therapies. In-frame fusions (IFFs) in the ATRX gene are associated with this phenotype. EZH2 inhibition is a promising new therapeutic strategy for ATRX-IFF neuroblastoma (Qadeer, Cancer Cell 2019). However, existing EZH2 small-molecule inhibitors lack potency and may contribute to second malignant neoplasms, emphasizing the importance of identifying more effective and selective EZH2 inhibitors. PF-06821497 is an orally available selective and potent inhibitor of EZH2 histone lysine methyltransferase enzymatic activity currently in Phase 1 clinical testing (NCT03460977).

Aims: Determine the activity of PF-06821497 in depleting H3K27me3 and killing ALT neuroblastoma cell lines at clinically relevant doses.

Methods: Long-term in vitro cell proliferation assays were performed on six ALT (C-circle positive) neuroblastoma cell lines (four ATRX-IFF, one isogenic ATRX-IFF, one isogenic ATRX-WT), one non-ALT neuroblastoma cell line, and one ALT triple-negative breast cancer (TNBC) cell line. Isogenic lines were generated with CRISPR-Cas9 gene editing. Proliferation assays included regularly splitting and re-drugging cells to prevent confluence. Cell viability was evaluated with CellTiter-Glo®. Histones were acid extracted and precipitated with trichloroacetic acid. H3K27me3 depletion was determined via immunoblotting.

Results: PF-06821497 was far more potent against ATRX-IFF neuroblastoma than tazemetostat, the only currently FDA-approved EZH2 small-molecule inhibitor. Across 4 ATRX-IFF neuroblastoma cell lines, PF-06821497 yielded a mean IC50 26x lower than tazemetostat (106.8+/-78.8nM vs. 2.7+/-1.7µM) and a considerably greater reduction in H3K27me3. Isogenic SK-N-FI neuroblastoma cells expressing ATRX-IFF treated with PF-06821497 had a lower IC50 than ATRX-WT SK-N-FI (19.2nM vs. 50.7nM), demonstrating fusion specificity. However, the IC50 for ATRX-WT SK-N-FI, which is C-circle positive, suggests potential activity of PF-06821497 against a wider range of ALT neuroblastomas and other cancers. Indeed, we demonstrated an IC50 of 5.9nM for PF-06821497 in TX-BR-100, an ALT, ATRX-WT TNBC cell line.

Conclusion: These data demonstrate activity and specificity of PF-06821497 for ALT cancer cell lines, with and without ATRX mutations, at likely clinically achievable concentrations. Preliminary data suggest PF-06821497 could enhance dinutuximab efficacy via tumor antigen-specific T-cell stimulation. We will report ongoing investigation of therapeutic PF-06821497 and dinutuximab combinations in neuroblastoma patient-derived xenografts.

P146

Refractory Adult-Onset Neuroblastoma with STRN-NTRK2 Fusion Treated with Larotrectinib and Dinutuximab

Mitchell M[1,2], Neff P[1,2], Harrod V[1,2], Morris J[2], Cohn S[1,2], Gonzales B[2], Widden H[3]
[1]Department of Pediatrics, Dell Medical School, University of Texas, Austin, United States, [2]Dell Children's Medical Center of Central Texas, Austin, United States, [3]Natera, Austin, United States

Background:

Despite improved outcomes for pediatric patients with high risk neuroblastoma over the past two decades, the prognosis for patients with adult-onset neuroblastoma remains dismal. More effective therapies for this subgroup of patients are urgently needed.

Aims:

We report a patient with metastatic adult-onset neuroblastoma whose tumor was found to have a STRN-NTRK2 fusion. Following a poor response to conventional induction chemotherapy and vorinostat, molecularly targeted therapy with the tropomyosin-related kinase inhibitor larotrectinib was initiated.

Methods:

A 20-year-old woman presented to medical care in December 2021 with right lower quadrant pain. She was found to have a left adrenal mass on abdominal imaging, as well as sclerotic lesions throughout the axial and appendicular skeleton. Biopsy of a bone lesion was consistent with neuroblastoma, and MIBG scan showed multiple metastatic sites, Curie score 9. Altera™ genomic profiling was performed on the adrenal mass, which revealed a STRN-NTRK2 fusion. She had a poor response to four cycles of multiagent induction chemotherapy with addition of vorinostat, Curie score decreased from 9 to 7. The patient also experienced severe malnutrition and prolonged myelosuppression, thus treatment was changed to larotrectinib monotherapy. Dinutuximab was added to larotrectinib three months later.

Results:

Following three months of larotrectinib monotherapy, the patient's Curie score decreased from 7 to 2, with negligible toxicity and resolution of malnutrition. Molecular residual disease has been monitored by Signatera™ bespoke circulating tumor DNA (ctDNA) assay and ctDNA levels declined but remained detectable with larotrectinib treatment. Treatment with dinutuximab was added to larotrectinib, and ctDNA is currently undetectable following one course of dinutuximab.

Conclusion:

Adult-onset neuroblastoma is a rare malignancy with poor outcomes and need for improved treatment strategies. To our knowledge, this is the first report of a neuroblastoma harboring a STRN-NTRK2 fusion. Genomic profiling should be offered to patients with high-risk neuroblastoma, especially those with onset in adulthood or those whose tumors are refractory to standard chemotherapy. Circulating tumor DNA may be a useful biomarker for molecular residual disease in neuroblastoma.

P147

The ultrahigh-risk subgroup in neuroblastoma cases identified through DNA methylation analysis and its treatment which exploit cancer metabolism

Watanabe K[1], Kimura S[1,2], Seki M[1], Isobe T[1], Kubota Y[1], Sekiguchi M[1], Sato-Otsubo A[1], Hiwatari M[1,3], Kato M[1], Oka A[1], Koh K[4], Sato Y[5], Tanaka H[6], Miyano S[6], Kawai T[7], Hata K[7,8], Ueno H[9,10], Nannya Y[10], Suzuki H[10,11], Yoshida K[10], Fujii Y[10], Nagae G[12], Aburatani H[12], Ogawa S[10,13], Takita J[1,9]

[1]Department of Pediatrics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, [2]Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan, [3]Department of Pediatrics, Teikyo University, School of Medicine., Tokyo, Japan, [4]Department of Hematology/Oncology, Saitama Children's Medical Center., Saitama, Japan, [5]Department of Urology, The University of Tokyo., Tokyo, Japan, [6]Department of Integrated Analytics, M&D Data Science Center, Tokyo Medical and Dental University., Tokyo, Japan, [7]Department of Maternal-Fetal Biology, National Research Institute for Child Health and Development., Tokyo, Japan, [8]Dpartment of Molecuar and Medical Genetics, Gunma University Graduate School of Medicine., Maebashi, Japan, [9]Department of Pediatrics, Graduate School of Medicine, Kyoto University., Kyoto, Japan, [10]Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University., Kyoto, Japan, [11]Division of Brain Tumor Translational Research, National Cancer Center Research Institute., Tokyo, Japan, [12]Genome Science & Medicine laboratory, Research Center for Advanced Science and Technology, The University of Tokyo., Tokyo, Japan, [13]Department of Pathology and Tumor Biology, Institute for the Advanced Study of Human Biology (WPI-ASHBi), Kyoto University., Kyoto, Japan

Background

Neuroblastomas require novel therapies based on the exploitation of their biological mechanism. Unfortunately, the approach for single-gene mutations has not been sufficiently practical for identifying therapeutic targets for the majority of patients.

Aims

The goal of this study was to better understand the nature of high-risk neuroblastoma and to identify a candidate gene that characterizes the aggressive features of intractable neuroblastomas. We also aimed to explore novel therapeutic approaches by focusing on the extracted candidate genes and their related pathways.

Methods

We analyzed the DNA methylation status of high-risk neuroblastoma samples in two cohorts using unsupervised consensus clustering. By combining expression datasets, we extracted candidate genes that characterize the cluster with poor prognosis. To investigate the potential of the candidate genes as therapeutic targets, we conducted in vitro experiments with siRNA and inhibitory compounds as well as in vivo experiments using tumor mouse models. Furthermore, we performed metabolomic and expression analysis using post-dosing cells to validate the effects of the drugs and to select the optimal combination drugs.

Results

Based on the DNA methylation data, we identified a subgroup of neuroblastoma with extremely poor prognosis in cases having 11q loss of heterozygosity. PHGDH, a serine metabolism-related gene, was extracted as a candidate which characterizes cases in this subgroup and cases with MYCN amplification. PHGDH inhibition suppressed neuroblastoma proliferation in vitro and in vivo, indicating that the inhibition of serine metabolism by PHGDH inhibitors is a therapeutic candidate. Inhibiting the arginine metabolism, closely related to serine metabolism using arginine deiminase, had a combined effect both in vitro and in

vivo, especially on extracellular arginine-dependent neuroblastoma cells with ASS1 deficiency. Expression and metabolome analyses of post-dose cells confirmed the synergistic impact of treatments targeting serine and arginine. It is also indicated that xCT inhibitors that inhibit cystine uptake could be candidates for further combinational treatment.

Conclusion
Our study revealed that DNA methylation profiling could help identify ultrahigh-risk neuroblastoma cases. Moreover, simultaneous inhibition of serine and arginine metabolism is a promising candidate approach for combination therapy for intractable neuroblastomas. Further clinical investigations are warranted for the practical use of these candidates.

P149

Degradation of XIAP as a novel targeting strategy for high-risk neuroblastoma

Choo Z[1,2], Koh X[3], Wong M[4], Ali Ahamed N[1,2], Kang C[3], Kuick C[5], Chang K[4,5,6], Larisch S[7], Loh A[4,6,8], Chen Z[1,2,4,9]

*[1]Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, [2]NUS Centre for Cancer Research, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, [3]Target and Assay Biology II, Experimental Drug Development Centre, A*STAR, Singapore, Singapore, [4]VIVA-KKH Paediatric Brain and Solid Tumour Programme, Children's Blood and Cancer Centre, KK Women's and Children's Hospital, Singapore, Singapore, [5]Department of Pathology and Laboratory Medicine, KK Women's and Children's Hospital, Singapore, Singapore, [6]Duke NUS Medical School, Singapore, Singapore, [7]Cell Death and Cancer Research Laboratory, Department of Human Biology and Medical Sciences, University of Haifa, Haifa, Israel, [8]Department of Paediatric Surgery, KK Women's and Children's Hospital, Singapore, Singapore, [9]National University Cancer Institute, Singapore, National University Health System, Singapore, Singapore*

Background:
XIAP, the most potent mammalian inhibitor of apoptosis protein (IAP), critically restricts developmental culling of sympathetic neuronal progenitors through apoptotic inhibition, and is overexpressed in most MYCN-amplified neuroblastoma tumors. Since ARTS is the only XIAP-antagonist that directly binds and degrades XIAP, we hypothesized that XIAP antagonism via ARTS mimetics may be effective against high-risk neuroblastoma.

Aims:
We sought to evaluate the preclinical effectiveness and tolerability of antagonists of XIAP and other IAPs in neuroblastoma tumor models, and elucidate the specific therapeutic mechanism of XIAP antagonism for this novel targeting approach.

Methods:
Pan-IAP (SMAC mimetics) and XIAP-specific inhibitors (ARTS mimetics) were screened against commercial and patient-derived neuroblastoma cell lines, and ranked by efficacy. IAP protein expression and cell death were assessed via Western blot, and clonogenic and biochemical apoptosis assays. Following CRISPR/Cas9-mediated XIAP knock-in, XIAP-compound interaction was evaluated using NanoBRET™ target-engagement, degradation and ubiquitination assays, and NMR spectroscopy. Pharmacokinetics and survival studies were performed with orthotopic patient-derived xenografts. Drug-interaction indices were computed using Chou-Talalay method.

Results:
Antagonism of XIAP (but not other IAPs), triggered apoptotic death in neuroblastoma cells. XIAP silencing induced apoptosis and over-expression conferred protection from drug-induced apoptosis. Among IAP antagonists tested, first-in-class ARTS mimetic A4 was most effective against high XIAP-expressing neuroblastoma cells (BE(2)-C, KELLY), and least toxic towards liver and bone marrow-derived control cells. NanoBRET™ assays showed XIAP engagement and degradation via the ubiquitin-proteasome system within 15 minutes of A4 exposure, in a dose-dependent manner. NMR analysis on ^{1}H-^{15}N-HSQC spectra of XIAP showed moderate binding with A4, supporting degradation rather than binding inhibition of XIAP by A4. In MYCN-amplified neuroblastoma xenografts, A4 10mg/kg twice-weekly was well-tolerated and significantly prolonged survival. A4 also showed synergism with second-line agents vincristine and topotecan, with 3- and 6.5-fold effective dose-reduction in BE(2)-C and KELLY, respectively.

Conclusion:
Engagement and degradation of XIAP by ARTS mimetics is a novel targeting strategy for neuroblastoma that may be especially effective against MYCN-amplified disease with intrinsically high XIAP expression. First-in-class ARTS mimetic A4 demonstrates preclinical efficacy as a single-agent and synergy in combination with current standard-of-care regimens, and warrants further development and study.

P150

Personalized therapy of patients with neuroblastoma: the experience of Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology

Andreeva N[1], Druy A[1], Kachanov D[1], Litvinov D[1], Myakova N[1], Rabaeva L[1], Zhukov N[1], Shamanskaya T[1]
[1]*Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology and Immunology, Moscow, Russian Federation*

Background
Patients with high-risk neuroblastoma (HRNB) and relapsed disease have an extremely unfavorable prognosis and need therapy intensification. Targeted therapies are considered. The results of ALK inhibitors (iALK) monotherapy showed good tolerability but limited effectiveness. The using of other targeted drugs is widely discussed.
Aim: To study the experience of personalized therapy (PT) in patients with NB.
Materials and methods: Since 2018, 155 patients with a diagnosis of HRNB or relapse/progression regardless of the risk group, have been searched for mutations using targeted high-performance sequencing of QIASeq (Qiagen, Germany) with 56 genes, including ALK. The ALK amplification was determined by MLPA and FISH.
PT was prescribed based on the literature data of the various drugs effectiveness at the time. The results of PT were evaluated to 19.12.2022.
Results: Markers of sensitivity to molecular-directed therapy were detected (above the IIC level by AMP/ASCO/CAP) in 16% (25/155): 17 – ALK mutations, 3 – ALK amplification, 3 - aberrations in the tyrosine kinase family genes (MAP2K1, BRAF, PDGFRA), 2 - high TMB (>10 mutations per Mb, determined using the QIAgen TMB panel). 16 patients were prescribed therapy: 13 – iALK (1 – alectinib, 3 – lorlatinib, 4 – crizotinib, 5 – ceritinib), 2 – pembrolizumab, 1 – imatinib. The median duration of PT was 4.4 months (0.2-42.6).
PT was prescribed in addition to the conventional therapy (in 5 cases – in the 1st line, 11 – subsequent lines). Grade 3-4 toxicity was observed in 3 cases (hepatic - 2, gastrointestinal and neurological - 1). Currently, 8 patients continue PT (in 3 cases monotherapy with iALK was continuing after ending program therapy), 7 – terminated therapy because of disease progression - 6 (2 - rapid progression) and toxicity - 1.
Conclusion: The most common target for therapy in NB are ALK aberrations. iALK are well tolerated and can be effectively used with various therapy regimens, including chemotherapy. Other targets were detected less frequently than ALK. To assess the efficiency of their blockade the collection of larger number of patients are needed.

P151

Standardization and validation of procedures for detection of the ALK genetic status in neuroblastoma samples by SIOPEN Biology reference laboratories

Saint-Charles A[1,2], Bellini A[1,2], Bobin C[1,2], Masliah-Planchon J[1], Iddir Y[1,2], Bhalshankar J[1,2], Taschner-Mandl S[3], Bernkopf M[3], Font De Mora J[4], Noguera Salvá R[5], Van Roy N[6], Goodman A[7], Tweddle D[7], Vicha A[8], Combaret V[9], Attignon V[9], Beiske K[10], Martinsson T[11], Schoumans J[12], Rossing M[13], Tops B[14], Tytgat G[14], Westermann F[15], Cotteret S[16], Fischer M[17], Birger Y[18], Hameiri-Grossman M[18], Shichrur K[18], Mazzocco K[19], Chesler L[20], George S[20], Betts D[21], Cowley M[22], Trahair T[22], Barahona P[22], Capasso M[23], Eggert A[24], Valteau-Couanet D[25], Delattre O[1], Schleiermacher G[1,2]

[1]Institut Curie, INSERM U830, Laboratoire de Génétique et Biologie des Cancers, Research Center, PSL Research University, Paris, France, [2]SiRIC RTOP « Recherche Translationelle en Oncologie Pédiatrique », Translational Research Department, Research Center, PSL Research University, Paris, France, [3]Children's Cancer Research Institute, Department of Tumor Biology, Vienna, Austria, [4]Instituto de Investigación Sanitaria La Fe, Clinical and Translational Research in Cancer, Valencia, Spain, [5]University of Valencia-INCLIVA Biomedical Health Research Institute, Department of Pathology, Medical School, Valencia, Spain, [6]Ghent University, Center for Medical Genetics, Ghent, Belgium, [7]Institute of Genetic Medicine, Newcastle Genetics Laboratory, Newcastle, United Kingdom, [8]Second Faculty of Medicine Charles University, Department of Paediatric Haematology and Oncology, Prague, Czech Republic, [9]Centre Léon-Bérard , Laboratoire de Recherche Translationnelle, Lyon, France, [10]The Norwegian Radium Hospital, Oslo University Hospital, Department of Pathology, Oslo, Norway, [11]University of Göteborg, East Hospital, Department of Clinical Genetics, Gothenburg, Sweden, [12]University Hospital CHUV, Pediatric Hematology-Oncology Research Laboratory, Pediatric Division, Lausanne, Switzerland, [13]Rigshospitalet, Copenhagen University Hospital, Centre for Genomic Medicine, Copenhagen, Denmark, [14]Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands, [15]German Cancer Research Center, Department of Neuroblastoma Genomics (B087), Heidelberg, Germany, [16]Gustave Roussy Cancer Campus, Department of Tumor Genetics, Villejuif, France, [17]University of Cologne Children's Hospital, Department of Experimental Pediatric Oncology, Cologne, Germany, [18]Schneider Children's Medical Center of Israel, Petah Tikva, Israel, [19]IRCCS Istituto Giannina Gaslini, Anatomia Patologica, Genova, Italy, [20]The Institute of Cancer Research, Division of Clinical Studies and Cancer Therapeutics, London, United Kingdom, [21]Children's Health Ireland at Crumlin, Department of Clinical Genetics, Dublin, Ireland, [22]Children's Cancer Institute, University of New South Wales, Randwick, Australia, [23]Università degli Studi Napoli Federico II, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Napoli, Italy, [24]Charité-Universitätsmedizin Berlin, Department of Pediatric Oncology/Hematology, Berlin, Germany, [25]Gustave Roussy Cancer Campus, Department of Oncology for Children and Adolescents, Villejuif, France

Background: The efficacy of a third generation ALK inhibitor (Lorlatinib) upfront, in combination with standard chemotherapy will be evaluated by SIOPEN and COG with the aim of improving survival of patients with ALK-altered neuroblastoma. The occurrence of distinct genetic alterations (amplifications, mutations, rearrangements), the resulting complexity of molecular diagnosis and the number of countries participating in SIOPEN high risk trials highlight the need for harmonisation of molecular diagnostic techniques and standardisation of output between SIOPEN reference laboratories, for identification of ALK alterations.

Aims: To standardize molecular diagnostic techniques and reporting results of the ALK genomic status (mutation/amplification) in neuroblastoma samples, in the 21 SIOPEN reference laboratories.

Results: 14 genomic DNA samples (NB cell lines or NB PDX models) were shared among 21 SIOPEN laboratories harbouring distinct ALK alterations: a) 10 samples had different ALK SNVs/mutations, in known hotspots of the tyrosine kinase domain (TKD), including also dilutions, resulting in different variant allele fractions (VAF) ranging from 91% to 1%; b) 3 samples had ALK genomic amplifications, over extended or

more restricted genomic regions; c) 1 DNA sample without any ALK alteration as a negative ALK control; d) FFPE tumour samples from PDX were also sent upon request.
ALK amplifications were detected using either a pan-genomic copy number technique (WES, WGS, CGHa, SNPa) or by FISH. ALK SNVs/mutations were characterized by NGS techniques to enable detection of mutations with lower VAF. The SIOPEN biology laboratories employed their own established techniques, but in accordance with consensus technical and reporting specifications.
All laboratories identified ALK mutations in a known TKD hotspot, with VAF >5% and large scale typical genomic ALK amplification.
Difference in interpretation and reporting was apparent when considering SNVs with a VAF <5% or outside known hotspots, which requires expert discussion prior to validation as inclusion criteria within future clinical trials.

Conclusion: Our results document the importance of established SOPs and the robustness of ALK genetic testing in the SIOPEN biology reference laboratories. Furthermore, our findings underline the importance of expert discussions regarding atypical ALK alterations, to validate eligibility for ALK targeted treatment.

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Preclinical PK/PD in Neuroblastoma of the MYCN-inhibitor BGA002 and modelling of dose prediction for clinical phase 1

Scarodvi A[1], Montemurro L[2], Bartolucci D[1], Bortolotti S[1], Cerisoli L[1], Oosterholt S[3], Della Pasqua O[3], Pession A[4], Hrelia P[5], Tonelli R[5]

[1]Biogenera SpA, R&D Department, Bologna, Italy, [2]IRCCS, Azienda Ospedaliero-Universitaria di Bologna, Pediatric Oncology and Hematology Unit, Bologna, Italy, [3]University College London, Clinical Pharmacology & Therapeutics Group, London, United Kindom, [4]University of Bologna, Department of Medical and Surgical Sciences, Bologna, Italy, [5]University of Bologna, Department of Pharmacy and Biotechnology, Bologna, Italy

Background/Introduction

About 25% of Neuroblastoma (NB) presents MYCN-amplification (MNA) which is linked to a poor prognosis. MYCN is an ideal target for therapy but is undruggable by traditional approaches. We have proposed an alternative approach by a MYCN-specific expression inhibition at the level of transcription through anantigene peptide-nucleic-acid (PNA) oligonucleotide (BGA002) (BGA002 received Orphan Drug Designation by EMA and FDA for Neuroblastoma Treatment).

Aims

To investigate BGA002 pharmacokinetic (PK) and pharmacodynamic (PD) profiles in MNA-NB mouse models. To model and predict the BGA002 dosage for the first-in-human phase 1 in NB.

Methods/Materials

BGA002 PK in tumors was conducted on MNA-NB xenograft mouse models. PK in healthy organs and blood was assessed in mouse and rabbit. To measure the amount of BGA002 a new hybridization-based ELISA assay has been developed. Biodistribution of radioactive [14C]-Ac-BGA002 was evaluated in mouse using Quantitative-Whole-Body-Autoradiography. MYCN PD was evaluated in-vitro and in-vivo.

Results

BGA002 PK after repeated systemic administration in MNA-NB mouse models, showed that it localized in tumors at high concentrations, comparable to those found in normal organs with highest concentration. PD showed a potent dose–response MYCN and tumor inhibition.These findings were also confirmed in two other MNA mouse models (Rhabdomyosarcoma and Small-Cell-Lung-Cancer). BGA002 biodistribution after a single administration to healthy mice showed broad distribution in organs and tissues. PK profile in blood of BGA002 in mice showed a similar exposure between male and female, and comparability between intravenous and subcutaneous route of administrations. BGA002 was quantifiable up to eight hours in plasma, and no accumulation was found after repeated administration. There was a linear correlation between dose and absorption. PK blood exposure of BGA002 in rabbit resulted up to ten times more than in mice. Moreover, the use of a model-based approach allowed integration of PK/PD data, providing dose prediction for the first-in-human phase 1 in NB.

Conclusion/Summary

PK/PD in MNA-NB mouse models showed the capacity of BGA002 to accumulate in tumors and exert potent anti-tumor activity. Modeling of PK/PD provided the rationale for BGA002 dosage in Phase 1 in NB.

P153

Exploring the combinatorial potential of the ROCK2-specific inhibitor KD025 for neuroblastoma therapy

Tümmler C[1], Pepich A[1], Vellema Q[1], Abu Ajamieh S[1], Sainero-Alcolado L[2], Park S[3], Ljungblad L[1], Seashore-Ludlow B[3], Karlsson K[3], Arsenian-Henriksson M[2], Kogner P[1], Johnsen J[1], Wickström M[1]
[1]Karolinska Institutet, Department of Women's and Children's Health, Division of Pediatric Oncology and Pediatric Surgery, Stockholm, Sweden, [2]Karolinska Institutet, Department of Microbiology, Tumor and Cell Biology, Stockholm, Sweden, [3]Karolinska Institutet, Department of Oncology-Pathology, Stockholm, Sweden

Background:
High-risk neuroblastoma remains a clinical challenge as the long-term survival rates for patients with recurrent disease are below 10%. Novel drug combinations are therefore needed to support existing therapies, improve survival, and reduce late effects. Signaling mediated by the Rho family of GTPases, and the downstream Rho kinases (ROCK), regulates important developmental processes and our research group has previously demonstrated that the Rho/ROCK signaling pathway is a promising therapeutic target in neuroblastoma.

Aims:
To explore novel drug combinations for neuroblastoma therapy with focus on the ROCK2-specific inhibitor KD025 (Belumosudil, Rezurock™).

Methods:
Drug combination screening was performed using KD025 together with a cancer drug library containing 528 drugs. Cell viability, clonogenicity, and protein expression/phosphorylation were evaluated in neuroblastoma cell lines. Furthermore, IncuCyte® LiveCell analysis was used to assess tumor spheroid growth and cell death. Agilent Extracellular Flux Analyzer was used to investigate metabolic changes in response to drug treatment. The in vivo efficacy of KD025 was evaluated in 9464D allografts and the transgenic TH-MYCN mouse model. RNA-sequencing and gene signaling enrichment analysis were applied to study transcriptomics.

Results:
KD025 impaired cell viability and growth of neuroblastoma cell lines, decreased N-MYC levels, and increased phosphorylation of p38 and Akt. Additionally, we observed that KD025 impaired tumor growth of 9464D allografts and in homozygous TH-MYCN mice but did not completely suppress tumor growth. RNA-sequencing of TH-MYCN tumors proposed downregulation of genes associated with metabolic processes. A drug combination screening revealed several synergistic combination partners for KD025 including TIC10 (ONC201) and the p97 inhibitor NMS-873. Synergistic effects of these drug combinations were confirmed in neuroblastoma cell lines grown in monolayer and tumor spheroids. Immunohistochemistry on tumor spheroids demonstrated that combinations of KD025 and TIC10 or NMS-873 reduced proliferation and increased the number of apoptotic cells compared to single drug treatment. Furthermore, we observed changes in glycolytic parameters in neuroblastoma cell lines treated with KD025, TIC10, and NMS-873, suggesting metabolic effects as one of the potential underlying molecular mechanisms of action.

Conclusion:
In conclusion, combination treatments using the ROCK2-specific inhibitor KD025 together with p97 inhibition or TIC10 may be promising therapeutic approaches for neuroblastoma.

P154

Adrenergic and mesenchymal signatures are identifiable in neuroblastoma cell free DNA and correlate with metastatic disease burden

Vayani O[1], Kaufman M[1], Moore K[2], Chennakesavalu M[1], TerHaar R[2], Chaves G[2], Chlenski A[2], He C[3], Cohn S[2], Applebaum M[2]

[1]The University of Chicago, Pritzker School of Medicine, Chicago, IL, United States, [2]The University of Chicago, Department of Pediatrics, Chicago, IL, United States, [3]The University of Chicago, Department of Chemistry, Chicago, IL, United States

BACKGROUND

We previously demonstrated that cell-free DNA (cfDNA) profiles of 5-Hydroxymethylcytosine (5hmC), an epigenetic marker of active gene expression, are correlated with metastatic burden, response to therapy and survival in patients with neuroblastoma. Here, we hypothesized that adrenergic (ADRN) and mesenchymal (MES) specific signatures in cfDNA 5-hmC profiles would improve the accuracy of this biomarker.

AIMS

We aimed to adapt our existing signatures of ADRN and MES cell lines derived from genomic DNA for use with cfDNA. We evaluated these gene sets in retrospectively collected samples to assess the utility of lineage specific signatures for monitoring disease burden in serially collected samples.

METHODS

We previously performed an integrative analysis to identify ADRN and MES specific genes (n=373 and n=159, respectively). Purified DNA from cell lines was serial diluted with healthy donor cfDNA. Using Gene Set Variation Analysis, ADRN and MES signatures were optimized using a leave-one-out analysis and a best fit correlation of signatures across dilutions. We then quantified signature scores, and our prior neuroblastoma signature, in cfDNA from 84 samples from 46 high-risk patients including 21 patients with serial samples. The receiver operatic characteristics (ROC) measured by area under the curve (AUC) for predicting subsequent relapse was assessed.

RESULTS

Initial correlation of signature scores with increasing percent tumor DNA was 0.93 for ADRN and 0.41 for MES genes. The optimized gene sets had 217 ADRN and 75 MES genes with correlations of 0.98 and 0.99, respectively. As expected, removed genes were enriched for pathways of endothelial and immune function. Samples from patients with higher metastatic burden had increased scores regardless of signature ($p<0.001$). ROC analysis showed similar AUCs for the neuroblastoma (AUC=0.76), ADRN (AUC=0.72), and MES (AUC=0.67) signatures ($p=0.14$). While neuroblastoma, ADRN, and MES signature scores tracked together in serial samples, we identified instances of patients with only increases in either MES or ADRN score at relapse.

CONCLUSION

It is feasible to identify ADRN and MES signatures using 5-hmC profiles of cfDNA to track metastatic burden and response to therapy. While ADRN and MES scores often track closely, discrepancies may help identify patients with residual disease.

P155

Amplicon structure creates collateral therapeutic vulnerabilities in neuroblastoma

Bei Y[1], Bramé L[1,2], Kirchner M[3], Fritsche-Guenther R[4], Kunz S[5], Gürgen D[5], Bhattacharya A[6], Köppke J[2], Proba J[1], Wittstruck N[1], Sidorova O[1], González R[1], Garcia H[1], Brückner L[4], Xu R[1], Giurgiu M[1], Rodriguez-Fos E[1], Koche R[7], Schmitt C[6], Schulte J[1], Eggert A[1], Haase K[1,2], Kirwan J[4], Hagemann A[1,2], Mertins P[2], Dörr J[1,2,8,9], Henssen A[1,2,5,8,9]

[1]Department of Pediatric Oncology/Hematology, Charité-Universitätsmedizin , Berlin, Germany, [2]German Cancer Consortium (DKTK), partner site Berlin, and German Cancer Research Center (DKFZ)., Heidelberg, Germany, [3]Core Unit Proteomics, Berlin Institute of Health at Charité- Universitätsmedizin Berlin and Max Delbrück Center for Molecular Medicine, Berlin, Germany, Berlin, Germany, [4]Core Unit Metabolomics, Berlin Institute of Health at Charité-Universitätsmedizin Berlin Germany., Berlin, Germany, [5]Max-Delbrück-Center for Molekulare Medizin (BIMSB/BIH), Berlin, Germany., Berlin, Germany, [6]Department of Hematology, Oncology and Tumor Immunology, Charité-Universitätsmedizin Berlin, Germany., Berlin, Germany, [7]Center for Epigenetics Research, Memorial Sloan Kettering Cancer Center, New York, NY, USA., New York, USA, [8]Berlin Institute of Health, 10178 Berlin, Germany., Berlin, Germany, [9]Experimental and Clinical Research Center (ECRC) of the MDC and Charité Berlin, Berlin, Germany., Berlin, Germany

Intra- and extrachromosomal DNA amplifications in cancer often encompass large genomic regions harboring oncogenes but can also contain passenger genes and gene regulatory elements. We sought to determine whether passenger co-amplifications can create amplicon structure-specific collateral therapeutic vulnerabilities in cancer. In high-risk neuroblastoma, around 25% of patients have a MYCN amplification. By analyzing copy number data from 238 MYCN-amplified patients, we identified the DEAD-Box Helicase 1 (DDX1) gene to be frequently co-amplified with MYCN on the same genomic fragment. A transgenic zebrafish model of DDX1-MYCN co-expression confirmed that DDX1 acts as a bona fide passenger gene in neuroblastic tumorigenesis. Analysis of CRISPR-Cas9 loss-of-function screens from the Cancer Dependency Map across over 700 human cancer cell lines revealed that the survival of MYCN-amplified cancer cells with DDX1 co-amplification depends on the enhanced activity of the mammalian target of rapamycin complex 1 (mTORC1). Interaction proteomics identified dihydrolipoamide S-succinyltransferase (DLST), a component of the tricarboxylic acid (TCA) cycle enzyme alpha-ketoglutarate dehydrogenase (alpha-KGDH) complex, as an interaction partner of DDX1 in mitochondria. Live-cell metabolomics suggested that this interaction can impair TCA activity and lead to the accumulation of alpha-ketoglutarate (alpha-KG) by interfering with its conversion to succinyl-CoA. Accumulation of alpha-KG, in turn, caused metabolic stress and triggered cell death, which was compensated for by enhanced mTORC1 activity in cancer cells. Consequently, disruption of mTORC1 function resulted in cell death, specifically in cells with aberrantly high copy number of DDX1. Thus, structurally linked co-amplification of a passenger gene (DDX1) and an oncogene (MYCN) on the same amplicon can result in collateral vulnerabilities in neuroblastoma.

P156

Treatment Strategies for Incidentally Found Neuroblastoma in Children younger than 18 Months Old: The Roles of 18F-FDOPA and 18F-FDG PET

Hung W[1,2], Liu Y[3], Hsu W[1]

[1]Division of Pediatric Surgery, Department of Surgery, National Taiwan University Hospital, Taipei, Taiwan, [2]Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei, Taiwan, [3] Department of Pediatrics, Taipei Medical University Hospital, Taipei, Taiwan

Background: Neuroblastoma (NB) in children younger than 18 months old especially for those identified incidentally usually but not always carries a favorable outcome.

Aims: This study aimed to evaluate whether 18F-FDOPA and 18F-FDG positron emission tomography (PET) having been shown to be able to predict NB patient prognosis and could help treatment decision in these patients.

Methods: From 2007 to 2021, NB patients younger than 18 months at our institute were reviewed. The patients were grouped into incidental or symptomatic according to their initial presentations. Symptomatic patients were subjected to standard treatments according to risk classifications of Taiwan Pediatric Oncology Group (TPOG). For the incidental group, patients were suggested to receive standard treatment if the 18F-FDG PET uptake is obvious, while those with minimal or no 18F-FDG PET uptake were suggested to receive regular image follow-up. The parents had the right to make final decision.
Results: Totally, 27 patients with symptomatic NB and 30 patients (36.8% by perinatal echo) with incidental finding and completed PET scans were identified. Only 7 symptomatic patients had received PET before initial treatment, and 6 of them revealed obvious PET uptake at the primary tumor. On the other hand, 12 (40%) patients in the incidental group had obvious PET uptake. The incidental group had a better overall survival than the symptomatic group (1/30 vs 5/27). Eight patients in the incidental group chose regular echo follow-up. Among them, 7 with minimal or no 18F-FDG PET uptake showed tumor regression during follow-up. However, the remaining 1 patient presenting with a right adrenal tumor at prenatal echo and having high 18F-FDG PET uptake showed tumor decrease in initial 3 months but then increase thereafter. As the parents refused to receive treatment at the whole course, the patient expired at 19-month-old due to tumor progression.

Conclusions: 18F-FDOPA and 18F-FDG PET together may serve as a useful tool to help identify the biology and tumor behavior of incidentally found NB. While NB tumors with minimal 18F-FDG PET uptake may be observed safely and avoid unnecessary treatment, tumors with high18F-FDG PET uptake warrant more aggressive treatments instead of image follow-ups only.

P157

Purine metabolism: a metabolic weakness of neuroblastoma and synergistic effects with APR-246.

Mlakar V[1], Oehme I[2,3,4,5], Lesne L[1], Peterziel H[2,3,4,5], Ansari M[1,6], Gumy-Pause F[1,6]

[1]Faculty of Medicine, Department of Pediatrics, Gynecology, and Obstetrics, Geneva, Switzerland, [2]Hopp Children's Cancer Center, Heidelberg, Germany, [3]German Cancer Research Center (DKFZ) and German Cancer Consortium (DKTK), Clinical Cooperation Unit Pediatric Oncology, Heidelberg, Germany, [4]National Center for Tumor Diseases, Heidelberg, Germany, [5]University Hospital Heidelberg, Department of Pediatric Oncology, Hematology, and Immunology, Heidelberg, Germany, [6]University Geneva Hospitals, Division of Pediatric Oncology and Hematology, Department of Women, Child and Adolescent, Heidelberg, Germany

Background: APR-246 (eprenetapopt) could be an efficient treatment option against neuroblastoma (NB), the most common pediatric extracranial solid tumor. We recently demonstrated that p53 and RAS-MAPK pathways determine the response to APR-246 treatment through the elevation of intracellular glutathione or cystine concentrations.

Aims: Here we investigated how exposure to APR-246 affects different metabolites of NB cells.

Methods: SK-N-DZ NB cells were treated with 60 M APR-246 and investigated for 250 metabolic products. CellTiter2.0 was used to determine IC50 of APR-246 alone or in combination with mycophenolate, allopurinol, 6-mercaptopurine, and guanosine. Proteome profiler apoptosis assays and western blot analysis were used to identify the molecular pathways involved in the responses to mycophenolate and APR-246.

Results: The most dysregulated metabolites belonged to the Krebs cycle, purine synthesis, and glutathione. Mycophenolate, an inhibitor of guanine nucleotide, demonstrated strong synergy with APR-246. Guanosine was able to completely revert the effects of mycophenolate. Other inhibitors of guanine metabolism, 6-mercaptopurine, and allopurinol, demonstrated additive effects with APR-246. Drug-drug interaction screens between APR-246 and 79 commonly used anticancer drugs demonstrated beneficial combinations for APR-246 and inhibitors of Bcl-xL, such as 1155463 and A1331852, and also with chemotherapeutic drugs involved in DNA replication. We identified Bcl-xL and phospho-p53 S392 as key apoptotic targets involved in the cellular response to mycophenolate. Combining mycophenolate with the SLC7A11 inhibitor sulfasalazine and APR-246 demonstrated strong synergistic effects on NB cells.

Conclusions: In this study, we identified purine metabolism as a significant metabolic vulnerability of NB cells. We demonstrated that this weakness could be exploited by combining APR-246 with mycophenolate (with or without sulfasalazine). The further molecular analysis demonstrates that APR-246 and mycophenolate converge on the same targets of the apoptotic pathway, Bcl-xL, and phosphorylation of p53 at S392, which might explain the strong synergistic behavior. The results also suggest that de novo purine synthesis is more important than the purine recycling pathway because no synergy was observed between APR-246 and 6-mercaptopurine or allopurinol. Overall, this finding points to additional treatment possibilities for NB patients by combining APR-246 with already clinically established mycophenolate/sulfasalazine therapy.

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Targeting PI3K and CDK4/6, or PARP and WEE1 in neuroblastoma cell lines

Lukoseviciute M[1], Holzhauser S[1], Mandal T[1], Dalianis T[1], Kostopoulou O[1]
[1]*Dept. of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden*

Background and aim: Neuroblastoma (NB) is the most frequent malignant extracranial tumor in children. Based on the tumor risk, NB patients are mainly treated with chemotherapy, surgery, radiation therapy and myeloblative therapy. However, recurrences, chemo-resistance and side effects still occur especially for high-risk NB, so new therapies are urgently needed. For this reason, we investigated if combined therapy with the recently Food and Drug Administration (FDA) approved Phosphatidylinositol 3-Kinase (PI3K), Cyclin-Dependent-Kinase-4/6 (CDK4/6), Poly-ADP-Ribose-Polymerase (PARP) and WEE1 inhibitors, could be useful for NB treatment.

Methods: Effects of single and combined treatment of the CDK4/6, PI3K, WEE1 and PARP inhibitors (PD-0332991, BYL719, MK-1775 and BMN673 resp.) on the 5 NB cell lines SK-N-AS, SK-N-BE(2)-C, SK-N-DZ, SK-N-FI and SK-N-SH were tested by viability, proliferation/cell confluence, cytotoxicity and apoptosis assays. Also, the effects on cell cycle were examined with FACS analysis.

Results: Single treatments in NB cell lines gave dose dependent responses and inhibited viability and cell confluence, while combined treatments of PI3K with CDK4/6 and PARP with WEE1 inhibitors gave synergistic effects in most of the cell lines. Furthermore, cytotoxicity and apoptosis were induced by MK-1775 and to a slightly lesser extent by PD-0332991, while for BYL719 and BMN673 the effects were rare. Moreover these effects were not enhanced by combining the drugs, except for some rare cases including PD-0332991. Preliminary data on cell cycle, showed additive effects when combining PARP with WEE1 inhibitor, and large increase in the percentage of cells in both G1 and G2 and a major decrease in the percentage of cells in S-phase. The combination of PI3K with CDK4/6 inhibitors showed mainly increase in the percentage of cells in G1 mostly due to the effect of CDK4/6 inhibitor.

Conclusion: To sum up our data showed that combination treatments with PI3K and CDK4/6 or PARP and WEE1 inhibitors exhibited promising anti-NB combined activity and lower doses of the drugs could be used, with less side effects.

P159

Exploring Dinutuximab Beta as a potential theragnostic vector for non-invasive molecular imaging and targeted radiotherapy of high-risk neuroblastoma

Gawne P[1,2,3], Kashani R[2], Sato M[2], Chupin J[2], Terry S[3], Sosabowski J[2], Gaze M[1,4]
[1]UCL Cancer Institute, University College London, London, United Kingdom, [2]Centre for Cancer Biomarkers and Biotherapeutics, Barts Cancer Institute, Queen Mary, University of London, London, United Kingdom, [3]School of Biomedical Engineering and Imaging Sciences, King's College London, London, United Kingdom, [4]Department of Oncology, University College London Hospitals NHS Foundation Trust, London, United Kingdom

Background
Molecular radiotherapy with the beta-emitting ^{131}I-meta-iodobenzylguanidine (^{131}I-mIBG), which targets the noradrenaline transporter (NET-1), has an established place in the management of high-risk neuroblastoma (HR-NB). Additionally, ^{177}Lu-DOTATATE which targets the somatostatin receptor (SSTR2) is currently in use in clinical trials. However, heterogeneity in target expression on tumours can affect treatment responses in patients. Hence, the development of a vector molecule aimed at targets other than NET and SSTR2 could potentially improve patient outcomes. Dinutuximab Beta (DB) – a monoclonal antibody against the di-sialo-ganglioside GD2 – previously developed as an immunotherapy, significantly improves survival in HR-NB, and could be used as a theragnostic vector.

Aims
The aim of this work was to investigate the use of Dinutuximab Beta (DB) as a platform for in vivo imaging using positron emission tomography (PET) and targeted molecular radiotherapy of neuroblastoma.

Methods
Dinutuximab Beta (DB) was chemically modified with the metal chelators DFO and DOTA, and radiolabelled with the PET isotope ^{89}Zr, and the beta-emitting ^{177}Lu respectively - using standard protocols. The radiolabelling efficiency and serum stability of ^{89}Zr-DB and ^{177}Lu-DB were evaluated using iTLC and radio-HPLC methods. In vitro binding of ^{89}Zr-DB was then tested in GD2-positive neuroblastoma cell lines. Finally, to assess the biodistribution of the radiolabelled DB and the in vivo stability of the radiolabel, preliminary in vivo experiments in athymic nude mice were performed.

Results
DB was successfully radiolabelled with both ^{89}Zr and ^{177}Lu with good radiolabelling efficiencies. Radio-HPLC analysis demonstrated that the attachment of the radiometals was stable in serum proteins. ^{89}Zr-DB was shown to bind to GD2-positive NB cell lines. After administration into athymic nude mice, ^{89}Zr-DB showed the expected biodistribution of a long-circulating monoclonal antibody. Importantly, relatively low bone uptake was observed, indicating good stability of the ^{89}Zr radiolabel in vivo.

Conclusion
We report the radiolabelling of Dinutuximab Beta to allow PET imaging and beta-particle based radiotherapy of neuroblastoma. This work strongly supports the future use of Dinutuximab Beta as a theragnostic vector for the in vivo mapping of NB tumours, as well as targeted radiotherapy, based on GD2 expression.

P160

Longitudinal evaluation of serum microRNAs as biomarkers for neuroblastoma burden and therapeutic p53 reactivation

Van Goethem A[1,2], **Deleu J[1,2]**, Yigit N[1,2], Everaert C[1,2], Moreno-Smith M[3], Vasudevan S[3], Zeka F[1,2], Demuynck F[1,2], Barbieri E[3], Speleman F[2,4], Mestdagh P[1,2], Shohet J[5], Vandesompele J[1,2], Van Maerken T[1,2,6]
[1]OncoRNALab, Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [2]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium, [3]Department of Pediatrics, Section of Hematology-Oncology, Texas Children's Cancer Center, Baylor College of Medicine, Houston, Texas, United States, [4]PPOL, Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [5]Department of Pediatrics, Division of Pediatric Hematology-Oncology, University of Massachusetts Chan Medical School, Worcester, Massachusetts, United States, [6]Department of Laboratory Medicine, AZ Groeninge, Kortrijk, Belgium

Background
Accurate assessment of treatment response and residual disease is indispensable for the evaluation of cancer treatment efficacy. However, performing tissue biopsies for longitudinal follow-up poses a major challenge in the management of solid tumours like neuroblastoma.

Aims
In the present study, we evaluated whether circulating miRNAs are suitable to monitor neuroblastoma tumour burden and whether treatment-induced changes of miRNA abundance in the tumour are detectable in serum.

Methods
We performed small RNA sequencing on longitudinally collected serum samples from mice carrying orthotopic neuroblastoma xenografts that were exposed to treatment with idasanutlin or temsirolimus.

Results
We identified 57 serum miRNAs to be differentially expressed upon xenograft tumour manifestation, out of which 21 were also found specifically expressed in the serum of human high-risk neuroblastoma patients. The murine serum levels of these 57 miRNAs correlated with tumour tissue expression and tumour volume, suggesting potential utility for monitoring tumour burden. In addition, we describe serum miRNAs that dynamically respond to p53 activation following treatment of engrafted mice with idasanutlin. We identified idasanutlin-induced serum miRNA expression changes upon one day and 11 days of treatment. By limiting to miRNAs with a tumour-related induction, we put forward hsa-miR-34a-5p as a potential pharmacodynamic biomarker of p53 activation in serum.

Conclusions
Our findings demonstrate that it is feasible to monitor both tumour burden and treatment response by measuring the levels of circulating miRNAs in serum and that expression changes in the tumour are reflected in serum. The identification of treatment-induced alterations of circulating tumour-related miRNAs is an unprecedented finding that holds promise for liquid biopsies as a tool for miRNA-based monitoring of treatment response in cancer patients.

P161

Evaluating the efficacy of Enhancer of Zeste Homolog 2 inhibitors in combination with anti-GD2/isotretinoin for the treatment of high-risk neuroblastoma

Gao J[1], Putnam C[1], Gibson J[1], Gray J[1], Walters Z[1]
[1]*School of Cancer Sciences, Faculty of Medicine, University of Southampton, Southampton, United Kingdom*

Background:
Isotretinoin or 13-Cis Retinoic Acid (RA), in combination with anti-GD2 immunotherapy, is widely used as the maintenance treatment for patients with high-risk neuroblastoma (HR NB). RA is a differentiating agent aimed at promoting differentiation of remaining tumour cells and reduce relapse. In addition, epigenetic factors can also control cell differentiation by altering gene expression. Enhancer of Zeste Homolog 2 (EZH2), a histone-modifying enzyme, is responsible for the trimethylation of Lysine 27 on Histone 3 (H3K27me3). This results in repressing the transcription of genes that are involved in normal embryological development and differentiation. Evidence from a few studies supports that NB has significantly higher EZH2 gene expression, and overexpression of EZH2 has been shown to promote tumorigenesis and maintain NB cells in an undifferentiated state. As inhibition of EZH2 has been shown to promote cell differentiation, we propose that combining an EZH2 inhibitor (EZH2i) with isotretinoin would further promote differentiation and improve the treatment outcome in HR NB.

Aims:
To determine if combining RA with EZH2i will enhance the efficacy and the mechanism of action in models of HR NB.

Methods:
2D and 3D cell model establishment, cell viability assays, immunoblotting, H&E, and IHC staining, and RNA-sequencing were performed in HR NB cell lines. For RNA-sequencing analysis, raw reads were assessed for quality before being aligned and quantified using STAR. Differential gene expression analysis was conducted using edgeR.

Results:
Combination therapy resulted in a significant decrease ($P<0.0001$) in 2D cell proliferation compared with RA alone. In 3D cell models, combination therapy resulted in phenotypic changes in H&E staining and a significant decrease in 3D cell viability compared to RA treatment alone. The RNA-sequencing analysis highlighted the upregulation of genes involved in NB differentiation.

Conclusions:
Initial results suggest that combination EZH2i + RA therapy is more effective than single agent treatment and therefore may represent a novel therapy for HR NB. Future work includes differentiation marker analysis, tests in murine mice models, and tests in combination with anti-GD2.

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BCLAF1 Functionalization Against Relapsed/Refractory Neuroblastoma

D'agostino E[1], Massaro C[1], Muro A[1], Sgueglia G[1], Lettiero I[1], Frattolillo V[1], Lania G[2], Bilio M[2], Capasso M[3], Morini M[4], Ponzoni M[4], Altucci L[1,5,6], Dell'Aversana C[1,6]

[1]Department of Precision Medicine, University of Campania "Luigi Vanvitelli" , Naples, Italy, [2]Institute of genetics and biophysics "Adriano Buzzati Traverso" (IGB), National Research Council (CNR), Naples, Italy, [3]CEINGE Biotecnologie Avanzate, Naples, Italy, [4]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [5]BIOGEM, Ariano Irpino, Italy, [6]Institute Experimental Endocrinology and Oncology 'Gaetano Salvatore' (IEOS)-National Research Council (CNR), Naples, Italy

Background-Aim: Neuroblastoma (NB) is the most common extracranial solid childhood tumor, with remarkably complex malignancy. It characterized by wide genetic, epigenetic, and phenotypic heterogeneity that impact upon diagnosis, therapeutic response and survival (PMID: 30116755; 10985139). While has been observed a substantial progress in NB cure with low and intermediate risk, new effective therapeutic strategies for high-risk NB constitute a urgent clinical need. Despite extensive studies and trials, outcomes have been stable, and the acquisition of multidrug resistance remain the major obstacle to the successful treatment of NB. We propose BCLAF1 as novel intracellular and circulating biomarkers in NB. Our recently published and new preliminary data provided the evidence of functional role for module miR-194-5p/BCLAF1 in intracellular and extracellular compartments in acute myeloid leukemia (AML) and NB (PMID: 28216661). BCLAF1 may act as intracellular player and extracellular messanger on recipient cells influencing tumorigenicity and drug-resistance in NB.

Methods: In NB cell lines, mouse models and primary samples: PCR&RT-PCR; Western blot; cell cycle and proliferation tests; immunofluorescence (IF) analysis; RNA-scope; RNA-seq.

Results: Our data suggest a significant correlation between BCLAF1 expression level, NB development and drug-resistance. BCLAF1 is over-expressed in different NB cell lines, especially in N-myc amplified, metastatic and cisplatin resistant cells. It is peculiar the BCLAF1 over-expression in NB cluster suspension subpopulations. Furthermore, we also verified the BCLAF1 differential expression (mRNA and Protein) inside NB derived-extracellular small vesicles (NB-SVs). We can impair the BCLAF1's mRNA and protein expression by Vorinostat/SAHA (HDACi) treatment in intracellular compartment and NB-SVs, causing a decrease of oncogenic BCLAF1 full-length (FL) isoform, an increase of tumor-suppressor exon5 truncated isoform (SL) and affecting the NB oncogenic potential. Interestingly, in in vivo C57/Bl6 models, BCLAF1 was identify in SNS progenitor cells at the roots of dorsal ganglia. Its expression is peculiar neural crest development in a specific subpopulation with a defined differentiation stage and commitment. The functional role of BCLAF1 in NB and NS development are the focus of current and future insights.

Conclusions: We speculate about BCLAF1 role in relapsed/refractory NB patients, suggesting its targeting for rationally designed diagnostic tools and new therapeutic approaches.

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Combined inhibition of EZH2 and EGFR results in neuroblastoma cell death

Martín Serrano J[1], García M[1], Rodriguez-Hernandez C[1], Lavarino C[1], Mora J[1], Silvia M[1]
[1]*Pediatric Cancer Center Barcelona. Developmental Tumor Biology laboratory. , Barcelona, Spain*

Background and aims: Neuroblastoma is the most common extracranial solid tumor in childhood and despite multimodal therapy, the long-term survival in high-risk neuroblastoma (HR-NB) is less than 50%. Thus, there is a need to develop novel strategies to increase survival. Enhancer of zeste homolog 2 (EZH2) silences transcription through trimethylation of histone H3 lysine 27 (H3K27me3) and is frequently overexpressed in HR-NB predicting clinical outcome. EZH2 mediates epigenetic silencing of NB suppressor genes and inhibition of H3K27 demethylation is effective against HR-NB. However, in vivo studies suggested that EZH2-inhibition may not be sufficient. In NB, expression of epidermal growth factor receptor (EGFR) has been correlated with cancer progression and resistance to treatment. However, monotherapy with EGFR-inhibitors rapidly produces resistance. Thus, we explored the putative synergistic effect of targeting EZH2 with GSK126 and EGFR with Canertinib, both in vitro and in vivo.

Methods: The anti-proliferative effect induced by GSK126, Canertinib, and their combination was evaluated by MTS cell proliferation and clonogenic assays. The apoptotic effect was evaluated by Western blot and annexin V staining by flow cytometry. Xenograft mouse models were generated to evaluate the in vivo effect.

Results: Treatment with either GSK126 or Canertinib decreased NB cell viability and colony formation. Notably, the combined treatment resulted in a more pronounced effect. Moreover, combination of GSK126 and Canertinib synergistically induced PARP-cleavage, strongly suggesting induction of apoptosis. The apoptotic effect was confirmed by annexin V staining. In vivo, although the inhibition of EZH2 or EGFR alone did not resulted in a significant delay of tumor growth, the combined treatment synergistically induced tumor growth inhibition.

Summary/conclusions: Our findings suggest that the combined use of GSK126 and Canertinib exerts a synergistic effect on tumor growth inhibition both in vitro and in vivo by inducing apoptosis. This data provides a novel therapeutic opportunity in neuroblastoma.

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Molecular and clinical impact of PTPN11/SHP2 in neuroblastoma

Nunes-Xavier C[1,2], Emaldi M[1], Zaldumbide L[3], Mosteiro L[3], Torices L[1], Rey E[1], Aguirre P[4], García de Andoin N[5], López-Almaraz R[6], Pulido R[1,7]

[1]Biomarkers in Cancer Unit, Biocruces Bizkaia Health Research Institute, Barakaldo, Spain, [2]Department of Tumor Biology, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, Oslo, Norway, [3]Department of Pathology, Cruces University Hospital, Barakaldo, Spain, [4]Department of Pathology, Donostia University Hospital, San Sebastian, Spain, [5]Unit of Pediatric Oncohematology, Donostia University Hospital, San Sebastian, Spain, [6]Pediatric Oncology and Hematology, Cruces University Hospital, Barakaldo, Spain, [7]IKERBASQUE, Basque Foundation for Science, Bilbao, Spain

Background

The protein tyrosine phosphatase PTPN11 gene is mutated with relative high frequency in the recurrent forms of high-risk neuroblastoma. PTPN11/SHP2 is a positive regulator of the RAS/MAPK pathway, and similar to other cancers, most PTPN11 mutations found in neuroblastoma are gain-of-function mutations, which supports its role as an oncogene in neuroblastoma. Pharmacological inhibitors of PTPN11 are currently under testing in cancer clinical trials, making PTPN11 a suitable therapeutic target in neuroblastoma.

Aims

To analyze the expression and function of PTPN11 in neuroblastoma, and to validate pre-clinically its potential as a novel therapeutic target in neuroblastoma.

Methods

We have performed in silico and experimental analyses of PTPN11 expression and mutational status in neuroblastoma tumors, in association with patient survival. The expression, localization, and function of PTPN11 has been evaluated in human neuroblastoma cell lines and in neuroblastoma tumor samples. Neuroblastoma cell lines resistant to therapeutic agents were generated. Sensitivity to chemotherapy agents and PTPN11 pharmacological inhibition was monitored in neuroblastoma cells by cell proliferation/viability assays.

Results

High expression of PTPN11, as well as the presence of PTPN11 mutations, are associated with a worse clinical outcome in neuroblastoma. Ectopic expression of PTPN11 in neuroblastoma cells, as well as the use of PTPN11 inhibitors, revealed an activating effect on the MAPK pathway. PTPN11 inhibition in combination with chemotherapy treatment resulted in decreased cell viability.

Conclusion

Our results highlight the potential of PTPN11 as a novel therapeutic target in high-risk neuroblastoma.

Acknowledgments

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P165

Targeted AURKA degradation: towards new therapeutic agents for neuroblastoma

Rishfi M[1,3], Krols S[2,3], Martens F[1,3], Bekaert S[1,3], Sanders E[1,3], Eggermont A[1,3], De Vloed F[1,3], Robert Goulding J[1,3], Risseeuw M[2,3], Molenaar J[4], De Wilde B[3,5], Van Calenbergh S[2,3], Durinck K[1,3]

[1]Department of Biomolecular Medicine, Faculty of Medicine, Ghent University, Ghent, Belgium, [2]Laboratory for Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium, [3]Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [4]Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands, [5]Department of Internal Medicine and Pediatrics, Faculty of Medicine, Ghent University, Ghent, Belgium

Background: Aurora kinase A (AURKA) is a well-established drug target in neuroblastoma (NB) due to both its catalytic role during mitosis and its kinase-independent functions regulating MYCN protein stability, DNA damage repair and mediation of replicative stress. Current small-molecule inhibitors effectively block AURKA's kinase activity but also trigger protein upregulation through feedback loops, which may drive the emergence and survival of drug resistant cells. Moreover, kinase inhibitors primarily target AURKA's mitotic/catalytic functions. AURKA degradation, as opposed to inhibition, may overcome upregulation and result in a more pronounced inhibitory effect by additionally targeting AURKA's scaffolding roles.

Aim: We aimed to develop small-molecule PROTACs (Proteolysis Targeting Chimeras) capable of inducing targeted AURKA degradation through the ubiquitin-proteasome pathway.

Methodology: PROTACs were synthesized by connecting the selective AURKA inhibitor MK-5108 with the E3-ligase recruiter thalidomide, via PEG-based linkers of varying lengths. The PROTACS were analyzed in a panel of NB cell lines using Simple Western and IncuCyte® Live-Cell Imaging which enabled high-throughput assessment of AURKA degradation potential and cell growth inhibition, respectively. We further validated the working mechanism of the PROTACs by co-inhibiting components of the ubiquitin-proteasome pathway and evaluated target-selectivity via a KINOME-scan and LC-MS shotgun-proteomics. Downstream effects of PROTAC treatment on MYCN protein levels, replicative stress and DNA damage were investigated through immunoblotting.

Results: PROTAC SK2188 induces the most potent AURKA degradation (DC50 3.9nM, Dmax 93%) in NB cell line NGP and displays excellent target-selectivity with limited off-target binding/degradation. Interestingly, AURKA degradation by SK2188 was associated with a subsequent reduction of MYCN protein, in keeping with AURKA's scaffolding role in MYCN stabilization. Furthermore, relative to inhibitor MK-5108, SK2188 efficiently induces elevated replicative stress and DNA damage markers, and triggers apoptosis at low nanomolar concentrations. Lastly, compared to inhibitor MK-5108, antiproliferative effects of AURKA-targeting PROTACs were significantly enhanced in NGP and IMR-32 cells (IC50 32nM and 22nM, respectively) and in NB patient-derived organoids [Rishfi & Krols et al.,].

Conclusion: We successfully synthesized PROTACs capable of inducing rapid and sustained degradation of AURKA followed by the subsequent depletion of MYCN and demonstrate the clinical potential of AURKA degradation as opposed to inhibition in neuroblastoma.

P166

Dual inhibition of ALK and SHP2 synergistically suppresses neuroblastoma cell proliferation

Uçkun E[1], Siaw J[1], Guan J[1], Anthonydhason V[1], Fuchs J[2], Wolfstetter G[1], Hallberg B[1], Palmer R[1]
[1]University of Gothenburg, Department of Medical Biochemistry and Cell Biology, Göteborg, Sweden, [2]University of Gothenburg, Proteomics Core Facility, Göteborg, Sweden

Background: Anaplastic lymphoma kinase (ALK) aberrations such as point mutations, intragenic deletions, genomic rearrangements, and gene amplifications have been described in various human malignancies including neuroblastoma (NB). Identification of signaling complexes in the context of wild-type and oncogenic ALK activation provides important information for the development of novel co-therapeutic approaches to target ALK-driven NB.

Aims: Our aim was to identify and characterize novel components of ALK signaling in NB cells by employing proximity-dependent biotin identification (BioID).

Methods: BioID is a powerful method for mapping protein-protein interaction networks. BirA is a biotin protein ligase which converts biotin to reactive biotinoyl-5'-AMP in the presence of ATP and labels lysine residues of substrates. BioID employs a mutant form BirA*(BirAR118G) which prematurely releases reactive biotin resulting in biotinylation of proximal proteins. Biotinylated proteins are isolated by streptavidin pull-down and further identified by TMT-based LC-MS3.

Results: Inducible Tet-On NB cell lines expressing ALK.BirA* fusion were generated. To identify the ALK proximitome, we compared biotinylated proteins in pull-downs from ALK-BirA* expressing cells compared to controls expressing BirA* upon ALK ligand stimulation or inhibitor treatment. Known ALK downstream signaling molecules were identified, as well as novel candidate proteins. Among them, interaction of SHP2 and PEAK1 with ALK were validated by anti-ALK immunoprecipitations in NB cells. These interactions were enhanced in the presence of ALK ligand and abrogated by ALK inhibitor. Furthermore, increased SHP2 phosphorylation in response to ALK activation and decreased SHP2 phosphorylation upon ALK inhibition confirmed that SHP2 is a downstream target of ALK in NB cells. Strikingly, combined ALK and SHP2 inhibition synergistically abrogated NB cell growth.

Conclusion: BioID identified previously known ALK interactors as well as novel potentially important components of ALK signaling. We further validated SHP2 as ALK interactor and show that SHP2 activity is important for the growth of ALK-driven NB cells. Importantly, co-inhibition of ALK and SHP2 acts synergistically in NB cells making it a potential therapeutic approach for ALK-driven NB. Supporting the role of SHP2 in NB, Cai et al. (2022) demonstrated that treatment of high-risk in vivo NB models with SHP2 inhibitor results in significant inhibition of tumor growth.

P167

ALK signaling primes the DNA damage response allowing exploitation of ATR inhibition in ALK-driven neuroblastoma.

Borenäs M[1], Lind D[1], Umapathy G[1], Lai W[1], Guan J[1], Kurhe Y[1], Johansson J[1], Aniszewska A[1], Zenténius E[1], Van den Eynden J[2], Hallberg B[1], Palmer R[1]

[1]University of Gothenburg, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden, [2]Ghent University, Department of Human Structure and Repair, Ghent, Belgium

Background/Aims

Oncogenes such as MYCN and ALK, which are often involved in high-risk NB, result in increased replication stress in cancer cells, offering therapeutically exploitable options. We previously identified an ATR/ALK inhibitor combination as an effective therapeutic approach that results in complete tumor regression in two independent genetically modified mouse NB models. In this work we identify an underlying molecular mechanism, in which ALK signaling leads to phosphorylation of ATR and CHK1, supporting an effective DNA damage response. Remarkably, the importance of ALK inhibition is supported by data in Alk-driven mouse neuroblastoma models, in which monotreatment with ATRi resulted in a robust initial response, but subsequent relapse, in contrast to a 14 day ALKi/ATRi combination treatment that resulted in robust and sustained responses. We also compare two independent ATR inhibitors, BAY1895344 (elimusertib) and AZD6738 (ceralasertib), showing that BAY1895344 is superior in inhibition of ATR in both NB cell lines and mouse models. We also investigate effect CD8+ T cell inhibition on response to ATRi/ALKi treatment in an Alk-driven mouse neuroblastoma model.

Methods

Neuroblastoma cell lines and Alk-F1178S;Th-MYCN mice harboring tumors were treated with either BAY1895344 (elimusertib) or AZD6738 (ceralasertib) BAY1895344 in the presence or absence of ALKi (lorlatinib) and tumor/cell responses were monitored. An additional arm investigated the effect of anti-CD8 antibodies on the Alk-F1178S;Th-MYCN tumor response to 14 day ALKi/ATRi combination. Complementary experiments in neuroblastoma cell lines were investigated at the level of phosphor- and total proteomics and with transcriptomics analyses.

Results

Treatment employing BAY1895344 exhibited increased efficacy compared with AZD6738 in all experimental setups. We observed a reduced response to ATRi/ALKi inhibition in the presence of anti-CD8 antibodies suggesting a role of the immune system in the robust response to this therapeutic intervention. At the molecular level we identify a novel molecular mechanism downstream of ALK underlying the increased response to ATRi/ALKi treatment.

Conclusion

Taken together, these results strongly motivate the continued exploration of ATR/ALK inhibition as a therapeutic approach in neuroblastoma.

P168

DEVELOPMENT OF A SWI/SNF INHIBITION STRATEGY FOR THE TREATMENT OF HIGH-RISK NEUROBLASTOMA

Molero Valenzuela A[1], Jiménez C[1], Boloix A[1], Nadal Ribelles M[2], Latorre P[2], Pérez García M[1], Solé C[2], Soriano A[1], Roma J[1], Posas F[2], de Nadal E[2], Moreno L[1,3], Segura M[1]

[1]Vall d'Hebron Research Institut, Barcelona, España, [2]Institute for Research in Biomedicine, Barcelona, España, [3]Pediatric Oncology and Hematology Department, Barcelona, España

Introduction: Neuroblastoma is an embryonal tumor developed from neural crest progenitor cells, which see their differentiation process block, giving rise a peripheral nervous system tumor. Neuroblastoma presents multiple epigenetic alterations that participate in tumor formation and progression. Thus, the understanding of the implicated epigenetic regulators is a crucial step, not only to understand its behavior, but also to develop new therapies. Previous results of our laboratory have demonstrated that SWI/SNF subtype BAF chromatin remodeling complex is a key player controlling master regulators of neuroblastoma cell proliferation and metastasis. In particular, ARID1A/B subunits are necessary to maintain the structure of the complex and sustain the oncogenic program of neuroblastoma cells. Our hypothesis is that disrupting the interaction of ARID1A/B with the rest of BAF subunits, we will repress the pro-oncogenic activity of the SWI/SNF complex, thus resulting in a therapeutic benefit.

Methods: An in silico druggability analysis was performed to find small molecules that can bind to ARID1A/B. In vitro functional screening was performed on a panel of different cell lines to test their impact on cell proliferation and BAF stability.

Results: A total of 7.5 million molecules were screened to find to potentially ARID1A/B binding molecules. The list of potential hits was shortlisted into 85 candidates after applying drug-like selection filters. The posterior in vitro functional screening identified 4 molecules with anti-proliferative activity. A “Hit to lead” strategy is ongoing to improve the efficacy and selectivity of the best candidates.

Conclusion: These findings represent a promising starting point for the development of a BAF inhibition therapeutic strategy against metastatic high-risk neuroblastoma.

P169

MYCN-amplified neuroblastomas are highly and selectively sensitive to inhibition of N-myristoyltransferases.

Zhang J[1,2,4], Poon E[4], Lueg G[1], Martins da Costa B[4], Falciani F[3], Solari R[3], Carr R[3], Chesler L[4], Tate E[1,2,3]
[1]Department of Chemistry, Imperial College London, London, United Kingdom, [2]Francis Crick Institute, London, United Kingdom, [3]Myricx Pharmaceuticals, London, United Kingdom, [4]Cancer Therapeutics, Institute of Cancer Research, London, United Kingdom

N-myristoyltransferases (NMTs) catalyse the protein N-terminal modification N-myristoylation, a lipidation event which affects >150 proteins and is involved in protein localisation, stability, and function. NMT has been suggested as a target in cancers, but there has been a lack of rationale to identify patients who may respond to NMT inhibition. Additionally, the mechanism of action of NMT inhibitors (NMTi) is difficult to dissect, as NMT substrates feature in multiple biological pathways, and many studies have focused only on a single substrate. Here, a combination of cellular biology, 'omics and bioinformatics techniques were combined to show that deregulation of MYC-family proteins sensitises cells to NMT inhibition. Cell lines sensitive to NMTi were identified through screening of hundreds of cancer cell lines, and the transcriptome of sensitive and insensitive lines compared to obtain a "Sensitive to NMTi" gene set. This gene set correlated well with MYC-related gene sets and mutation, amplification, or chromosomal rearrangement in MYC(N) were predictive for NMTi sensitivity, suggesting that MYC deregulated cancer cells are sensitive to NMTi. Screening of a panel of neuroblastoma lines, including the tet-off MYCN SHEP-21N system, revealed that the effects of NMT inhibitors diverged depending on MYCN-amplification status. Additionally, proteomic profiling of neuroblastoma cell lines identified multiple pathways are affected by NMTi and likely contribute to their MoA. In particular, an impact on Complex I formation was seen, in line with results in alternative cancer types. Furthermore, application of an orally bioavailable NMTi suppressed tumours in the Th-MYCN mouse model in vivo and caused no changes in body weight, suggesting that NMT inhibitors are well tolerated and efficacious in vivo.

P170

Regulation of oncogenic MYCN transcription and tumor growth in neuroblastoma by the CDK9 inhibitor KB-0742

Saffran D[1], Poon E[2], Heuss S[2], Amara S[1], Noe C[1], Tsai M[1], Hood T[1], Kumar P[1], DiMartino J[1], Chesler L[2], Lin C[1]
[1]Kronos Bio, Inc., San Mateo, USA, [2]Division of Clinical Studies, The Institute of Cancer Research, London, London, United Kingdom

Background:
Disruption of transcriptional regulatory networks that drive normal cellular differentiation and development can result in oncogenic transformation and transcriptional addiction. In neuroblastoma, MYCN, a member of the MYC family of transcription factors (TF), is often amplified and localizes to super enhancer regions, where it rewires lineage-specific transcriptional programs driving oncogenesis.
Oncogenic TFs have proven difficult to target directly; we and others have proposed targeting associated transcriptional co-regulators to inhibit their activity. Cyclin-dependent kinase 9 (CDK9) interacts with many oncogenic TFs and is essential for TF-mediated transcription elongation through phosphorylation of the C-terminal domain of RNA polymerase II (RNA pol II). Here, we developed a potent, selective, and orally bioavailable inhibitor of CDK9, KB-0742, and present preclinical activity in models of neuroblastoma.

Materials and Methods:
Cell lines were tested for antiproliferative effects of KB-0742, using either Cell Titer Glo (CTG; Promega) or Alamar Blue cell viability reagent (Bio-Rad). Pharmacodynamic (PD) markers of KB-0742 treatment, including phospho-SER2 (pSER2) on RNA pol II, MYCN, MYC, and cleaved poly ADP ribose polymerase (PARP), were measured by Western blot. The transgenic TH-MYCN model of neuroblastoma was used to study antitumor effects of KB-0742. Tumor samples and plasma were collected to determine PD effects and drug concentrations, respectively. All animal experiments were approved by The Institute of Cancer Research Animal Welfare and Ethical Review Body and performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

Results:
Neuroblastoma cell lines with MYCN amplification were more sensitive to KB-0742 treatment than non MYCN amplified cell lines. KB-0742–treated neuroblastoma cells had decreased pSER2, loss of expression of MYCN and MYC, and an induction of cleaved PARP. KB-0742 treatment of a TH-MYCN transgenic mouse model resulted in regression of established tumors. Analysis of tumor samples revealed decreases in pSER2 and expression and function of the oncogenic TF.

Conclusions:
CDK9 targeting by KB-0742 inhibits growth of MYCN-amplified neuroblastoma via selective modulation of oncogenic MYCN expression and activity. KB-0742 is being evaluated in a phase I dose-escalation trial in patients with relapsed or refractory solid tumors or non-Hodgkin lymphoma (NCT04718675).

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Identification of MYCN non-amplified neuroblastoma subgroups points towards molecular signatures for precision prognosis and therapy stratification

Hu X[1,3,5], Zhou Y[2,4], Hill C[2], Chen K[1,5], Wu Y[1,5], Li Z[3], Ewing R[2,4], Cheng C[1,5], Liu X[1,5], Gu Y[1,5], Duan P[1,5], Wu Z[1,5], Wang Y[2,4]

[1]Department of Pediatric Surgery, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China, [2]Biological Sciences, Faculty of Environmental and Life Sciences, University of Southampton, Southampton , The United Kingdom, [3]Department of Pediatric Surgery, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, China, [4]Institute for Life Sciences, University of Southampton, Southampton , Southampton, The United Kingdom, [5]Division of Pediatric Oncology, Shanghai Institute of Pediatric Research, Shanghai, China

Background

Neuroblastoma is a highly heterogeneous disease ranging from spontaneous regression to drug resistance and metastasis ultimately resulting in death. Despite the extensive study of MYCN-amplified neuroblastomas, there is a significant unmet clinical need in MYCN non-amplified neuroblastomas.

Aims

To investigate whether MYCN non-amplified neuroblastomas can identify transcriptional subgroups with discrete clinical characteristics and therapeutic vulnerabilities.

Methods

A comprehensive search of microarray samples was screened in GEO (Gene Expression Omnibus) and ArrayExpress. Identification of subgroups was performed by ConsensusClusterPlus using the top 50% varying genes. Clinical and molecular characterizations of the subgroups were compared using Kaplan-Meier analyses, single-sample Gene Set Enrichment Analysis (ssGSEA), co-expression module analyses and CibersortX. The combination of MetaIntegrator and machine learning algorithms was performed to identify predictors and develop the classifier, which was further validated by the test set and another 3 independent datasets (TARGET microarray, TARGET RNA-seq and GSE49711). Finally, we compared the prognostic impact of our classifier to other published transcriptional classifiers.

Results

Totally 1,566 microarray samples from 16 datasets were collected in GEO and ArrayExpress after excluding poor quality and duplicate data. We demonstrated that MYCN non-amplified neuroblastomas were heterogeneous and can be classified into 3 subgroups based on transcriptional signatures. Within these groups, subgroup 2 had the worst prognosis and showed a similar molecular signature to MYCN-AMP cases; Whilst subgroup 3 was characterized by an "inflamed-mesenchymal" gene signature and may benefit from anti-PD-1 therapy. 53 genes were identified as predictors and successfully constructed a machine learning classifier with area under curve (AUC) larger than 0.9 for all three subgroups on the test set. The multivariate analysis illustrated our stratification as an independent classifier after being corrected by previous classifiers. Importantly, distinguishing patients stratified by previous algorithms to our classifier still displayed significant survival differences.

Conclusion

MYCN non-amplified neuroblastoma is heterogeneous, and the subgroup classification we defined has important clinical significance because each subgroup has a unique prognosis and molecular pattern, which will guide more precise individualized treatment.

P172

Translational precision medicine in childhood cancer – a representative NBL case

Aesoey R[1,2], de Montlaur C[1,2], Popa M[1], Brenner A[2], Forthun R[3], Hovland R[3], McCormack E[1,4], Herfindal L[1], Gunnes M[2,5]

[1]University of Bergen, Department of Clinical Science, Bergen, Norway, [2]Haukeland University Hospital, Department of Paediatrics, Bergen, Norway, [3]Haukeland University Hospital, Section for Cancer Genomics, Bergen, Norway, [4]Centre for Cancer Biomarkers, Department of Clinical Science, Bergen, Norway, [5]Oslo University Hospital, Paediatric Haematology and Oncology Department, Oslo, Norway

Background

Although over 80% of paediatric cancer patients show long-term survival, a portion of survivors suffer from relapsed disease and late-effects after intensive cancer treatment. Thus, there is a great need for more personalized therapy approaches for paediatric cancer patients in order to improve their long-term outcome.

Aims

Through translational research, combining clinical findings with preclinical cancer models, we aim to improve the outcome for patients with relapsed, refractory, or rare childhood cancers.

Methods

- Clinical examinations and blood tests
- Next-Generation Sequencing (NGS) of the tumour material
- Serial measurements of patient cell-free DNA by droplet digital PCR (ddPCR)
- Establishment of tumour-derived cell cultures and drug-screening
- Establishment of patient-derived xenograft (PDX) models and drug-testing
- Proteomics on the tumour material
- Treatment selection for the individual patient

Results

Among our paediatric patients is a 4-year-old girl who was diagnosed with neuroblastoma (NBL) of the left adrenal gland with extensive metastases in the bone marrow and skeleton. After induction chemotherapy, tumour resection was performed, followed by tandem high-dose chemotherapy with autologous stem cell support, irradiation, and immunotherapy. NGS identified a rare case of MYCN amplification and 11q deletion in the primary tumour, which are usually mutually exclusive in NBL. Liquid biopsy was performed in order to monitor the tumour burden, and a MYCN amplification was confirmed by ddPCR at inclusion followed by a normal MYCN copy number in the successive measurements. A PDX model was established from the primary tumour and a cell culture was derived from the PDX. Based on the genetic profile, diverse therapies have been tested to identify drug candidates for individualized therapy. Through both in vitro and in vivo experiments, we found that Cabozantinib had high efficacy towards the neuroblastoma cells. Additionally, proteomic results from Cabozantinib treated PDX-derived cells will be presented. Other potentially effective drugs are currently being tested on the PDX model.

Conclusion

Through the course of this study, we managed to establish a representative model of a rare high-risk NBL enabling in vitro and in vivo drug-testing to identify effective patient treatment options.

P173

131-I-MIBG therapy in combination with PARP inhibitors for relapsed neuroblastoma with DNA repair pathway alterations

Cohen-gogo S[1,4], Mills D[1], Vali R[2], Shammas A[2], Kanwar N[3], Davidson S[3,4], Lo W[3], Layeghifard M[4], Villani A[1], Shlien A[3,4], Irwin M[1], Morgenstern D[1]

[1]Division of Hematology/Oncology, The Hospital for Sick Children, Toronto, Canada, [2]Division of Nuclear Medicine, Department of Diagnostic Imaging, The Hospital for Sick Children, Toronto, Canada, [3]Department of Pediatric Laboratory Medicine, The Hospital for Sick Children, Toronto, Canada, [4]Genetics and Genome Biology, The Hospital for Sick Children Research Institute, Toronto, Canada

Background: We present the case of an 18-year-old patient diagnosed with metastatic high-risk neuroblastoma (MYCN-non-amplified, 11q LOH) at age 5, who had multiple relapses and exhausted available therapeutic options. Tumour and normal DNA profiled at latest relapse identified a pathogenic germline variant in PALB2 (p. Lys346Thrfs*13), somatic PALB2 LOH, additional one copy loss of ATM and CHEK1, and presence of COSMIC DNA signature SBS3. PARP inhibition is a promising approach for patients with tumours harboring defective homologous recombination repair (HRR). Pre-clinical data also indicate that PARP inhibitors can enhance 131-I-MIBG efficacy.

Aims: We examined the feasibility, safety, tolerability and outcome of 131-I-MIBG therapy in combination with PARP inhibitors in a single patient with the described HRR pathway alterations.

Methods: 131-I-MIBG (18 mCi/kg) was administered in combination with olaparib (90 mg/m2 b.i.d. for 10 days). Following PBSC administration and bone marrow recovery, talazoparib was administered at starting dose 600 µg/m2 once daily continuously. Access to talazoparib occurred through a single patient study, approved by Health Canada and the Hospital's Research Ethics Board, with written consent for reporting of results.

Results: Our patient did not experience any unexpected adverse events. Disease reassessment 43 days post 131-I-MIBG showed an improvement in Curie score (from 20 to 13). The patient recovered from expected myelotoxicity and started maintenance talazoparib therapy on day 78. As a result of grade 4 thrombocytopenia, talazoparib dose was reduced. The patient experienced prolonged stable disease until progressive disease was detected after 18 cycles of maintenance therapy (18 months from 131-I-MIBG administration).

Conclusion: The administration of 131I-MIBG therapy in combination with a PARP inhibitor, followed by a prolonged period of maintenance therapy was safe and feasible. This novel treatment approach provided prolonged stable disease in a patient with multiply relapsed disease. Of note, this patient had previously experienced periods of disease response of 9 and 8 months, following two prior courses of single agent MIBG therapy. This case report, together with pre-clinical studies, provides rationale for future early phase trials utilizing molecular selection of patients based on HRR pathway alterations for delivery of MIBG with PARP inhibition.

P174

RUVBL1 and RUVBL2 are prognostic biomarkers and putative therapeutic targets for high-risk neuroblastoma.

Siaw J[1,2,3], Claeys A[1,2], Lai W[3], Borenäs M[3], Hilgert E[2,4], Eggermont A[2,4], Bekaert S[2,4], Durinck K[2,4], Speleman F[2,4], Hallberg B[3], Palmer R[3], Van den Eynden J[1,2]

[1]Ghent University, Department of Human Structure and Repair, Anatomy and Embryology Unit., Ghent, Belgium, [2]Cancer Research Institute Ghent, Ghent, Belgium, [3]University of Gothenburg. Department of Medical Biochemistry and Cell Biology., Gothenburg, Sweden, [4]Ghent University. Department of Biomolecular Medicine., Ghent, Belgium

Background

High-risk neuroblastoma accounts for about 50% of all neuroblastoma cases and has a relapse rate of more than 50% despite intensive therapies. Life-threatening toxicities occur in many of high-risk patients during and after treatment with cytotoxic chemotherapy and radiotherapy. Hence, safer, and more effective drugs are urgently needed to treat high-risk neuroblastoma. MYCN amplification, one of the strongest prognostic biomarkers, together with high MYC expression are believed to drive substantial proportion of high-risk neuroblastoma. While strategies for direct pharmacological targeting of MYC proteins have been elusive, targeting proteins and/or pathways that modulate MYC transcription factors could provide a promising alternative.

Aims:

To identify novel biomarkers and therapeutic targets in high-risk neuroblastoma.

Methods

Molecular signature score and network analyses of the Hallmark gene sets were performed on cohorts of publicly available neuroblastoma tumour samples and cell line RNA transcriptomic data to identify novel biomarker and druggable interaction gene nodes. Kaplan-Meier survival and multivariate Cox regression analyses were performed to assess prognostic values of novel gene candidates. Neuroblastoma cell culture models, siRNAs, small molecule inhibitors, qPCR, RNA-seq and immunoblotting were used to validate gene candidates as therapeutic targets.

Results

MYC targets were strongly associated with high-risk neuroblastoma and we identified RUVBL2 as a notable MYC interacting gene in the MYC/MYCN network. We showed that high expression of RUVBL2 and its homolog RUVBL1 were associated with unfavourable prognostic markers such as stage 4, age >18 months and MYCN amplification. Interestingly, a multivariate Cox regression analysis indicated that RUVBL1/2 expression are strong and independent predictors for both overall and event-free survival in two independent cohorts of neuroblastoma patients (overall survival: RUVBL1 [HR = 1.70 – 1.94] and RUVBL2 [HR = 2.12 – 2.97]). We then performed an experimental validation and found that inhibition of RUVBL1/2 ATPase activity with siRNA or pharmacologically, with CB-6644, led to significant abrogation of growth of several neuroblastoma cell lines, and induction of S phase arrest, DNA damage and apoptosis. Mechanistically, RUVBL1/2 regulated ATR and ATM protein stability and transcriptionally regulated MYCN and MYC mRNA expression.

Conclusion.

RUVBL1/2 are promising new biomarkers and putative therapeutic targets for high-risk neuroblastoma.

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The two-drug regimen panobinostat/lorlatinib is effective against neuroblastomas driven by genomic TERT rearrangements and ALK mutations

Linke R[1], Krienelke-Szymansky A[1], Wünschel J[1,2], Finkler S[1], Toedling J[1], Kopp-Schneider A[3], Astrahantseff K[1], Lodrini M[1,2], Eggert A[1], Deubzer H[1,2]

[1]Department of Pediatric Hematology and Oncology, Charité - Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany, [2]Experimental and Clinical Research Center (ECRC) of the Charité and the Max-Delbrück-Center for Molecular Medicine (MDC) in the Helmholtz Association, Lindenberger Weg 80, 13125 Berlin, Germany, [3]Department of Biostatistics, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

Background: Prognosis of patients with high-risk neuroblastoma remains poor with survival rates of 50% at initial diagnosis and as low as 10% at relapse. Thus, new treatment strategies are urgently required. Telomere maintenance mechanisms including genomic TERT rearrangements have recently been identified as major oncogenic drivers. Neuroblastomas harboring both telomere maintenance mechanisms and mutations in the RAS-MAPK pathway present a subgroup among high-risk tumors with particularly dismal prognosis. The histone deacetylase inhibitor panobinostat displays strong antitumoral activity in preclinical models of TERT-driven neuroblastoma.

Aims: Drug combinations are essential to increase therapeutic efficacy and reduce therapy resistance. Here, we aimed at identifying synergistically acting drug partners with panobinostat in eight neuroblastoma cell lines harboring TERT rearrangements and/or ALK aberrations.

Methods: Neuroblastoma cells were treated with the combination of panobinostat and one of four chemotherapeutics (doxorubicin, irinotecan, oxaliplatin, temozolomide) or one of five targeted therapeutics (bortezomib, dasatinib, IBET-762, lorlatinib, rapamycin). Metabolic activity was measured with the CellTiter-Glo assay and analyzed with the SynergyFinder software. The top six drug combinations were evaluated with the Caspase-3/7 Glo assay using a linear mixed-effects model. The top two drug combinations were further examined for PARP cleavage by Western blotting and cell viability by Trypan blue staining. Cell viability data were analyzed by one-way ANOVA and Tukey post-hoc tests.

Results: Doxorubicin, temozolomide, bortezomib, dasatinib, lorlatinib and rapamycin synergistically reduced metabolic activity up to 95% in CLB-GA and GI-ME-N cells when combined with panobinostat. Lorlatinib and rapamycin most effectively and synergistically induced caspase activity. PARP cleavage and Trypan blue staining displayed the strong induction of apoptosis by the two-drug regimen panobinostat/lorlatinib over a wide range of drug concentrations. Findings were confirmed in six cell lines harboring the ALK p.R1275Q or ALK p.F1174L hotspot mutations or an ALK amplification. Metabolic activity was reduced by >82% in ALK mutated neuroblastoma cell lines (LAN-5, Kelly, SK-NSH) and by >95% in ALK amplified cell lines (IMR-5, IMR-32, NB-1).

Conclusion: Combining the histone deacetylase inhibitor panobinostat with the ALK inhibitor lorlatinib is strongly effective against ALK-driven high-risk neuroblastoma with and without TERT rearrangements warranting further validation in neuroblastoma in vivo models.

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Targeting the extracellular matrix in therapy-resistant pediatric tumors

Emperador M[1,2,3], Nafaji S[1,2,4], Herter S[1,2,3], Witt O[1,2,4], Oehme I[1,2]

[1]Hopp Children's Cancer Center Heidelberg (KiTZ), Heidelberg, Germany, [2]Clinical Cooperation Unit Pediatric Oncology, German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany, [3]Faculty of Biosciences, Heidelberg University, Heidelberg, Germany, [4]Department of Pediatric Oncology, Hematology and Immunology, University of Heidelberg Medical Center, Heidelberg, Germany

Background: The development of effective therapies for relapsed and metastasized cancer is very challenging, in part due to the complexity of the tumor microenvironment (TME). The TME can promote alterations in components of the extracellular matrix (ECM), leading to collagen accumulation and an increase of ECM stiffness, which contributes to tumor growth and progression. Our preliminary results indicate that ECM-related oncogenic signaling pathways are deregulated in therapy-resistant neuroblastomas compared to primary neuroblastomas, making the ECM a potential target to overcome therapy resistance in relapsed tumors.

Aim: With this project, we aim to address the novel druggable vulnerability of deregulated ECM components in therapy-resistant relapsed neuroblastomas, which will also allow the elucidation of the pathways involved.

Methods: To do so, inhibitors of ECM proteins overexpressed in relapsed tumors were tested alone and in combination with a drug library containing 78 clinically relevant (mostly approved) drugs with patient-derived neuroblastoma cultures (either fresh or so-called long-term cultures kept organoid-like in serum-free stem cell medium) obtained through the INFORM study. Hit combinations were tested for their effect on oncogenic ECM pathways and on migration, invasion and metastasis, and validated in vivo with several neuroblastoma zPDX models.

Results: We have identified that several ECM components are overexpressed in relapsed neuroblastoma patients in comparison to the primary tumor at the gene and protein levels. Between those proteins, matrix metalloproteinases (MMPs; e.g., MMP9 and MMP13) and different types of collagens were the most significantly deregulated genes in relapsed patients. The treatment of patient-derived cultures with inhibitors of these ECM proteins alone decreased proliferation and induced apoptosis of neuroblastoma cells, which could be further enhanced with the combination of neuroblastoma-relevant drugs. The effects on in vivo migration are currently under investigation using an established zebrafish embryo xenograft model.

Conclusion: ECM components are overexpressed in relapsed neuroblastomas and are potential targets to overcome therapy resistance. We aim to find novel combination treatments for the treatment of relapsed neuroblastoma patients by targeting the ECM combined with drug hits identified by functional drug testing of patient-derived cultures.

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Succesful targeting of ALK in relapsed or refractory metastatic neuroblastoma with lorlatinib monotherapy

Ek T[1,2], Georgantzi K[3,4], Vogt H[5,6], Fransson S[7], Träger C[8,9,4], Øra I[10,11], Treis D[4], Hansson L[12], Chuang T[13], Palmer R[13], Hallberg B[13], Dalin M[1,2], Martinsson T[7], Kogner P[4,14]

[1]Children´s Cancer Centre, Sahlgrenska University Hospital, Gothenburg, Sweden, [2]Institute for Clinical Sciences, University of Gothenburg, Gothenburg, Sweden, [3]Pediatric Oncology, Karolinska University Hospital, Stockholm, Sweden, [4]Childhood Cancer Research Unit, Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden, [5]Pediatric Oncology, Crown Princess Victoria's Child and Youth Hospital , Linköping, Sweden, [6]Division of Children's and Women's Health, Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden, [7]Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden, [8]Pediatric oncology, Academic Children`s Hospital, Uppsala, Sweden, [9]Department of Women´s and Children`s Health, Uppsala University, Uppsala, Sweden, [10]Pediatric oncology, Skane University Hospital, Lund, Sweden, [11]Clinical Sciences, Lund University, Lund, Sweden, [12]Department of Oncology, Sahlgrenska University Hospital, Gothenburg, Sweden, [13]Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden, [14]Pediatric Oncology, Karolinska University Hospital, Stockholm, Sweden

Background

Relapsed or refractory neuroblastoma portends poor prognosis, without general agreement on the best second line treatment. ALK-mutations are linked to worse prognosis, more prevalent in refractory/relapsed neuroblastomas and targetable with ALK-inhibition. First generation ALK-inhibitor produced disappointing results, but preliminary data indicate that the third-generation ALK-inhibitor lorlatinib may be more effective.

Aims

To establish a national program of analyzing and targeting ALK in neuroblastoma, implement national guidelines on insertion, surveillance and completion of ALK-inhibitor treatment.
To evaluate lorlatinib monotherapy and collect clinical and biological data from a cohort with refractory/relapsed neuroblastoma and ALK-mutations.

Methods

All neuroblastoma patients were analyzed for ALK-aberrations at diagnosis and at relapse/progression/refractory disease. Five patients treated with lorlatinib monotherapy were followed with collection of clinical, radiological and laboratory data, genomic tumor analyses (SNP-arrays, ALK Sanger-sequencing, WGS). A Neuroblastoma ALK sequencing panel was developed (NB-ALK, detecting 11 activating ALK-mutations, and 21 known or potential resistance mutations) and circulating tumor DNA was analyzed in blood samples collected at regular intervals during and after lorlatinib treatment.

Results

Five children with refractory/progressive/relapsed (N=2/N=1/N=2) metastatic neuroblastoma harboring ALK R1275Q (N=4, one constitutional de novo mutation) or ALK F1174L mutations (N=1, MYCN-amplified). The mean lorlatinib dose was 79 mg/m2/d (range 56-98), and was well tolerated with expected manageable side effects (hyperlipidemia, weight gain, neurocognition). Best response was metastatic CR (INRC criteria, N=4) or PR (N=1), and reached within 2-6 months. The duration of response was 36+ months for 3 patients with CR (two maintain CR 6-12+ months after concluded therapy), and 20+ months for 1 patient with metastatic CR. The patient with ALK F1174L and MYCN-amplification relapsed after 10 months of lorlatinib treatment. No patient revealed novel ALK-resistance mutations, but the child with relapse acquired a novel HRAS–

mutation (c.182A>T, p.Q61L). Longitudinal analysis of NB-ALK sequencing-panel indicated that the mutational load in plasma could be used to guide treatment decisions.

Conclusions
Lorlatinib monotherapy can induce prolonged remission in ALK-mutated relapsed or refractory metastatic neuroblastoma with manageable adverse effects. Our results further suggest that ALK R1275Q may respond favourably over an extended time, and that the subclinical course can be monitored with ctDNA.

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Dual ROCK inhibitor RKI-1447 promotes cell death and emerges as a synergistic partner for BET inhibitors in neuroblastoma

Pepich A[1], Tümmler C[1], Abu Ajamieh S[1], Tries D[1], Boje A[3], Vellema Q[1], Åkerlund E[2], Seashore-Ludlow B[2], Kogner P[1], Johnsen J[1], Wickström M[1]

[1]Division of Pediatric Oncology and Surgery, Department of Women's and Children's Health, Karolinska Institutet , Stockholm , Sweden, [2]Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden, [3]Division of Antibody-Based Immunotherapy, Department of Internal Medicine II, Christian Albrechts University and University Hospital Schleswig-Holstein, Kiel, Germany

Background: Despite intensive treatment, high-risk neuroblastoma has a poor prognosis, demonstrating the need for new therapeutic strategies. Genes in the Rho/Rac pathway are frequently genetically altered in neuroblastoma, leading to active Rho/ROCK signaling. BET inhibitors target MYCN transcription and have demonstrated therapeutic efficacy against neuroblastoma, however clinical trials have suggested that resistance limits their therapeutic benefit. Notably, cytoskeletal remodeling and changes in ROCK activity have been proposed to play a role in therapy resistance.

Aims: To investigate the therapeutic potential of dual ROCK-BET inhibition in neuroblastoma.

Methods: RKI-1447 efficacy was studied in vitro in cell lines through viability and clonogenic assays, and in vivo using the transgenic TH-MYCN mouse model. Transcriptomic profiling was performed with RNA-sequencing and gene signaling enrichment analysis. A drug combination screening with RKI-1447 and the FIMM oncology drug library (n=528) was performed. Combinations were validated using cell viability assays, western blots, and multicellular tumor spheroids (MCTS). Synergy was quantified using several models, including ZIP and MuSyC.

Results: Treatment with pan-ROCK inhibitor RKI-1447 in a panel of neuroblastoma cell lines, resulted in decreased cell viability, reduced clonogenic ability and increased apoptotic cell death. In accordance, treatment of homozygous TH-MYCN mice with RKI-1447 repressed tumor growth and was shown to reduce expression of MYC targets. The combination screening revealed synergistic effects between RKI-1447 and several BET inhibitors. BET inhibitors ABBV075/Mivebresib or JQ1 with RKI-1447 demonstrated synergy in both MYCN-amplified and non-MYCN-amplified neuroblastoma cell lines, while MuSyC analyses of combinations indicated an RKI-1447 induced synergistic effect on potency. Treatment with ABBV075 increased ROCK activity, measured as phosphorylated myosin light chain 2 (MLC2), an effect that was blocked when combining RKI-1447 and ABBV075. Additionally, combination treatment decreased N-MYC or C-MYC protein expression. Finally, studies in MCTS validated combinational effects, showing decreased spheroid growth and increased cell death compared to single treatment.

Conclusion: ROCK and BET inhibitors have previously demonstrated potential for the treatment of neuroblastoma; here we reveal that the combination of ROCK and BET inhibitors offer a promising treatment approach that can potentially mitigate resistance to BET inhibitors and reduce toxicity.

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Design of Retinoid-Responsive Suicide Gene Systems For Neuroblastoma Treatment

Giardina E[1], Stoker A[1], Hart S[2]

[1]UCL Great Ormond Street Institute of Child Health, Department of Developmental Biology and Cancer, London, United Kingdom, [2]UCL Great Ormond Street Institute of Child Health, Department of Genetics and Genomic Medicine, London, United Kingdom

Background

Retinoic acid (RA) has been shown to improve clinical outcomes for high-risk neuroblastoma patients due to its ability to induce differentiation in tumour cells. However, its use has been limited by tumour-acquired resistance after prolonged treatment, following which patients relapse and this is often fatal.

Aims

To increase the efficacy and decrease the necessary treatment window of RA, we aim to design a "suicide gene" therapeutic that is highly sensitive to transcriptional activation by retinoic acid. When tumour cells take up the DNA-based drug, they will be bathed in exogenous RA and be rapidly killed. This will effectively re-purpose RA by converting the differentiation response into a lethal, cytotoxic response. Moreover, the drug will be delivered to cells in culture and in vivo using our liposome-based nanotechnology.

Methods

Using recombinant DNA technology, we have linked the toxin gene diphtheria toxin A (DTA) to RA-responsive promoters in plasmids and tested their cytotoxic capabilities at a range of RA concentrations in vitro. MicroRNA-response-elements (MREs) were incorporated into the plasmids to knockdown expression in off-target tissues, primarily the liver. Several fusion constructs of the suicide gene are being generated and tested on their ability to provide tumour-selectivity and allow protein secretion to generate a strong bystander effect in non-transfected neighbouring tumour cells.

Results

The RA-driven DTA vector is highly sensitive to RA, requiring minimal exogenous RA (<50 nM) for maximal activity, and decreases cell survival sufficiently in neuroblastoma cell lines. Interestingly, the vector shows greater toxicity at all concentrations of RA compared to RA alone. Liver-specific MREs inserted into the three prime untranslated region of the plasmid knocked down transcription by 98% in the hepatoma-derived Huh7 cell line, and reduced off-target cytotoxicity; however, decreased on-target activity was also seen in some neuroblastoma cell lines.

Conclusion

We have developed an inducible suicide gene therapy that is highly sensitive and cytotoxic in culture. The next steps are to improve the selectivity of the suicide gene and test our plasmids in vivo in combination with our liposome-based delivery system. If successful, this novel approach could decrease relapse frequencies and improve long-term survival of high-risk patients.

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Targeting of the polyamine transporter ATP13A3 sensitises neuroblastoma cells to DFMO

Azfar M[1], Gao W[2,3], Murray J[2,3], Karsa M[2,3], Ronca E[2], Xiao L[2,3], Pandher R[2,3], Bongers A[2], Verhelst S[1], Norris M[2,3], Haber M[2,3], Somers K[2,3], Vangheluwe P[1]

[1]Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium, [2]Children's Cancer Institute, Lowy Cancer Research Centre, UNSW Sydney, Sydney, NSW, Australia, [3]School of Women's and Children's Health, UNSW Sydney, Sydney, NSW, Australia

Background: High-risk neuroblastomas are characterised by elevated levels of polyamines, small polycations that play key roles in cell proliferation and survival. DFMO, an irreversible inhibitor of polyamine synthesis, is currently in clinical trial in combination with chemotherapy in children with neuroblastoma (NCT03794349), and we recently showed that DFMO-induced neuroblastoma growth inhibition is enhanced by addition of the polyamine transport inhibitor AMXT 1501 (Gamble et al, Sci Trans Med, 2019). Although DFMO/AMXT 1501 is currently in clinical trial for advanced adult solid tumours (NCT05500508), and paediatric trials of this combination are planned, very little is known about the polyamine transport system in neuroblastoma. We previously identified the role of SLC3A2 as a neuroblastoma polyamine transporter (Gamble et al, 2019), but its silencing does not completely abrogate polyamine uptake, suggesting the role of other polyamine transporters in this disease.

Aims: To investigate the role of two recently described polyamine transporters, ATP13A2 and ATP13A3 (van Veen et al, Nature 2020, Hamouda et al. J Biol Chem, 2021) in neuroblastoma.

Methods: si/mi-RNA knockdown and/or overexpression of ATP13A2, ATP13A3 and SCL3A2 were compared in human neuroblastoma Kelly and SH-SY5Y cells. Polyamine uptake was measured using radio-labelled or fluorescently labelled polyamines/analogues. Cell proliferation was determined by Incucyte proliferation assay or colony assay.

Results: High expression of SLC3A2 and ATP13A3, but not ATP13A2, was associated with poor survival in a cohort of 498 neuroblastoma patients ($P<0.0001$). Silencing of ATP13A3 in Kelly or SH-SY5Y cells decreased polyamine uptake by 80-90% (similar to treatment with AMXT1501) compared to only 30-50% or 15-25% reduction after silencing of SLC3A2 or ATP13A2, respectively. Overexpression of wild-type ATP13A3, but not of a catalytically dead ATP13A3 mutant, significantly increased polyamine uptake in SH-SY5Y cells ($P < 0.0001$), an effect that was completely abolished by treatment with AMXT1501. Furthermore, increased polyamine uptake normally observed in neuroblastoma cells following DFMO treatment was reduced ($P< 0.001$) by ATP13A3 knockdown, which also resulted in increased sensitivity of neuroblastoma cells to DFMO ($P<0.0001$).

Conclusion: Our data suggest that ATP13A3 plays a critical role in mediating polyamine transport in neuroblastoma cells and is the major polyamine transporter in this disease.

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Preclinical evaluation of the MDM2/p53 binding antagonist idasanutlin and PARP inhibitor olaparib alone and in a novel combination in neuroblastoma

Hartley H[1], Watson J[1], Southgate H[1], Allinson L[1], Tweddle D[1]
[1]*Wolfson Childhood Cancer Research Centre, Institute of Translational & Clinical Medicine, Newcastle University, United Kingdom, ,*

Background: Prognosis for high-risk neuroblastoma patients remains poor, thus development of novel therapeutics is important. An attractive strategy is activation of the wild-type p53 tumour-suppressor by antagonism of its inhibitor, MDM2, leading to cell cycle arrest and apoptosis. Additionally, Poly (ADP-ribose) polymerase (PARP) inhibition increases replication stress, whilst stabilising p53 expression. We hypothesise that a combination of an MDM2/p53 antagonist and a PARP inhibitor may enhance p53 pathway activation, giving synergistic therapeutic benefit.

Aims:

1. To determine single and combined agent sensitivity of idasanutlin and olaparib.

2. To test the downstream effects of p53 and mediators of its function on cell cycle arrest and apoptosis.

Methods: Five human neuroblastoma cell lines expressing wild-type p53 (SHSY5Y, NGP, SJNB1, NB-1691) or mutant p53 (SK-N-BE(2C)), were treated with idasanutlin and olaparib as single or combined agents. XTT inhibition assays were used to determine cell viability, and concentrations required to cause growth inhibition (GI50) were calculated. Combination Index (CI) values were determined to assess synergy. Western blotting was used to determine p53, p21, MDM2 and ɣH2AX protein expression. Effects on cell cycle arrest were assessed using cell cycle analysis. Immunofluorescence was used to further analyse p21 protein expression.

Results: All four wild-type p53 cell lines demonstrated concentration dependent sensitivity to idasanutlin, consistent with p53 pathway activation. Three of four demonstrated sensitivity to olaparib. However, mutant p53 cells were resistant to both single agents. In three of four wild-type p53 cell lines, idasanutlin and olaparib combinations enhanced growth inhibition compared to either agent alone; this effect was strongly synergistic (<0.25 CI). In all wild-type p53 cell lines, idasanutlin increased G1 cell cycle arrest and olaparib increased S phase content, whilst the combination gave varying cell cycle effects. In two wild-type p53 cell lines, single and combined agents induced p21 expression, although expression was not induced in mutant p53 cells.

Conclusion: Combined idasanutlin and olaparib produced synergistic growth inhibition, with dose lowering potential, in three of four wild-type p53 cell lines. Moreover, these results indicate that p21 expression may be a promising pharmacodynamic biomarker. This data supports further preclinical evaluation of this combination in neuroblastoma models.

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LAROTRECTINIB AS SOLE ALTERNATIVE TREATMENT FOR NEUROBLASTOMA PRESENTING NTRK3-SCAPER GENE FUSION

Fonseca M[2,3], Halley N[1], Prandini A[1,2], Vince C[1,2], Brumatti M[1,2], Pereira P[1,2], Milone M[3], Sanglard W[5], Cristofani L[2], Bendit I[5,6], Gimenez T[2,4], Novak E[2,4,5], Odone Filho V[1,2]

[1]Hospital Israelita Albert Einstein , São Pauo , Brazil, [2]Instituto Tratamento Cancer Infantil -ITACI/ Faculdade de Medicina da Universidade de Sao Paulo (FMUSP) , São Paulo , Brazil , [3]Centro de Tratamento Infantojuvenil Fabiana Macedo de Moraes/GACC SJC, São paulo, Brazil, [4]Laboratório de Investigação Médica (LIM36), Instituto da Criança, HC/FMUSP , São paulo, Brazil, [5]Fundação Pró-Sangue Hemocentro de São Paulo, São paulo, Brazil, [6]Laboratorio de Biologia Molecular - Universidadade de São Paulo (USP), São Paulo, Brazil, [7]ACKNOWLEDGMENTS This work was supported by grants from bayer and Associação Beneficente Stella Demarco, Fundação Criança, and SH Formas., ,

Background

A 8-month-old child presenting with cough was found to have a posterior mediastinal mass, crossing the midline, pushing the aorta away from vertebral bodies, and invading the vertebral canal (T6-T7 to T8-T9). Biopsy revealed a non-amplified NMYC and non-mutated ALK neuroblastoma (NB). Tc scintigraphy demonstrated a femoral lesion and bone marrow biopsies were negative. Tumor molecular testing identified a NTRK3-fusion product. There was no volumetric response to initial chemotherapy (topotecan, cyclophosphamide, etoposide, doxorubicin, vincristine, and carboplatin), and a new biopsy again revealed active NB. Taking advantage of the NTRK-fusion product found, the treatment was modified to Larotrectinib monotherapy. Following 4 months of therapy, although there was no volumetric change, a new biopsy showed a mature, differentiated tumor.

Aims

Report the importance of molecular testing in NB and the benefits of target therapy in refractory tumors.

Results

NTRK3 (TRK family: Neurotrophic Tyrosine Receptor Kinase) is a transmembrane receptor and a member of the neurotrophic receptor family of tyrosine kinase. Activation of NTRK3-triggering autophosphorylation results in activation and subsequent downstream of signaling pathways that control proliferation, survival, and cell differentiation. The protein encoded by the SCAPER gene (S Phase Cyclin A-Associated Protein In The Endoplasmic Reticulum) has both nucleic acid binding activity and ion binding activity zinc, being located mainly in the cytosol. The gene fusion SCAPER-NTRK3 preserves the protein tyrosine kinase domain of NTRK3, by having the same chromosomal breakpoint (chr15:88,483,984) found in fusions oncogenic strains of NTRK3, such as ETV6-NTRK3. This finding has not been described in the literature so far.

Conclusion

Understanding the context of neural crest development helps explain the heterogeneity of this complex tumor, with varied pathogenesis depending on age, location, and interactions between the patient's immune system and the tumor. This results shows the importance of early molecular testing and benefit of target therapy in some situations. This case report shows that an NTRK positive fusion NB may be treated with Larotrectinib and highlights the importance of early molecular testing.

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Utilization of Liquid Biopsy To Identify Genomic Alterations for Pediatric High-Risk Neuroblastoma

Martinez M[1], O'Halloran K[1,2], Marachelian A[1,2], Frazer B[1], Onyeama S[1], Prorok R[1,3]
[1]Children's Hospital Los Angeles, Department of Hematology and Oncology, Los Angeles, United States, [2]University of Southern California, Department of Pediatrics, Los Angeles, United States, [3]University of South Florida, Morsani College of Medicine, , United States

BACKGROUND
With the advent of molecular technology, the utility of identifying genomic alterations by ctDNA technology in patients with relapsed/refractory high-risk neuroblastoma (HRNBL) is feasible but remains underutilized in the clinical setting.

METHODS
A retrospective chart review at a single institution was performed with patients who had clinical testing with Foundation One Medicine liquid biopsy assay from 5/2019-1/2022. Aims included describing the clinical and practical implication of ctDNA results in the HRNBL population.

RESULTS
A total of 63 samples (n= 32 patients) were collected (range: 1-11 samples per patient), and 60 reports were generated. Fifty-four samples were obtained when disease was present. Aberrations were detected in 39/60 (65%) of samples (n=19/31) when clinical/radiographic disease was present. Five samples (n=5) were obtained simultaneously with tissue biopsies that underwent tumor genomic testing. Four of those ctDNA results matched with their corresponding tissue genomics, but in 3 of those cases ctDNA detected additional variants. The fifth sample under-detected aberrations compared to its corresponding tissue genomics. ALK variants were most commonly detected in 12 samples (n=9). MAP-kinase pathway variants were also implicated, including NRAS, MEK, and NF1. Additional potentially actionable variants included ATRX, TSC2, and CDK. In some cases targeted therapies were implemented with varying degrees of response. Tumor mutation burden was also utilized for therapy selection, and Tumor Fraction was used in conjunction with disease evaluations. For some cases, the ctDNA results led to consideration of tumor biopsy required for eligibility of clinical trials if sufficient tissue could be obtained. No aberrations were detected in all 6 samples (n=5) when disease was absent, and none of these patients (n=2 no prior progression, n=3 relapsed disease) have had a further recurrence (16-29 months from last follow up).

CONCLUSION
Liquid biopsy is able to detect ctDNA in patients with HRNBL. Results can be useful for clinical decision making particularly when tissue sampling is challenging. It may also detect additional mutations beyond what can be captured from a single tissue biopsy and has the advantage of serial monitoring to aid in therapy selection. Optimal use needs to be further characterized in prospective studies.

P184

In vivo identification of active drugs and drug combinations for high-risk neuroblastoma using zebrafish models

He S[1], Martel M[1], Zimmerman M[1], Look A[1]
[1]*Dana-Farber Cancer Institute, Pediatric Oncology, Boston, United States*

Background: Most children with high-risk neuroblastoma respond initially to chemotherapy, but a large proportion will experience therapy-resistant relapse. In addition, due to intensified therapies, survivors of high-risk neuroblastoma often face long-term toxicities such as skeletal dysplasia, cardiac dysfunction and premature mortality.

Aims: We aim to identify drugs and drug combinations with selectively activity against high-risk neuroblastoma cells in vivo, without undue toxicity to normal tissues.

Methods: We capitalize on the advantages of zebrafish as a model organism to address pressing questions relevant to the generation of effective and selective therapies for high-risk neuroblastoma. We have developed zebrafish models of high-risk neuroblastoma driven by overexpression of MYCN or MYC, as well as refractory neuroblastomas harboring hyperactivation of RAS-MAPK pathway or inactivation of p53. These zebrafish develop aggressive neuroblastoma with up to 80% tumor penetrance by 3 weeks of age, ideal for the rapid analysis of small-molecule drugs by adding the drugs to fish water in 12-well plates. The neuroblastoma tumor cells are visualized in living fish using fluorescent stereomicroscope. The drug effects are quantified by comparing the fluorescent tumors before and after the treatment. Post-treatment fish can be followed for tumor relapse, normal development and long-term toxicities.

Results: We screened a panel of kinase inhibitors and identified entrectinib (a RTK inhibitor that is active against NTRK1/2/3, ALK and ROS1) as the most active candidate drug. We validated its anti-neuroblastoma activity in human neuroblastoma cell lines. Our data showed that entrectinib is more active than the ALK inhibitor lorlatinib or the NTRK inhibitor larotrectinib, and less toxic than the multi-RTK inhibitor crizotinib. Importantly, we found that the activity of entrectinib is independent of MYC/MYCN, ALK, NF1, P53 mutation status, suggesting entrectinib as a potential anti-neuroblastoma drug.

Conclusions: We have established zebrafish neuroblastoma models for preclinical basic science studies, as multiple dose levels of each drug alone and in combination with other drugs can be readily tested at low cost and in high-throughput. The effective and safe drugs and drug combinations identified in our zebrafish platforms will inform follow-up testing in murine neuroblastoma PDX, before moving forward in clinical trials for neuroblastoma patients.

P185

TP53-dependent synergism of BET and IDH2 inhibitors in MYCN/cMYC-driven Neuroblastomas

Martínez Alarcón N[1], Sanmartín E[1], Cañete A[2], Font de Mora J[1]

[1]Health Research Institute Hospital La Fe, Laboratory of Cellular and Molecular Biology. Clinical and Translational Research., Valencia, Spain, [2]La Fe University Hospital, Clinical and Translational Research in Cancer. Pediatric Oncology Unit. , Valencia, Spain

Background:
MYCN amplification is the most significant genetic alteration in neuroblastoma (NB), which correlates with an aggressive phenotype and treatment failure. Despite the fact that bromodomain and extra-terminal domain protein inhibitors (BETi) have demonstrated antitumoral activity in MYC-overexpressing cancers, including MYCN-driven NB, the monotherapy efficiency of these inhibitors studied in multiple clinical trials remains limited by their severe adverse events, including thrombocytopenia. Therefore, finding MYCN-alternative effective targets for optimal therapy combinations is urgently needed to fight against this disease.

Aims:
Identify actionable genes subrogated to MYCN amplification whose targeted treatment may improve efficacy of BETi (JQ1) in NB.

Methods:
Data for in silico correlation of expressed genes with MYCN were retrieved from R2 Genomics platform. Luciferase assays were performed with IDH2 promoter and Ebox mutants. SK-N-BE(2), IMR-5, SH-SY5Y and SK-N-AS were treated with different concentrations of JQ1 and IDH2 inhibitor AG-221. IC50 and coefficient of drug interaction was determined for each cell line. Proliferation/survival was determined by XTT colorimetric assay. Apoptosis induction and cell cycle phase alterations were determined by flow cytometry and western blotting after 48h of treatment. Autophagy induction was analyzed by western blotting and immunofluorescence assays.

Results:
MYCN expression strongly correlated with IDH2 expression in NB cohorts. MYCN is an IDH2 transcriptional activator through three Eboxes located in IDH2 promoter. Highly synergistic effect was observed between BET and IDH2 inhibitors in TP53 wild-type cells, but limited or absent synergism in p53-mutated cells. Combined treatment also increased the apoptosis ratio, sub-G0 population, G0/G1 arrest and caspase 3 and PARP cleavage in a TP53-WT background. Notably, combined treatment also increased LC3βII expression and autophagy flux in a p53-dependent manner.

Conclusion:
IDH2 links AG-221 with JQ1 synergism through TP53-mediated autophagy in MYCN/cMYC expressing neuroblastoma. Our results reveal that this combination treatment is more effective than monotherapy alone and supports its further investigation in preclinical studies.

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Molecular-Guided Therapy for the Treatment of Relapsed/Refractory Neuroblastoma: Results from the Beat Childhood Cancer Research Consortium Trial MGT009

Kraveka J[1], Ferguson W[2], Bergendahl G[3], Lewis E[3], Nagulapally A[3], Dykema K[3], Brown V[4], Isakoff M[5], Junewick J[6], Mitchell D[6], Rawwas J[7], Roberts W[8], Eslin D[9], Oesterheld J[3], Wada R[10], Pastakia D[11], Harrod V[12], Ginn K[13], Saab R[14], Bielamowicz K[15], Glover J[16], Chang E[17], Hanna G[18], Enriquez D[19], Izatt T[19], Halperin R[19], Byron S[19], Hendricks W[19], Trent J[19], Saulnier Sholler G[3]

[1]Medical University of South Carolina, MUSC Shawn Jenkins Children's Hospital, Hollings Cancer Center, Charleston, United States, [2]Cardinal Glennon Children's Medical Center, St. Louis University School of Medicine, St Louis, United States, [3]Levine Children's Hospital, Atrium Health, Charlotte, United States, [4]Penn State Milton S. Hershey Medical Center and Children's Hospital, Hershey, United States, [5]University of Connecticut School of Medicine, Connecticut Children's Medical Center, Hartford, United States, [6]Helen DeVos Children's Hospital, Grand Rapids, United States, [7]Children's Hospitals and Clinics of Minnesota, Minneapolis, United States, [8]University of California San Diego, Peckham Center for Cancer and Blood Disorders, Rady Children's Hospital, San Diego, United States, [9]Bayless Children's Cancer Institute, St. Joseph's Children's Hospital, Tampa, United States, [10]University of Hawaii, Honolulu, United States, [11]Vanderbilt-Ingram Cancer Center, Nashville, United States, [12]Dell Children's Medical Center, University of Texas Dell Medical School, Austin, United States, [13]Children's Mercy Hospital, Kansas City, United States, [14]Stanford Medicine Children's Health, Palo Alto, United States, [15]Arkansas Children's Hospital, Little Rock, United States, [16]Randall Children's Hospital, Portland, United States, [17]St Luke's Cancer Institute, Boise, United States, [18]Orlando Health Cancer Institute, Orlando, United States, [19]Translational Genomics Research Institute, Phoenix, United States

Background

Children with relapsed/refractory neuroblastoma (NB) face poor outcomes. The hypothesis that genomic alterations and transcriptomic perturbations can be identified via genomic sequencing and can be matched with specific inhibitors that target pathways resulting in effective targeted therapy and reduced toxicities is the basis of precision medicine.

Aims

To determine the feasibility, efficacy, and safety of evaluating genomic profiling of tumor samples within a molecular tumor board (MTB) to make real‑time treatment decisions for children with relapsed/refractory neuroblastoma.

Methods

Tumor samples were sent for whole exome (WES) and whole transcriptome sequencing and the genomic data were used in a MTB to make real‑time treatment decisions. The MTB recommended plan allowed for a combination of up to 4 agents. Feasibility was measured by the time to completion of genomic sequencing and analysis, MTB and initiation of treatment. Response was assessed after every two cycles. Grade 3 and higher related and unexpected adverse events (AEs) were tabulated for safety evaluation.

Results

Neuroblastomas (n=31) were enrolled as a stratum on MGT009. The average time from biopsy to completion of DNA/RNA sequencing was 10 days (range 4-19 days); to completed analysis and drug prediction report, 17 days (8-26); 22 days (13-42) to MTB decisions; and 31 days (18-75) to initiation of the 4-drug combination agreed upon by the MTB. Treatments were selected on DNA and RNA findings in 26% of cases and in 74% on RNA alone. WES of 37 NB tumors revealed MYCN amplification in 30%, ATRX deletion in 38%, TP53 alterations in 5%, and ALK mutations in 11%. The response rates for the 27 evaluable NB subjects

were as follows: 14.8% (4/27) CR, 66.7% (18/27) SD, and 14.8% (4/27) PD; one patient (3.7%) was NED after biopsy and remained so. Two Grade 3 SAEs were reported.

Conclusions
Here, we have demonstrated the feasibility, efficacy, and safety of a comprehensive sequencing model to guide targeted therapy for patients with relapsed/refractory neuroblastoma. Molecular tumor board guided targeted therapy was well tolerated, and the response benefit rate in this heavily pretreated population suggests that this method of treatment paradigm selection may be an effective option.

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Identification of drug resistance mechanisms by single cell imaging of neuroblastoma reporter cells.

Lebedev T[1], Bogomolov M[1], Tabiev R[1], Sementsov M[1], Mikheeva A[1], Prassolov V[1]
[1]*Engelhardt Institute of Molecular Biology, Moscow, Russia*

Background: High-throughput single cell imaging and analysis of morphology features are becoming powerful tools for understanding and discovering drug mechanisms of action. Here we expanded this approach by using genetically encoded fluorescent reporters of ERK1/2 kinase activity and cell cycle in neuroblastoma cells and focused on studying resistance mechanisms.
Methods: To generate a reporter cell line panel we used five neuroblastoma cell lines: SH-SY5Y, SK-N-AS, SK-N-SH, SK-N-BE and Kelly. We used lentiviral vectors to stably express ERK reporter (ERK-KTR-mClover) along with H2B-mRuby to measure ERK activity, and Fucci-PiP reporter for the cell cycle. We trained image recognition models using Cellpose to segment single cells in bright and fluorescence images and used CellProfiler to extract morphological features and reporter signal for single cells.
Results: To selected most relevant drugs to test, we developed an algorithm based on gene fitness data from DepMap which selects genes on those expression neuroblastoma cells are most dependent. Then we used ChEMBL and DSigDB databases to select targetable genes and respective drugs. We tested inhibitors of different mechanisms of action, including drugs used for neuroblastoma treatment, and drugs identified by our algorithm, such as HDAC2, PIM1/3, ALK, KIT, MYT1 and PIK3 inhibitors, measuring cell response at 2, 24, 72 and 144h after treatment. Overall we collected data for more than 180 unique conditions (drugs x concentrations) and 100000 individual cells per cell line. For each cell we measured 200 morphological, ERK activity and cell cycle. Correlation of cell cycle and ERK activity changes with drug-induced toxicity allows us to identify potential mechanisms for drug action and resistance, and time-dependent changes can tell us which changes preceded cell death or formation of drug resistance. We show that measuring dose-dependent ERK response accurately predicts synergy for drugs with ERK inhibitors. Using clustering of single cell morphology data we identified distinct cell phenotypes during in cell populations that survived drug treatments.
Conclusion: We showed that cell morphology analysis coupled with measuring ERK activity and cell cycle changes can be a powerful tool for studying drug mechanisms of action and drug resistance and exploring combinational therapies.

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Usefulness of WB MRI with diffusion-weighted sequences for neuroblastoma characterization, monitoring in combination with precision medicine programs: a case report.

Tondo A[1], Carra F[1], Peri M[2], Trevisan F[1], Bongini U[3], Ponzoni M[4], Pastorino F[4], Garaventa A[5], Amoroso L[5], Aveic S[6], Grimaldi C[7], Olianti C[8], Perrone A[3]

[1]Department of Pediatric Oncology and Hematology, Meyer Children's Hospital IRCCS, Florence, Italy, [2]Meyer Children's Hospital IRCCS, University of Florence, Florence, Italy, [3]Department of Radiology, Meyer Children's Hospital IRCCS, Florence, Italy, [4]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [5]IRCCS Istituto Giannina Gaslini, Genova, Italy, [6]Laboratory of Target Discovery and Biology of Neuroblastoma, IRP, Padua, Italy, [7]Department of Surgery, Meyer Children's Hospital IRCCS, Florence, Italy, Florence, Italy, [8]Nuclear Medicine Unit, Careggi University Hospital, Florence, Italy

Background: 123I-mIBG scintigraphy is widely used in the assessment of primary and secondary neuroblastoma with high sensitivity (83-92%) and specificity (88-92%). WB MRI may represent a radiation-free technique in the evaluation of the biological features of the tumor. In particular, DWI sequences, based on ADC maps, could be useful to distinguish between different patterns of neuroblastic tumors.
Case description: female, 15 months old, with abdominal unresectable non-metastatic mass. CT-guided tumor biopsy was performed: poorly differentiated neuroblastoma, MYCN non-amplified; no genomic profile result. She had significant life-threatening symptoms (LTS). The patient was classified as low-risk group, according to the LINES guidelines. LTS disappeared after 2 courses of Carboplatin/Etoposide. WB MRI and MIBG scans showed good response with dimensional and uptake reduction of the tumor.
The Italian Advisory Panel for Neuroblastoma recommended to perform a second tumor biopsy with the aim of identifying the presence or absence of segmental chromosomal aberrations, but it failed. Therefore, she underwent a close follow-up. After 2 years WB MRI showed increasing size of the tumor but high ADC values and reduced levels of shrinkage, as in areas of differentiating cells.
123I-mIBG avidity was absent and 68Ga PET/CT scan excluded the receptor switch of the tumor; hence a watchful waiting was pursued. The dimensional increase of the tumor led to a further biopsy. The pathological assessment was peripheral neuroblastic tumor schwannian stroma-rich, mature ganglion cells. Genomic profile of the tumor sample showed no evidence of pathological aberrations. Residing tumor material has been as well challenged in the 3D in vitro spheroid growth. A long-term cultivation confirmed a low proliferative capacity of the residual mass and lack of cancer stem cells with renewal potential.

Conclusion: Neuroblastoma has heterogeneous clinical behavior, from spontaneous regression or differentiation into a benign neuroblastic tumor, to malignancy aggressiveness despite multimodality therapy. ADC maps are useful as imaging markers: a lower ADC value with the increased levels of shrinkage may suggest an undifferentiated tumor.
In this case we noted a high concordance between the radiological and molecular biology methods.

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The splicing inhibitor isoginkgetin sensitizes MYCN-amplified neuroblastoma cells to vincristine

Maugeri L[1], Egan D[1], Wynne K[1], Zhernovkov V[1], Blanco A[2], **Halasz M[1,2]**
[1]Systems Biology Ireland, School of Medicine, University College Dublin, Dublin, Ireland, [2]Conway Institute, University College Dublin, ,

Background: Neuroblastoma (NB) is one of the deadliest childhood cancers. High-risk cases are associated with poor survival despite aggressive and highly toxic multimodal therapy. Therefore, more effective, and less toxic treatments are desperately needed. Half of the high-risk cases are driven by amplification of MYCN. Previously, we reported a link between MYCN and splicing, and showed the anti-tumour effects of the splicing inhibitor isoginkgetin in MYCN-amplified neuroblastoma.

Aims: Here, we aim to evaluate if isoginkgetin synergizes with vincristine, a currently used anti-neuroblastoma chemotherapy drug. This would allow us to decrease the doses of toxic chemotherapy and therefore this would limit the side effects.
Methods: MYCN-amplified neuroblastoma cells were treated with various concentrations of vincristine and isoginkgetin alone or in combination and we measured cell viability. Then we used SynergyFinder to calculate synergy scores. We also performed mass-spectrometry based proteomics with Be(2)-C cells treated with vincristine and isoginkgetin alone or in combination to explain the mechanism of synergy. We used gene set enrichment analysis with the top regulated genes in the combined vs single treated NB cells. After the single or combination drug treatments, we measured the expression of cell cycle phase specific proteins by Western blotting and analysed the cell cycle distribution by flow cytometry.

Results: Isoginkgetin synergizes with vincristine in MYCN-amplified NB. We identified the differentially expressed proteins in NB cells treated with the drug combination. Gene enrichment analysis revealed that the top regulated genes are participating in the cell cycle. Based on single-cell RNA-Seq data of Be(2)-C cells, these genes are mainly expressed in cells in the G2 phase of the cell cycle. In addition, we analysed pharmacogenomics data of vincristine treated NB cells, and we found a correlation between high expression of the top regulated genes (from combined treatment) and increased sensitivity to vincristine. We then experimentally confirmed that the combined treatment increases G2/M arrest (Western blotting and flow cytometry).

Conclusion: We observed synergy between isoginkgetin and vincristine in MYCN-amplified NB. Mechanistically, isoginkgetin sensitises the NB cells to vincristine. This synergistic drug combination could reduce the toxic side effects of vincristine.

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Targeting TERT activation in high risk neuroblastoma

Behura C[1], Dovat S[1], Uzun Y[1], Duke K[1], Mohammad D[1]
[1]*Pennsylvania state University College of medicine , Hershey, United States*

Background: NBL biological subgroups with robustly active Telomerase Maintenance Mechanisms (TMM) show poor response to therapy, high relapse rate, and poor survival. Increased Telomerase reverse transcriptase (TERT) expression due to TERT gene promoter rearrangements or MYCN amplification confers aggressive phenotype and worst overall survival among all high-risk (HR) NBL patients. In HR-NBL, high expression of Protein Kinase CK2 (CK2) is associated with significantly worse overall survival, as noted in two different data sources (R2 and TARGET database). Although CK2 is being pursued as a therapeutic target in other cancers, the robust association of CK2 overexpression with poor outcomes in HR-NBL has never been reported. Several CK2 inhibitors are currently in preclinical and clinical development for solid tumors.

Aims: 1. to establish the therapeutic efficacy of the combination of CK2 inhibitor with temozolomide, and 2. to analyze the functional role of CK2 in NBL cell survival and proliferation in the context of MYCN, and TERT. Methods and results: We analyzed eight different datasets with primary NBL samples associated with survival outcomes. We analyzed the HR-NBL dataset (Fischer 394,) where tumors were categorized into four groups according to evidence of an activated Telomere Maintenance Mechanism (TMM pos), which includes alternative lengthening of telomeres (ALT+), MYCN amplification, and TERT gene rearrangements or the absence of such a mechanism (TMM neg) (7). TMM positive group had significantly high expression of CSNK2A1 (Figure 3B). More importantly, TERT high NBL patients that are CK2α high expressers had significantly lower overall survival than CK2α low expressers. These findings suggest that CK2α is overexpressed in TMM-positive NBL patients and correlates with worse overall survival. CK2 inhibitor showed modest cytotoxic activity in TERT+ HR-NBL PDX and showed synergistic cytotoxic activity when combined with temozolomide. Genetic depletion of CK2 using ShRNA, sensitized NBL cells to temozolomide therapy.

Conclusion: These preliminary findings suggest that inhibition of CK2 in neuroblastoma, especially HR NBL with TERT activation, can be beneficial in combination with currently used cytotoxic therapy. These Further validation of Ck2 anti-tumor activity using PDX models and dissection of the mechanism of TERT suppression by CK2 inhibitors is needed.

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Mutational topography reflects clinical neuroblastoma heterogeneity

Rodriguez-fos E[1,2], Planas-Fèlix M[1,10], Burkert M[3], Puiggròs M[4], Toedling J[2], Thiessen N[5], Blanc E[5], Szymansky A[2], Hertwig F[2], Beule D[5], Torrents D[4], Eggert A[1,2], Koche R[7], Schwarz R[3,8,9], Haase K[1,10], Schulte J[2], Henssen A[1,2,3,5,10]

[1]Experimental and Clinical Research Center (ECRC) of the MDC and Charité Berlin, Berlin, Germany, [2]Department of Pediatric Oncology and Hematology, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin, Germany, [3]Berlin Institute for Medical Systems Biology (BIMSB), Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin, Germany, [4]Barcelona Supercomputing Center, Joint Barcelona Supercomputing Center-Centre for Genomic Regulation-Institute for Research in Biomedicine Research Program in Computational Biology, Barcelona, Spain, [5]Berlin Institute of Health, Berlin, Germany, [6]Institució Catalana de Recerca i Estudis Avançats (ICREA), , Barcelona, Spain, [7]Center for Epigenetics Research, Memorial Sloan Kettering Cancer Center, New York, NY, USA, [8]Center for Integrated Oncology (CIO), Cancer Research Center Cologne Essen (CCCE), Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany, [9]BIFOLD - Berlin Institute for the Foundations of Learning and Data, Berlin, Germany, [10]German Cancer Consortium (DKTK), partner site Berlin, and German Cancer Research Center (DKFZ), Heidelberg, Germany

Background and aims

The activity of different endogenous and/or exogenous mutational processes, including replication errors, exposure to DNA damaging agents, and errors in DNA repair mechanisms, imprint characteristic patterns of mutations in the genome defined as mutational signatures. Recent analyses in neuroblastoma have extracted different mutational signatures associated with single-nucleotide variants, small insertions and deletions, and specific patterns of structural variants such as extrachromosomal circular DNA (ecDNA), chromothripsis, and breakage-fusion-bridge cycles. However, our current understanding of the whole catalogue of complex rearrangement patterns and mutational processes, their co-occurrence, as well as their impact on clinical outcomes in neuroblastoma remains limited.

Methods and results

Applying variant detection algorithms and signature analyses to Illumina short-read whole-genome sequencing from a cohort of 114 neuroblastoma tumor-normal pairs, we extracted 10 catalogued mutational signatures associated with single-nucleotide variants and small insertions and deletions as well as 13 recently defined copy number alterations and structural variant signatures. Interestingly, we observed that specific mutational processes were differentially active across neuroblastoma's risk groups. Results were validated in a cohort of 36 ultra-deeply sequenced whole neuroblastoma genomes. The study of this novel cohort allowed us to describe the subclonal distribution of mutational processes and infer the mutational history in these tumors. Using statistical and graph-based methods, we integrated all the different mutational signatures with 9 complex structural variant pattern classes as well as clinical annotations, identifying previously unrecognized co-occurring mutational footprints, which we termed mutational scenarios.

Conclusion

We demonstrate that clinical neuroblastoma heterogeneity is linked to differences in the processes driving these mutational scenarios. Whereas high-risk MYCN-amplified neuroblastoma genomes were characterized by signs of damage caused by ROS, replication slippage and stress, and ecDNA presence, homologous recombination-associated signatures along with non-circular complex rearrangements defined high-risk non-MYCN-amplified patients. Non-high-risk neuroblastomas, on the other hand, were marked by footprints of chromosome missegregation and TOP1 mutational activity. This analysis provides a systematic

perspective on the repertoire of mutational patterns that contribute to clinical neuroblastoma heterogeneity.

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Comprehensive allelic series of ATRX In-Frame-Fusions in neuroblastoma reveals neomorphic protein functions

Jostes S[1,2,3], Cooke A[1,2,3,5], Rosemann F[1,2,3,5], Cook A[1,2,3], Hasson D[1,2,3,4,5], Bernstein E[1,2,3,5]
[1]Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, New York, United States, [2]Department of Dermatology, Icahn School of Medicine at Mount Sinai, New York, United States, [3]Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, United States, [4]Bioinformatics for Next Generation Sequencing (BiNGS) Shared Resource Facility, Icahn School of Medicine at Mount Sinai, New York, United States, [5]Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, United States

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BACKGROUND: The chromatin remodeler ATRX is frequently mutated in high-risk neuroblastoma, particularly in older children. These often present as large genomic deletions that produce ATRX in-frame fusion (IFF) proteins primarily affecting the N-terminal chromatin binding domains, but can also include the DAXX interaction domain. ATRX regulates heterochromatin formation, DNA repair, and transcription. When in complex with DAXX, ATRX serves to deposit the histone variant H3.3 into chromatin at telomeres, pericentric chromatin, and retrotransposons for silencing.
AIMS: Previous work on ATRX IFF proteins in neuroblastoma is scarce due to limited availability of ATRX IFF neuroblastoma cell lines. While we identified a vulnerability of ATRX IFF cells to EZH2i (Qadeer et al, 2019), the generation of an isogenic ATRX IFF model system allows us to study the direct molecular and functional effects of the IFFs, and aids in uncovering new vulnerabilities for therapeutic intervention.
METHODS: Using CRISPR/Cas9 genome editing in ATRX wildtype neuroblastoma cells, we generated an isogenic panel of representative ATRX IFFs (fusions of exons 1-11, 2-10 and 9-13) found in patients. Using this allelic series with distinct domain deletions, we performed genomic, epigenomic, biochemical and functional assays.
RESULTS: The isogenic ATRX IFF proteins recapitulate molecular and biochemical properties of IFFs found in cell lines, such as EZH2i sensitivity. The 1-11 and 2-10 IFFs localize to active chromatin and the cytoplasm compared to IFF9-13 and wildtype ATRX which are found in heterochromatin. Interestingly, the retention of the ATRX IFFs in the cytoplasm is accompanied by a dramatic accumulation of cytoplasmic DAXX. RNA-seq analysis revealed a significant upregulation of inflammatory pathways and epithelial-to-mesenchymal signatures in ATRX IFF1-11 cells. This is in line with our corresponding ATAC-seq data, demonstrating that open chromatin regions in IFFs (vs. WT) are enriched for mesenchymal transcription factor motifs, whereas open chromatin regions in WT (vs. IFF) are enriched for adrenergic transcription factor motifs. Thus, IFF proteins are not loss-of-function, but rather neomorphic, and their function depends on the specific deletion.
CONCLUSION: Our studies will reveal how distinct ATRX IFF mutations alter the epigenome and cellular states of neuroblastoma, and how these may contribute to therapy resistance.

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The C57 BL/6 Th-ALKF1174L/MYCN genetically-engineered murine model of high-risk neuroblastoma enables the preclinical study of metastatic drug resistant relapse.

Tucker L[1], Piapi A[2], Martins da Costa B[1], Barker K[1], Straathof K[2], Chesler L[1]
[1]The Centre for Paediatric Oncology Experimental Medicine, The Institute of Cancer Research, London, United Kingdom, [2]UCL Great Ormond Street Institute of Child Health, London, United Kingdom

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Background
Translational studies of Anaplastic Lymphoma Kinase (ALK) inhibitors for neuroblastoma have been strengthened by the Th-ALKF1174L/MYCN genetically engineered murine model (GEMM). The Th-ALKF1174L/MYCN predicted the inherent resistance of ALKF1174L-driven clinical neuroblastoma to the first generation ALK inhibitor, crizotinib. Subsequently, this model also demonstrated survival benefit to the late generation ALK inhibitor lorlatinib, which could be enhanced by the addition of combination chemotherapy. However, limitations of this model are short tumour latency and rapid tumour progression, making preclinical studies of drug resistance more challenging. We therefore backcrossed the Th-ALKF1174L/MYCN model from the existing 129svj strain into the C57 BL/6 strain, in an effort to discover if the relatively enhanced cell-mediated immunity of the C57 BL/6 would enable optimized study of drug response and resistance.

Aims
To characterize neuroblastoma GEMM tumour development and response to therapeutics in the C57 BL/6 Th-ALKF1174L/MYCN

Methods
Th-ALKF1174L and Th-MYCN 129svj were back-crossed into C57 BL/6 over 10 generations, and bred to generate double heterozygote Th-ALKF1174L/MYCN animals. Tumour penetrance and latency were measured, in addition to evidence of metastatic spread and circulating tumour DNA (ctDNA) in the plasma. The immune microenvironment of abdominal neuroblastomas was interrogated by multi-channel FACS and the response to lorlatinib treatment was monitored by non-invasive magnetic resonance imaging.

Results
The C57 BL/6 ThALKF1174L/MYCN double heterozygous model develops abdominal neuroblastoma tumours with 100% penetrance, with a lengthened period of tumour progression, compared to 129svj Th-ALKF1174L/MYCN abdominal tumours. No neuroblastoma tumours were found in C57 BL/6 Th-ALKF1174L or Th-MYCN animals. C57 BL6 Th-ALKF1174L/MYCN mice frequently develop second primary tumours in the thorax, and metastatic neuroblastoma cells can be identified in the bone marrow. ctDNA can be isolated in the plasma from tumour-bearing animals. C57 BL/6 Th-ALKF1174L/MYCN tumours are exquisitely sensitive to lorlatinib treatment, with tumour regrowth occurring after treatment ends, in contrast to studies in the original 129svj GEMM, in which tumour re-growth occurred during continuous lorlatinib treatment.

Conclusion

The C57 BL/6 ALK mutant, MYCN amplified neuroblastoma GEMM, with a lengthened period of indolent tumour progression, provides an optimized preclinical tool to study metastatic drug resistant relapse in an immunocompetent model.

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Nucleosome footprinting enables inference of expression profiles in circulating tumor DNA from neuroblastoma patients

Chicard M[1,2], **Iddir Y[1,2]**, Bhalshankar J[1,2], Saberi-Ansari E[1,2], Saint-Charles A[1,2], Bellini A[1,2], Butterworth C[1,2], Baulande S[3], Bohec M[3], Pierron G[4], Geoerger B[5], Combaret V[6,7], Delattre O[1,3,4], Waterfall J[1,2], Schleiermacher G[1,2,8]

[1]INSERM U830, CANCER, HÉTÉROGÉNÉITÉ, INSTABILITÉ ET PLASTICITÉ - CHIP, PSL Research University, Institut Curie, Paris, France, Paris, France, [2]SiRIC RTOP « Recherche Translationelle en Oncologie Pédiatrique », Translational Research Department, Research Center, PSL Research University, Institut Curie, Paris, France, Paris, France, [3]Institut Curie Genomics of Excellence (ICGex) Platform, Research Center, Institut Curie, Paris, France, Paris, France, [4]Unité de Génétique Somatique, Service de Génétique, Hospital Group, Institut Curie, Paris, France, Paris, France, [5]Department of Pediatric Oncology, Institut Gustave Roussy, Villejuif, France, Paris, France, [6]Plateforme de Génomique des Cancers, Centre Léon Bérard, Lyon, France, Paris, France, [7]Laboratoire de Recherche Translationnelle, Centre Léon-Bérard, Lyon, France, Paris, France, [8]Department of Pediatric Oncology, Hospital Group, Institut Curie, Paris, France, Paris, France

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Background: In neuroblastoma (NB), cell-free DNA (cfDNA) and the circulating tumor DNA (ctDNA) component, can be used to detect tumor cell-specific genetic alterations. Expression profiles might also constitute important prognostic/predictive biomarkers.

Aims: In cfDNA extracted from plasma of NB patients, we sought to infer expression profiles based on nucleosome footprints. Nucleosome-free transcription start sites (TSS) of expressed genes are cut more readily than nucleosome-occupied TSS of unexpressed genes, with distinct nucleosome footprints and differential coverage upon sequencing of cfDNA.

Methods: In 51 NB patients, paired germline/tumor WES, WGS and/or lcWGS and/or cfDNA WES/lcWGS analysis were performed for calling of SNVs and copy number alterations. From these patients, in 53 cfDNA samples (including 3 matched diagnosis-relapse pairs, and 3 healthy controls) TSS nucleosome footprinting was analysed. A target capture panel to encompass 21140 TSS was applied to sequence cfDNA (expected coverage: 1000x). Normalized coverage scores were calculated around the TSS using deeptools to generate footprint profiles and perform kmeans clustering (two groups) to classify genes as expressed or silent.

Results: Comparison of tumor/cfDNA WES, filtered on paired germline, resulted in a mean of 27 SNVs common to the tumor and cfDNA (range 3-107), with 20 (range 1-80) specific to tumor and 15 (range 1 – 62) specific to cfDNA, respectively, indicating genetic heterogeneity.
In each cfDNA, based on the coverage across the TSS and attribution to a kmeans cluster, genes could be classified as expressed or silent. Prediction of the MYCN status was based on the comparison of the nucleosome footprints of MYCN TSS for patients with MYCN amplified (N=22) versus MYCN non-amplified (N=24) tumors. Nucleosome footprinting enabled to establish MYCN amplification status, and differential expression of genes according to the MYCN amplification status, and modifications of gene expression between diagnosis and relapse could be established.

Conclusion: We were able to distinguish two distinct nucleosome footprint patterns for genes corresponding to expressed versus silent genes, and to discriminate the MYCN status of patients according to the MYCN nucleosome footprint. Inferring expression profiles based on nucleosome TSS occupancy constitutes a compelling and novel tool for sequential expression analysis in NB.

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Detection of epigenetic cell states in cell-free DNA of high-risk neuroblastoma patients

Cornelli L[1,2], Santos M[1,2], Roelandt S[1,2], Westerhout E[3], Van Nes J[3], Speleman F[2,4], De Wilde B[5], Van Roy N[2,4], De Preter K[1,2]

[1]Center for Medical Biotechnology & Department of Biomolecular Medicine, VIB-Ghent University, Ghent, Belgium, [2]Cancer Research Institute Ghent, Ghent, Belgium, [3]Department of Oncogenomics, Cancer Center Amsterdam, Amsterdam UMC, Amsterdam, The Netherlands, [4]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium, [5]Department of Internal Medicine and Pediatrics, Ghent University, Ghent, Belgium

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Background
In high-risk neuroblastoma, treatment resistance and refractory disease are responsible for the death of half of all patients. Mutational profiling through targeted resequencing of circulating cell-free DNA (cfDNA) is an important approach for genomic monitoring of treatment response and has convincingly been shown to allow in time detection of tumor recurrence. However, genomic mutations in neuroblastoma tumors are rare. Moreover, epigenetic plasticity has been shown to play an important role in acquiring treatment resistance, including adrenergic-mesenchymal switches. At present, minimally invasive methods assays lack sensitivity and the possibility to detect epigenetic plasticity of the tumor during therapy.

Aims
Our aim is to establish a DNA-methylation based assay that allows detection of epigenetic cell state switches in the blood plasma of high-risk neuroblastoma patients.

Methods
To analyze DNA methylation, we used cell-free reduced representation bisulfite sequencing (cfRRBS), a method optimized to profile DNA methylation patterns of fragmented cell-free DNA (cfDNA) molecules. Computational deconvolution of the methylation patterns (using MethAtlas or MethylCIBERSORT) is used to estimate the fractions of the different cell states that contribute to the complex cfDNA mixtures in blood plasma.

Results
In a first step, we determined the lower limit of tumoral fraction that can be detected using cfRRBS in conjunction with computational deconvolution. To this end, we mixed healthy cfDNA with MNase treated neuroblastoma cell line DNA (that resembles cfDNA fragments) and observed a good performance of tumor fraction estimation for fractions around 1.5%. In the next step, we compared the DNA methylation patterns in several adrenergic versus mesenchymal neuroblastoma cell lines and identified cell state-specific methylation patterns. Currently, we are applying these cell state-specific patterns to estimate the different cell state fractions in methylation data of cfDNA of high-risk neuroblastoma patients.

Conclusion
cfRRBS is a promising technology that allows accurate and sensitive detection of neuroblastoma tumoral fractions in blood plasma. The cfRRBS-computational deconvolution pipeline will allow minimal invasive monitoring of cell state switches through longitudinal blood sampling and link these switches with tumor recurrence and therapy resistance.

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Adrenergic-lineage genes are epigenetically repressed in immature MES-type neuroblastoma cells

Jridi I[1], Koster J[2], A. Zwijnenburg D[2], Hasselt N[1], Haneveld F[1], M.F. Wolthuis R[1], Versteeg R[3], Valentijn L[1]
[1]Amsterdam UMC, University of Amsterdam, Department of Human Genetics, Amsterdam, Netherlands, [2]Amsterdam UMC, University of Amsterdam, Center for Exp. Molecular Medicine, Amsterdam, Netherlands, [3]Charité University Medicine Berlin, Department of Oncology and Hematology, Berlin, Germany

Background/introduction:
Neuroblastoma tumors include at least two types of tumor cells with diverging characteristics. Typically, the majority of cells are adrenergic (ADRN) cells, which are chemosensitive and characterized by lineage markers like PHOX2A, PHOX2B and DBH. A more minor proportion of the cells are mesenchymal (MES) and have immature features and lack neuroblast-like lineage genes. Interestingly, the 2 different types of cells can spontaneously interconvert into one another, which may have far reaching consequences towards clinical intervention of neuroblastoma tumors. During reprogramming, MES and ADRN cells undergo a major shift in epigenetic modification status.

Aims
An important mechanism for large scale inhibition of gene expression is Polycomb repression, whose activity is characterized by accumulation of H3K27me3 histone modification at target sites in the genome. In the current study, we investigated differential chromatin repression in MES- vs ADRN-type neuroblastoma cells, using H3K27me3 as a proxy.

Methods/Materials:
Four isogenic pairs of MES and ADRN cell lines were analyzed by ChIP-sequencing with anti-H3K27me3 and anti-H3K4me3. The HiSeq sequencing results were analyzed and visualized on the R2 genomics analysis and visualization platform (http://r2.amc.nl/).

Results:
ADRN-specific lineage commitment genes like ASCL1, DBH and neuritogenesis genes are silent in MES-type cells. We observed that the transcription start site (TSS) of these genes have a H3K27me3 silencing mark in MES cells. The reverse pattern was not apparent: MES-specific genes that are silenced in ADRN-type cells do not have H3K27me3 marks at their TSS site in ADRN cells.
In addition to H3K27me3, the activating histone mark H3K4me3 is present at the same TSS sites. This bivalent activating (H3K4me3) and inactivating (H3K27me3) signal was seen in MES-type cells (where the expression of those ADRN genes is inhibited). Bivalent histone modifications are considered to prepare cells for lineage development. The bivalent pattern in MES cells and absence in ADRN cells suggest that also at the histone modification level neuroblastoma follows part of the normal neuroblasts development.

Summary/conclusion:
We conclude that the neuroblastic lineage commitment program is actively repressed via H3K27me3 at TSS sites in MES-type neuroblastoma cells, resembling normal neuronal development.

P198

Sequential cfDNA studies by ddPCR and WES enable early detection of relapse and clonal evolution during ALK-inhibitor treatment in neuroblastoma

Bobin C[1], Iddir Y[2], Butterworth C[3], Saint-Charles A[4], Bellini A[5], Bhalshankar J[6], Masliah Planchon J[7], Pierron G[8], Combaret V[9], Attignon V[10], Shufang R[11], Baulande S[12], Raynal V[13], Bozec L[14], Bieche I[15], Delattre O[16], Schleiermacher G[17]

[1]SiRIC RTOP (Recherche Translationelle en Oncologie Pédiatrique); Translational Research Department, Institut Curie Research Center, PSL Research University, Institut Curie, Paris, France, [2]SiRIC RTOP (Recherche Translationelle en Oncologie Pédiatrique); Translational Research Department, Institut Curie Research Center, PSL Research University, Institut Curie, Paris, France, [3]SiRIC RTOP (Recherche Translationelle en Oncologie Pédiatrique); Translational Research Department, Institut Curie Research Center, PSL Research University, Institut Curie, Paris, France, [4]SiRIC RTOP (Recherche Translationelle en Oncologie Pédiatrique); Translational Research Department, Institut Curie Research Center, PSL Research University, Institut Curie, Paris, France, [5]SiRIC RTOP (Recherche Translationelle en Oncologie Pédiatrique); Translational Research Department, Institut Curie Research Center, PSL Research University, Institut Curie, Paris, France, [6]SiRIC RTOP (Recherche Translationelle en Oncologie Pédiatrique); Translational Research Department, Institut Curie Research Center, PSL Research University, Institut Curie, Paris, France, [7]Somatic Genetics Unit, Institut Curie, Paris, France, [8]Somatic Genetics Unit, Institut Curie, Paris, France, [9]Laboratoire de Recherche Translationnelle, Centre Léon-Bérard, Lyon, France, [10]Laboratoire de Recherche Translationnelle, Centre Léon-Bérard, Lyon, France, [11]Circulating Biomarkers Unit, Institut Curie, Paris, France, [12]Institut Curie Genomics of Excellence (ICGex) Platform, Research Center, Institut Curie, Paris, France, [13]Institut Curie Genomics of Excellence (ICGex) Platform, Research Center, Institut Curie, Paris, France, [14]Department of Medical oncology, Institut Curie, Paris, France, [15]Department of Medical oncology, Institut Curie, Paris, France, [16]INSERM U830, Equipe Labellisée Ligue contre le Cancer, PSL Research University, Institut Curie Research Center, Paris, France, [17]INSERM U830, Equipe Labellisée Ligue contre le Cancer, PSL Research University, Institut Curie Research Center, Paris, France

Background

The study of cell free DNA (cfDNA), and its circulating tumor DNA (ctDNA) fraction provides an important surrogate for sequential analysis of tumor cell-specific genetic alterations in neuroblastoma (NB). We applied a combination of molecular techniques to sequential liquid biopsies in patients with ALK-altered NB receiving ALK-targeted therapy.

Methods

Genomic fragmented DNA was serially diluted, and used for ALK-mutation specific ddPCR directly, or for whole genome sequencing (WGS) library construction followed by ddPCR. Comparison of mutated allele fractions (MAF) revealed a high concordance, validating the approach of WGS library construction followed by both ALK-targeted ddPCR (MAF LOD 0.1%) and WES (MAF LOD 1%). In 5 NB patients receiving the ALK inhibitor Lorlatinib for resistant/progressive ALK-altered NB, cfDNA was extracted from 1-5 sequential plasma samples (blood/bone marrow) , WGS sequencing libraires were constructed and then subjected to: 1) ddPCR targeting the known ALK hotspot mutation (F1174L, R1275Q, I1170N) for evaluation of disease burden; 2) cfDNA WES for study of clonal evolution, following comparison with tumor/germline WES.

Results

ALK mutation MAFs measured by ddPCR correlated with the clinical disease status, with a MAF < 1% in clinical remission, versus higher MAFs (10-46%) at disease progression in all patients. Importantly, the follow-up of ALK MAF by ddPCR could detect relapse earlier than clinical imaging, suggesting a higher

sensitivity for relapse detection. Following lorlatinib treatment, new disease progression occurred in 4/5 patients (time between begin of lorlatinib treatment and disease progression 1 month – 2.5 years). cfDNA WES revealed new SNVs, not seen in the primary tumor at diagnosis, in all instances of disease progression after lorlatinib treatment, indicating clonal evolution with impact of cellular pathways including cellular growth, cellular death, cell-cell adhesion as well as epigenetic regulation such as DNA methylation as resistance mechanisms. The MAF dynamics enabled to distinguish SNVs of somatic clonal evolution from clonal hematopoiesis events.

Conclusions

We validated the use cfDNA WGS libraries for concomitant ddPCR/WES analysis. Our approach underlines the clinical utility of ddPCR for the follow-up of ALK MAF during ALK-targeted treatment of NB patients, and WES for the study of clonal evolution and clonal hematopoiesis.

P199

YAP1-TEAD2 mediates therapy resistance in RAS-driven neuroblastoma

Shim J[1,2], Ho A[1], Jonus H[1], Pilgrim A[3], Barwick B[4,5], Tang T[6], Boise L[4,5], Goldsmith K[1,2]
[1]Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia, United States, [2]Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Atlanta, Georgia, United States, [3]Cancer Biology Program, Laney Graduate School, Emory University, Atlanta, Georgia, United States, [4]Department of Hematology and Medical Oncology, Emory University School of Medicine, Atlanta, Georgia, United States, [5]Winship Cancer Institute, Emory University, Atlanta, Georgia, United States, [6]Vivace Therapeutics, San Mateo, California, United States

Background: Despite intensive multimodal therapy, greater than 50% of children with high-risk neuroblastoma (HR NB) relapse with incurable disease. Gene expression analyses showed a significant decrease in expression of genes suppressed by the Yes-Associated Protein1 (YAP1) at relapse, suggesting increased YAP1 transcriptional repression. YAP1 binds with TEAD family transcription factors to regulate gene expression. We have shown that YAP1 promotes chemotherapy and MEK inhibitor resistance in RAS-mutated NB tumors in vivo by suppressing the expression of Harakiri (HRK), a BH3-only pro-apoptotic protein activated in response to tumor environmental stress such as serum starvation.

Aims: Our overall objective is to elucidate how YAP1 represses HRK and tumor suppressor genes globally, and to enhance MEK inhibitor potency by combining MEK inhibition with agents that inhibit YAP1 or induce HRK to restore in situ apoptosis and therapy response in RAS-mutated NB.

Methods: To identify the specific TEAD (1-4) binding partner to YAP1, we performed siRNA and co-immunoprecipitation studies. We used publicly available databases to identify TEAD binding sites on the HRK gene locus in NB. To assess the global state of methylation, we treated NB cells, SK-N-AS (NRASQ61K mutation, MYCN non-amplified) and NLF (NF1 deletion, MYCN amplified), with demethylating agent azacitidine and evaluated HRK expression. We further tested novel YAP1-TEAD small molecule inhibitors with varying TEAD1-4 inhibition specificity in SK-N-AS and NLF cells in vitro.

Results: We identified TEAD2 as the specific binding partner to YAP1 in NB and found that TEAD2 is necessary for HRK regulation by YAP1. We observed that TEAD2 binds near cis-regulatory regions on the HRK gene locus in NBs. We found that HRK expression is restored when SK-N-AS and NLF cells are treated with azacitidine despite YAP1 expression increasing. Novel YAP1-TEAD small molecule inhibitors affect NB cell viability under serum-deprived conditions in vitro, especially the inhibitor with highest specificity against TEAD2, and affect YAP1-TEAD downstream targets.

Conclusion: YAP1-TEAD2 interaction is essential for HRK regulation in RAS-mutated NB and thus is a logical therapeutic target to enhance MEK inhibitor response. Further studies are ongoing to test YAP1-TEAD small molecule inhibitors in combination with MEK inhibitors in RAS-driven NB.

P200

Characterizing Chemoimmunotherapy Response in Patients with ALK-mutated Relapsed or Refractory Neuroblastoma

Prorok R[1,2], Onyeama S[2], Martinez M[2], Frazer B[2], Marachelian A[2,3]
[1]University of South Florida, Morsani College of Medicine, Tampa, United States, [2]Children's Hospital Los Angeles, Department of Hematology and Oncology , Los Angeles, United States, [3]University of Southern California, Department of Pediatrics, Los Angeles, United States

Background
High-risk relapsed neuroblastoma remains a therapeutic challenge. At relapse, the frequency of ALK mutation is around 26%, compared to 10% at diagnosis. Therapy for first relapsed refractory neuroblastoma (RR-NB) with chemoimmunotherapy with Dinutuximab, Irinotecan, Temozolomide, and cytokines (ANBL1221), has impressive response and progression-free survival (PFS). However, for nearly a third of patients with ALK-aberration RR-NB, it is unknown how mutation status impacts response to chemoimmunotherapy and rates of PFS.

Aims
This study evaluates response rates to chemoimmunotherapy, PFS, and overall survival (OS) in patients with ALK-mutated RR-NB.

Methods
A single-center, retrospective chart review was conducted on patients who received chemoimmunotherapy for RR-NB at Children's Hospital Los Angeles between 2012 to 2022. Patient demographic, chemoimmunotherapy, and outcome data were collected.

Results
12 patients were identified as having ALK-mutated RR-NB (seven refractory/four relapsed) who received chemoimmunotherapy. Median age of diagnosis was 4 (range 1-13) years. ALK mutations occurred in one of three loci: F1174(64%), F1245(27%), and R1275(9%). 3/11 patients had MYCN amplification. Median cycles of chemoimmunotherapy received was 4 (range 1-14). Best overall response (BOR) after was Complete Response(CR) for one patient (9%), Partial Response(PR) for two patients (18%), Minor Response(MR) for one patient (9%), Stable Disease(SD) for two patients (18%), and Progressive Disease(PD) for five patients (45%). Three of four CR/PR/MR patients had either ALK mutation identified on ctDNA following chemoimmunotherapy or were noted to have some tumor sites without ALK aberration. Progression in patients with BOR of PD occurred during the first two cycles of chemoimmunotherapy. Four additional patients had progression after a median of 9 chemoimmunotherapy cycles (range 5-11). One-year PFS was 27%, median of 76 days (range 10-637), and one-year OS was 40%, median of 338 days (range 111-2271). There were no significant differences in response in patients with different ALK mutations, relapse/refractory status, or MYCN amplification, albeit with small numbers.

Conclusion
Patients with ALK-mutated RR-NB may have increased rates of progressive disease during chemoimmunotherapy and reduced one-year PFS compared to patients without ALK mutations. Future studies of chemoimmunotherapy should collect ALK aberration status and if confirmed, could use as stratification variables for future studies.

P201

EXTRACELLULAR VESICLES FROM DRUG-RESISTANT NEUROBLASTOMA CELLS REGULATE THE METABOLISM OF NONCANCEROUS CELLS THROUGH THE TRANSFER OF GLYCOLYTIC ENZYMES

Frawley T[1,2], Ma L[1], Piskareva O[1,2]

[1]Cancer Bio-Engineering Group, Department of Anatomy and Regenerative Medicine, RCSI University of Medicine and Health Sciences, Dublin, Ireland, [2]National Childrens Research Centre, Dublin, Ireland

Background

The development of drug resistance is considered the main contributor to neuroblastoma relapse. Multiple mechanisms and genetic pathways are involved in acquiring this phenotype, one of which is cell-cell communication mediated by exosomes or small extracellular vesicles (sEV). The bioactive sEV cargo contains proteins, lipids, nucleic acids or other cellular components that can be transferred between cells.

Aims

We hypothesise that the development of cisplatin resistance in neuroblastoma alters the proteome and function of neuroblastoma-derived sEV, resulting in the propagation of a chemo-resistant phenotype in its microenvironment.

Methods

To test our hypothesis, we utilized a cisplatin-resistant model of neuroblastoma developed in our lab, comprising Kelly and its cisplatin-resistant subline KellyCis83. We first performed the characterisation of exosomes isolated from Kelly and KelliCis83 cell lines coupled with mass spectrometry analysis, NTA, TEM and western blotting. Metabolism was examined using the Seahorse XF Glycolytic Rate Assay.

Results

NTA and TEM revealed no significant difference in morphology between Kelly and KellyCis83-derived exosomes with a mode particle size of 150nm. The western blotting confirmed sEV markers: positive for HSP70, TSG101 and negative for VDAC1.

Next, we integrated vesicle proteomes with whole-cell molecular profiles and identified high similarities closely reflecting the proteome of the cell of origin. The pathway enrichment analysis found distinct protein sets in the EVs secreted by Kelly and KellyCis83 cells, involved in the Warburg effect and proliferation in target noncancerous cells. We reasoned that the cancer-promoting activity of EVs could be attributed to the glycolytic enzymes enriched in EVs secreted by drug-resistant KellyCis83. Our data indicated that hexokinase I (HK1), pyruvate dehydrogenase and LDHA levels were >5.8, 2.36 and >2.49 fold-change higher in KellyCis83 compared to Kelly sEV, respectively, accompanied by an increased level of intracellular pyruvate to lactate conversion by 25-33% ($p = 0.0284$). KellyCis83 sEVs also promoted an anchorage-dependent differentiation of HUVECs by 30% compared to Kelly sEV.

Conclusion

Our findings highlight that sEVs promoted the Warburg effect in the drug-resistant neuroblastoma microenvironment. The EV proteome analysis demonstrated its great translational benefit as a rich source of diagnostic and prognostic markers for tumour, therapy response, and disease monitoring.

P202

MOLECULAR MECHANISMS OF CHEMORESISTANCE IN NEUROBLASTOMA PATIENTS

Garcia Lopez M[1], Gómez-González S[1], Muñoz Ó[2], Lemos I[2], Suñol M[3], Mora J[2], Lavarino C[1]
[1]Translational Genomics Group, Developmental Tumor Biology Laboratory, SJD Pediatric Cancer Center (PCCB), Institut de Recerca Sant Joan de Déu, Barcelona, Spain, [2]SJD Pediatric Cancer Center Barcelona (PCCB), Hospital Sant Joan de Déu, Barcelona, Spain, [3]Department of Pathology, Hospital Sant Joan de Déu, Barcelona, Spain

Background
The emergence of drug-resistance is the major cause of cancer treatment failure. Patients with high-risk neuroblastoma tumors (HR-NB) are treated with intensive multimodal therapy, however, approximately 60% suffer disease relapse. Achieving cure after relapse is challenging due to tumor heterogeneity and the development of drug resistance.

Aims
We aim to investigate the molecular mechanisms of therapy-induced resistance in HR-NB, to identify cellular pathways promoting survival as potential therapeutic targets.

Methods
Drug-resistant NB cells were generated using cytotoxics included in current chemotherapy protocols for HR-NB patients. Drug-resistant cells were characterized using gene expression and DNA methylation profiling, ChIP-seq assays and NGS sequencing. Functional in-vitro and in-vivo assays were performed. Paired diagnosis-relapse tumor samples were used for validation. High-throughput screening (HTS) was performed to test cytotoxicity of 2,400 FDA approved compounds (at 100nM) in both drug-resistant and native NB cells.

Results
Drug-resistant cells showed changes of expression levels of genes involved in cell differentiation, migration and DNA damage repair processes. A significant portion of these genes have been reported previously in NB associated with mesenchymal state and drug-resistance. Functionally, drug-resistant cells showed defects on cell proliferation, cell cycle and colony-forming capability whereas showed enhance invasion and in-vivo tumor growth capacities. DNA methylation profiling identified extensive hypermethylation across the genome of drug-resistant cells, whereas hypomethylation affected super-enhancers previously described in NB mesenchymal phenotype. HTS identified 34 compounds with high cytotoxicity effect (>60% of cell death) in three different drug-resistant cell lines. Compounds with high-activity target molecules associated with cell cycle and DNA damage (25%), apoptosis (16%), and autophagy (16%) pathways, among others.

Conclusion
We have identified a set of differentially expressed genes and signalling pathways potentially underlying acquired resistance in our drug-resistant model. HTS identified active compounds against drug-resistant cells, providing insight into potential vulnerabilities of interest for therapeutic opportunities.

P203

Chemotherapy response of neuroblastoma patient-derived xenografts correlated with patient outcome and identified gene expression patterns associated with drug resistance

Kang M[1], Noureen N[1], McCoy K[1], Nance J[1], Ixlamati-Nava D[1], Irwin M[2], Hogarty M[3], Creighton C[4], Zhang F[5], Naranjo A[5], Reynolds C[1]

[1]Texas Tech Univ. Health Sciences Ctr. Cancer Center, Lubbock, United States, [2]University of Toronto, Hospital for Sick Children, Pediatrics, Toronto, Canada, [3]Children's Hospital of Philadelphia and Perelman School of Medicine, UPENN, Pediatrics, Philadelphia, United States, [4]Baylor College of Medicine, Human Genome Sequencing Center, Houston, United States, [5]University of Florida, Children's Oncology Group Statistics and Data Center, Gainsville, United States

Background: Approximately 15% of high-risk neuroblastoma patients develop progressive disease (PD) during induction chemotherapy. Laboratory models are needed to understand the mechanisms of early neuroblastoma PD.
Methods: We evaluated 31 subcutaneous patient-derived neuroblastoma xenografts (PDX) established at diagnosis (Dx, n=15) and PD (n=16) from the Children's Oncology Group/ALSF repository (www.CCcells.org). Twenty-eight PDXs expressed TERT, two the alternative lengthening of telomeres biomarker DNA C-circles, and one was TERT and C-circle negative; 21 had MYCN gene amplification, 10 non-amplified. Response over 175 days was assessed to 3 cycles of cyclophosphamide (cyclo, 30 mg/kg) + topotecan (topo,0.6 mg/kg) daily x 5 days every 21 days. RNA sequencing of PDXs prior to therapy in three batches was corrected for batch effect using Combat. Differentially expressed genes were identified using DESeq2 and limma.
Results: Six PDXs (1 established at Dx, 5 at PD) were non-responders (NR), two (Dx and PD) stable disease (SD), eleven (5 Dx, 6 PD) partial responders (PR), four (1 Dx, 3 PD) complete responders (CR), and eight (7 Dx, 1 PD) maintained CRs (MCR). Median patient event-free survival (EFS) for seventeen PDXs established at Dx was 418 days for 9 CR/MCR PDXs vs 236 days for 8 PR/NR PDXs ($p = 0.05$). We identified 79 differentially expressed genes (DEGs) between NR/SD and responding (PR/CR/MCR) PDXs with log2 fold-changes > 0.5 and $p < 0.05$ using two independent analyses. Gene set enrichment analysis identified three major pathways significantly enriched in the NR group: Hallmark MYC targets V1/V2, hallmark E2F targets, and hallmark G2M checkpoint genes. CDK4, and CDKN2A are genes highly expressed in the NR/SD group common to the three pathways. Using the logistic regression classifier we identified an RNA expression signature that segregated NR/SD from responding PDXs.
Conclusions: Response of PDXs established at Dx to cyclo/topo correlated with patient EFS. The gene expression signature of non-responding PDXs can potentially provide a biomarker to identify patients destined to have suboptimal responses to induction chemotherapy. Genes overexpressed in non-responding PDXs provide potential molecular targets to reverse drug resistance. This well-characterized panel of PDXs will be valuable in preclinical testing of novel therapies.

P204

The impact of MYCN depletion on neuroblastoma cell survival and immune evasion

Torres-Rodriguez K[1,2], Salvacion M[1,2], Scolaro L[1], Felipe I[3], Lindsay J[1,2], Wingrove C[1,2], Betts M[2], Wolpaw A[1], Maris J[1]

[1]Division of Oncology and Center for Childhood Cancer Research, Children's Hospital of Philadelphia, Philadelphia, USA, [2]Perelman School of Medicine, University of Pennsylvania, Philadelphia, USA, [3]Epithelial Carcinogenesis Group, Spanish National Cancer Research Centre-CNIO, Madrid, Spain

Background:
Neuroblastoma is poorly responsive to immune checkpoint inhibition. This is hypothesized to be due to low MHC-I expression, a low tumor mutation burden, and scarce basal T-cell infiltration, all features that are further enhanced in MYCN-amplified tumors. However, the precise role that MYCN plays in coordinating immune escape, particularly on MHC-I expression, remains poorly understood. As our laboratories seek to develop MYCN degrader compounds for the clinic, there is an urgent need to understand the degree of depletion required to exert a therapeutic effect.

Aim:
To define the biologically relevant degree of MYC(N) depletion required to impact neuroblastoma cell survival and immune evasion in neuroblastoma using genetically diverse cellular models.

Methods:
We first investigated the impact MYC(N)-MAX dimerization disrupters MYCi975 and OmoMYC on MYCN protein levels and activity by immunoblotting for MYCN and MYCN-target genes. This was done daily for 5 days in MYCN-amplified cell lines with high levels of MYCN protein such as Kelly, IMR-5, and SK-N-BE(2). We then performed ATP bioluminescence assays to determine viability after MYCN targeting. To understand the role of MYCN on MHC-I silencing, we performed flow cytometry for surface MHC-I after MYCi975 treatment as well as following pharmacological inhibition of the PRC2 complex, an epigenetic repressor of MHC-I.

Results:
MYCi975 resulted in rapid and consistent degradation of MYCN protein and downregulation of established MYCN target genes such as PHOX2B and EZH2 in neuroblastoma cell lines at IC50 concentration or higher. At doses that result in greater than 30-50% decrease in MYCN protein, cell viability was impacted as measured by ATP levels. These results were validated with the MYC inhibitor OmoMYC. Even though epigenomic analysis showed MYCN binding to HLA-A, -B and -C loci, MYCi975 treatment alone or in combination with an EZH2 inhibitor did not acutely alter MHC-I surface expression.

Conclusions:
MYCi975 effectively decreases MYCN abundance and transcriptional activity, and complete degradation is not necessary for growth inhibition of neuroblastoma cells. Furthermore, MYCN degradation does not lead to an abrupt increase in surface MHC-I, but later timepoints are being studied and parallel investigations on immune cell recruitment and function will be reported.

P205

The impact of MYCN on therapy-induced senescence in neuroblastoma

Montuori G[1,2], Qin D[3], Popp O[4], Bosco B[1,2], Gürgen D[5], Mertins P[4], Eggert A[1], Coscia F[3], Henssen A[1,2,4,6,7], Dörr J[1,2,4,7]

[1]Department of Paediatric Oncology/Hematology, Charité-Universitätsmedizin Berlin, Berlin, Germany, [2]Experimental and Clinical Research Center (ECRC) of the MDC and Charité Berlin, Berlin, Germany, [3]Spatial Proteomics Group, Max Delbrück Center, Berlin, Germany, [4]German Cancer Consortium (DKTK), partner site Berlin, and German Cancer Research Center (DKFZ), Heidelberg, Germany, [5]Experimental Pharmacology & Oncology GmbH, Berlin, Germany, [6]Max-Delbrück-Center for Molekülare Medizin (BIMSB/BIH), Berlin, Germany, [7]Berlin Institute of Health, 10178 Berlin, Germany

Background/Aims: The curative treatment of high-risk neuroblastomas with MYCN amplification remains a major obstacle in pediatric oncology. Therefore, a better mechanistic understanding of how MYCN influences treatment effector mechanisms, such as apoptosis or senescence, and thereby undermines treatment success is needed to identify novel treatment approaches. Senescent tumor cells undergo dynamic structural and functional changes, which impact on tumor biology. While therapy-induced senescence (TIS) has been investigated in different adult cancers, the relevance of TIS in neuroblastoma remains incompletely defined. Here we analyze the impact of MYCN and of MYCN-induced tumor heterogeneity on TIS and the TIS phenotype in neuroblastoma with the goal to identify new senescence-targeting neuroblastoma therapies.

Methods: Cell viability (e.g. Annexin-V/PI staining) and proliferation (e.g. BrdU/PI incorporation and MKI67 staining) was analyzed in neuroblastoma cell lines -/+ MYCN amplification before and after exposure to doxorubicin in vitro. Similarly, TIS was assessed by senescence-associated β–galactosidase reactivity, by the induction of senescence regulators (CDKN1A, CDKN2A, H3K9me3) with immunoblot, immunofluorescence and immunohistochemistry analyses, and RNA sequencing in neuroblastoma cell lines, PDX and high-risk neuroblastoma patient samples -/+ MYCN amplification. TIS-associated reprogramming in neuroblastoma cells with different MYCN status as defined by fluorescence in-situ hybridisation (FISH) was analyzed by label-free quantitative proteomics and spatially resolved proteomics in vitro and in vivo.

Results: We demonstrate that MYCN status impacts on TIS and the TIS phenotype in neuroblastoma cells in vitro as well as in neuroblastoma PDX and high-risk neuroblastomas in vivo. In MYCN-amplified neuroblastomas we revealed by ImmunoFISH analyses as well as by genetic and pharmacological perturbations that neuroblastoma cells with high MYCN expression induced apoptosis while neuroblastoma cells with low MYCN expression entered senescence. Label-free and spatially resolved proteomics showed that the senescence phenotype differs in neuroblastoma cells -/+ MYCN amplification and is heterogeneous within the same neuroblastoma sample depending on MYCN expression. These unique MYCN-dependent senescence functionalities can be exploited by novel senolytic therapies using metabolic inhibitors or immunotherapies.

Conclusion: We uncover MYCN-dependent senescence remodeling as a source for intertumor and intratumor heterogeneity in high-risk neuroblastomas with the potential to identify new senescence-based treatment approaches.

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Pharmacogenetics of treatment response and toxicity in French patients with metastatic High-Risk Neuroblastoma

Barthoulot M[1], Dumont A[2], Martignene N[1], Le Deley M[1,3], Revillion F[2], Poetschger U[4], Schleiermacher G[5], Combaret V[6], Valteau-Couanet D[7], Defachelles A[8]

[1]Methodology and biostatistics unit, Centre Oscar Lambret, Lille, France, [2]Laboratoire d'oncogénétique , Centre Oscar Lambret , Lille, France, [3]Université Paris-Saclay, UVSQ, CESP, INSERM, Villejuif, France, [4]St Anna Children's Cancer Research Institute, Vienna, Austria, [5]SIREDO: Care, Innovation and Research for Children, Adolescents and Young Adults with Cancer, Institut Curie, Paris, France, [6]Translational Research Laboratory, Centre Léon Bérard, Lyon, France, [7]Department of Pediatric and Adolescent Oncology, Gustave-Roussy, Villejuif, France, [8]Pediatric and AYA Oncology Unit, Centre Oscar Lambret, Lille, France

Background. Neuroblastoma (NB) is the most common malignant extra-cerebral solid tumor in children. Growing evidence suggests that polymorphisms may affect NB treatment efficacy and/or toxicity.

Aims. We aimed to identify Single Nucleotide Polymorphisms (SNPs) which may contribute to heterogeneity in treatment efficacy and toxicity in metastatic high-risk neuroblastoma (M-HR-NB) children.
Methods. SNP genotyping was performed in children with M-HR-NB enrolled in France within HRNBL1-trial, on selected genes involved in the pharmacokinetic pathways. Efficacy outcomes were tumor response to induction chemotherapy, progression-free survival (PFS) and overall survival (OS). Toxicity outcome was severe extra-hematological toxicity, defined as NCI-CTCAE V2 grade ≥3 toxicity reported during induction. Associations of each SNP with outcomes were estimated using logistic and Cox regression models, as appropriate, with additive and dominant models, adjusted for age, disease stage at study entry, NMyc status, number of metastatic sites and TVD-regimen (for PFS and OS only). To account for multiple comparisons, we used Bonferroni correction with a p-value threshold set at 5.10-4.

Results. Final population comprised 256 patients, and 109 SNPs were selected. Overall, 129 (51%) patients had a good response to HR-NBL induction. We observed no significant association between SNP and tumor response; the lowest p-value was obtained for SNP-rs12826 (ABCC2 gene) with the dominant coding (Adjusted Odds Ratio=1.39; 95%CI, 1.05-1.85; p= 0.022). With a median follow-up of 5.6 years, 126 progressions and 123 deaths were observed. The 5-year PFS and OS were estimated to 37.7% [30.9–44.4] and 50.2% [43.1–56.8], respectively. We detected no significant association with PFS and/or OS. Safety data were available for 220 patients; among them, 117 (53.2%) presented a severe acute extra-hematological toxicity. We detected no significant association with severe acute extra-hematological toxicity.

Conclusion. We investigated association between pharmacogenetic profiles from SNPs and treatment efficacy and toxicity outcomes in high-risk NB French children. We detected no significant association with tumor response to induction chemotherapy, survival (PFS and OS) or acute toxicity; further studies are necessary to confirm or infirm our results

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IgA3.0 ch14.18 antibodies are effective in preclinical neuroblastoma models without inducing neuropathic pain

Stip M[1], Evers M[1], Nederend M[1], Reiding K[2,3], Damen M[2,3], Heck A[2,3], Koustoulidou S[4], Ramakers R[4], Krijger G[5], de Roos R[5], Souteyrand E[1], Cornel A[1,6], Dierselhuis M[6], Jansen M[1], Chan C[1], de Boer M[7], Valerius T[8], van Tetering G[1], Leusen J[1], Meyer-Wentrup F[6]

[1]Center for Translational Immunology, UMC Utrecht, Utrecht, Netherlands, [2]Biomolecular Mass Spectrometry and Proteomics Bijvoet Center for Biomolecular Research and Utrecht Institute for Biopharmaceutical Sciences, University of Utrecht, Utrecht, Netherlands, [3]Netherlands Proteomics Center, Utrecht, Netherlands, [4]MIlabs, UMC Utrecht, Utrecht, Netherlands, [5]Radionuclide Pharmacy, UMC Utrecht, Utrecht, Netherlands, [6]Princess Máxima Center for Pediatric Oncology, Utrecht, Netherlands, [7]De Boer Biotech Consultancy B.V., Blaricum, Netherlands, [8]Section for Stem Cell Transplantation and Immunotherapy, Department of Medicine II, Christian Albrechts University and University Hospital Schleswig-Holstein, Kiel, Germany

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Background

Immunotherapy targeting GD2 is effective against high-risk neuroblastoma, though administration of dinutuximab induces dose-limiting neuropathic pain by binding GD2-expressing sensory neurons. We have reformatted the IgG1 antibody dinutuximab (IgG1 ch14.18) into the IgA isotype, which engages a different set of immune cells via the Fc-alpha receptor (FcαRI/CD89). Additionally, IgA ch14.18 lacks the C1q binding domain, hence we hypothesized it will not be able to initiate the complement cascade nor induce pain.

Methods and Results

First, we engineered a novel IgA ch14.18 molecule (named IgA3.0) to improve stability and increase half-life. It contains the following mutations: N45.2G and P124R (CH1 domain), C92S, N120T, I121L and T122S (CH2 domain) and a deletion of the tailpiece P131-Y148 (CH3 domain). Antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis assays (ADCP) showed that in particular neutrophils and macrophages are able to kill neuroblastoma tumor cells efficiently upon IgA3.0 ch14.18 stimulation. Peripheral blood mononuclear cells (PBMCs) from neuroblastoma patients performed less ADCC with IgG1 ch14.18 compared to healthy control PBMCs, corresponding to a lower number of NK cell in the PBMC fraction and a lower FcγRIIIa (CD16) expression. However, IgA3.0-stimulated patient neutrophils killed neuroblastoma cells almost as well as neutrophils from healthy donors.

Von Frey nociception tests revealed that indeed IgG1 ch14.18 induced pain in mice, whereas IgA ch14.18 did not. Further characterization of IgA3.0 ch14.18 showed that the introduced mutations resulted in increased antibody half-life in mice, due to reduced overall glycosylation and increased terminal sialylation at the remaining glycosylation site. IgA3.0 ch14.18 therapy significantly improved survival in vivo in both a subcutaneous xenograft model with human IMR32 cells and in an intraperitoneal model with murine 9464D cells. Moreover, therapeutic efficacy could be further enhanced by blocking the CD47/SIRPα axis both in vitro and in vivo.

Conclusion

Our results indicate that IgA3.0 ch14.18 antibody therapy has two major benefits compared to traditional IgG1 ch14.18: (1) it halts antibody-induced neuropathic pain and (2) induces killing of neuroblastoma tumors by both healthy donor and patient-derived neutrophils and macrophages. Thus, we expect that patients with high-risk neuroblastoma could strongly benefit from IgA3.0 ch14.18 immunotherapy.

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Best of two worlds: Engineering NKT-cells to generate an alternative adaptive cell therapy strategy against neuroblastoma

Cornel A[1,2], Forbes C[1], Dunnebach E[1,2], Nierkens S[1,2]
[1]Princess Máxima Center, Utrecht, The Netherlands, [2]UMC Utrecht, Center for Translational Immunology, Utrecht, The Netherlands

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Background
The current high-risk neuroblastoma (HR-NBL) immunotherapy regimen does not live up to its full potential due to the low immunogenicity of NBL, which is a derivative of its embryonal origin. One of the most notable immunomodulatory processes is the absence of MHC-I display in NBL, which greatly limits cytotoxic T-cell engagement.

We previously showed that MHC-I expression can be induced by IFNy secretion by activated NK-cells. Consequently, it is suggested that the high degree of plasticity in MHC-I expression in NBL allows alternate evasion of both cytotoxic T- and NK-cells. We hypothesize that generation of a cell product from CD3+CD56+ cells (NKT-cells) that can both engage in missing-self cytotoxicity and in recognition of malignant transformation in MHC-I context will circumvent immune evasion by MHC-I plasticity.

Aims
To generate NBL-specific T-cell receptor (TCR)-engineered NKT-cells and study their potential to target MHC-I plasticity-mediated immune evasion in NBL.

Method/Materials
NKT-cells were isolated from healthy-donor peripheral blood. Cells were extensively phenotyped with flow cytometry and functionally assessed on their capacity to elicit NK receptor-mediated cytotoxicity. Subsequently, NKT-cells were lentivirally engineered to express a NBL-specific PRAME TCR, after which transduction efficacy, cognate peptide recognition, and antigen-dependent and missing-self cytotoxicity was confirmed. Finally, the cytotoxic capacity of wild-type and TCR-engineered NKT- and T-cells was compared to evaluate the potential of TCR-engineered NKT-cells.

Results
Phenotypical analysis of the CD3+CD56+ NKT-cell fraction revealed NKG2D expression on all, and NKp46 and KIR2D expression on a subset of NKT-cells. We show that NKT-cells, like NK-cells, can engage in NK receptor-mediated cytotoxicity, recognize and kill a panel of NBL cells, and induce MHC-I expression on surviving NBL cells. An NBL-specific TCR was subsequently effectively introduced in NKT-cells, after which peptide recognition and antigen-dependent cytotoxicity was confirmed. Finally, we observed a superior cytotoxic capacity of TCR-engineered NKT-cells compared to wildtype NKT-cells and TCR-engineered T-cells.

Conclusion
We provide groundwork for the use of NKT-cells as a therapy source to improve outcome for children with HR-NBL. This study supports our hypothesis that combining NK receptor-mediated cytotoxicity with MHC-I restricted targeting is a promising strategy to overcome MHC-I plasticity-related immune evasion by NBL.

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GPC2-directed CAR T cells are safe and efficacious in preclinical models of neuroblastoma

Bosse K[1,2], Gladney W[3], Giudice A[1], Pascual-Pasto G[1], McIntyre B[1], Foster J[1,2], Buongervino S[1], Lane M[1], Chagin K[3], Fountaine T[3], Peddareddigari V[3], Martinez D[4], Assenmacher C[5], Radaelli E[5], Pawel B[6], Grupp S[1,2], Barrett D[1,2], Maris J[1,2]

[1]Division of Oncology and Center for Childhood Cancer Research, Children's Hospital of Philadelphia, Philadelphia, PA, United States, [2]Department of Pediatrics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, United States, [3]Tmunity Therapeutics, Inc., Philadelphia, PA, United States, [4]Department of Pathology, Children's Hospital of Philadelphia, Philadelphia, PA, United States, [5]Comparative Pathology Core, Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA, United States, [6]Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles and the Keck School of Medicine at the University of Southern California, Los Angeles, CA, United States

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Background: We identified glypican 2 (GPC2) as a MYCN-regulated neuroblastoma oncoprotein and immunotherapeutic target and developed a D3 antibody that binds a conformational, tumor-specific epitope shared between mouse and human GPC2.

Aims: To develop GPC2 chimeric antigen receptor (CAR) T cells and test their efficacy and safety in neuroblastoma.

Methods: GPC2 expression was quantified using immunohistochemistry (IHC) in human neuroblastoma (n=61) and patient-derived xenograft (PDX; n=35) tumor microarrays and by flow cytometry (n=5 cell lines). D3-based GPC2 CAR T cells were generated from lentiviral constructs with either CD28 or 4-1BB costimulatory domains. GPC2 CAR pharmacology was tested in vitro with proliferation studies, cytokine ELISAs, and cytotoxicity assays, and in vivo with murine preclinical trials. GPC2 CAR T cell safety was assessed with primary human cell line co-incubation studies and comprehensive murine necropsies.

Results: IHC showed that GPC2 is expressed on most neuroblastomas [97% (59/61) of human tumors and 91% (32/35) of PDXs] and flow cytometry confirmed high levels of cell surface GPC2. GPC2 CAR T cells were activated (released IL-2/INF-γ) and proliferated in response to GPC2-high neuroblastoma cells (NB-SD, SMS-SAN, NB-EbC1; 8,184-16,430 GPC2 molecules/cell) but not GPC2-low neuroblastoma (SK-N-AS) or control (BxPC-3) cells (460-1,479 GPC2 molecules/cell). GPC2 CAR T cells induced selective GPC2-high neuroblastoma cytotoxicity and apoptosis, inducing 95-100% cell killing after 24-72 hours in luciferase-based assays at a 5:1 E:T ratio, 96-100% and 84-100% specific cytotoxicity at 1:1 or 1:5 E:T ratios in cell-impedance-based assays, and robust elevation of caspase-3/-7 levels (11-68-fold-increase vs. non-targeting CARs). Very limited GPC2 CAR T cell induced cytotoxicity or apoptosis was observed in GPC2-low SKNAS or BxPC-3 cell co-incubations. Similarly, no significant release of 38 cytokines or cytotoxicity was observed in co-incubation studies with 9 primary human cell lines. Finally, GPC2 CAR T cells induced significant regression of GPC2-high COG-N-421x PDXs and NB-1643 xenografts compared to non-targeting CARs ($p<0.0001$) and no GPC2 CAR-related toxicities were noted in mouse necropsies performed 16-27 days after CAR administration.

Conclusion: GPC2 CAR T cells are potently efficacious against a diverse panel of neuroblastoma preclinical models without any signs of toxicity. A phase 1 study is planned for 2023.

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Immunomodulatory effect of the MEK pathway inhibitor enhanced the efficacy of GD2-CAR-T cell therapy against neuroblastoma

Tomida A[1], Yagyu S[1,2], Kubo H[1], Nakazawa Y[3], Hosoi H[1], Iehara T[1]
[1]Department of Pediatrics, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan, [2]Center for Advanced Research of Gene and Cell Therapy, Shinshu University Innovative Research & Liaison Organization, Matsumoto, Japan, [3]Department of Pediatrics, Shinshu University School of Medicine, Matsumoto, Japan

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[Background] Disialoganglioside (GD2)-specific chimeric antigen receptor (CAR)-T cells (GD2-CAR-T cells) have been developed and validated in early clinical trials in patients with relapsed/refractory neuroblastoma (NB). However, the limited efficacy of immunotherapy using these cells has prompted various studies to elucidate and overcome the underlying mechanisms. Combinatorial therapy with CAR-T cells and molecular-targeted drugs could be a promising strategy to enhance the antitumor efficacy of CAR-T cell immunotherapy. Recent studies have revealed that the excessive activation of CAR-T cells induces effecter-like CAR-T cells that exhibited rapid but transient cytotoxicity and impair sustained anti-tumor effect. Since Raf/MAPK pathway is an important downstream pathway of TCR signaling, we hypothesized that transient inhibition of Raf/MAPK pathway would prevent excessive activation of TCR signaling and following exhaustion of CAR-T cells.

[Aims] In this study, we investigated whether the combination therapy of GD2-CAR-T cells and Raf/MAPK pathway inhibitor would improve the killing activity against NB cells.
[Methods] We have established GD2-CAR-T cells by piggyBac transposon-based gene transfer, and evaluated their phenotypes using flow cytometry. Then we evaluated the combinatorial effect of trametinib, an inhibitor of the Raf/MAPK pathway, on GD2-CAR-T cells against Ras/MAPK pathway mutated (SK-N-AS) and intact (SH-SY5Y, and IMR32) NB cells in vitro and in vivo.

[Results] GD2-CAR-T cells exhibited rapid and potent in vitro antitumor effects against NB, but the activation of GD2-CAR-T cells was suppressed by the combination of trametinib. The expression of immune exhaustion markers, including PD-1/Tim-3/LAG3, was downregulated in the trametinib/GD2-CAR-T cell combination group. Interestingly, trametinib did not suppress the proliferative activity of antigen-stimulated GD2-CAR-T cells. Furthermore, the combinatorial treatment of trametinib and GD2-CAR-T cells demonstrated superior tumor control with improved CAR-T cell persistence in NB-bearing immunodeficient xenograft, regardless of the mutation status of Raf/MAPK pathway, suggesting an immunomodulatory function of trametinib on GD2-CAR-T cells.

[Conclusion] Trametinib could not only directly inhibit tumors with MEK pathway but it also has some immunomodulatory function that could enhance the efficacy of T cell-mediated immunotherapy. The results presented here provide new insights into the feasibility of combinatorial treatment with CAR-T cells and MEK inhibitors in patients with neuroblastoma.

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Antigen-driven cytokine expression systems to enhance the function and overcome challenges of CAR T cell therapy in neuroblastoma

Okada R[1], Reyez Gonzalez J[1], Abbas S[1], Li N[2], Kelly M[3], Ho M[2], Gulley J[4], Hinrichs C[5], Zhang X[1], Nguyen R[1]
[1]Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, United States, [2]Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, United States, [3]Center for Cancer Research Single Cell Analysis Facility, Cancer Research Technology Program, Frederick National Laboratory, Bethesda, United States, [4]Center for Immuno-Oncology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, United States, [5]Section of Cancer Immunotherapy, Rutgers Cancer Institute of New Jersey, New Brunswick, United States

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Background: Despite remarkable successes in patients with hematological malignancies, chimeric antigen receptor (CAR) T-cell therapy has shown disappointing results in clinical trials of children with neuroblastoma. The clinical development of effective CAR T-cell therapies in solid tumors, including neuroblastoma, is in part impeded by 1. The lack of tumor-specific antigens, 2. The immunosuppressive tumor microenvironment (TME), and 3. Large tumor burden. We have optimized a CAR that targets tumor-specific exons of a new antigen, glypican-2 (GPC-2), in neuroblastoma. We leveraged constitutively expressed membrane-tethered (m-teth) cytokines to further enhance CAR T-cell function in the TME and against large tumor burden and designed antigen-driven conditional cytokine expression systems to mitigate future cytokine-driven toxicities.

Methods: We used in vitro and in vivo systems of neuroblastoma to model large disease burden and test our CAR products.

Results: CAR T cells were outperformed by those armored with constitutive m-teth cytokines both in vitro and in vivo. However, animals treated with CAR + m-teth cytokines experienced significant toxicities despite significant tumor regression. In contrast, mice treated with CAR T cells with conditional antigen-driven cytokine expression systems exhibited superior anti-tumor activity against large tumor burden but lacked signs of toxicity. Correlative analysis by multiplex cytokine analysis, Cellular Indexing of Transcriptomes and Epitopes by Sequencing, and multi-color flow cytometry corroborated these observations.

Conclusion: The use of antigen-driven cytokine expression systems in conjunction with a new GPC2-targeted CAR offer an effective and safe cell therapy against neuroblastoma.

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Desensitizing the Autonomic Nervous System to mitigate anti-GD2 Monoclonal Antibody Side Effects.

Mora J[1], Climent A[1], Roldan M[1], Jayaprakash N[2], Flores M[1], Varo A[1], Perez-Jaume S[1], Jou C[1], Celma M[1], Lazaro J[1], Cheung I[3], Castañeda A[1], Gorostegui M[1], Rodriguez E[1], Chamorro S[1], Muñoz J[1], Zanos S[2], Cheung N[3]
[1]Pediatric Cancer Center Barcelona. Hospital Sant Joan de Déu, Barcelona, Spain, [2]Institute of Bioelectronic Medicine. Feinstein Institutes for Medical Research. , Manhasset, 11030 New York., USA, [3]Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, 10021 New York, USA

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Background: Anti-GD2 monoclonal antibodies (mAbs) have shown to improve the overall survival of patients with high-risk neuroblastoma (NB). Serious adverse events (SAEs) within hours of antibody infusion have limited the development of these therapies.
Aims: To provide evidence of Autonomic Nervous System (ANS) activation by naxitamab.

Methods: Confocal microscopy provided super-resolution immunostaining of nerves (Imaris software, Bitplane). Electrophysiology of vagus (VN) and splenic nerves (SN) following 18 mg/kg naxitamab was studied in rats using a tripolar Flex electrode (Intan technologies), while electrocardiography was recorded. In patients, ANS assessment was performed using the Sympathetic Skin Response (SSR) test. Naxitamab pharmacokinetics (PK) was studied by ELISA. A novel infusion protocol (Step-Up) to dissociate on-target off-tumor side effects while maintaining serum drug exposure was modelled and tested in the clinic.

Results: Selective GD2 expression on the cell membranes of human Schwann cells of peripheral nerves (PN) was significantly lower (Student's T test $p< 0.05$) than on NB. In rats, infusion of naxitamab caused an increase in spontaneous activity of the VN and SN accompanied by an increase in heart rate. Electro activity of SN reverted to baseline 90-minute post infusion.
In patients, changes in the SSR were observed 4 minutes into naxitamab infusion. SSR latency quickly shortened followed by gradual decrease in the amplitude before disappearance. SSR response did not recover for 24 hours consistent with tachyphylaxis and absence of side effects in the clinic. To exploit tachyphylaxis, the Step-Up protocol used quantile increase of the dose over 75 minutes (16% of total dose) completing the target dose of 3 mg/kg over 45 minutes for an infusion time of 2h (cycle day one). The Step-Up infusion protocol was tested in 42 patients. The frequency of ≥G3 AE reduced from 7.8% (23/294) to 2.9% (6/204) with Step-Up vs standard 30-minute infusion ($p=0.037$). Naxitamab serum levels were identical between protocols.

Conclusions: Naxitamab activated the ANS followed by tachyphylaxis. ANS activation explained the main side effects of anti-GD2 mAbs. The Step-Up protocol exploited tachyphylaxis to significantly reduce SAEs. Effective mitigation of autonomic side effects should expand the utility of anti-GD2 therapies.

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Peptide-centric CAR T cell therapy targeting the tumor-associated antigen PRAME

Runbeck E[1], Hartnett N[1], O'Reilly K[1], Marshall Q[1], Kiefel B[2], Beasley M[2], Yarmarkovich M[1], Maris J[1,3]
[1]Center for Childhood Cancer Research, Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, United States, [2]Myrio Tx, Melbourne, Australia, [3]Perelman School of Medicine at the University of Pennsylvania, Philadelphia, United States

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Background: The 5-year survival rate of high-risk neuroblastoma remains below 50%. Recently, the Maris laboratory has demonstrated the targeting of aberrantly expressed nonmutated peptides derived from intracellular oncoproteins and complexed with Human Leukocyte Antigen (HLA) in neuroblastoma with peptide-centric CAR (PC-CAR) T cells. The restriction of PReferentially Expressed Antigen in MElanoma (PRAME) to tumor tissue has been shown across multiple cancer types, including neuroblastoma, and thus would be an appropriate target for PC-CAR therapy. Here, we aimed to show proof-of-concept for PC-CAR T cells targeting the PRAME-derived peptide SLLQHLIGL presented by HLA A*02.

Methods: Candidate scFvs were developed through the ReD platform at Myrio Tx and screened for specificity and activity as second-generation CARs. The second-generation CAR included a CD3ζ signaling domain and CD28 hinge/transmembrane and costimulatory domains. This construct was expressed in human primary T cells using lentiviral transduction and tested for specific binding to the target using flow cytometry. Cytotoxicity of the PC-CAR T cells was determined by analyzing loss of GFP signal from stably expressing target cells on the Incucyte platform in a panel of 7 HLA A*02 cell lines.

Results: Eight PRAME-directed scFvs were screened and the clone PR2294 was prioritized. PR2294 showed specific binding to the PRAME-HLA*A02+ colorectal carcinoma cell line SW620 pulsed with target peptide and not to SW620 pulsed with irrelevant peptide. Additionally, primary T cells expressing the PC-CAR construct PR2294-28z exhibited binding to an SLLQHLIGL-A*02 dextramer, but not an A*02 dextramer loaded with an irrelevant peptide. PR2294-28z showed potent cytotoxicity against exogenously and endogenously PRAME-expressing A*02+ cell lines across effector to target (E:T) ratios of 10:1 to 1:1. Cytotoxicity was accompanied by IFNγ release. Two neuroblastoma cell lines were resistant to PRAME PC-CAR mediated killing. It was hypothesized that this was due to lower levels of MHCI and PRAME expression. Pre-treatment with the demethylator decitabine increased MHCI and PRAME expression and sensitized these cells to PR2294-28z T cells.

Conclusion: The PC-CAR construct PR2294-28z is effective at specifically targeting the pan-cancer antigen PRAME in A*02+PRAME+ neuroblastoma, glioblastoma and melanoma cell lines. Ongoing preclinical trials of this PC-CAR in murine models will be presented.

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Novel immune interventions for neuroblastoma from simultaneous single-cell RNA and T cell receptor sequencing of tumors

Zappa E[1], Wienke J[1], Molenaar J[1]
[1]*Prinses Maxima Centrum, Utrecht, Netherlands*

Introduction: There is a great need for new treatment options for high-risk neuroblastoma patients, which could increase their survival while decreasing the side effects associated to current therapy. Immunotherapy represents a promising solution as suggested by the improvement of patients' outcome following the implementation of anti-GD2 antibodies in standard care. However, neuroblastoma tumors are poorly immunogenic and they are suspected to exploit many immunosuppressive mechanisms that lead to immune cell dysfunctionality. Targeting these immunosuppressive pathways could strengthen the immune cell response, resulting in enhanced tumor killing and increased patients' survival.

Aims: This project aims at typing the tumor microenvironment (TME) of neuroblastoma with combined single-cell RNA and T cell receptor sequencing (scRNAseq; scTCRseq), to unveil T cell dynamics, dysfunctionality and immunosuppressive mechanisms that could dampen immune cell response.

Methods: Tumors from 10 patients at diagnosis, after treatment and at relapse will be digested, dissociated into single cells and enriched for CD45+CD3+ T cells. Blood-derived immune cells from the same patients and from age-matched healthy donors will be analyzed as controls. scRNAseq and scTCRseq will be then performed in parallel on the samples with the 10x platform. Through the analysis of the obtained data we will identify clonally-expanded T cells and mechanisms underlying their dysfunctionality. We will validate single-cell data with high-dimensional flow cytometry and with co-culture systems of tumor-specific T cells + patient-derived organoids to pinpoint therapeutic drugs that could increase T cell killing activity.

Results: Collectively, these data will contribute to the generation of an unprecedented database of the neuroblastoma TME. In particular, we will determine: 1) T cell clones that are expanded in the TME, 2) dynamics of the immune cell compartment throughout the course of the therapy, 3) dysfunctionality markers and their relationship with T cell clones, 4) targetable mechanisms to restore immune cell function.

Conclusion: The obtained results will generate new insights on T cell dynamics and mechanisms that lead to their dysfunctionality in neuroblastoma, which will be at the base for the generation of new immunotherapies to implement in clinic with the final goal to increase survival of high-risk patients.

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T-cell inflammation is prognostic of survival in high-risk, MYCN-non-amplified neuroblastoma

Kaufman M[1], Vayani O[1], Moore K[1], Chlenski A[1], Wu T[2], Chavez G[1], Lee S[3], He C[2], Desai A[1], Cohn S[1], Applebaum M[1]

[1]University of Chicago, Department of Pediatrics, Chicago, United States, [2]University of Chicago, Department of Chemistry, Chicago, United States, [3]University of Chicago, Department of Statistics, Chicago, United States

Background: Recent insights into neuroblastoma biology have identified mesenchymal (MES) cell lineage and T-cell inflammation as markers of therapy resistant and responsive disease, respectively. Here, we hypothesized that these features could serve as novel biomarkers for improving risk stratification in high-risk patients.

Aims: The aims of this study were 1) to identify cell lineage-specific single-stranded super-enhancers in MES and adrenergic (ADRN) cell lines, 2) to characterize the relationship between mesenchymal cell lineage and T-cell inflammation in neuroblastoma tumors, and 3) to describe the impacts of cell lineage and T-cell inflammation on overall survival (OS) and event-free survival (EFS).

Methods: We identified lineage-specific single-stranded super-enhancers to define ADRN and MES specific genes. Publicly available RNA-seq of diagnostic tumor biopsies was used in a Discovery (GSE49710) and Validation (TARGET NBL, dbGaP: phs000467.v21.p8) cohort. Using Gene Set Variation Analysis, each tumor was assigned a relative MES score and inflammation score. Survival was assessed using the Kaplan-Meier method, and differences were assessed by the log-rank test and 3-year point estimates of OS and event-free survival EFS.

Results: We identified 159 MES genes and 373 ADRN genes. Inflammation scores were correlated with relative MES scores (R=0.56, p<0.001 and R=0.38, p<0.001) and anticorrelated with MYCN-amplification (R=-0.29, p<0.001 and -0.18, p=0.03) in both cohorts. In the Discovery cohort, among patients with high-risk tumors (n=176), those with T-cell inflamed (TCI) tumors (n=74) had a trend towards improved OS compared to non-inflamed (NI) tumors (n=102) (74%; CI=64-85% versus 44%; CI=35-56%, p=0.05). Among patients with high-risk, MYCN-non-amplified tumors (n=84), those with TCI tumors (n=50) had superior OS to those with NI tumors (n=34) (87%; CI=78-97% versus 45%; CI=30-67%, p=0.03) which was confirmed in the Validation cohort (65%; CI=52-80% versus 37%; CI=20-66%, p=0.04). Patients with high-risk, MYCN-amplified tumors (n=92) had no difference in survival according to TCI or cell lineage.

Conclusion: High inflammation and relative mesenchymal scores were both correlated and associated with improved survival in patients with high-risk, MYCN-non-amplified but not MYCN-amplified neuroblastoma. These findings have implications for risk stratification and treatment of high-risk neuroblastoma.

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The immunocytokine FAP-IL-2v enhances anti-neuroblastoma efficacy of the anti-GD2 antibody dinutuximab beta

Siebert N[1], Leopold J[1], Zumpe M[1], Troschke-Meurer S[1], Biskupski S[1], Zikoridse A[1], Lode H[1]
[1]*University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany*

Treatment of high-risk neuroblastoma (NB) patients with the anti-GD2 antibody (Ab) dinutuximab beta (DB) improved survival by 15%. Ab-dependent cellular cytotoxicity (ADCC) is the major mechanism of action and is primarily mediated by NK cells. Since IL-2 co-treatment did not show a therapeutic benefit but strongly induced Treg, we investigated here a DB-based immunotherapy combined with the immunocytokine FAP-IL-2v, that comprises a fibroblast activation protein α (FAP)-specific Ab linked to a mutated IL-2 variant (IL-2v) with abolished binding to the high-affinity IL-2 receptor, thus stimulating NK cells without induction of Treg. Effects of FAP-IL-2v on NK cells, Treg and ADCC mediated by DB as well as FAP expression in NB were investigated by flow cytometry, calcein-AM-based cytotoxicity assay and RT-PCR analysis. Moreover, impact of soluble factors released from tumor cells on FAP expression by primary fibroblasts was assessed. Finally, a combined immunotherapy with DB and FAP-IL-2v was evaluated using a resistant syngeneic murine NB model.
Incubation of leukocytes with FAP-IL-2v enhanced DB-specific ADCC without induction of Treg. FAP expression on NB cells and myeloid-derived suppressor cells (MDSC) in tumor tissue was identified. A tumor cell-dependent enhancement of FAP expression by primary fibroblasts was demonstrated. Combination with DB and FAP-IL-2v resulted in reduced tumor growth and improved survival. Analysis of tumor tissue revealed increased NK- and cytotoxic T cell numbers and reduced Treg compared to controls.
Our data show that FAP-IL2v is a potent immunocytokine that augments the efficacy of DB against NB providing a promising alternative to IL-2.

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Dinutuximab beta combined with chemotherapy in patients with relapsed or refractory neuroblastoma

Wieczorek A[1,2], Zaniewska-Tekieli A[2], Ehlert K[3], Pawinska-Wasikowska K[1,2], Balwierz W[1,2], Lode H[3]
[1]Department of Pediatric Oncology and Hematology, Jagiellonian University Medical College, Krakow, Poland, [2]Department of Pediatric Oncology and Hematology, University Children's Hospital, Krakow, Poland, [3]Pediatric Hematology and Oncology, University Medicine Greifswald, Greifswald, Germany

Background. Prognosis in children with refractory and relapsed (RR) high-risk neuroblastoma (HR-NBL) is poor. Chemotherapy combined with anti-GD2 antibodies has previously been shown to increase response and survival rates.

Methods. We carried out a retrospective review of 25 patients with RR HR-NBL, who received dinutuximab beta (DB) immunotherapy combined with temozolomide and irinotecan (TEMIRI) as part of compassionate use programs at one of two centers, Krakow, Poland, and Greifswald, Germany, between December 2017 and October 2021. The data cut-off was January 31, 2022. DB was given as continuous long-term infusion of 10 mg/m2/day on days 2–6 of each 21-day cycle. Chemotherapy was given on days 1–5 of each cycle. Patients received 1–10 cycles (mean 3).

Results. The median age of the patients was 35.1 months (range 6.7–99.7), 11 (44%) patients had MYCN amplification, and 16 (64%) had unfavorable histology. The majority of patients (84%) had metastatic disease at diagnosis. Twenty patients (80%) received chemoimmunotherapy for relapsed and five (20%) for refractory disease. Objective response rate was 64%, with 32% of patients achieving a complete response. Fourteen of our 25 patients had previously received dinutuximab beta, four of whom achieved complete and six partial response (objective response rate 71%). Median OS and PFS from the initiation of chemoimmunotherapy were 10.3 months (range 0.7–43.0) and 6.3 months (range 0.2–37.0), respectively. The 1- and 3-year OS was 47% and 35%, and 1- and 3-year PFS 48% and 36%, respectively. One-year OS and PFS rates were significantly better in patients who achieved CR or PR than in those who did not (1-year OS 77% vs 11%, p=0.0001; 1-year PFS 63% vs 22%, p=0.003) No severe or unexpected toxicities were observed. Conclusions. Our findings show that combination therapy with DB and TEMIRI in patients with relapsed or refractory neuroblastoma is feasible and well tolerated, with encouraging response rates and survival data. No severe side effects were observed in heavily pre-treated patients, including those who had previously been treated with DB. This chemoimmunotherapy combination is a promising treatment option for refractory and relapsed NBL and should be further explored in clinical studies.

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Antitumor Activity of the Investigational B7-H3 Antibody-drug Conjugate, Vobramitamab Duocarmazine, in Preclinical Neuroblastoma Models

Brignole C[1], Calarco E[1], Bensa V[1], Giusto E[1], Perri P[1], Ciampi E[1], Corrias M[1], Astigiano S[2], Cilli M[2], Loo D[3], Bonvini E[3], Pastorino F[1], Ponzoni M[1]

[1]Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genoa, Italy, [2]Animal Facility, IRCSS Ospedale Policlinico San Martino, Genoa, Italy, [3]MacroGenics, Rockville, MD & Brisbane, CA, USA

Background: Vobramitamab duocarmazine (MGC018) is an antibody-drug conjugate (ADC) composed of a humanized anti-B7 H3 monoclonal antibody conjugated via a cleavable to a synthetic DNA alkylating agent (vc-seco-duocarmycin-hydroxybenzamide-azaindole). Vobramitamab has shown preliminary clinical activity in B7-H3-expressing tumors. Owing to its expression in several childhood tumors, B7-H3 is a potential target for pediatric cancers, including neuroblastoma (NB).
Aims: To investigate on the effectiveness of Vobramitamab against NB in vitro and in vivo.

Methods: B7-H3 expression was evaluated by flow-cytometry in a panel of human NB cell lines. Cytotoxicity was evaluated in monolayer and in multicellular tumor spheroid models. Apoptotic cell death was investigated by the Annexin V staining. Pseudometastatic, orthotopic, and resected mouse NB models were developed via tumor cells tail vein injection, implantation in the adrenal gland, or implantation followed by surgical resection of the primary tumor mass, respectively, to mimic disease conditions related to circulating tumor cells and metastases, primary tumor growth, and minimal residual disease.

Results: All cell lines expressed cell surface B7-H3 in a unimodal fashion. Vobramitamab was cytotoxic in a dose- and time-dependent manner against all NB cell lines. Vobramitamab was inactive against a murine NB cell line (NX-S2) that did not express human B7-H3; however, NX-S2 cells were killed in the presence of vobramitamab when co-cultured with human B7-H3-expressing cells, demonstrating by-stander activity. In the pseudometastatic and in five orthotopic NB mouse models, weekly iv treatments with 1 mg/kg vobramitamab for 3 weeks resulted in delayed tumor growth and increased survival rates compared to animals treated with an irrelevant (anti-CD20) duocarmycin-ADC or an anti-GD2 antibody (dinutuximab beta). A 4-week treatment (1 course) further ameliorated vobramitamab antitumor effect in both orthotopic and resected NB models and increased the survival of NB-bearing mice co-treated with TOpotecan-TEMozolomide, the standard-of-care therapy for NB relapsed disease. In the orthotopic model, tumor relapse was temporarily or completely arrested by 2 or 3 courses of vobramitamab, respectively. Vobramitamab treatment was not associated with body weight loss, hematological toxicity, or clinical chemistry abnormalities.

Conclusion: Vobramitamab exerts relevant antitumor activity in pre-clinical NB models and may represent a potential candidate for future clinical translation.

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Naxitamab combined with granulocyte-macrophage colony-stimulating factor as end-induction consolidation for high-risk neuroblastoma patients in first complete remission.

Mora J[1], Castañeda A[1], Mañe S[1], Krauel L[1], Chamorro S[1], Varo A[1], Muñoz J[1], Simao M[1], Gorostegui M[1], Garraus M[1], Santa-Maria V[1], Larrosa C[1], Perez-Jaume S[1], Lavarino C[1]
[1]Pediatric Cancer Center Barcelona. Oncology, Barcelona, Spain

Background: Naxitamab is a humanized anti-GD2 monoclonal antibody approved for treatment of bone/bone marrow (BM) refractory high-risk neuroblastoma (HR-NB). Compassionate use permitted treatment in complete remission (CR).
Aims: We report the survival, toxicity, and relapse pattern of patients in first CR treated with naxitamab and sargramostim (GM-CSF).

Methods: From June 2017 until April 2022, 82 patients (pts) were treated with 5 cycles of immunotherapy. Treatment consisted of subcutaneous (SC) GM-CSF for 5 days at 250 µg/m2/day, followed by naxitamab + SC GM-CSF for 5 days at 500 µg/m2/day. Naxitamab was infused over 30-60 minutes at 3 mg/kg/day, days 1, 3, and 5, outpatient.

Results: At diagnosis all but one patient were >18 months and had stage M; 21 (25.6%) were MYCN-amplified (A); median age 3 years (range, 0.6 – 13). Before immunotherapy, all pts had received at least 5 cycles of induction chemotherapy and surgery; 11 (13.4%) had received high-dose chemotherapy and autologous stem cell transplant (ASCT); 26 (31.7%) radiotherapy; and 12 (14.6%) had detectable minimal residual disease (MRD) in the BM. Median age at treatment initiation is 3.9 (range, 1.4 – 13.8). Fifteen (18%) pts did not complete therapy: four experienced grade 4 toxicities (3 apnea and one stroke) and 11 early relapse. Overall, thirty-one (37.8%) pts have relapsed including 6/21 (28.6%) MYCN A and 25/61 (41%) MYCN non-A. The pattern of relapse is predominantly (77.4%) of an isolated organ, mainly bone (14/31) and soft tissue (8/31). Three (3.6%) pts relapsed in the CNS (all MYCN A). Five-year event-free survival (EFS) and overall survival (OS) from time of immunotherapy were 57.9% (71.4% for MYCN A) 95% CI = (47.2, 70.9%); and 78.6% (81% for MYCN A) 95% CI = (68.7%, 89.8%), respectively. Prior ASCT (log-rank test, p=0.037) and detectable MRD (log-rank test, p=0.0011. Univariate Cox model HR=3.29) were significant predictors of EFS but not OS. No variable was associated with OS.

Conclusion: Consolidation with naxitamab and GM-CSF resulted in excellent survival rates for HR-NB patients after end-induction complete response. These results strongly support the revision of the role of ASCT in this subgroup of HR-NB.

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Chemotherapy used in induction for high-risk neuroblastoma improves efficacy of anti-GD2 therapy with dinutuximab beta in preclinical spheroid models.

Troschke-Meurer S[1], Zumpe M[1], Meißner L[1], Siebert N[1], Grabarczyk P[2], Forkel H[2], Lode H[1]
[1]*University Medicine Greifswald, Department of Pediatric Oncology and Hematology, Greifswald, Deutschland,*
[2]*University Medicine Greifswald, Internal Medicine Clinic C, Greifswald, Deutschland*

Background
GD2-directed immunotherapy with dinutuximab beta (DB) improved outcome of patients (pts) with high-risk neuroblastoma (NB) when used post consolidation.

Aims
Here, we investigated immunomodulatory effects of induction chemotherapy and its anti-tumor efficacy combined with DB-based immunotherapy.

Methods
Chemotherapeutics used during induction treatment of patients with high-risk NB and dinutuximab beta were investigated using concentrations that were achieved in paediatric Phase II clinical trials. We analyzed the impact of vincristine, etoposide, carboplatin, cisplatin and cyclophosphamide on checkpoint- (PD-L1, Gal9, CD86, CD112, CD115), and stress ligand (B7-H6, ULBP2/5/6, ULBP-3, MICA/B) abundance on tumor cells and on activation markers on NK cells (NKp30, NKp44, NKp46, NKG2 and CD226) using flow cytometry. Long-term cytotoxicity effects of chemoimmunotherapy was analyzed in a live-cell assay with spheroids derived of GD2 positive NB cell lines (LAN-1; CHLA 20; CHLA 136) expressing fluorescent near-infrared protein iRFP680 (NIR) using the Incucyte® live-cell analysis system for a time span of 10 days. Chemotherapeutic effects on NK cell activity was measured with a CD107a/IFN-γ assay and by deletion of B7-H6 using CRISPR/CAS9 method followed by viability analysis.

Results
Chemotherapeutics combined with DB in the presence of peripheral mononuclear cells mediating antibody-dependent cellular cytotoxicity (ADCC) significantly improved cytotoxic efficacy up to 17-fold compared to the monotherapies. Etoposide and cisplatin had the strongest effects on surface abundance of B7-H6, ULBPs and MICA/B activating stress ligands. However, most checkpoint ligands were also strongly elevated during chemotherapy. Despite of this, chemoimmunotherapy significantly improved cytotoxic efficacy against all cell lines compared to the monotherapies. In LAN-1 etoposide combined with ADCC showed the highest anti-tumor effects of chemoimmunotherapy compared to ADCC and etoposide alone (17.6 and 8.3-fold decrease in viability, respectively). Knock-out rescue experiments showed that ADCC was partially dependent on B7-H6 in CHLA-20. However, CD107a/IFN-γ assay revealed no additional activation of NK cells after chemotherapy suggesting an inhibitory role of observed increased checkpoint ligand abundance during chemotherapy.

Conclusions
The synergistic effect of DB with chemotherapeutics may therefore primarily attributed to the combined toxicity of the cytotoxic compounds and ADCC. Our data support further clinical evaluation of using DB during frontline induction therapy.

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Pilot clinical trial of DNA vaccination against neuroblastoma

Praliaskouskaya I[1], Meleshko A[1], Konoplya N[2]
[1]Belarusian Research Center for Pediatric Oncology, Hematology and Immunology, Minsk, Belarus, [2]N.N. Alexandrov national cancer center of Belarus, Minsk, Belarus

Background
Effective treatment of high risk neuroblastoma (NB) remains a serious problem for pediatric oncology. A promising therapy option for this group is immunotherapy, including antitumor vaccination.
Aims
Assessment of safety and immunogenicity, preliminary outcome.

Methods/Materials
Patients in remission for 2-4 months after completion of relapse treatment were included.
Patients received the vaccine against three NB-associated antigens of 6 candidates: tyrosine hydroxylase, Phox2B, Survivin, MAGE-A1, MAGE-A3, PRAME, fused to potato virus X coat protein (PVXCP) gene. IHC and RQ-PCR confirmation determines the choice of antigen for each patient.
Intramuscular DNA-PEI injection (500µg) was boosted with oral attenuated Salmonella enterica vaccine carrier (1010 CFU). The vaccination course included three administrations weekly for each antigen.
Patients received cyclophosphamide 300 mg/m2 three days and lenalidomide 25 mg/day starting 7 days before the first vaccination, propranolol and celecoxib according to the metronome therapy regimen.
Monitoring included MRD assessment by RQ-PCR; circulating tumor cells; production of INF-γ in ELISpot test.

Results
The protocols received ethical approval January 9, 2019. To date seven patients are currently enrolled and six have completed the full course. Patients tolerated the vaccine well without adverse symptoms.
Four patients (B2, K3, M5, S6) are free of tumor by MRT/MIBG scintigraphy and MRD PCR-negative in BM except one (S6). 6 months after vaccination, patient B1 had isolated CNS relapse. M4 – had a residual MIBG-positive site in paravertebral area. Both foci of the tumor were completely surgically removed and CD8 infiltration was observed. Immune response was negative for patient K3, all other patients had definite CD4/CD8 cellular immune response against the PVXCP/antigens peptide library. Three patients were re-vaccinated with an increased immune response.

We compared event-free survival after relapse of patients with relapsed neuroblastoma who received chemotherapy +autoHSCT+/- MIBG-therapy plus vaccine therapy (group 1, n=6) and patients who received chemotherapy +autoHSCT +/- MIBG-therapy (group 2, n=17) (groups were comparable by age of patients, stage of disease, received therapy) on September 1, 2022. EFS in group 1 was 82+18% vs. 29+11% in group 2 (p=0.03).

Conclusion: DNA vaccination against neuroblastoma is well tolerated by patients and improves response to second-line treatment.

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Adverse Reaction (AR) Reporting in Naxitamab Chinese Early Access (EA) Treatment Administered by Children's Hospital of Fudan University

Qian X[1], Zhang D, Li K[1], Chen W[1], Zhuang P[1], Wang H[1], Lei Z[2], Li Y[2], Chen M[3], Eldridge J[4], Dong K[1], Zhai X[1]
[1]Children's Hospital of Fudan University, Shanghai, China, [2]Hainan Branch of Children's Hospital of Fudan University, Haikou, China, [3]Sciclone Pharmaceutical (China) Co., Ltd., Shanghai, China, [4]Y-mAbs Therapeutics, New York, United States

Background
Naxitamab is a humanized GD2-binding monoclonal antibody conditionally approved in China (30-Nov-2022) in combination with granulocyte-macrophage colony-stimulating-factor (GM-CSF), for the treatment of pediatric patients (1-year and older) and adult patients with refractory/relapsed high-risk neuroblastoma in the bone or bone marrow who have demonstrated a partial response, minor response, or stable disease to prior therapy. Naxitamab EA was started prior to approval by physicians from Children's Hospital of Fudan University at the Weilai Hospital in Boao, China in August 2021.

Aims
To characterize the patient demographics and safety profile of naxitamab over multiple treatment cycles in EA treatment in China.

Methods
A review of the Y-mAbs Argus Global Safety Database for adverse reactions (AR) reported from patients treated under EA in China from August 1, 2021, to July 31, 2022.

Results
At data cutoff, 41 patients had received a total of 150 treatment cycles (451 infusions) of naxitamab. The median age of patients was 3 years (range: 1-7 years), median weight was 13kg (range: 7-24kg), and 26 patients were female (63%). The following percent of patients had completed cycles 1, 2, 3, 4, and 5, respectively: 100%, 85%, 66%, 51%, and 42%. The median number of cycles completed was 3 and 13 patients (32%) were still in ongoing naxitamab treatment. During cycles 1, 2, 3, 4, and 5, patients experienced the following, respectively: any AR (68%, 63%, 63%, 55%, 47%), serious AR (24%, 31%, 7%, 0%, 0%). The rolling reporting rates for all AR, serious AR, hypotension, flushing, cough, and pain, respectively, were 8.7, 0.9, 1.4, 0.7, 0.6, and 0.5 AR per cycle after 3 months of EA and 4.6, 0.3, 1.0, 0.5, 0.3, and 0.2 AR per cycle after 12 months of EA.

Conclusion
During the first 12 months of EA treatment in China, a reduction in the rate of AR, including serious AR, hypotension, flushing, cough, and pain was observed as more patients were initiated and proceeded to later treatment cycles. Decrease in AR rates over time with continued naxitamab treatment may be attributed to clinician's increased knowledge of AR management and hands-on experience with the naxitamab-treated patient.

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Effect of Prior Anti-GD2 Immunotherapy on Efficacy and Safety for Patients Treated with Naxitamab in Trial 201

Kushner B[1], Chan G[2], Morgenstern D[3], Amoroso L[4], Nysom K[5], Faber J[6], Wingerter A[6], Bear M[7], Rubio San Simon A[8], Martinez De Las Heras B[9], Tornøe K[10], Settergren Sørensen P[10], Mora J[11]
[1]Memorial Sloan Kettering Cancer Center, New York, United States, [2]Queen Mary Hospital & Hong Kong Children's Hospital, The University of Hong Kong, Pokfulam, Hong Kong, [3]The Hospital for Sick Children, Toronto, Canada, [4]IRCCS Istituto Giannina Gaslini, Genoa, Italy, [5]Department of Paediatrics and Adolescent Medicine, Rigshospitalet, Copenhagen, Denmark, [6]Center for Pediatric and Adolescent Medicine, University Medical Center of the Johannes Gutenberg University, Mainz, Germany, [7]Riley Hospital for Children, Indianapolis, United States, [8]Hospital Infantil Universitario Niño Jesús, Madrid, Spain, [9]Hospital Universitario y Politecnico La Fe, Valencia, Spain, [10]Y-mAbs A/S, Hørsholm, Denmark, [11]Pediatric Cancer Center Barcelona, Hospital Sant Joan de Déu, Barcelona , Spain

Background:
GD2-targeted monoclonal antibodies (mAb) have shown efficacy in neuroblastoma. Naxitamab is a humanized GD2-binding mAb approved by the FDA in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF), for the treatment of pediatric patients (1-year and older) and adult patients with refractory/relapsed (R/R) high-risk (HR) neuroblastoma (NB) in the bone or bone marrow (BM) who have demonstrated a partial response, minor response, or stable disease to prior therapy. Assessing efficacy and safety of naxitamab in patients previously treated with GD2-binding mAbs is warranted.

Methods:
Trial 201 (ongoing phase II, NCT03363373) evaluating naxitamab+GM-CSF in patients with R/R HR-NB in bone/BM. Naxitamab (3mg/kg/dose) was administered intravenously (30-60 min) (Days 1,3,5) with subcutaneous GM-CSF (Days -4 to 5); cycles were repeated monthly. Efficacy endpoints: overall response rate (ORR) and complete response (CR). Efficacy was evaluated by independent pathology and radiology review per revised International Neuroblastoma Response Criteria. Here we report efficacy and safety of naxitamab+GM-CSF in patients previously treated with GD2-binding mAbs.

Results:
The preplanned interim analysis (data cut-off: 31-Dec-2021) evaluated efficacy (n=52) and safety (n=74) of naxitamab+GM-CSF. Outcomes in the efficacy cohort showed ORR 50% with CR 38%. Baseline characteristics included history of relapse in 92% of patients with prior anti-GD2 therapy and 36% of patients without prior GD2 therapy. Patients who received prior anti-GD2 therapy (n=13) achieved ORR 31% [95%CI: 9%-61%] and CR 23% [95%CI: 5%-54%]. Patients without prior anti-GD2 therapy (n=39) had ORR 56% [95%CI: 40%-72%] and CR 44% [95%CI: 28%- 60%]. The frequency of naxitamab-related SAEs was 40% in patients with prior anti-GD2 therapy and 26% in patients without prior anti-GD2 therapy. Grade ≥3-related adverse events occurred in 85% of patients with prior exposure and 78% of patients without prior exposure; patients with prior exposure had higher incidence of hypotension, pain, hypoxia, and abdominal pain and had increased alanine aminotransferase.

Conclusion:
The preplanned interim data analyses support the efficacy, and document an acceptable safety profile, of naxitamab+GM-CSF in patients previously treated with GD2-binding mAbs. Baseline disease characteristics (prior relapse) may have contributed to the outcomes.

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Chemotherapy resistant neuroblastoma cells with stem-like properties are characterized by increased GD2 surface abundance

Meissner L[1], Troschke-Meurer S[1], Zumpe M[1], Siebert N[1], Maletzki C[2], Lode H[1]
[1]Department of Pediatric Oncology and Hematology, University Medicine Greifswald,, Greifswald, Germany, [2]Department of Medicine, Clinic III - Hematology, Oncology, Palliative Medicine, Rostock University Medical Center, Rostock, Germany

Background:
Cancer stem-like cells (CSC) have been reported to be GD2-positive and highly resistant to chemotherapy. Therefore, we investigated whether chemotherapy can induce epithelial-to-mesenchymal transition (EMT) that leads to enriched CSC frequency and increased GD2 expression in neuroblastoma.

Methods:
We investigated the impact of chemotherapeutics used in high-risk neuroblastoma induction regimens on EMT marker (E-Cadherin, N-Cadherin, Vimentin) and CSC marker (nestin, CD44, SOX2) as well as GD2 using flow cytometry. LAN-1 cells were treated with carboplatin, cisplatin, etoposide, and vincristine for 24 h with pharmacologically relevant concentrations and cultivated for another 72 h. The correlation between CSC/EMT marker and GD2 expression was analyzed. The impact of cisplatin on relative gene expression (2-ΔΔCT, endogenous control: UBC) of transcription factors FOXC2 and NFκB, known to bind promotor of GD2 synthase, and GD2 synthase was determined using real-time PCR. Anti-tumor toxicity of chemotherapy in combination with anti-GD2 antibody dinutuximab beta and PBMCs (antibody-dependent cellular cytotoxicity, ADCC) was assessed with a long-term live cell viability spheroid assay using near-infrared protein iRFP680 expressing LAN-1 cells.

Results:
Carboplatin, cisplatin, etoposide, and vincristine induced EMT, shown by strongly increased frequency of N-cadherin-positive cells compared to medium control (73±2%, 68±2%, 53±5%, 42±3%, and 32±5%, respectively). Accordingly, the frequency of nestin-positive CSC was also increased compared to control (68±5%, 54±3%, 48±4%, 36±2% and 25±4%, respectively). Importantly, after cisplatin treatment, N-cadherin-positive cells showed an up to 2.3-fold higher GD2-abundance compared to N-cadherin-negative cells (13402±2302 gMFI and 5879±756 gMFI, respectively, p =0.0015). This was also observed in nestin-positive but not CD44- and SOX2-positive cells. In line with the increased frequency of GD2high cells, elevated expression of FOXC2 and NFκB was observed (2-ΔΔCT were 5.9±1.3 and 10.9±6.3, respectively). We also observed an increased expression of GD2 synthase (2-ΔΔCT = 3.4±1.4). Finally, ADCC was significantly improved when combined with carboplatin, cisplatin, etoposide, or vincristine (3.1-, 3.3-, 6.4-, and 2.7-fold decrease in viability compared to chemotherapy and PBMCs alone, p = 0.0075 and p = 0.0059, p = 0.0178 and p = 0.0337, respectively).

conclusion:
Chemotherapy enriches cancer-stem-like cells, that are characterized by high GD2 abundance that may be addressed with an anti-GD2 treatment.

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Long term follow-up of treatment with anti-GD2 antibody (ch14.18/SP2/0) in children with metastatic high-risk neuroblastoma: Long-term toxicity and quality-of-life

Flaadt T[1], Rehm J[1], Simon T[2], Hero B[2], Klingebiel T[3], Lode H[4], Ebinger M[1], Erdmann F[5], Grabow D[5], Greil J[6], Bader P[3], Schlegel P[7], Eyrich M[7], Lang P[1], Handgretinger R[1]

[1]Department of Hematology and Oncology, University Children's Hospital, Eberhard Karls University Tuebingen, Tuebingen, Germany, [2]Department of Pediatric Oncology and Hematology, University Hospital, University of Cologne, Cologne, Germany, [3]Department of Children and Adolescents, University Hospital, Goethe University, Frankfurt, Germany, [4]Department of Pediatric Hematology and Oncology, University Medicine Greifswald, Greifswald, Germany, [5]Division of Childhood Cancer Epidemiology, German Childhood Cancer Registry, Institute of Medical Biostatistics, Epidemiology and Informatics, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany, [6]Department of Pediatric Oncology, Hematology and Immunology, University Hospital Heidelberg, Heidelberg, Germany, [7]Department of Pediatric Hematology and Oncology, University Children's Hospital, University Medical Center, Wuerzburg, Germany

Background/Aims:
Treatment with ch14.18/SP2/0 significantly improves prognosis in high-risk neuroblastoma (HR-NB) patients. Studies assessing long-term outcomes and sequelae and quality of life are lacking.

Methods:
Between 1989 and 2002, a cohort of 65 children (aged 0.3-17.7 years, median 3.1 years) received ch14.18/SP2/0 for primary HR-NB (INSS-stage 4: 64 patients, 1 stage 3 with MYCN-amplification) at Tuebingen children's hospital. All patients received chemotherapy according to German NB90/NB97-protocol, 56 received myeloablative therapy + autologous stem cell transplantation (ASCT). In post-consolidation, four to nine cycles of ch14.18/SP2/0 (since 2015 named dinutuximab) were administered, 100mg/m²/cycle.

We evaluated overall and event-free survival (OS/EFS), risk factors and conducted questionnaire-based surveys in long-term survivors to evaluate the proportion of severe toxicity and quality-of-life (EORTC-QLQ-C30).

Results:
OS and EFS, 25 years after initial diagnosis, were 49% and 47%; 32 patients died, causes were progression/relapse (n=27), secondary neoplasm (myelodysplastic syndrome n=3; Adenocarcinoma n=1; brain tumor n=1)

Thirty-three long-term survivors were identified with a median follow-up of 24.6 years (15.5-32.2 years). Long-term survivors underwent ASCT (n=23), tandem-transplant (n=2), allogenic SCT (n=1), radiotherapy (n=13), and/or I131-mIBG-therapy (n=21).
Twenty-five patients were evaluated for QoL and long-term effects. In all survivors, long-term sequelae occurred: Ototoxicity in 19/25 (76%), cardiac toxicity in 7/25 (28%), nonreproductive endocrine toxicity in 19/25 (76%), renal toxicity 4/25 (16%). Height/weight z-scores were less than -1.7 in 3/25 (12%). Seven patients developed autoimmune diseases: Hashimoto thyroiditis (2), diabetes mellitus (1), inflammatory bowel disease (3), chronic thrombocytopenia (1, meanwhile resolved), no neuro-immunological long-term sequelae occurred.
Three long-term survivors developed secondary malignancies: MPNST, colon cancer, basalioma.

All QoL-scores were comparable to those of the general population, with women showing slightly lower functioning and higher symptom levels. All patients attained secondary education, eight graduated from university. Three patients have children of their own.

Conclusions:
Multimodal treatment, including ch14.18/SP2/0, can induce long-term survival in HR-NB patients. EORTC-QLQ-C30 scales were comparable to the general population. The high incidence of long-term side effects underlines importance of careful follow-up. With the limitation of non-randomization, no long-term sequelae were attributable to ch14.18/SP2/0. Nevertheless, the relatively high number of autoimmune diseases should be investigated in further long-term follow-up studies.

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Safety and efficacy of naxitamab plus modified GM-CSF dosing in treating primary refractory or high-risk neuroblastoma in first complete remission

Kushner B[1], Modak S[1], Basu E[1], Iglesias Cardenas F[1], Kramer K[1], Roberts S[1,2], Cheung N[1]
[1]Memorial Sloan Kettering Cancer Center, Department of Pediatrics, New York, United States, [2]Doernbecher Children's Hospital, Portland, United States

Background: In an international trial, the humanized anti-GD2 antibody naxitamab+GM-CSF was effective against chemo-resistant HR-NB, leading to approval by the Food and Drug Administration. We subsequently modified GM-CSF dosing based on a shortened priming period and pharmacokinetics showing prolonged T½ of naxitamab.

Aims: To describe safety and efficacy of naxitamab plus GM-CSF administered by a novel schedule in HR-NB patients treated in 1st CR (Group 1) or with primary refractory disease in bones and/or bone marrow (BM) (Group 2).

Methods: Immunotherapy was administered q1-2 months up to 5 cycles in Group 1 and >5 cycles after major responses in Group 2. Naxitamab was infused intravenously (30-90") on days 1/Monday-3/Wednesday-5/Friday (i.e., 3 doses/cycle), 3mg/kg/infusion (9mg/kg/cycle, i.e., ~270mg/m2/cycle). Previously, priming doses of GM-CSF 250µg/m2/day were subcutaneously-administered x5 (days -4 to 0), followed by a step-up to 500µg/m2/day x5 (days 1-to-5), but now priming doses were x3 days (days -2 to 0 [Friday-Saturday-Sunday]) and stepped-up dosing was x7 (days 1-to-7 [Monday-to-Sunday]), i.e., through 2 days after the last dose of naxitamab. Patients did not receive isotretinoin. Follow-up was from start of naxitamab.

Results: From 2/22/2021-to-12/15/22, 59 patients (32 Group 1, 27 Group 2) received 1-10 (median 5) cycles, total 250. No unexpected toxicities occurred. No patient had to discontinue GM-CSF. Treatment was outpatient, except 3 patients with hypertension. Cycles were aborted because of viral symptoms (n=3), suspected small bowel obstruction (n=3), hypoxia (n=2), and hyperbilirubinemia (n=1). Four patients received only ½ of dose 1 in cycle 1 because of agitation or hypotension. In Group 1, 4/32 patients relapsed at 4-5 months while 28 patients remain in CR at 1+-to-22+ (median 15+) months. In Group 2, disease sites and responses were: BM alone (n=7): CR in 6 (1 later relapsed); MIBG alone (Curie scores 1-11) (n=8): 1 partial response, 1 progressive disease, 2 stable (1 later relapsed), 4 non-evaluable because of radiotherapy; MIBG (Curie scores 1-26) and BM(+) (n=12): 7 CR (3 later relapsed), 4 PR, 1 stable disease (subsequently progressed).

Conclusions: This large experience confirms that the new schedule is safe and, despite short follow-up, appears as effective as the prior schedule.

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Stage 4N neuroblastoma before and during the era of anti-GD2 immunotherapy

Kushner B[1], Modak S[1], LaQuaglia M[2], Basu E[1], Gerstle J[2], Iglesias Cardenas F[1], Kramer K[1], Roberts S[1,3], Wolden S[4], Cheung N[1]

[1]Memorial Sloan Kettering Cancer Center, Department of Pediatrics, New York, United States, [2]Memorial Sloan Kettering Cancer Center, Department of Surgery, New York, United States, [3]Doernbecher Children's Hospital, Portland, United States, [4]Memorial Sloan Kettering Cancer Center, Department of Radiation Oncology, New York, United States

Background: Patients with stage 4N neuroblastoma (distant metastases limited to lymph nodes) stand out as virtually the only survivors of high-risk neuroblastoma (HR-NB) before myeloablative therapy (MAT) and immunotherapy with anti-GD2 monoclonal antibodies (mAb) became standard. No report presents results with contemporary treatment programs for 4N. The only large study to date (from the International NB Risk Group) analyzed patients diagnosed 1990-2002, found 4N to have significantly better event-free (EFS) and overall survival (OS) compared to other stage 4 patients, and recommended considering less intensive therapy for 4N.

Aims: To analyze our large experience to fill the gap in the literature on treatment of 4N, namely, results with contemporary multi-modality therapy including MAT and anti-GD2 mAbs.

Results: All 48 pediatric 4N patients (<18 years old) treated 1985-2021 at our center were reviewed. Biological features included segmental chromosomal aberrations in 21/21 MYCN-non-amplified patients, mutations of ALK in 4/22 (18%), ATRX in 4/16 (25%), and TERT in 4/12 (33%) patients. Among 33 MYCN-non-amplified high-risk patients (>18 months old), 19 are relapse-free 1.6+-to-36.9+ (median 12.2+) years post-diagnosis, including 13 without prior MAT and 3 with no mAb, while 14 patients (7 without prior immunotherapy) relapsed (all in soft tissue initially). Eleven of the latter died, including 3 who developed late osteomedullary metastases after 3-5 relapses in soft tissue alone. Of 13 MYCN-amplified 4N patients, 6 are relapse-free 4.5+-to-25.8+ (median 11.7+) post-diagnosis (all received mAbs; 3 underwent MAT) and 3 are in 2nd remission 4.4+-to-21.1+ years post-relapse (all soft tissue). For all high-risk 4N patients, 5/10-year EFS was 55%/52%, and OS was 76%/64%. MAT was not prognostic. The 2 patients with intermediate-risk 4N (14 months old) are relapse-free 7+ years post-resection of primary tumors; distant disease spontaneously regressed.

Conclusions: The natural history of 4N is marked by NB confined to soft tissue without early relapse in bones or bone marrow, sites where mAbs have proven efficacy. These findings plus the possibility of survival without MAT and/or mAb immunotherapy, as seen elsewhere and at our center, support consideration of treatment reduction for selected MYCN-non-amplified 4N patients.

P230

High-dose naxitamab plus stepped-up dosing of GM-CSF for patients with high-risk neuroblastoma in first complete remission: A phase II trial

Kushner B[1], Modak S[1], Mauguen A[2], Basu E[1], Roberts S[1,3], Cheung I[1], Cheung N[1]

[1]Memorial Sloan Kettering Cancer Center, Department of Pediatrics, New York, United States, [2]Memorial Sloan Kettering Cancer Center, Department of Epidemiology and Biostatistics, New York, United States, [3]Doernbecher Children's Hospital, Portlan, United States

Background: Naxitamab is a humanized form of the murine anti-GD2 monoclonal antibody (MoAb) 3F8. In an international trial, naxitamab+GM-CSF (administered outpatient) was effective against chemo-resistant high-risk neuroblastoma (HR-NB), leading to approval by the Food and Drug Administration. We now report results with patients treated in 1st complete remission (CR).

Aims: The primary objective of the Memorial Sloan Kettering phase II protocol 16-1643 (Clinicaltrials.gov NCT03033303) was to assess relapse-free survival (RFS) of HR-NB patients in 1st CR treated with naxitamab+GM-CSF plus isotretinoin. HR-NB was defined as MYCN-amplified tumor (any stage) or metastatic disease diagnosed at age >18 months.

Methods: Cycles of immunotherapy were administered monthly up to 5 cycles and comprised: 1) subcutaneously-administered priming doses of GM-CSF 250µg/m2/day on days -4 to 0 (Wednesday-Sunday), followed by a step-up to 500µg/m2/day on days +1 to +5 (Monday-Friday), and 2) naxitamab infused intravenously (30-90") on days +1, +3, and +5 (Monday-Wednesday-Friday, i.e., 3 doses/cycle). Naxitamab was 3mg/kg/infusion (9mg/kg/cycle, i.e., ~270mg/m2/cycle). Oral isotretinoin started post-cycle 2 of immunotherapy and was 160mg/m2/day, x14 days/course, x6 courses.

Results: The 59 patients (enrolled 2/2017-7/2020) were 5.5 months – 17.8 years old (median 3.5 years) and 4 months – 3.8 years (median 7 months) post-diagnosis of stage 4 (n=53) or MYCN-amplified stage 3 (n=6) HR-NB. At 24 months, RFS was 73% and OS was 97%. 5/16 relapses were isolated in central nervous system. Although the study was underpowered to show statistical significance, better outcome was seen with stage 3, shorter time from diagnosis, no prior 2nd-line therapy, and previous MoAb treatment for refractory disease. Prior autologous stem-cell transplantation (ASCT) was not prognostic. 21/59 patients took no isotretinoin. After this trial, 50 patients received additional anti-NB therapy: vaccine (n=44), difluoromethylornithine (DFMO) (n=4), or vaccine followed by DFMO (n=2).

Conclusions: Naxitamab+GM-CSF is a good option for HR-NB patients in 1st CR, including those who did not undergo ASCT. Efforts to prevent CNS relapse are warranted.

P231

Apoptosis-Promoting Drugs in Combination with Chimeric Antigen Receptor Cell Therapies to Treat High-Risk Neuroblastoma

Hakoum M[1,2], Earl N[2], LaCasse E[2], McComb S[1,3], Beug S[1,2]

[1]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Canada, [2]Apoptosis Research Centre, Children's Hospital of Eastern Ontario Research Institute, Ottawa, Canada, [3]Human Health Therapeutics Research Centre, National Research Council of Canada, Ottawa, Canada

Background: Chimeric antigen receptor (CAR)-cell therapy has revolutionized the therapeutic landscape of many malignancies, including Neuroblastoma (NB). However, the limited persistence and efficacy of CAR cells, loss of tumour antigens, tumour resistance to death signals, and an immune suppressive microenvironment are all limitations that contribute to frequent tumour resistance and relapse. Capitalizing on the success of monoclonal antibody-based therapies targeting the glycolipid GD2 expressed on NB tumours in treating high-risk patients, we propose generating anti-GD2 CAR cells. The antitumor potential of these CAR cells could be further advanced through combination treatment modalities. Combining CAR-T cells and CAR-Natural Killer (NK) cells with apoptosis-inducing drugs can potentially exploit different cell death pathways and synergize to produce robust responses against resistant NB tumours. We hypothesize that anti-GD2 CAR cells, combined with these compounds, will provide a new effective treatment against high-risk NB.

Aims: Generate anti-GD2 primary CAR-T cells and anti-GD2 CAR-NK cells. Determine their efficacy in vitro and in vivo in combination with various apoptosis-sensitizing drugs.

Methods: We will assess the killing activity of these anti-GD2 CAR cells and control cells on a panel of GD2 negative and positive NB cell lines in the presence and absence of a panel of apoptosis-sensitizing compounds. We will perform coculture experiments and measure cell viability using the Incucyte, a live time-lapse microscope. We will use an orthotopic syngeneic model whereby a Luciferase tagged GD2 positive 9464D cell line will be used to evaluate the ability of murine-derived anti-GD2 CAR cells to eradicate tumours in combination with the best-identified apoptosis-sensitizing drug. Further tumour immunity and microenvironment analysis will be performed using flow cytometry and immunohistochemistry.

Significance: There is a high demand for novel combinatorial treatment regimens that offer better clinical outcomes for patients with high-risk NB. Treatment regimens using CAR cells may provide an effective strategy against NB-specific tumour antigens and, once combined with adjuvant agents, produce a significant and long-lasting immune response.

P232

Photoimmunotherapy using anti-GD2 antibody for neuroblastoma

ZHAO J[1,2], Ueki M[1], Nakajima K[3], Terashita Y[1], Hirabayashi S[1], Cho Y[1], Nakajima M[4], Ogawa M[3], Manabe A[1]
[1]Department of Pediatrics, Hokkaido University Hospital, Sapporo, Japan, [2]Department of Pediatric Dentistry, Hokkaido University Hospital, Sapporo, Japan, [3]Laboratory of Bioanalysis and Molecular Imaging, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan, [4]Department of Pediatric Oncology, National Cancer Center Hospital, Tokyo, Japan

Background
Photoimmunotherapy (PIT) is a novel cancer therapy that specifically kills cancer cells. In PIT, cancer cells were targeted by antibody-photoabsorber conjugates (APCs) specific for cancer antigens. When APCs bind to antigens and are irradiated with near-infrared light, they kill targeted cancer cells. PIT has been clinically applied to squamous cell carcinoma of the head and neck in Japan, but not to neuroblastoma. It is widely known that neuroblastoma expresses GD2 antigen, and immunotherapy using anti-GD2 antibodies has been conducted. Thus, PIT targeting GD2 would be an additional treatment option to patient who cannot be saved by conventional therapies.

Aims
To investigate whether PIT using anti-GD2 antibody can be applied to neuroblastoma, we conducted basic experiments in vitro and in vivo.

Methods
Anti-human Ganglioside GD2 monoclonal antibody (clone 3F8, a gift from Dr. Cheung) was conjugated with IR700. Human neuroblastoma cells expressing GD2 (SK-N-SH) were used. In vitro experiments, cell death induced by PIT was examined 1 hour following treatment by microscopy and flow cytometry (FCM). In microscopy, morphological changes in cells were observed, and calcein-AM and ethidium homodimer (EthD-1) were used to identify live and dead cells. In FCM, dead cells were labeled with propidium iodide and the percentage of labeled cells were evaluated. To perform in vivo experiments, SK-N-SH-Tumor-bearing mice were assigned to two groups: APC-injected/light-irradiated (PIT) group and non-treatment group. In the PIT group, mice were intravenously injected with APC (100 ug) and irradiated with 100 J/cm2 of light. The tumors were resected one day after treatment to evaluate therapeutic effects histologically.

Results
In vitro experiments, cell swelling and bleb formation were observed during PIT. These morphological changes of cells were consistent with previously reported cell death induced by PIT. Cells were labeled by calcein-AM before PIT, though they were labeled by EdhD-1 1 hour after PIT. Quantification using FCM also showed a significant increase in the percentage of dead cells. In xenograft experiments, destroyed tumor tissue was histologically confirmed only in the PIT group mice (n=2).

Conclusion
Our study suggested that photoimmunotherapy with anti-GD2 antibody is effective for neuroblastoma in vitro and in mouse xenograft model.

P234

Defining the mechanisms of B7-H3 overexpression and role in neuroblastoma metastasis and immune evasion

Wingrove C[1], Lindsay J[1], Kendsersky N[1], Torres-Rodriguez K[1], Maris J[1,2]
[1]Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, United States, [2]Division of Oncology and Center for Childhood Cancer Research; Children's Hospital of Philadelphia, Philadelphia, PA, United States

Background: B7-H3 is a type 1 transmembrane protein in the B7 family of immunoregulatory proteins and is highly expressed in neuroblastoma. In addition to being implicated in immuno-inhibition, B7-H3 may also mediate tumor migration and metastasis. Preclinical efficacy of several immunotherapeutic strategies directed toward B7-H3, including CAR-T cells and antibody drug conjugates, have been and will continue to be translated into several pediatric clinical trials. Therefore, it is critical to understand the oncogenic functions of B7-H3 and how its expression is regulated to anticipate mechanisms of anti-tumor efficacy and therapy resistance.

Aims: To characterize the role of B7-H3 in neuroblastoma cell growth, identify regulatory mechanisms of B7-H3 expression, and define the role of B7-H3 in neuroblastoma immune evasion.

Methods: We used CRISPR interference (CRISPRi) to deplete B7-H3 from neuroblastoma cell lines with varied genetic backgrounds (SK-N-SH, SK-N-AS, SK-N-DZ) and monitored their rate of cellular proliferation using the IncuCyte SX5 Live-Cell Analysis System. We treated IMR-5 with TNF-α (0-20ng/mL) for 48 hours and treated SK-N-FI and Be2 with the MYC(N)-MAX disrupter MYCi975 (10nM) for 7 days and measured B7-H3 expression in each cell line using immunoblotting. Finally, we performed T cell activation assays using CD3/CD28 antibody stimulation (1ug/mL and 5ug/mL respectively) in the presence of recombinant human B7-H3. TNF-α and IFN-y release was quantified using ELISAs and expression of T cell activation markers was determined using flow cytometry.

Results: Cellular proliferation was inhibited by B7-H3 depletion in SK-N-SH and SK-N-AS ($p<0.05$) but not in SK-N-DZ. IMR-5 cells showed upregulation of B7-H3 expression following TNF-α exposure in a dose-dependent fashion. Unexpectedly, MYCN depletion (88%) with MYCi975 led to significant upregulation of B7-H3. Finally, recombinant human B7-H3 mediated dose-dependent inhibition of T cell activation, TNF-α, and IFN-y release.

Conclusion: B7-H3 has both neuroblastoma cell-intrinsic and -extrinsic influences on cellular proliferation and immune evasion. Further definition of mechanisms promoting immune evasion and metastasis in neuroblastoma in immune competent isogenic preclinical models of neuroblastoma are planned and will be reported.

P235

Dual GD2 and PTK7 targeted γδ T cell immunotherapy for neuroblastoma

Jonus H[1], Lee J[1], Silva J[1], Spencer T[1], Goldsmith K[1]
[1]*Emory University School of Medicine, Department of Pediatrics, Atlanta, United States*

Background: Adoptive cell therapy (ACT) for neuroblastoma (NB) has primarily utilized autologous products of alpha beta (αβ) T cells, that while tolerable, have been relatively unsuccessful. Gamma delta (γδ) T cells offer a potentially superior and non-alloreactive alternative with innate NB cytotoxicity. We demonstrated preclinical potency of unengineered allogeneic γδ T cells in combination with anti-GD2 antibody and chemotherapy against NB, supporting a first-in-human clinical trial (NCT05400603). In our second generation γδ T cell therapy, we propose to introduce chimeric antigen receptor (CAR) expression to enhance efficacy. While GD2-targeted ACTs are well-tolerated, GD2 may downregulate following relapse leaving a need for novel NB-specific immunotherapy targets. We therefore identified protein tyrosine kinase 7 (PTK7) to be robustly expressed on the NB cell surface with minimal normal tissue expression using a novel pediatric normal tissue microarray.

Aims: Our primary objective is to bioengineer γδ T cells with dual CAR expression targeting both PTK7 and GD2 to improve tumor trafficking, minimize toxicity, and circumvent therapy resistance due to antigen escape.

Methods: Transgenes containing either GD2 or PTK7 scFv targeting domain, CD8 hinge, CD28 co-stimulatory domain, and CD3ζ signaling domain were designed for mRNA transfer to γδ T cells via co-electroporation. CAR expression was determined using flow cytometry and anti-NB cytotoxicity by annexinV-7AAD apoptosis assay.

Results: Simultaneous expression of both CARs appears highest 24 hours following electroporation, with ~70% of cells dually modified. Compatible with the in vivo lifespan of allo γδ T cells, CAR expression can be detected up to 72 hours after mRNA introduction. Anti-GD2/PTK7 γδ T cells are potent against IMR5 (GD2+PTK7+) with ~50% NB death at a 1:1 effector:target ratio. Dual-targeted γδ T cells show antigen-specific activity against GD2+PTK7+, GD2+PTK7-, and GD2-PTK7+, but not GD2-PTK7- NB models engineered to represent potential clinical heterogeneity of target expression.

Conclusion: Dual PTK7/GD2 CAR expression in γδ T cells is feasible and yields strong anti-NB potency. Multiple infusions of the allogeneic donor-derived therapy can counteract a finite γδ T cell lifespan. Ongoing efforts include optimizing CAR signaling domains for maximal efficacy and confirming efficacy in vivo with the ultimate goal of rapid clinical translation.

P236

Anti-GRP-R monoclonal antibody antitumor therapy against neuroblastoma

Qiao J[1], Liu J[2], Jacobson J[1], Clark R[1], Lee S[1], Liu L[3], An Z[2], Zhang N[2], Chung D[1,4]
[1]UT Southwestern Medical Center; Department of Pediatic Surgery, Dallas, United States, [2]Texas Therpeutics Institute, Brown Foundation Institute of Molecular Medicine, McGovern Medical School, UT Health Science Center, Houston, United States, [3]UT Southwestern Medical Center; Department of Radiology, Dallas, United States, [4]Children's Health, Dallas, United States

Background. High-risk neuroblastoma remains difficult to cure despite advances in multimodal therapy including chemoradiation, surgical resection, and autologous stem cell rescue. Immunotherapy has demonstrated success in treating many types of cancers; however, its use in pediatric solid tumors has been limited by low tumor mutation burdens. Gastrin-releasing peptide receptor (GRP-R) is overexpressed in numerous malignancies, including poorly-differentiated neuroblastoma.

Aims. To develop monoclonal antibodies (mAbs) against GRP-R as a potential novel immunotherapy for treating patients with neuroblastoma.

Methods. Four peptides of GRP-R extracellular domains were mixed and used for panning phage single-chain variable (scFv) library. The positive phage clones were constructed into full IgG1 heavy and light chain backbones in a mammalian expression vector for expression in HEK293 cells. GRP-R mAb candidates were evaluated against GRP-R expressing neuroblastoma cell lines. The inhibitory function of GRP-R mAbs was assessed in vitro. Antibody-dependent cellular cytotoxicity (ADCC) of GRP-R mAbs was examined both in neuroblastoma cells and in a mouse xenograft tumor growth model.

Results. We established four candidate anti-GRP-R mAbs by screening an scFv library. GRP-R mAb-1 demonstrated the highest efficacy with the lowest EC50 at 4.607 ng/ml against GRP-R expressing neuroblastoma cells, and blocked the GRP-ligand activation of GRP-R and its downstream PI3K/AKT signaling. This resulted in functional inhibition of cell proliferation and anchorage-independent growth, indicating that mAb-1 has an antagonist inhibitory role on GRP-R. To examine the ADCC of GRP-R mAb-1 on neuroblastoma, we co-cultured neuroblastoma cells with natural killer (NK) cells versus GRP-R mAb-1 treatment alone. GRP-R mAb-1 mediated ADCC effects on neuroblastoma cells and induced release of IFNγ by NK cells under co-culture conditions in vitro. The cytotoxic effects of mAb-1 were confirmed with the secretion of cytotoxic granzyme B from NK cells and the reduction of mitotic tumor cells in vivo using a murine tumor xenograft model.

Conclusion. GRP-R mAb-1 demonstrated efficacious anti-tumor effects on neuroblastoma cells in preclinical models. Importantly, GRP-R mAb-1 may be an efficacious, novel immunotherapy in the treatment of high-risk neuroblastoma patients.

P237

Case report: Successful therapy of multiply relapsed neuroblastoma patient with poor organ function with escalating doses of ANBL1221

Frazer B[1], Martinez M[1], Onyeama S[1], Marachelian A[1,2]
[1]Children's Hospital Los Angeles, Los Angeles, United States, [2]University of Southern California, Los Angeles, United States

Background: Relapsed or refractory(RR) high risk neuroblastoma (HR-NBL) is challenging to manage with a poor overall prognosis. Chemoimmunotherapy with dinutuximab is well accepted for RR disease and given generally as per ANBL1221.Therapeutic challenges in advanced progressive relapsed disease include consideration of whether potential benefits of therapy outweigh risk of toxicity.

Aims: To describe a case of chemoimmunotherapy used in the palliative setting with dose escalation and novel combination.

Case: Patient diagnosed with HR stage M, unfavorable NBL at age of 6. Upfront therapy consisted of multiagent induction chemotherapy, single transplant, resection, local radiation, and immunotherapy with no active disease at end of therapy. Patient relapsed numerous times thereafter. Patient treated with chemoimmunotherapy as per ANBL1221 at first relapse, MIBG therapy at second relapse. At third relapse was given palliative radiation.
Patient presented after 7 months of progressive disease with pain, debilitating fractures, visible/palpable masses, thrombocytopenia, and hyperfibrinolysis. Urine catecholamines(UC) were > 200x upper limit normal (ULN). X-Ray showed skeletal metastases with pathologic fractures. CT showed disseminated soft tissue masses in the calvaria, iliac bones, liver, and lungs with diffuse lymphadenopathy. MIBG scan not obtained. Given prior response, palliative treatment initiated with ANBL1221 therapy. Due to tenuous clinical status including minimal marrow function, patient received 50% dosing of temozolomide/irinotecan and 40% dosing of dinutuximab. Patient tolerated therapy with supportive care and pain was managed with high dose narcotics. UC post Cycle 1 decreased by 40% and symptoms improved. Given improved clinical status, patient received 3 additional cycles at 75% dosing for all agents. After cycle 4, UC reduced to <12xULN, MIBG obtained with Curie score 24. Patient weaned off all narcotics and regained strength/mobility.
Ctdna was obtained after cycle 4 and pembrolizumab (anti-PD-1) was added to all subsequent cycles to target high tumor mutation burden (11Muts/Mb). Patient has now received 20 cycles of 75% dose chemoimmunotherapy and pembrolizumab with continued response (MIBG score 1) with non-transfusion dependent profound thrombocytopenia.

Conclusion: ANBL1221 therapy may be given at reduced doses for patients with poor organ function and escalated as tolerated, and in combination with pembrolizumab even in the face of very advanced disease.

P238

THE IDENTIFICATION OF GAMMA DELTA T CELLS AS CELLS WITH IMMUNOTHERAPY POTENTIAL IN HUMAN NEUROBLASTOMA

Verhoeven B[1], Ye K[2], Olsen T[1], Stenman J[1], Kogner P[1], Johnsen J[1], Kreslavskiy T[2], Baryawno N[1]
[1]*Childhood Cancer Research Unit, Department of Women's and Children's Health, Karolinska Institutet , Solna, Sweden,*
[2]*Department of Medicine Solna, Karolinska Institutet, Solna, Sweden*

Background
A comprehensive functional immune cell overview at the single-cell level in neuroblastoma is currently lacking and previous studies have mainly focused on a single cell type. Conflicting results remain in terms of numbers and function of different immune cells despite them being studied extensively both in vitro and in vivo. Furthermore, despite the advances in anti-GD2 immunotherapy, significant side effects and low survival rates remain.

Aims
We previously characterized the immune cell composition of neuroblastoma, and here we aimed to identify cell types with immunotherapy potential.

Methods
We performed single-cell RNA-sequencing (scRNA-seq) on nineteen human neuroblastoma samples coupled with multiplex immunohistochemistry (IHC) and survival analysis. We complemented our data with scRNA-seq data from normal fetal adrenal gland and additional neuroblastoma datasets to increase the power of our study and characterize cell-state changes from normal to cancerous. Furthermore, we performed a pilot scRNA/VDJ-seq experiment to characterize clonal composition and functional states of alpha beta and gamma delta T cells infiltrating neuroblstoma.

Results
Twenty-seven immune cell subtypes were revealed including distinct subpopulations of myeloid, NK, B and T cells not identified in neuroblastoma before. In contrast to adult cancers and previous neuroblastoma studies, we demonstrated an increase in inflammatory monocyte cell-state in tumor tissue, whereas no differences in cytotoxicity and exhaustion score for cytotoxic T cells, nor in Treg activity were detected. The pilot scRNA/VDJ-seq experiment revealed prominent clonal expansion of both alpha beta and gamma delta T cells in the tumor. In both cases expanded clones exhibited clear cytotoxic signature. Clonal expansion was particularly prominent for gamma delta T cells where nearly half of the cells represented one of the two top expanded clones with private Vdelta1 and Vdelta3 TCRs.

Conclusions
We have significantly broadened the understanding of the immune landscape in neuroblastoma, provided important insights into the clinical relevance of the different cell types and created a resource for further investigation. Drastic clonal expansion of gamma delta T cells suggests that they may recognize antigens present in the tumor environment and therefore may have immunotherapy potential.

P239

Anti-tumor reactivity of αβ T cells engineered to express a γ9δ2TCR (TEG A3) towards solid tumor in vitro models

Pscheid R[1,2], Wienke J[2], Strijker J[2], Drent E[1], Bisso A[1], Throsby M[1], Molenaar J[2,3]
[1]Gadeta B.V., Utrecht, Netherlands, [2]Princess Máxima Center for Pediatric Oncology, Utrecht, Netherlands, [3]Department of Pharmaceutical Sciences, Utrecht University, Utrecht, Netherlands

Background: Neuroblastoma accounts for 15% of all cancer-related deaths worldwide. Despite of aggressive treatments with surgery, radiotherapy and chemotherapy, over 50% of patients relapse and eventually die. An alternative with less side effects might be T cell based immunotherapy, such as T cell receptor (TCR) engineered T cells. Compared to conventional antigen-specific and MHC-restricted αβ TCRs, γδ TCRs may provide more universal recognition independent of MHC expression. TEG A3 expresses a γ9δ2 TCR, which recognizes CD277J, an isoform of CD277 which is triggered by the accumulation of phosphoantigens (pAG) induced by bisphosphonates (N-BPs) e.g. pamidronate (PAM).

Aim: We investigate the cellular anti-tumor reactivity and safety of αβ T cells engineered to express a defined γδ T cell receptor (TEG A3) in (hybrid) 3D in vitro models.

Methods: We developed a 3D cytotoxicity assay consisting of patient-derived organoids formed in serum free media without the need of matrigel. TEG A3 response and efficacy was monitored using the Incucyte 3S live cell imaging system with the apoptosis marker caspase 3/7 green, and endpoint readouts of IFNγ secretion in the supernatant. Furthermore, hybrid assays mixing tumor cells and (patient-matched) fibroblasts were developed to evaluate TEG safety and tumor-specificity.

Results: All used neuroblastoma organoids expressed CD277. 75% of all tested neuroblastoma organoids triggered an anti-tumor reactivity of TEG A3 after incubation with pamidronate within 48 hours, whereas untransduced αβ T cell (UNTR) had no effect. IFNγ secretion in the supernatant was in line with the caspase 3/7 green observations. In hybrid 3D in vitro models, where patient-derived organoids were mixed with unmatched patient-derived fibroblasts or matched cancer-associated fibroblasts (CAF), the TEG A3 cytotoxicity was tumor specific, leaving fibroblasts unharmed. Moreover, the presence of patient-matched CAF in mixed hybrids significantly influenced killing of tumor cells by TEG A3.

Conclusion: Our study demonstrated TEG A3 reactivity and specificity towards 3D CD277+ neuroblastoma cells in the presence of PAM, even when mixed with other cell types. We established an in vitro 3D cytotoxicity assay model system for the functional evaluation of T cell based adoptive immunotherapy providing a useful platform for early stage preclinical development of immunotherapies.

P240

SHED L1CAM IMPAIRS CAR T CELL EFFICACY AGAINST NEUROBLASTOMA

Andersch L[1,2], Schwiebert S[1], Klaus A[1], Jensen M[3], Eggert A[1,2,4], Anders K[1], Künkele-Langer A[1,2,4]
[1]Charité-Universitätsmedizin Berlin, Department of Pediatric Oncology and Hematology, Berlin, Germany, [2]German Cancer Consortium (DKTK), Berlin, Germany, [3]Ben Towne Center for Childhood Cancer Research, Seattle, USA, [4]Berlin Institute of Health (BIH), Berlin, Germany

L1CAM-directed CAR T cell therapy has shown limited efficacy in the treatment of neuroblastoma patients. The type of target antigen plays a crucial role for CAR T cell therapy success against solid tumors. L1CAM is overexpressed in neuroblastoma, and the cancer-specific glycosylation of the CE7 epitope makes it a suitable target antigen. Constitutive cleavage of the L1CAM extracellular domain by the ADAM metalloproteases sheds the CE7 epitope-containing L1CAM ectodomain into blood as found in ovarian, gastrointestinal and ovarian cancer patients as well as ascites of ovarian cancer patients. In addition, soluble L1CAM can bind via its RGD motif to extracellular matrix or integrins expressed by many normal tissues. We hypothesized that shed L1CAM, present in its soluble or re-bound form could trap CAR T cells outside the tumor or limit CAR T cell function by inducing exhaustion through repetitive antigen encounter before even reaching the tumor site.

We demonstrated that soluble L1CAM levels were elevated in serum from children with neuroblastoma and mice harboring neuroblastoma xenografts, confirming in vivo L1CAM shedding. We found that recombinant plate-bound and soluble L1CAM activated CAR T cells in vitro, but activation by the soluble form required 30-fold higher L1CAM concentrations. Activation of CAR T cells with long spacer element was more pronounced compared to those harboring a short spacer. Interestingly, plate-bound and soluble L1CAM upregulated inhibitory receptors on L1CAM-CAR T cells and induced apoptosis as analyzed via flow cytometry. Pre-exposing L1CAM-CAR T cells to plate-bound or soluble L1CAM induced IFNG release and pre-exposure to plate-bound L1CAM diminished subsequent in vitro cytotoxicity against L1CAM-positive neuroblastoma cell lines. Exposure of L1CAM-negative tumor cells to soluble recombinant L1CAM confirmed binding on the cell surface, presumably via integrins, as analyzed by flow cytometry. Co-culture with L1CAM-bound tumor cells activated CAR T cells and induced IFNG-release.

Our findings suggest that shed L1CAM, either soluble, sequestered in extracellular matrix or bound to integrins on non-tumor tissue negatively affects L1CAM-CAR T cell efficacy, and may explain the poor L1CAM-CAR T cell persistence and efficacy in the clinic.

P241

Functional characterization of CAR-T cells derived from different sources for the treatment of Neuroblastoma

García L[1], Pastora K[1], Ivanova M[1], Martín-Antonio B[2], Ramírez M[3], González-Murillo Á[1,3]
[1]Oncohematology Research Unit, Fundación para la Investigación Biomédica, Hospital Infantil Universitario Niño Jesús, Madrid, Spain, [2]Department of Experimental Hematology, Instituto de Investigación Sanitaria-Fundación Jiménez Díaz, Madrid, Spain, [3]Advanced Therapies Unit, Oncology, Hospital Infantil Universitario Niño Jesús, Madrid, Spain

Background: The clinical success with chimeric antigen receptors-modified T cells (CAR-T cells) for haematological malignancies has not been reproduced for solid tumours, in part due to the lack of cancer-type specific antigens. Here, we propose a strategy based on anti-fluorescein (anti-FITC) CAR-T cells combined with a FITC-conjugated specific antibody for neuroblastoma (NB), anti-GD2 (Dinutuximab).

Aims: To test the efficacy of CAR-T and GD2-FITC combination against neuroblastoma.
Methods: In a first set of experiments, we compared two sources of clinically available naïve T cells (cord blood-CB- and CD45RA enriched peripheral blood leukapheresis product-45RA-) cultured under two cytokine conditions (IL-2 versus IL-7+IL-15+IL-21) with or without CD3/CD28 stimulation in order to choose the best condition for anti-FITC CAR-T production. Peripheral blood, a current source of T cells for CAR-T cell manufacturing, was used as control. Immunophenotype, vector copy number and genomic integrity of the final products were done as characterization and quality controls. Functionality and antitumoral capacity of CB/45RA-derived anti-FITC CAR- T were analysed in coculture with different anti-GD2-FITC labelled NB cell lines.

Results: The cocktail IL-7+IL-15+IL-21 in addition to co-stimulation signals resulted in favorable cell proliferation rate and maintained less differentiated immunophenotypes in both CB and 45RA T cells, therefore, it was used for CAR-T cells manufacturing and their further characterization. Both, CB and CD45RA-derived anti-FITC CAR-T cultured with IL-7+IL-15+IL-21 retained a predominated naïve phenotype compared with control. In the presence of the NB-FITC targeting, CD4+ CB-derived anti-FITC CAR-T showed the highest values of co-stimulatory receptors OX40 and 4-1BB and CD8+ CAR-T cells exhibited high levels of PD-1 and 4-1BB, while TIM3 and OX40 were low, comparing with the other sources studied. CB-derived anti-FITC CAR-T also released the highest amounts of cytokines (IFNγ and TNFα) into coculture supernatants. Viability of NB target cells decreased to a 30% when cocultured with CB-derived CAR-T cells during 48h. In vivo experiments are currently on-going to test the efficacy of CAR-T cells in combination with GD2 treatment.

Conclusion: Our strategy may complement the current use of Dinutuximab in the treatment of NB through its combination with a targeted CAR-T cell approach.

P242

Induction of telomere dysfunction as a therapeutic strategy to target neuroblastoma by immune checkpoint inhibition

Höppner S[1,2,3], Werr L[1,3], Bartenhagen C[1,3], Hellmann A[1,3,4], Szilagyi B[1,2], Kahlert Y[1], Hemstedt N[1], Pakulla I[1], Hölzel M[5], Fischer M[1,3]

[1]Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany, [2]Mildred Scheel School of Oncology, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany, [3]Center for Molecular Medicine Cologne, University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany, [4]Else Kröner Forschungskolleg Clonal Evolution in Cancer, University Hospital Cologne, Cologne, Germany, [5]Institute of Experimental Oncology, University Hospital Bonn, University of Bonn, Bonn, Germany

Background: High-risk neuroblastoma is mechanistically defined by the presence of telomere maintenance mechanisms. Treatment with the telomerase substrate precursor 6-thio-2'-deoxyguanosine (6-thio-dG) induced telomere dysfunction in mouse models of telomerase-expressing cancers, leading to an activation of the cGAS/STING pathway in the tumor and surrounding dendritic cells. As a result, the tumor is infiltrated by immune cells releasing interferon γ which upregulates PD-L1 expression on the tumor cells to protect the tumor against immune cell attacks.

Aim: We aimed to investigate whether induction of telomere dysfunction by treatment with 6-thio-dG sensitizes immunological cold high-risk neuroblastoma to checkpoint inhibitors.

Methods: A MYCN amplified neuroblastoma mouse cell line (NHO2A) was treated with increasing 6-thio-dG concentrations only (200nM, 1µM, 5µM) or in combination with interferon γ to simulate cytotoxic T cell activation in the tumor microenvironment. A transgenic neuroblastoma mouse model expressing MYCN and ALK-F1174L under a tyrosine hydroxylase promoter was treated with 6-thio-dG in combination with PD-L1 antibody or IgG control, or with 6-thio-dG, PD-L1 antibody, IgG control or dilution buffer only. Mouse MRI was performed weekly to monitor treatment response. Immunoblotting was performed to detect cGAS/STING pathway activation and PD-L1 expression in NHO2A cells and in mouse tumors. HE and immunohistochemical staining with CD45, CD19, CD4 and CD8 antibodies was performed to analyze tumor histology and immune cell infiltration, respectively.

Results: Treatment of the neuroblastoma cell line NHO2A with 6-thio-dG led to upregulation of cGAS and PD-L1 protein expression and STING pathway activation. Similarly, elevated cGAS expression and activation of the STING pathway was noted in mouse tumors after treatment with 6-thio-dG. Prolonged overall survival as well as tumor regression was observed in mice in both, the combined and PD-L1 only treatment groups. Regressive tumors harbored a higher grade of immune cell infiltration and differentiation of tumor cells in comparison to progressive and undifferentiated tumors in the control groups.

Conclusion: 6-thio-dG treatment activates the cGAS/STING pathway and upregulates PD-L1 expression in a neuroblastoma cell line and a transgenic mouse model, suggesting that induction of telomere dysfunction may trigger a vulnerability to checkpoint inhibitors in high-risk neuroblastoma.

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Organoid-specific optimization of killing assays to test novel immunotherapies in a high-throughput system

Van Den Ham F[1], Kholosy W[1], Ober K[1], Cornel A[1,2], Nierkens S[1,2], Anderson J[3], Molenaar J[1], Wienke J[1]
[1]Princess Maxima Center for Pediatric Oncology, Molenaar Group, Utrecht, Netherlands, [2]University Medical Center Utrecht, Center for Translational Immunology, Utrecht, Netherlands, [3]Great Ormond Street Hospital & Institute of Child Health, London, United Kingdom

Background: Immunotherapy offers a great promise in the treatment of high-risk neuroblastoma. Current neuroblastoma in vitro models lack the ability to study novel immunotherapies with High-Throughput compound Screening (HTS).

Aim: We aimed to optimize neuroblastoma organoid-lymphocyte cocultures for HTS, and possibly personalized testing, of novel antibody-mediated and cellular immunotherapies.

Material and methods: Two patient-derived organoids (691B: GD2-pos MHC-I-neg and 691T: GD2-neg MHC-I-pos) were transduced with an endogenous luciferase-construct to use D-luciferin-induced bioluminescence as a readout for cell growth. The growth rate, optimal seeding density and optimal pre-culture time per organoid were determined by density curves. The number of needed cells was downscaled to facilitate HTS. Luciferase-transduced organoids were cocultured with primary PBMCs from healthy donors, PRAME-TCR transduced T cells or CAR-T cells to study Dinutuximab-induced antibody-dependent cytotoxicity (ADCC) and T-cell mediated cytotoxicity.

Results: Density screens showed an optimal seeding density of 5000-10.000 organoid cells per well, yielding a high luminescence signal while minimizing the number of cells needed. Already at the lowest effector to target (E:T) ratio (1:3), we observed killing of the MHC-I expressing 691T organoid, which increased with higher E:T ratios and co-culture time. Pre-culturing of organoids for 72 hours before addition of effector cells resulted in formation of larger 3D spheres, which reduced killing efficacy for all E:T ratios. ADCC effects of Dinutuximab in GD2+MHC-I- cells resulted in 25% increase of killing after 24 hours and reached up to 70% increase after 72 hours for 10:1 and 20:1 E:T ratios. Dinutuximab did not increase killing of the GD2- organoid, confirming specificity of the antibody. T cell mediated killing was almost 100% for MHC-I+ 691T organoids after 24 hours of culturing with PRAME-TCR transduced T cells or B7-H3 CAR-T cells at a 1:3 E:T ratio, showing the high anti-tumor cytotoxicity of these cells and potential for HTS at very low E:T ratios.

Conclusions: We have developed a robust in vitro bioluminescence-based organoid/lymphocyte co-culture assay with a low cell input, to facilitate high-throughput screening of novel antibody-based or cellular immunotherapies, possibly combined with chemotherapeutic or targeted compounds. In the future this method may be applied for personalized drug screens.

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B7-H3 immune checkpoint protein in neuroblastoma therapy response

Emaldi M[1], Zaldunbide L[2], Mosteiro L[2], Torices L[1], Rey E[1], Aguirre P[3], García de Andoin N[4], López-Almaraz R[5], Pulido R[1,6], Nunes-Xavier C[1,7]

[1]Biomarkers in Cancer Unit, Biocruces Bizkaia Health Research Institute, Barakaldo, Spain, [2]Department of Pathology, Cruces University Hospital, Barakaldo, Spain, [3]Department of Pathology, Donostia University Hospital, San Sebastian, Spain, [4]Unit of Pediatric Oncohematology, Donostia University Hospital, San Sebastian, Spain, [5]Pediatric Oncology and Hematology, Cruces University Hospital, Barakaldo, Spain, [6]IKERBASQUE, Basque Foundation for Science, Bilbao, Spain, [7]Department of Tumor Biology, Institute for Cancer Research, Oslo University Hospital Radiumhospitalet, Oslo, Norway

Background

B7 family proteins are important regulators of antitumor immune response, and can mediate oncogenic signaling and cancer development. B7 immune checkpoint proteins, including B7-H1 (PD-L1), B7-DC (PD-L2), B7-H2, B7-H3, B7-H4, B7-H5, B7-H6 and B7-H7, constitute prognostic and therapeutic relevant proteins in cancer.

Aims

We aim to analyze the expression and function of B7 protein family as prognostic and therapeutic relevant proteins in neuroblastoma.

Methods

We have analyzed the expression of B7 proteins in Neuroblastoma, and found highest expression of B7-H3 in neuroblastoma tumor cells. We have used neuroblastoma cell lines with different expression levels of B7-H3, or treated with an inhibitory B7-H3 antibody, to evaluate the effects on the sensitivity to anticancer compounds, including chemotherapy and targeted-therapy agents.

Results

Neuroblastoma cells treated with the alkylating agents cisplatin and carboplatin showed enhanced decrease of cell viability and proliferation in B7-H3 knockdown tumor cells, compared to their B7-H3 expressing counterparts. Similar enhanced decrease in viability was observed in neuroblastoma cells treated with an anti-B7-H3 monoclonal antibody. On the other hand, in B7-H3 overexpressing cells, the effect of chemotherapy on cell viability was reduced. We also found a modest increase in sensitivity to Lorlatinib ALK inhibitor in B7-H3 knockdown neuroblastoma cells.

Conclusion

In conclusion, we have unveiled an effect of B7-H3 expression on neuroblastoma therapy response, suggesting that B7-H3 may confer resistance to chemotherapies and targeted therapies. The results provide novel insights into the function of B7-H3 in neuroblastoma, and suggest that targeting of B7-H3 may be a suitable immunotherapeutic approach to improve current anticancer therapies.

Acknowledgments

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End of Induction Response and Toxicities in Children with High-Risk Neuroblastoma Treated with Dinutuximab Chemoimmunotherapy in all Cycles of Induction

Cupit-Link M[1], Federico S[1]
[1]*St. Jude Children's Research Hospital, Department of Oncology, Memphis, United States*

BACKGROUND
The addition of an anti-GD2 monoclonal antibody (hu14.18K322A) to 6 cycles of Induction chemotherapy for the treatment of patients with high-risk neuroblastoma (HRNBL) led to an end of Induction (EOI) objective response rate (ORR; ≥ partial response [PR]) of 93.8% in a single institution phase 2 clinical trial (NB2012, NCT01857934). Chemoimmunotherapy using dinutuximab (DIN) has been effective in the treatment of patients with relapsed/refractory neuroblastoma and successfully administered to newly diagnosed (HRNBL) patients during Induction cycles 3-5 in a Children's Oncology Group (COG) study (ANBL17P1, NCT03786783). We report the results of 24 patients with HRNBL treated with DIN in all cycles of Induction therapy.

AIMS
The primary aim was to determine the EOI ORR (≥ PR) of patients diagnosed with HRNBL treated with DIN in all cycles of Induction. The secondary aim was to describe the common toxicities experienced by the patients during Induction chemoimmunotherapy.

METHODS
This is a single-center retrospective study of patients with HRNBL treated with DIN (17.5mg/m2/dose, IV Days 2-5) and GM-CSF (250mcg/m2/dose, subcutaneous Days 6-count recovery), with or without subcutaneous interleukin 2 (IL-2), with COG Induction chemotherapy. Data was abstracted from the electronic medical record. Toxicities experienced during Induction were graded by CTCAE v.5.0. EOI ORR was evaluated using the Revised International Neuroblastoma Response Criteria (INRC).

RESULTS
Twenty-four patients with HRNBL (21 newly diagnosed, 3 previously treated with intermediate-risk therapy including 2 recurrent and 1 refractory, 15 females, median age 3.1 years; range, 0.35-8.1 years) received Induction chemoimmunotherapy from 1/27/2017 to 12/28/2022. All patients received DIN with all cycles of Induction. The most common DIN-related grade ≥ 3 toxicities recorded during Induction included fever (46%), hypoxemia (21%), and hypoalbuminemia (13%). All 24 patients completed EOI evaluations, including 17 with complete response, 7 with PR, 0 with minor response, 0 with stable disease, and 0 with progressive disease. The EOI ORR was 100%.

CONCLUSION
The administration of DIN and GM-CSF to COG Induction for patients with HRNBL had an encouraging EOI ORR. A randomized phase 3 study of Induction chemoimmunotherapy is warranted.

P246

Tumor irradiation and anti-GD2 immunocytokine (hu14.18-IL2) drive curative responses to CAIR therapy in a murine model of high-risk, treatment-resistant neuroblastoma

Zebertavage L[1], Erbe A[1], Aiken T[1,2], Schopf A[1], Nielsen M[1], Katz S[1], Morris Z[1], Rakhmilevich A[1], Sondel P[1,3]
[1]University Of Wisconsin, Department of Human Oncology, Madison, United States, [2]University of Wisconsin, Department of Surgery, Madison, United States, [3]University of Wisconsin, Department of Pediatrics, Madison, United States

Background:
Currently, there are no effective treatment options for high-risk neuroblastoma (HR-NBL) patients that are refractory to, or relapse following, initial treatment once they have failed salvage therapy of chemotherapy and anti-GD2 therapy. Previously, our group developed a Combination Adaptive and Innate immunotherapy Regimen (CAIR) to treat a murine model of HR-NBL (9464D-GD2). This therapy, in contrast to all other treatments we know to have been tested, is able to cure some 9464D-GD2 tumors.

Aims:
Due to the complex nature of CAIR (radiotherapy [RT], anti-GD2 immunocytokine [hu14.18-IL2], anti-CD40, CpG, and anti-CTLA4) and the challenges this may pose to clinical translation, we are interested in determining which components of CAIR drive curative responses. We tested: 1) if 9464D-GD2 models the resistance to salvage therapy (temozolomide and irinotecan [TEM+IRI] and anti-GD2) seen in clinical HR-NBLs, and 2) if all components of CAIR are necessary for treatment efficacy.

Methods:
To model clinical salvage therapy, 9464D-GD2-bearing mice were injected with temozolomide, irinotecan, and/or hu14.18-IL2 and were administered 12Gy RT. For studies testing variations of CAIR, mice were treated with 12Gy RT, anti-CD40, anti-CTLA4, CpG, and/or hu14.18-IL2.

Results:
Treatment with TEM+IRI and/or hu14.18-IL2 slightly extended the survival of 9464D-GD2-bearing mice ($p<0.03$) but did not cure tumors. Adding RT further improved survival ($p<0.0001$), but not more than RT alone ($p=0.29$), and also did not cure tumors.

CAIR, conversely, cured ~40% of 9464D-GD2 tumors. When RT or hu14.18-IL2 were removed from CAIR, survival was dramatically reduced ($p<0.0001$) and few tumors were cured (1/16 and 0/16). Removing anti-CD40, anti-CTLA4, or CpG did not alter survival ($p=0.81$, $p=0.85$, $p=0.70$) and resulted in similar rates of tumor cures as CAIR (8/16, 9/21, and 7/16 versus 13/30).

Conclusion:
These data suggest that 9464D-GD2 may act as a model for human HR-NBLs that fail to be cured by salvage therapy. These tumors, in contrast, can be cured by CAIR therapy and by several versions of reduced CAIR, but not those lacking RT or hu14.18-IL2. These results demonstrate that RT and hu14.18-IL2 are necessary treatments for CAIR-mediated 9464D-GD2 tumor cures and suggest that similar regimens might be tested in patients with therapy-resistant HR-NBL.

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Induced innate CD8+T cells engineered to express a new class of anti-GD2 chimeric antigen receptors for neuroblastoma immunotherapy

Lode B[1,2], Grabarczyk P[2], Troschke-Meurer S[1], Forkel H[2], Depke M[2], Zumpe M[1], Siebert N[1], Schmidt C[2], Lode H[1]

[1]Department of Pediatric Oncology and Hematology, University Medicine Greifswald, Greifswald, Germany, [2]Internal Medicine Clinic C, University Medicine Greifswald, Greifswald, Germany., University Medicine Greifswald, Greifswald, Germany

Background:
Despite continuous progress, chimeric antigen receptor bearing T cell therapies for neuroblastoma face challenges related to efficacy and side effects.

Aim:
To improve anti-tumor toxicity and to reduce side effects, we used induced innate CD8+ T cells (BCL11B-KO/IL-15, iiT8). The iiT8 cells are characterized by the expression of innate activating receptors that recognize transformed cells similar to NK cells. Such iiT8 cells equipped with a chimeric antigen receptor (CAR) designed with an innate costimulatory domain only (Co-CAR) are activated by a combined engagement of innate- and chimeric antigen-receptors, thereby reducing the on-target-off-tumor side effects.

Methods:
To yield iiT8 cells, BCL11B was deleted in human CD8+ T cells using the CRISPR-Cas9 method. Expression of innate receptors Nkp30, Nkp44 and Nkp46 was determined by flow cytometry. Innate receptor functionality was confirmed with the CD107a/IFN-γ assay. Co-CAR constructs were generated using a GD2 specific variable domain of anti-GD2 antibody ch14.18 linked to CD28, OX40 and 41BB and NK cell specific costimulatory domains DAP10, DAP12 and combined DAP10/DAP12. A classical second-generation CAR with the same antigen recognition domain, a 41BB costimulatory and a CD3 zeta stimulatory domain was used as a comparator. Anti-tumor efficacy was determined against spheroids derived of the GD2 positive human neuroblastoma cell lines LAN-1 and CHLA-136 transfected with near infrared protein for live-cell viability analysis. The stress ligand dependency was determined using CHLA-136 neuroblastoma cells engineered to lack stress ligand B7-H6.

Results:
iiT8 cells were characterized by high abundance of Nkp30, Nkp44 and Nkp46 innate-receptors and showed superior cytotoxicity compared to CD8 T cells. Co-CARs containing DAP12 showed the highest GD2-specific anti-tumor activity compared to DAP10, CD28, OX40 and 41BB domains when expressed on iiT8 cells. Importantly, the DAP12 containing CAR showed improved cytotoxicity compared to 2nd generation CAR T cells (2.28-fold change). Deletion of B7-H6 in CHLA-136 resulted in a decreased activity of iiT8 cells with costimulatory CAR compared to wild type CHLA-136 cells.

Conclusion:
The iiT8-Co-CAR-DAP12 promises improved efficiency accompanied by superior specificity against GD2 and stress ligand positive tumor cells.

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MYCN-driven neuroblastoma utilizes extracellular cyst(e)ine to promote immune suppression and tumor progression

Wolf A[1], Tao L[1], Li L[1], Hernandez B[1], Dysthe M[1], Badachhape A[2], Ghaghada K[2], Bettini M[3], Patel T[1], Coarfa C[4], Putluri N[4], Mamonkin M[5], Parihar R[1], Barbieri E[1]

[1]Department of Pediatrics, Section of Hematology-Oncology, Texas Children's Cancer and Hematology Centers, Baylor College of Medicine, Houston, United States, [2]Department of Radiology, Baylor College of Medicine, Houston, United States, [3]Department of Pathology, University of Utah, Salt Lake City, United States, [4]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, United States, [5]Department of Pathology and Immunology, Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, United States

Background: High-risk neuroblastoma (NB) patients desperately need novel therapies. MYCN amplification (MNA) drives high-risk NB, reprogrammed tumor metabolism, and immune-suppression. On-going research has shown that MYCN promotes NB cysteine metabolism. Cysteine is also critical for immune cells. Activated T cells upregulate cysteine (ASCT1/2) and cystine (xCT) transporters to acquire exogenous cyst(e)ine, suggesting metabolic competition between NB and T cells.

Aim: We aim to elucidate and exploit MYCN-driven tumor-immune cell competition for cyst(e)ine to sensitize NB to immunotherapy.

Methods: TH-MYCN GEM tumors and healthy kidneys/adrenal glands were subjected to untargeted metabolomics (H4 platform, Metabolon) and mRNA-Sequencing at different stages of tumor development. The immune-microenvironment was phenotyped by high-parameter flow cytometry. Tumor-infiltrating and splenic T cells, isolated by CD4/CD8 Miltenyi Biotec MicroBeads, were stimulated ex vivo (anti-CD3/anti-CD28/IL-2 for 24-96h) to measure proliferation (CellTrace Violet), cytokine production (intracellular antibody staining), and gene expression (RT-qPCR). MYCN-activated SK-N-AS MYCN-ER™ cell supernatant (4-OHT for 72h) was analyzed by targeted LC-MS. NB cell lines were treated with Erastin (xCT inhibitor) for cell viability assessment.

Results: Advanced-stage TH-MYCN+/+ tumors are enriched for immune-suppressive polymorphonuclear myeloid-derived suppressor cells and inactive T cells. Overlapping TH-MYCN+/+ and MNA patient gene expression (GSE45547) revealed that MYCN represses immune-related gene sets. Compared to control tissue, THMYCN+/+ tumors have reduced intratumoral pools of cyst(e)ine and precursor metabolites (methionine and serine) with corresponding upregulation of GSH, suggesting oncogenic cyst(e)ine consumption to meet redox balance needs. Moreover, MYCN-activated SK-N-AS MYCN-ER™ cells consume exogenous cyst(e)ine and MNA cells are more sensitive to Erastin than non-MNA cells, suggesting MYCN-driven tumor dependency on exogenous cyst(e)ine. T cells also depend on exogenous cyst(e)ine and upregulate xCT and ASCT1/2 upon ex vivo stimulation. Naïve T cells cannot proliferate in cyst(e)ine-free media. However, cystine supplementation promotes naïve and tumor-exposed T cell proliferation and CD8+ T cell cytokine production.

Conclusions: These data suggest that MYCN depletes extracellular cyst(e)ine, which is essential for T cell expansion and activation. Our working model is that MYCN reprograms NB cysteine metabolism to promote a cyst(e)ine poor, immune-suppressive microenvironment. On-going efforts will elucidate in vivo competition for exogenous cyst(e)ine and develop therapeutic interventions combining metabolic and immune-based therapies.

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Preclinical evaluation of O-acetylated GD2-specific CAR γ9δ2T cells targeting neuroblastoma cells

Alliot H[1], Thomas P[1], Ollier J[1], Machy P[1], Gratas C[1], Fougeray S[1], Clémenceau B[1], Faraj S[1], BIRKLE S[1]
[1]Nantes Université, Inserm UMR 1307, CNRS UMR 6075, Université d'Angers, CRCI2NA, F-44000 Nantes - France, Nantes, France

Background: Autologous chimeric antigen receptor (CAR) T cell therapy targeting the GD2 tumor antigen has been recently proposed to improve neuroblastoma (NB) outcome. Their efficacy has been limited due to several obstacles: T cell homing and persistence, immunosuppressive tumor microenvironment and costly manufacturing process. A solution for these limitations is the use of donor-derived Vγ9Vδ2T cells, highly cytolytic effectors that recognized and kill tumor cells through major histocompatibility complex (MHC)-independent antigens upregulated under stress. The Vδ2Vγ9 subset can be expanded and engineered to express a tumor-specific CAR to further enhance intrinsic antitumor activity. O-acetylated-GD2 (OAcGD2) ganglioside represents a solution to minimize off-tumor on-target toxicity associated with anti-GD2 immunotherapies.
Aims: The aim was to generate OAcGD2-specific CAR Vγ9Vδ2T cells and to assess their anti-tumor potency in pre-clinical NB models.

Methods: OAcGD2 expression was studied on primary patient-derived NB cells and NB cell lines using flow cytometry analysis (FCM). Peripheral Blood Mononuclear Cells (PBMC) from healthy donors were stimulated using zoledronic-acid and IL-2 to expand γδT cells. This population was next retrovirally transduced to express a second-generation OAcGD2-specific CAR (8B6-ScFv-huIgG1Fc-CD28-CD3ζ). Control CAR γδT cells were generated expressing a CD3ζ-signaling domain-deleted CAR. CAR expression was analyzed by FCM and CAR γδT cells activation assessed by CD107a degranulation assay. CAR specific cytotoxicity was analyzed through a chromium-51 release assay. GFP-expressing NB cells cultured in 3D system, referred as spheroids, were used to study CAR γδ T's long-term anti-NB activity using video-microscopy analysis. CAR γδT's phenotype and cytokine secretion were evidenced using FCM.

Results: OAcGD2 was expressed in the primary patient-derived and NB cell lines tested. Vγ9Vδ2T cells were expanded and reached >80% purity and CAR transduction efficacy was >75%. Compared to CD3ζ deleted CAR T cells, OAcGD2-CAR γδT cells were effective against OAcGD2+ tumor cell spheroids, showing CD107a cell surface expression, cytokine production and direct target cell killing over a period that exceeded 96 hours.

Conclusion: OAcGD2-CAR Vγ9Vδ2T cell production is feasible and leads to pure and efficient effector cells. OAcGD2 CAR-Vγ9Vδ2 T cells may provide a promising platform for HR-NB therapy and potentially for other OAcGD2-expressing pediatric solid tumors.

P250

Ex-vivo activation and expansion of NK cells to generate "memory-like" NK cells for the treatment of relapsed or refractory neuroblastoma

DeSantes K[1], Forsberg M[1], Shahi De A[1], Sondel P[1], Capitini C[1]
[1]*University of Wisconsin - American Family Children's Hospital, Department of Pediatrics, Madison, United States*

Background:
We previously characterized NK cells that were activated/expanded, ex-vivo, utilizing irradiated K562 cells modified to express transmembrane IL-15 and 41BBL, for use in a clinical trial for relapsed/refractory neuroblastoma (NCT03209869). Given the substantial cost and complexity using feeder cells to generate a GMP grade cellular product, we investigated a cytokine-only culture system to manufacture “memory-like” NK cells for clinical use.

Methods:
NK cells were isolated from PBMCs using the Miltenyi Biotec NK Cell Isolation Kit and autoMACS ® Pro Separator. Cells were incubated with IL-15 alone (1 ng/ml, control), or preactivated with IL-12 (10ng/ml) + IL-15 (50ng/ml) + IL-18 (50ng/ml) for 16 hours. Cells were then incubated for 7 days with half the media replaced every 2 days with addition of IL-2(100 IU/ml) and IL-15(10 ng/ml). Control and preactivated cells were then compared for expression of different cell surface markers. In a second set of experiments, control or preactivated NK cells were cultured for 21 days in NK cell optimized Enable media with addition of either IL-15 (10 ng/ml) alone, or with IL-15 combined with IL-2 (100 IU/ml). NK cells generated under these different conditions were then compared for their level of expansion, and ability to mediate cytotoxicity against a neuroblastoma cell line (CHLA-20), with or without addition of an anti-GD2 antibody, using the IncuCyte Live Cell Analysis system.

Results
NK cells preactivated with IL-12+IL-15+IL-18 showed higher expression of NKG2A, CD62L, NKp30, NKp46, perforin, granzyme, and Ki-67, compared to control NK cells. The greatest degree of expansion was achieved when NK cells were preactivated with IL-12, IL-15 and IL-18, and then cultured with IL-15 alone (32-fold and 54-fold expansion on days 14 and 21, respectively). Preactivated NK cells cultured with IL-15 alone also mediated the greatest level of antibody dependent, and antibody independent cytotoxicity (68.9% and 45.7%, respectively).

Conclusions
We have identified a culture system achieving > 50-fold expansion of NK cells without utilizing a feeder cell line. These NK cells express a “memory-like” phenotype and demonstrate potent cytotoxicity against a neuroblastoma cell line. These cells may be ideally suited for clinical use in combination with an anti-GD2 mAb.

P251

Pharmacologically inhibiting MYCN improves L1CAM-directed CAR T cell efficacy against MYCN-amplified Neuroblastoma

Grunewald L[1,2], Andersch L[1,2], Jensen M[3], Henssen A[1,2,4], Eggert A[1,2,4], Schulte J[1,2], Anders K[1], Künkele A[1,2,4]
[1]Charité Universitätsmedizin, Dept. of Pediatric Oncology, Berlin, Germany, [2]DKTK, German Cancer Consortium, Berlin, Germany, [3]Ben Towne Center for Childhood Cancer Research, Seattle, USA, [4]Berlin Institute of Health, Berlin, Germany

Current treatment protocols have only limited success in pediatric patients with neuroblastoma harboring amplifications of MYCN. Adoptive T cell therapy presents an innovative strategy to improve cure rates, however, L1CAM-targeting CAR T cells achieved only limited response against neuroblastoma in an ongoing clinical trial. One possible factor limiting success in trials might be MYCN amplification, that is always associated with a “cold” tumor microenvironment and repression of the immune response.

Here we investigated how oncogenic MYCN levels influence tumor cell response to CAR T cells with the aim to illuminate mechanisms inhibiting CAR T cell success against neuroblastoma.

High MYCN levels were determined in SK-N-AS cells harboring the normal diploid MYCN complement using a tetracycline-inducible system. The cell model with different MYCN expression was cocultured with L1CAM-CAR T cells. L1CAM-CAR T cell effector function was assessed by activation/inhibitory receptor expression, cytokine release and cytotoxicity. Using RNA sequencing the cell line was characterized and our data was compared to publicly available RNA and proteomic data sets of neuroblastoma patients. ChIP-sequencing data was used to determine transcriptional regulation by MYCN on L1CAM (public data sets). Combination of CAR T cells with the indirect MYCN inhibitor, MLN8237, was assessed in vitro via combinatorial cytotoxic effects and in vivo using an immunocompromised mouse model.

MYCN induction impaired L1CAM-CAR T cell activation, tumor cytotoxicity and cytokine release by 14-fold in vitro. Interestingly, MYCN induction caused a significant downregulation of L1CAM expression on tumor cells. We confirmed diminished L1CAM mRNA and protein expression in different cohorts of primary neuroblastomas with high MYCN expression. Inverse correlation of MYCN and L1CAM can be caused by transcriptional repression by MYCN on L1CAM. Indirectly inhibiting MYCN in vitro using MLN8237 increased L1CAM expression on the neuroblastoma cells and restored L1CAM-CAR T cell effector function. MLN8237 synergistically enhanced L1CAM-CAR T cell-directed killing of neuroblastoma cells overexpressing MYCN.

We shed new light on a possible resistance mechanism of neuroblastoma controlled by MYCN against L1CAM-CAR T cells by target antigen downregulation. These data suggest that combining L1CAM-CAR T cell therapy with pharmacological MYCN inhibition may benefit patients with high-risk neuroblastomas harboring MYCN amplifications.

P252

Secondary hematologic malignancies after treatment including compartmental antibody-based radioimmunotherapy for solid tumors with CNS metastases

Troullioud Lucas A[1], Spitzer B[1], Kushner B[1], Modak S[1], Basu E[1], Iglesias F, Friedman D[1], Kramer K[1]
[1]*Memorial Sloan Kettering Cancer Center, MSK Kids, Department of Pediatrics, New York, United States*

Background: Pediatric patients with solid tumor CNS metastases have a poor prognosis. Compartmental antibody-based radioimmunotherapy (cRIT) aims to improve CNS disease control. The impact of cRIT on the development of treatment-related myeloid neoplasms (tMN) is unknown.

Aims: To describe the incidence of tMN in pediatric patients with solid tumors with CNS metastases who received intraventricular cRIT with 131I-8H9 targeting B7H3.

Methods: Data from pediatric patients treated at Memorial Sloan Kettering Cancer Center (New York, NY, USA) with cRIT for CNS metastases of solid tumors, was retrospectively assessed. Routine surveillance bone marrow evaluations for the primary malignancy were examined for morphology, flow cytometry, cytogenetics/FISH and molecular studies. For cumulative incidences Fine-Gray competing risk methods were used.

Results: Of 177 patients, 4 developed isolated cytogenetic changes and 10 developed tMN. Primary diagnoses were neuroblastoma (n=11), and one patient each with rhabdomyosarcoma, medulloblastoma, and choroid plexus carcinoma. Two patients had germline TP53 mutations. Median time from start of cRIT to cytogenetic changes without progression to tMN was 7.5 (range 1.9-16.2) months. One patient died of neuroblastoma, the others remain alive without progression to hematologic malignancy. One patient with neuroblastoma had MDS identified at initiation of cRIT. For the 9 patients who developed tMN after cRIT, median time from first treatment to presentation with tMN was 20.5 (range 2.3-68.2) months. Cytogenetic changes associated with tMN were KMT2A-associated (n=3), complex karyotype (n=4), normal karyotype (n=2), and EVI-rearranged (n=1). Six of these 9 patients underwent allogeneic hematopoietic cell transplant (allo-HCT), of which 2 are long-term survivors, 2 died of refractory/relapsed tMN and 2 died of treatment-related mortality after HCT. Cumulative incidence of tMN in patients who received cRIT treatment was 3.7% (95% CI 2-8.9%) at 5 years since initial oncologic diagnosis.

Conclusion: Although we have identified cytogenetic changes with and without tMN in some patients treated with cRIT, the cumulative incidence is comparable to other reports of heavily treated pediatric patients with solid tumor malignancies who did not receive cRIT, suggesting that the addition of cRIT to other conventional therapies does not increase the risk of secondary hematologic malignancies.

P253

The Yes-Associated Protein (YAP) regulates GD2 immunotherapy response in high-risk neuroblastoma

Pilgrim A[1,2,3], Jonus H[1,3], Ho A[1,3], Cole A[2], Shim J[1,3], Goldsmith K[1,3]

[1]Department of Pediatrics, Emory University School of Medicine, Atlanta, United States of America, [2]Laney Graduate School, Emory University, Atlanta, United States of America, [3]Division of Hematology/Oncology, Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Atlanta, United States of America

Background/ Aims: Patients with high-risk neuroblastoma (HR NB) receive intense multimodal therapy yet 50% still relapse with chemotherapy-resistant disease. Relapsed NBs harbor increased RAS/MAPK pathway mutations and increased expression and activity of the transcriptional co-regulator YAP. We have previously shown that YAP mediates resistance to chemotherapy and MEK inhibitors in RAS mutant NBs. Patients with relapsed NB are treated with the GD2-targeting antibody dinutuximab in combination with chemotherapy. Given the increased expression and activity of YAP in relapsed HR NB, we posited that YAP might be involved in GD2 immunotherapy response.

Methods/ Results: We stably knocked down YAP in SK-N-AS cells, and co-cultured these shYAP1, shYAP2, and control SK-N-AS cells with gamma delta (γδ) T cells which mediate antibody-dependent-cellular cytotoxicity in the presence of dinutuximab. YAP knockdown sensitized SK-N-AS shYAP cell lines to γδ T cell killing in the presence and absence of dinutuximab. To investigate the mechanism of increased dinutuximab sensitivity, we evaluated a panel of NB cell lines for YAP protein and GD2 cell surface expression and noted an inverse relationship. We therefore assessed GD2 expression following YAP knockdown in SK-N-AS and show that GD2 increased on the cell surface. In the GD2 biosynthesis pathway, GM3 is converted into GD3 by GD3 synthase (GD3S); then, GD2 synthase catalyzes GD3 into GD2. GD3S gene (ST8SIA1) expression significantly increased (>100-fold) upon YAP knockdown. In primary HR NB tumor datasets, we noted an inverse relationship between ST8SIA1 and YAP expression. PRRX1 induces a mesenchymal NB phenotype characterized by high YAP expression and low/ no GD2. Interestingly, PRRX1 expression increased in YAP knockdown cells yet GD2 expression also increased, suggesting YAP regulates GD2 more directly than PRRX1. Furthermore, stable inhibition of GD3S in shYAP NB cells reverted the phenotype, decreasing GD2 expression. We then treated established SK-N-AS control or shYAP xenografts with human γδ T cells, dinutuximab, and cyclophosphamide. Results show significantly extended survival in mice harboring SK-N-AS shYAP tumors.

Conclusion: These results support YAP regulation of GD2 expression through transcriptional suppression of GD3 synthase and identify YAP as a therapeutic target to augment GD2 immunotherapy responses in HR and relapsed NB.

P254

Combined chemotherapy and immune checkpoint blockade treatment enhances antitumor immunity by remodelling the tumor immune landscape in neuroblastoma

Lucarini V[1], Melaiu O[1], D'Amico S[1], Pastorino F[2], Tempora P[1], De Ninno A[3], De Ioris M[1], Alaggio R[4], Businaro L[3], Ponzoni M[2], Locatelli F[1], Fruci D[1]

[1]Department of Paediatric Haematology/Oncology and of Cell and Gene Therapy, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [2]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genoa, Italy, [3]CNR Institute for Photonics and Nanotechnology, Rome, Italy, [4]Pathology Unit, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

Background

Recent findings on the administration of drug combinations capable both of stimulating the recruitment of immune cell into the tumor microenvironment (TME) and of reinvigorating the T-cell-mediated immune response, prompted us to investigate new strategies to enrich the TME of the neuroblastoma (NB) with functional TILs.

Aims

Here, we identified combinatorial in situ immunomodulation strategies based on the administration of selected immunogenic drugs and immune checkpoint inhibitors to sensitize the poorly T-cell-infiltrated NB to the host antitumor immune response.

Methods

In vivo and ex vivo approaches were used to study combined drug treatments to enhance the antitumor immune response against 975A2 and 9464D NB cells derived from spontaneous tumors of TH-MYCN transgenic mice. The migration of immune cells towards murine-derived organotypic tumor spheroids (MDOTS) treated with drugs was assessed by microfluidic devices. Activation status of immune cells co-cultured with drug-treated MDOTS was assessed by flow-cytometry analysis. The effect of drug treatment on the immune content of subcutaneous or orthotopic tumors was analyzed by flow-cytometry, immunohistochemistry and multiplex immunofluorescence. The chemokine array assay was used to detect soluble factors released into TME. The efficacy of the most promising drugs in inducing migration and activation of immune cells was tested in cocultures of patient-derived organotypic tumor spheroids (PDOTS), generated from human NB specimens, and autologous PBMC.

Results

We found that treatment with low-doses of anthracycline recalled immune cells into TME and promoted CD8+ T and NK cell activation in MDOTS when combined with TGFβ and PD-1 blockade. These combined immunotherapeutic strategy curbed NB growth, resulting in the enrichment of a variety of both lymphoid and myeloid immune cells, particularly intratumoral dendritic cells (DC) and IFNγ and granzyme B-producing CD8+ T cells and NK cells. A concomitant production of inflammatory chemokines involved in remodeling the tumor immune landscape was also detected. Interestingly, this treatment was also effective in inducing the activation and recruitment of CD8+ T cells and NK cells to PDOTS.

Conclusions

Combined treatment with low-doses of chemotherapy, anti-TGFβ and PD-1 blockade improves antitumor immunity by remodeling the tumor immune landscape and overcoming the immunosuppressive microenvironment of aggressive NB.

P255

CHRNA3 as a super-enhancer-driven oncoprotein and potential immunotherapeutic target in high-risk neuroblastoma

Schuerch P[1], Zecchino V[1], Mcintyre B[1], Weiner A[1], Groff D[1], Garrigan E[1], Bosse K[1,2]
[1]Children's Hospital Of Philadelphia Division of Oncology and Center for Childhood Cancer Research, Philadelphia, United States, [2]Children's Hospital Of Philadelphia Department of Pediatrics, Philadelphia, United States

Background
We recently conducted an RNA sequencing-based screen in neuroblastoma to identify differentially expressed cell surface molecules suitable for immunotherapeutic therapeutic targeting (Cancer Cell 2017) and prioritized the nicotinic acetylcholine receptor (NACHR) CHRNA3 for further evaluation.

Aims
To elucidate the mechanisms of deregulated CHRNA3 expression and function and validate CHRNA3 as an immunotherapeutic target in neuroblastoma.

Methods
RNA expression and chromatin immunoprecipitation sequencing (ChIPseq) data for CHRNA3 and other NACHRs were queried from publicly available databases. CHRNA3 levels were validated in neuroblastoma patient-derived xenograft (PDX) models (n=35) and primary tumors (n=64) and pediatric normal tissues (n=41) using immunohistochemistry. Binding of NACHRs was assessed via coimmunoprecipitation in neuroblastoma cell lines. To investigate the role of the NACHR subunits on neuroblastoma cell growth, we targeted either CHRNA3, CHRNB4, or CHRNA5 with RNAi. To validate the immunotherapeutic targetability of CHRNA3 in neuroblastoma, scFvs specific for CHRNA3/CHRNB4 epitopes were evaluated for binding to neuroblastoma and control isogenic cell lines and utilized to generate chimeric antigen receptor (CAR) T cells.

Results
We confirmed high tumor-restricted CHRNA3 expression using immunohistochemistry in neuroblastoma primary and PDX tumors versus pediatric normal tissues. We next discovered the presence of a super-enhancer using H3K27 acetylation ChIP-data upstream of the CHRNA3 locus in 10 of 10 neuroblastoma cell lines that is also occupied by multiple core regulatory circuit transcription factors. CHRNA3 shares a locus with CHRNB4 on chromosome 15q and expression of these NACHRs is robustly correlated in 6 neuroblastoma tumor datasets (n = 2,600 total neuroblastomas; r = 0.6-9, p<3x10-22). Coimmunoprecipitations showed that CHRNA3 binds CHRNB4 in Kelly and SK-N-FI neuroblastoma cells and genetic depletion of CHRNA3 or CHRNB4 significantly inhibited growth of these cell lines. Finally, scFvs specific for CHRNA3/CHRNB4 complex epitopes bind neuroblastoma cell lines and PDXs and the testing of CHRNA3 CAR T cells in neuroblastoma preclinical models is ongoing.

Conclusion
CHRNA3 is a super-enhancer driven oncoprotein that complexes with CHRNB4 and is robustly differentially expressed between neuroblastomas and normal pediatric tissues. CAR T cells targeting CHRNA3/CHRNB4 complex epitopes in neuroblastoma are being explored for their anti-tumor efficacy and safety.

P256

Neuroblastoma Cells Modulate Macrophage Differentiation and Polarization in 3D Co-Culture Models

Coronado E[1,2], Martinez N[1,2], Burgos R[1,2], Castel V[2,3], Cañete A[2,3], Font de Mora J[2,3]
[1]Laboratory of Cellular and Molecular Biology, Health Research Institute Hospital La Fe, Valencia, Spain, [2]Clinical and Translational Research in Cancer, Health Research Institute Hospital La Fe, Valencia, Spain, [3]Pediatric Oncology Unit, La Fe University Hospital, Valencia, Spain

Background: Although neuroblastoma (NB) is considered an immunologically 'cold' tumor, the high-risk subtype displays high infiltration of macrophages.
Aims: We hypothesize a signaling crosstalk between NB cells and monocytes leading to their macrophage differentiation and subsequent polarization that could be exploited therapeutically.

Methods: We established 5 high-risk NB patient-derived organoids (4 MYCN-amplified) and 3D cultures of 4 NB cell lines (SH-SY5Y, SK-N-BE(2), IMR-32, SK-N-AS) and co-cultured them with THP-1 cells and THP-1-derived macrophages (MΦM0). Co-culture was performed using Transwell inserts (1.0 µm pore size membrane). The conversion rate of THP-1 to MΦM0 and MΦM0 to MΦM1 (pro-inflammatory macrophages) and MΦM2 (immunosuppressive macrophages) was assessed after 72h. Expression of specific population markers CD86 (MΦM0), HLA-DR (MΦM1), CD206 and CD163 (MΦM2), was measured by digital droplet PCR (ddPCR). Expression of immune-checkpoint molecules IL10, TGFb, IDO1, and PD-L1 was also quantified to validate their interplay in NB immune niche.

Results: In vitro 3D co-culture models showed that NB induced THP-1 differentiation into MΦM0, irrespective of NB genetic alterations. Notably, MYCN-amplified NBs promoted higher polarization into MΦM2 than the rest NB subtypes, supporting their relevance in the immune escape of the MYCN subtype.

Conclusion: There is an immunosuppression crosstalk in high-risk NB with preferential M2 polarization by MYCN amplification. Identifying the underlying signaling pathways could provide valuable information into the mechanisms of immune evasion exploited by NB and the design of targeted therapies for specific NB subgroups.

P257

Preliminary real-world data study of Dinutuximab β in the treatment of Chinese children with high-risk or recurrent/ refractory neuroblastoma

Yuan X[1], Wu Y, Li J[3], Wang J[4], Qian X[5], Zhang W[6], Zhao W[10], Gao Y[7], Mao J[8], Fang Y[9]
[1]Dept. of Paediatric Hematology/Oncology, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2]Dept. of Paediatric Surgery, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China, [3]Tianjin Medical University Cancer institute & Hospital, Tianjin, China, [4]Shandong Cancer Hospital and Institute, Jinan, China, [5]Children's Hospital of Fudan University, Shanghai, China, [6]Beijing Tongren Hospital, Beijing, China, [7]Shanghai Children's Medical Center, Shanghai, China, [8]The Children's Hospital, Zhejiang University School of Medicine, Hanzghou, China, [9]Children's Hospital of Nanjing Medical University, Nanjing, China, [10]Beijing Children's Hospital, Beijing, China

Bacground The prognosis of children with high-risk or recurrent/refractory neuroblastoma (NB) is gloomy, even though receiving strong chemotherapy, aggressive resection, radiotherapy, stem cell transplantation or tretinoin.

Objective To evaluate the safety and short-term efficacy of Dinutuximab β (DB) in the treatment of children with high-risk or relapsed/refractory NB in China, and to provide some basis for the clinical study design and dose determination of DB after its marketing in China.

Methods To objectively evaluate the tolerability, incidence of adverse events and initial response to treatment with DB based on real-world clinical study data.

Results Thirty-seven NB patients received DB immunotherapy, including 26 males and 11 females. There were 26 high-risk cases, 5 recurrent and 6 refractory patients. The pain scores of CRIES in this group were ≤3 points, and the major manifestations were limb or abdominal pain. Hundred percent (37/37 cases) of the children suffered fever, the average peak temperature was 39.4°C. Three children (8.1%) younger than 3 years old were diagnosed as severe capillary leakage syndrome. Two cases abandoned subsequent immunotherapy after 2 cycles of DB treatment due to progressive disease or repeated intestinal obstruction. The median follow-up time was 15 months, Twenty-six patients (70.3%) got a complete response, and one patient (2.7%) had a very good partial response. Nine patients (24.3%) relapsed, of which 3 died and 6 remained stable disease after re-treatment. And notably, intracranial metastasis occurred in 4 cases. One patient abandoned the following treatment due to progressive disease.

Conclusion Dinutuximab β was well tolerated in high-risk and relapsing/refractory NB patients in China. The overall incidence and severity of adverse effect were lower than those reported in foreign literature, but more attention should be paid to the safety of young children ≤3 years old and prevention of intracranial metastasis. This study designed a reasonable administration regimen and showed good short-term efficacy.

P258

Butyrophilin 3A2 plays a critical role in phosphoantigen-mediated gamma delta (γδ) T cell cytotoxicity of neuroblastoma

Ho A[1], Jonus H[1], Goldsmith K[1]
[1]*Emory University School of Medicine, Department of Pediatrics, Aflac Cancer and Blood Disorders Center, Atlanta, United States*

Background: The use of antibodies targeting the diasylganglioside GD2 improved survival and identified a role for immunotherapy in neuroblastoma (NB). However, high-risk NB survival remains 50%, warranting novel immunotherapeutic approaches. Given their MHC-independent innate cytotoxicity towards cancer, gamma delta (γδ) T cells are attractive candidates for adoptive cellular immunotherapy. γδ T cell cytotoxicity is mediated by the γδ-TCR. Elevated intracellular phosphoantigens (pAg) found in tumors bind to tumor-resident butyrophilin-3 (BTN3A) proteins to alter extracellular BTN3A conformation, leading to γδ TCR recognition and T cell activation. Furthermore, zoledronate (ZOL) stimulation of pAg production can promote γδ T cell recognition/killing. We find γδ T cells are differentially cytotoxic towards NBs, suggesting variations in expression of key players responsible for γδ T cell activity.

Aim: We aimed to explore the impact of NB BTN3A expression on γδ-TCR mediated killing.

Methods: BTN3A family expression was determined by immunoblot in human-derived NBs. NBs were pre-treated with vehicle or ZOL for 24hrs and then co-incubated with expanded γδ T cells for 4hrs at various effector:target (E:T) ratios. NB apoptosis was determined using Annexin-V/7-AAD. BTN3.2 was knocked down by shRNA in SK-N-AS to determine functional role.

Results: At baseline, Kelly, NB-1643, NGP, SK-N-AS, SH-SY5Y, NLF, and SMS-SAN cells were resistant to γδ T cells while IMR5 underwent apoptosis at a 5:1 E:T. Pre-treatment with ZOL enhanced cytotoxicity for all above NBs except SK-N-AS. We identified NBs with differential γδ T/ZOL susceptibility: SK-N-AS<NLF<IMR5. Using a NB gene expression dataset, we queried BTN3.1/BTN3.2 expression, revealing IMR5 (low/low), NLF (low/mod), and SK-N-AS (high/high) expression. It has been postulated that BTN3.2 acts as a decoy receptor, interacting with γδ T cells/TCR without cytotoxic response. Due to high BTN3.2 expression in γδ T cell resistant SK-N-AS, BTN3.2 was knocked down. SK-N-A-S non-target control had 21-24% lysis at a 5:1 E:T, regardless of ZOL pre-treatment. shBT3.2 models had 48-55% lysis at a 5:1 E:T when untreated and 65-72% lysis when pre-treated with ZOL.

Conclusion: Zoledronate pre-treatment augments anti-NB cytotoxicity by γδ T cells. BTN3.2 expression may be responsible for differential sensitivity of NBs to γδ T cells and thus a biomarker of resistance.

P259

Omalizumab for management of urticaria in patients receiving anti-GD2 immunotherapy.

Lentini-Rivera A[1], D'Andrea L[1], Durney C[1], Kasper C[1], Lin Y[1], Andre-Jones L[1], Glincher R[1], Shrager L[1], Iglesias-Cardenas F[1], Basu E[1], Kramer K[1], Lacouture M[1], Noor S[1], Kushner B[1], Modak S[1]
[1]*Memorial Sloan Kettering Cancer Center, New York , United States*

Background: Anti-GD2 antibodies are considered standard-of-care for high-risk neuroblastoma. Common side effects include allergic reactions that sometimes manifest as skin rashes including urticaria. Rash has been reported in approximately 10% of patients receiving naxitamab and 20-25% in those receiving dinutuximab and is thought to be IgE-mediated. Therapy for allergic skin rashes includes antihistamines and steroids. However, severe dermatologic reactions might not respond to the above and rarely result in discontinuation of anti-GD2 antibody therapy. Omalizumab, a monoclonal antibody that inhibits IgE binding, is FDA-approved for treatment of asthma and urticaria.

Aims: To report on the management of severe naxitamab-associated urticaria with omalizumab.

Methods: After IRB approval, medical records of patients receiving omalizumab for naxitamab-associated urticarial rash were reviewed.

Results: Two patients with high-risk neuroblastoma aged 12 (patient#1) and 16 years (patient#2) developed grade 3 urticarial rash while receiving, respectively, a 5th cycle of naxitamab + GM-CSF, and a 3rd cycle of naxitamab + irinotecan + temozolomide +GM-CSF (NCT03189706). Rashes occurred during the 2nd and 3rd doses of naxitamab of respective cycles and were initially treated with maximum doses of antihistamines; patient 2 also received methyl prednisone. Patients 1 and 2 received 150mg and 300mg subcutaneous omalizumab respectively. There was near complete resolution of rash in both within 24 hours of omalizumab. Omalizumab was not repeated during the initial rash presentation, no omalizumab-related adverse reactions were noted and both patients were able to complete anti-GD2 immunotherapy without recurrence of rash. Patient#2 continued an antihistamine regimen for 1 week post-immunotherapy. Both patients continued monthly omalizumab during subsequent cycles of immunotherapy. <grade 3 adverse events in subsequent cycles were: generalized macular rash and facial redness (patient#2); sneezing, cough, GM-CSF-related rash, and mild urticaria (patient#1). These events were well controlled by the addition of antihistamines. Patient#1 received 4 more cycles of naxitamab+GMCSF and patient#2 received 5 more cycles of chemoimmunotherapy.

Conclusion:
Omalizumab was effective in controlling severe naxitamab-associated urticaria and permitted continuation of naxitamab therapy. It should be considered for the management of severe urticaria associated with anti-GD2 antibody therapy.

P260

Neuroblastoma extracellular vesicles expose GPC2 and activate GPC2 CAR T cells in an antigen-dependent manner

Giudice A[1], Pascual-Pasto G[1], Matlaga S[1], Rouin G[1], Mcintyre B[1], Schürch P[1], Bosse K[1,2]
[1]Division of Oncology and Center for Childhood Cancer Research; Children's Hospital of Philadelphia, Philadelphia, United States, [2]Department of Pediatrics; Perelman School of Medicine at the University of Pennsylvania, Philadelphia, United States

Background: GPC2 chimeric antigen receptor (CAR) T cells are safe and efficacious in neuroblastoma. Tumor-derived extracellular vesicles (TEVs) are nanosized vesicles secreted by cancer cells and enriched in immunoregulatory proteins, such as PD-L1. The presence of tumor-associated antigens, such as GPC2/GD2, on neuroblastoma TEVs and interaction with CAR T cells has not been explored.

Aims: To define the levels of GPC2 on TEVs and how GPC2+ TEVs modulate CAR T cell efficacy in neuroblastoma.

Methods: TEVs were isolated by differential ultracentrifugation and characterized by Nanoparticle-Tracking Analysis, flow cytometry, and western blotting. TEV effects on CAR T cells were quantified by flow cytometry for markers of T cell activation, IL-2/INF-γ ELISAs, and luciferase-based cytotoxicity assays. RAB27A KO xenografts, generated using CRISPR/Cas9, with significantly decreased TEV secretion, were utilized to validate TEV modulation of CAR T cell efficacy in vivo.

Results: High-levels of GPC2 were found on TEVs isolated from neuroblastoma models (7 cell lines, circulating EVs from 4 patient-derived xenograft (PDX)-bearing mice) and from the peripheral blood from 8 neuroblastoma patients, but not on circulating EVs from non-tumor bearing mice or 8 healthy human donors. The level of circulating GPC2+ TEVs positively correlated with PDX size ($R=0.96$; $P<0.0001$) and parent cell GPC2 expression. We also found that GPC2+ TEVs bind GPC2 CAR T cells proportionally to TEV-GPC2 surface levels and induce T cell activation (CD69/Granzyme B expression and release of IL-2/IFN-γ) and enhance target neuroblastoma cell cytotoxicity. We next observed that injection of GPC2+ TEVs into a xenograft genetically altered to have significantly decreased TEV production (RAB27A KO SK-N-AS xenografts) significantly increased the efficacy of co-infused GPC2 CAR T cells compared to treatment with CAR T cells alone or intra-tumor injection of GPC2-low TEVs. Finally, we have also found high levels of other neuroblastoma immunotherapeutic target molecules on TEVs and performed similar studies with GD2+ TEVs and GD2 CAR T cells, which will be reported.

Conclusion: GPC2+ TEVs are secreted from neuroblastomas and bind and activate GPC2 CAR T cells in an antigen-specific manner that enhances their anti-tumor cytotoxicity. Neuroblastoma TEVs should be further validated as liquid biopsy-derived biomarkers for targeted-immunotherapy.

P261

Controlled RRM2 protein degradation for pharmacological target validation and functional analysis in MYCN-driven neuroblastoma

Leys S[1,2], Hilgert E[1,2], Loontiens S[1,2], Sanders E[1,2], Depestel L[1,2], Goulding J[1,2], He S[3,4], Look T[3,4], Poon E[5], Chesler L[5], Vanhauwaert S[1,2], Bek J[1,2], Durinck K[1,2], Speleman F[1,2]

[1]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium, [2]Cancer Research Institute Ghent (CRIG), Ghent University, Ghent, Belgium, [3]Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, USA, [4]Division of Pediatric Hematology/Oncology Boston Children's Hospital, Boston, USA, [5]Division of Cancer Therapeutics, Institute of Cancer Research, London, UK

Background. MYC(N) overexpressing neuroblastoma (NB) cells undergo enhanced replication stress marked by elevated ATR-CHK1 signaling. It was recently shown that MYC-multimers associate with stalled forks to suppress DNA double strand break formation (Solvie et al., 2022). We identified RRM2 (Nunes et al., 2022) and BRIP1 (unpublished) as important replicative stress resistance factors in NB, further underscoring the importance of fork dynamics for NB cell fitness and survival that provide new therapeutic avenues.

Aims. We aim to (1) assess temporal dynamics of RRM2 dependency in MYCN-driven NB to gain deeper insights into the role of RRM2 in NB initiation and tumor maintenance and (2) further validate RRM2 as a target for combinatorial drugging of the ATR-CHK1-RRM2 axis.

Methods/materials. We follow a unique cross-species approach of combined mouse and zebrafish modeling to explore a novel dTAG (protein degrader tagging) approach for selective targeting and reversible tuning of RRM2 protein expression using an FKBP12F36V degrader in frame with RRM2, allowing degradation in a tightly regulated manner.

Results. We have designed an in vitro approach for rapid and reversible RRM2 degradation using dTAG-13 or dTAGV1 ligands for respective cereblon (CRBN) or von Hippel-Lindau tumor suppressor (VHL) recruitment to the fused FKBP12F36V degron in a NB cell line. In addition, we have initiated selection of stable dbh-hRRM2-FKBP12F36V, hCRBN and hVHL overexpressing zebrafish lines. The effects of RRM2 protein degradation in established zebrafish NB tumors will be evaluated by immunostaining for proliferation markers, DNA damage markers and R-loop formation as well as single-cell RNA-sequencing and spatial transcriptomics. Based on the data generated from in vitro and zebrafish experiments, we will develop a mouse dTAG model to further probe the effects of RRM2 degradation.

Conclusion. Our controlled RRM2 protein degradation approach will allow for the first time to assess temporal dynamics of RRM2-dependency in MYCN-driven NB. This will provide a powerful platform for further integrative functional and genomics-proteomics analyses and will aid in the deeper understanding of the mechanistic basis of replicative stress resistance factors to explore novel drugging strategies.

Nunes et al., Science Advances, 2022. doi:10.1126/sciadv.abn1382
Solvie et al., Nature, 2022. doi: 10.1038/s41586-022-05469-4

P263

The role of immunosuppressive tumor-associated macrophages in Mycn-driven neuroblastoma

Yoda H[1], Menard M[1], Ray A[2], Krummel M[2], Weiss W[1]

[1]Department of Neurology, University of California, San Francisco, San Francisco, United States, [2]Department of Pathology, University of California, San Francisco, San Francisco, United States

The TH-MYCN mouse model is representative of human high-risk neuroblastoma and recapitulates the tumor microenvironment of high-risk neuroblastoma. Immune profiling demonstrates a tumor microenvironment by tumor-associated macrophages. Since TH-MYCN tumors rarely arise in the C57BL6/J strain, it is challenging to leverage powerful immunological genetic tools available primarily in C57BL6/J backgrounds. Therefore, we created a non-germline genetically engineered mouse model in which primary trunk neural crest cells isolated from C57BL6/J embryos were modified genetically by transducing mouse them with murine Mycn (Mycn-nGEMM). We transduced Mycn-nGEMM tumor-derived cell lines with Pmel (mouse premelanosome protein), a strong antigen. Surprisingly, the progression of Mycn-nGEMM tumors was not suppressed in the Pmel-1 T cell receptor transgenic mouse model that specifically recognizes the MHC class I-mouse pmel-derived peptide complex, indicating that the tumor microenvironment of Mycn-nGEMM tumors is highly immunosuppressive. Moreover, mass cytometry characterization of the tumor microenvironment showed mostly immunosuppressive tumor-associated macrophages, suggesting that M2-like tumor-associated macrophages support the immunosuppressive tumor microenvironment in Mycn-nGEMM tumors. To assess the effects of immune checkpoint inhibitors on Mycn-nGEMM, we administrated anti-PD-1/anti-CTLA-4 and anti-PD-L1 antibodies and found that anti-PD-L1 antibody suppressed tumor progression and improved survival rates by which anti-PD-L1 antibody decreased M2-like tumor-associated macrophages and elicited infiltration of cytotoxic T cells. These findings prompted us to clarify further the role of immunosuppressive tumor-associated macrophages in Mycn-nGEMM tumors. We employed a novel CD206-lox-stop-lox-Venus-diphtheria toxin receptor (DTR) mouse model in which the endogenous pro-tumorigenic immunosuppressive-marker CD206 promoter drives the expression of a Venus fluorescent reporter. After crossing to the Csf1r-Cre mouse model, the CD206-DTR mouse model selectively depleted myeloid-derived immune cells expressing CD206. After diphtheria toxin exposure, we evaluated the tumor microenvironment of Mycn-nGEMM tumors and the draining lymph nodes using flow cytometry. While we confirmed massive infiltration of neutrophils, tumors were growing with no increase in tumor-infiltrating lymphocytes (monocytes, conventional dendritic cells (cDC1), cDC2, B, natural killer, CD4+ T and CD8+ T cells) despite a decrease in CD206+ tumor-associated macrophages, suggesting that CD206+ tumor-associated macrophages also play an essential role in the recruitment and maintenance of cDC1 and CD8+ T cells in the tumor microenvironment of Mycn-nGEMM tumors.

P264

Repression of the STING pathway by MYCN contributes to the cold immunophenotype of MYCN amplified neuroblastoma

Di Giulio S[1], Belardinilli F[1], Belloni L[1], Rullo E[1], Petroni M[1], Fabretti F[1], La Monica V[1], Battaglini D[1], Augusto M[1], Schirano R[1], Manfredi C[1], Gerardi L[1], Licursi V[2], Liu Z[3], Thiele C[3], Giannini G[1,4]

[1]Dept. of Molecular Medicine, University La Sapienza, Rome, Italy, [2]Dept. of Biology and Biotechnology "Charles Darwin", University La Sapienza, Rome, Italy, [3]Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethasda, USA, [4]Istituto Pasteur-Fondazione Cenci Bolognetti, Rome, Italy

MYCN amplification dramatically affects tumor behavior and associates with refractoriness to standard therapy in MYCN amplified neuroblastoma (MNA NB), raising the need for more effective interventions. The STING pathway has gained interest in cancer immunotherapy due to its ability to stimulate type-I interferon (I-IFN) transcription and immune/inflammatory responses against tumor cells, by sensing cytosolic DNA derived from viral or endogenous sources (DNA damage).
Whilst MNA NBs are characterized by high levels of oncogene-derived DNA damage, they are typically associated with a 'cold' phenotype, that is lack of tumor infiltrating lymphocytes, low I-IFN transcriptomics and chemokine expression. Whether this might depend on STING pathway attenuation has remained so far unexplored.
Transcriptomics, methylome and ChIP-Seq analyses, as well as treatments with 5-aza-2'-deoxycytidine (DAC) and DNMT inhibitors were performed to assess the epigenetic state of the STING pathway on primary tumors and/or a panel of non-MNA and MNA NB cell lines and MYCN-inducible models. The expression and activation of the STING pathway were evaluated by WB and qPCR. STING agonist (cGAMP/diABZI) and other STING pathway inducers (dsDNA90/olaparib) were used to assess its activity under different conditions.
The STING pathway is significantly less expressed in MNA compared to non-MNA NBs and cGAS and STING promoters are significantly more methylated in MNA NBs. Consistently cGAS and STING are undetectable in all MNA NB cells and variably expressed in non-MNA cells. Inducible MYCN expression transcriptionally repressed cGAS and STING, supporting the idea that MYCN enforces the suppression of this pathway. Mechanistically, cGAS/STING repression occurs through DNA methylation. STING pathway activation and I-IFN response are functionally impaired in MYCN-driven cells. Restoring cGAS/STING by transgene expression or epigenetic derepression via DAC/DNMT inhibitors is sufficient to confer responsiveness to STING pathway inducers in MNA NBs. The induction of I-IFN transcriptional responses in these settings indicate that STING pathway reactivation might be sufficient to restore the secretion of pro-inflammatory cytokines in MNA NB. These data reveal that the STING pathway is silenced in MNA NB through epigenetic mechanisms enforced by MYCN. Understanding and counteracting these mechanisms may provide new therapeutic opportunities for MNA NB based on STING pathway reactivation.

P265

In-depth phenotypic and functional characterization of Natural Killer Cells in Bone Marrow of High-Risk neuroblastoma patients

Vitale C[1], Colonna A[1], Morini M[2,3], Ardito M[2,3], Altosole T[4], Conte M[5], Garaventa A[5], Bottino C[1,6], Dondero A[1], Castriconi R[1]

[1]Department of Experimental Medicine (DIMES), University of Genoa, Genoa, Italy, [2]Laboratory of Molecular Biology, IRCCS Istituto Giannina Gaslini, Genoa, Italy, [3]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genoa, Italy, [4]IRCCS Ospedale Policlinico San Martino, Genoa, Italy, [5]Pediatric Oncology Unit, IRCCS Istituto Giannina Gaslini, Genoa, Italy, [6]Laboratory of Clinical and Experimental Immunology, IRCCS Istituto Giannina Gaslini, Genoa, Italy

Background: high-risk neuroblastoma (HR-NB) still have a low 5-year overall survival despite the use of multiple therapeutic approaches. These include immunotherapy with the anti-GD2 monoclonal antibody, which can also act by activating Natural Killer (NK) cells through the engagement CD16 (FcγRIII).
Aims: i) providing a wide phenotypic and functional analysis of Bone Marrow (BM) and peripheral blood (PB) NK cells in HR-NB patients; ii) monitoring BM-NK cell properties at different diseases's stages, onset, post Autologous Hemopoietic Stem Cell Transplantation (aHSCT), and post-immunotherapy.

Methods: we set up a cytofluorimetric multiparametric panel to identify and characterize NK cells in PB and BM of HR-NB (n=11 patients recruited at the onset). NK cells, identified as CD56+ CD127- CD3- CD19- CD14- B7-H3- lymphocytes, were analyzed for the expression of activating, inhibitory receptors (including immune checkpoints), activation markers (including CD69) and chemokine receptors (including CXCR6). The cytofluorimetric data of BM- and PB-NK cells were concatenated and analyzed by the unsupervised t-distributed Stochastic Neighbor Embedding (t-SNE) method. BM-NK cell degranulation was induced using different stimuli, and analyzed by flow cytometry using the CD107a assay.

Results: in addition to the conventional CD56bright CD16+/- and CD56dim CD16+ NK cell subsets, CD56intermediate (int) CD16low/- NK cells were observed that co-expressed CD69 and CXCR6. This CD56int CD16low/- CD69+ CXCR6+ NK cell subset was undetectable in PB, while was present in BM with different percentages (from 5 to 54% of all NK cells) in the various patients analyzed. Longitudinal studies in 4 patients showed that this population was enriched in the post aHSCT, particularly in the time window suitable for starting immunotherapy. Importantly, this population showed an altered expression of receptors involved in NB recognition, accompanied by a significant increase of NKp46 expression. Functional analysis showed that the CD56int BM-NK cell subset had degranulation capability when properly activated but could not be stimulated by the anti-GD2 mAb.

Conclusions: the in-depth analysis of BM-NK cells in HR-NB patients highlights which NK cell receptor could be engaged or blocked in immunotherapeutic protocols, with the aim of potentiating the NK-mediated immune surveillance of BM metastasis.

P266

"Exploring the Potential of Non-Canonical ORFs as Immunotherapeutic Targets in Neuroblastoma"

Hofman D[1], van Dinter J[1], Engels S[1], Cornel A[1], Kok L[1], Oostenbach E[1], Nierkens S[1,2], van Heesch S[1]
[1]Princess Máxima Center for Pediatric Oncology, Utrecht, Netherlands, [2]UMCU, Center of Translational Immunology, Utrecht,

BACKGROUND: While immune therapies targeting tumor-specific neoantigens have shown significant successes in the treatment of adult cancers, their potential in the treatment of neuroblastoma (NBL) remains largely untapped. An important reason for this is the scarcity of NBL-specific neoantigens, as these tumors often have low mutational burdens and lack expression of MHC-1.

AIM: To investigate the potential of non-canonical open reading frames (ORFs) from NBL-specific genes and transcripts as immunotherapeutic targets.

METHODS: We developed an integrated 2-step workflow that combines deep RNA sequencing and ribosome profiling of NBL tumor tissues and organoids to visualize the translation of previously unannotated transcripts into tumor-specific proteins.

RESULTS: We reconstructed novel transcripts from RNA-seq data from 202 patient samples, yielding 42 recurrently transcribed novel genes that are absent from RNA expression datasets of healthy tissues or other childhood cancers. Using ribosome profiling on 15 NBL patient tumor samples, we identified the translation of at least 2 ORFs derived from these novel NBL-specific genes. In addition, we identified translation of multiple non-canonical NBL-specific transcripts derived from canonical parent genes.

CONCLUSIONS: Our results suggest that previously undiscovered proteins are synthesized in a NBL-specific manner, which may represent unknown early developmental genes or tumor-activated molecules. These proteins could potentially serve as a new resource of targetable antigens for NBL immunotherapy.

P267

A Phase I Study of Expanded Autologous Natural Killer Cells with Dinutuximab for Relapsed/Refractory Neuroblastoma: A NANT Consortium Study.

Marachelian A[1,2], Villablanca J[1,2], Asgharzadeh S[1,2], Nakkula R[4], Cash A[4], Troy E[4], Thakkar A[4], O'Donnell L[5], Bradbury H[5], Daneault B[5], Ghazarian S[1], Chi Y[1,3], Mody R[6], Park J[7], Matthay K[8], Seeger R[1,2], Lee D[4,5]
[1]Children's Hospital Los Angeles, Cancer and Blood Disease Insititute, Los Angeles, United States, [2]University of Southern California, Department of Pediatrics, Los Angeles, United States, [3]University of Southern California, Department of Preventive Medicine, Los Angeles, United States, [4]Center for Childhood Cancer & Blood Diseases, The Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, United States, [5]The Ohio State University James Cancer Hospital Cell Therapy Laboratory, Columbus, United States, [6]C.S. Mott Children's Hospital, Michigan, United States, [7]Seattle Children's Hospital Division Hematology/Oncology, Department of Pediatrics, Seattle, United States, [8]UCSF Benioff Children's Hospital, Department of Pediatrics, San Francisco, United States

Background: Natural Killer (NK) cell-mediated antibody-dependent cell cytotoxicity by dinutuximab is an established therapeutic modality in neuroblastoma. However, NK cell numbers and functions are often abnormally impacted in these patients, providing rationale for the use of adoptive transfer of expanded autologous activated NK cells to improve efficacy.

Aims: To determine feasibility, maximum tolerated dose, and immunological effects of NK cell adoptive transfer with dinutuximab in children with relapsed/refractory high-risk neuroblastoma (RR-HRNB).

Methods: A phase 1 trial of 3+3 design was conducted in eligible patients ≤30 years old, with evaluable tumor, and regardless of prior anti-GD2 therapy. Autologous NK cells were expanded and activated (aNK) for 14 days (using CSTX-002 feeder cells (IL-21/4-1BBL)) from 3cc/kg (Dose level (DL)1-2) or 10cc/kg (DL3) of peripheral blood. aNK cells were infused at respective DLs (3x10^7, 1x10^8, 3x10^8 cells/kg/course) on Day 5 post-dinutuximab infusion (17.5 mg/m2/day on Days 1-4) in 28-day cycles. Safety for dose escalation (DE) was determined by course 1 toxicity.

Results: Thirteen patients aged 4-22.9 (median 11.2) years were enrolled. All had prior anti-GD2 therapy. There were no dose limiting toxicities (DLTs) among 18 total courses of therapy. Three patients were evaluable for DE at DL1 and DL2 and 4 at DL3. Reasons for DE inevaluability were inadequate NK yields (n=2, treated at DL1 and 2), and progressive disease (PD) prior to therapy (n=1). aNK cells expanded 137-7393 fold (median 1694) with final yield 3.82x10^7-2.6x10^9 with variable starting NK cell numbers. Expansion yielded sufficient aNK cells for more than one course for 2 patients on DL1, none on DL2 and for two patients on DL3 (range 7-9). Twelve patients evaluable for response had 1 Minor Response, 5 Stable Disease, and 6 PD. No statistical difference ($p>0.05$) in serum cytokines were observed post-aNK infusion. aNK demonstrated high variability of in vitro cytotoxicity against NB cell lines.

Conclusions: We demonstrate feasibility of manufacture and multi-site distribution of aNK cells, and safety of combining with dinutuximab in patients with RR-HRNB. Variable aNK yield limited overall duration of therapy, supporting future trials using banked universal-donor allogeneic NK products.

P268

Targeting PHOX2B with CAR T cells: Progress from peptide discovery to clinical trial

Yarmarkovich M[1], Marshall2 Q[2], Premaratne3 R[3], Gupta S[2], Li P[2], Groff D[2], Mycek E[2], Sun Y[2], Gupta S[2], Garfinkle S[2], Young M[2], Papadaki G[2], Malik L[2], Kiefel B[3], Sgourakis N[2,4], Maris J[2,4]
[1]New York University, Perlmutter Cancer Center, Grossman School of Medicine, New York, United States, [2]Center for Childhood Cancer Research, Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, USA, [3]Myrio Therapeutics, Melbourne, Australia, [4]Perelman School of Medicine at the University of Pennsylvania,, Philadelphia, USA

Background: We previously reported efforts for discovering and targeting intracellular neuroblastoma-specific drivers across multiple HLAs using CAR-T cells (Nature, 2021).
Aims: As a first-in-class therapeutic slated for clinical trials, we are developing processes for optimizing PC-CAR function, screening for safety, understanding the structural basis of binding, and characterizing the principles of cross-HLA binding such as to maximize population coverage.

Methods: To characterize activity and persistence, we constructed and tested our lead PC-CAR targeting PHOX2B (10LH) using various combinations of transmembrane and co-stimulatory domains. To evaluate cross-reactivity, we performed X-scanning, mutating each position off the PHOX2B 9mer to each other amino acid. We also tested 10LH PC-CARs against a panel of 27 primary normal tissue and 12 cancer cell lines expressing HLA-A*24:02 and/or HLA-A*23:01. To expand population coverage, we transduced cell lines with HLAs predicted to present PHOX2B and measured 10LH-PC-CAR cytotoxicity. To understand the basis of cross-HLA binding, we solved the crystal structure of the 10LH/PHOX2B-scFv/pMHC complex.

Results: CD28 hinge and transmembrane domains coupled with the CD28 co-stimulatory domain resulted in the most potent cytotoxicity but resulted in premature T cell exhaustion. We found that the CD8 transmembrane/hinge coupled with the 41BB co-stimulatory domain performed best over time, resulting in complete tumor ablation, while maintaining long-term persistence. 10LH PC-CARs potently ablate neuroblastoma cells but showed no significant cytotoxicity in any of the primary normal tissue and 11/12 cancer cell lines but did kill one glioblastoma cell line. The crystal structure reveals docking on conserved residues of the HLA alpha helices to allow for cross-HLA docking of the PC-CAR receptor and predicts additional HLAs that can be targeted using 10LH. We have completed GMP plasmid manufacturing and lentiviral vector manufacturing is underway.

Conclusions: As the first PC-CARs progress to the clinic, we are developing methods for optimizing receptor selectivity and function, developing safety screens, and employing structural predictions to maximize population coverage across HLAs. A phase 1/1b clinical trial is planned for Q4-2023.

P269

Characterizing the effects of polyamine synthesis inhibition and TGFb inhibition on the neuroblastoma immune microenvironment

Turn C[1], Vu A[1], Ferry G[1], Benavides A[1], Karageorgos S[1], Gandhi O[1], Bassiri H[1,2], Hogarty M[1,2]
[1]Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, United States, [2]University of Pennsylvania, Department of Pediatrics, Philadelphia, United States

Background: MYCN coordinately upregulates polyamine pools in high-risk neuroblastoma. Ornithine decarboxylase (Odc), the rate-limiting enzyme in polyamine synthesis, is a direct Myc target that is irreversibly inhibited by difluoromethylornithine (DFMO), an FDA-approved drug with preclinical activity in neuroblastoma mouse models, including TH-MYCN+/+ transgenic mice. Polyamines are members of an immunomodulatory metabolite network, and preclinical work supports immune mechanisms as relevant to DFMO activity. We hypothesize that in addition to its mechanistic effects, DFMO's polyamine inhibition alters the immune microenvironment (TME) to promote antitumor immunity.

Aims: To assess DFMO and TGFβ inhibition in TH-MYCN+/+ mice for effects on survival and the tumor microenvironment.

Methods: TH-MYCN+/+ neuroblastoma-prone mice (n=6/arm) were administered galunisertib (a selective TGFβR1 inhibitor) and/or 1% DFMO in drinking water in a four-arm trial. Tumor onset and survival were monitored. Multiparameter flow cytometry determined frequencies and functional markers for tumor-infiltrating leukocytes (TILs). Gene expression profiling used the nCounter Immuno-Oncology 360 platform (770 analytes). Multiplex cytokine analysis was evaluated via the Luminex xMap platform (33 analytes) and ELISA (TGFβ). Cell line TGFβ signaling was evaluated in tissue culture.

Results: In vitro, MYCN-amplified neuroblastoma cell lines produced more TGFβ and activated phospho-Smad2. Galunisertib reliably inhibited the TGFβ-Smad2/3 signaling cascade. In vivo, DFMO increased intra-tumoral NK cells, partially restored NK-activating NKG2D expression, and upregulated tumor cell NK ligand Rae1-γ. TGFβ was abundant in tumors and may decrease NKG2D expression. TH-MYCN+/+ mice treated with DFMO had increased survival (as shown previously); galunisertib alone accelerated tumor penetrance, and progression, and no synergistic anti-tumor effect was seen with combined DFMO/galunisertib. DFMO markedly upregulates the activity level of T cells and, to a lesser extent, NK cells. Ongoing studies are further characterizing the cytokine levels of the neuroblastoma TME under these treatment conditions.

Conclusions: DFMO administration creates a pro-inflammatory cellular and cytokine milieu in high-risk NB. TGFβR1 inhibition with galunisertib accelerates tumor onset when administered from an early age, but its in vitro effects suggest that later TGFβ inhibition with DFMO may better reverse intratumoral immunosuppression.

P270

Development of murine Gpc2 CAR T cells and syngeneic preclinical models to identify mechanisms of immunotherapeutic resistance in neuroblastoma

Giudice A[1], Pascual-Pasto G[1], Matlaga S[1], Schürch P[1], Bosse K[1,2]

[1]Division of Oncology and Center for Childhood Cancer Research; Children's Hospital of Philadelphia, Philadelphia, PA, 19104, United States, [2]Department of Pediatrics; Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, 19104, United States

Background: Glypican-2 (GPC2)-directed chimeric antigen receptor (CAR) T cells are safe and efficacious in neuroblastoma. However, how the tumor microenvironment (TME) modulates GPC2 CAR T cell efficacy has not been explored.

Aims: To develop murine Gpc2 CAR T cells as a tool to study the role of the TME in GPC2 CAR efficacy in neuroblastoma.

Methods: Mouse neuroblastoma cell lines 9464D and NXS2 were engineered to overexpress Gpc2. Murine Gpc2 CAR T cells, with either CD28 or 4-1BB costimulatory domains and a truncated murine CD19 (mCD19t) to enable CAR T cell tracking, were generated by retroviral transduction. Gpc2 CAR T cells were tested for activation, cytokine secretion, and cytotoxicity in vitro, and for in vivo efficacy using 9464D-Gpc2 allograft models. TME profiling was achieved using Cytometry by Time of Flight (CyTOF), cytokine profiling, and flow cytometry.

Results: Murine Gpc2 CAR T cells were selectively activated (IL-2 and INF-γ release) and induced target-specific cell cytotoxicity when co-incubated with 9464D/NXS2-Gpc2 cell lines but not empty vector-transduced paired control cells. Specifically, near complete 9464D/NXS2-Gpc2 cell killing was achieved at 10:1 and 5:1 E:T ratios (24h; $p<0.001$). In vivo, murine Gpc2 CAR T cells significantly induced 9464D-Gpc2 allograft regression and prolonged murine survival compared to mock transduced-T cells ($P<0.01$); however, durable tumor regressions were limited. Analysis of the CAR-treated 9464D-Gpc2 allograft TME revealed robust CAR T cell infiltration (45%±6.7 mCD19t+ of total lymphocytes), accompanied by increased intratumor recruitment of endogenous T cells (11.8-fold increase; $p<0.01$), macrophages (6.1-fold increase; $p<0.05$), NK cells (6.1-fold increase; $p<0.05$) and myeloid-derived suppressor cells (MDSCs; 4.7-fold increase; $p<0.05$) compared to control CAR T cells 5 days after CAR infusion. Consistent with immune activation, the levels of pro-inflammatory cytokines IL-6, IL-2 and IFN-γ were increased in the Gpc2 CAR-treated TME; however, the top-five enriched cytokine/chemokines (MMP-9, Chitinase 3-like 1, Lipocalin-2, CCL6/C10, MMP-3) were MDSC-related.

Conclusion: Immune-profiling of murine syngeneic neuroblastomas show that Gpc2 CAR T cells significantly reprogram the TME and suggest that recruitment of immunosuppressive-myeloid cells may limit sustained CAR T cell efficacy. Validation of combination immunotherapeutic approaches addressing the immunosuppressive TME and MDSC infiltration are ongoing.

P271

Persistence of Racial and Ethnic Disparities in Risk and Survival for Patients with Neuroblastoma: An International Neuroblastoma Risk Group Project

Chennakesavalu M[1], Pudela C[2], Applebaum M[2], Lee S[3], Che Y[3], Naranjo A[4], Park J[5], Volchenboum S[2], Henderson T[2], Cohn S[2], Desai A[2]

[1]Pritzker School of Medicine, University of Chicago, Chicago, USA, [2]Department of Pediatrics, University of Chicago, Chicago, USA, [3]Department of Public Health Sciences, University of Chicago, Chicago, USA, [4]Children's Oncology Group Statistics and Data Center, Department of Biostatistics, University of Florida, Gainesville, USA, [5]St. Jude Children's Research Hospital, Memphis, USA

Rapid Fire session 1B, May 16, 2023, 11:30 AM - 11:45 AM

Background:
We previously reported that Black and Native American patients with neuroblastoma have a higher prevalence of high-risk disease and inferior event-free survival (EFS) compared to White patients.

Aims:
We sought to validate our prior findings and investigate racial/ethnic survival disparities in the current era of therapy using the International Neuroblastoma Risk Group Data Commons.

Methods:
Two patient cohorts were identified (Cohort 1: diagnosed 2001-2009, n=4359; Cohort 2: diagnosed 2010-2019, n=4891). Chi-squared tests were used to assess the relationship between race/ethnicity and clinical and biologic features. EFS and overall survival (OS) were estimated by the Kaplan-Meier method from the time of diagnosis. Cox proportional hazards regression analyses were performed.

Results:
Cohort 2 patients had significantly higher 5-year EFS (P<0.001) and OS (P<0.001) compared to Cohort 1. Black patients in both cohorts had a significantly higher prevalence of high-risk disease (Cohort 1: P<0.001; Cohort 2: P<0.001) and worse EFS (Cohort 1: P<0.001; Cohort 2 P<0.001) and OS (Cohort 1: P<0.001; Cohort 2: P<0.001) compared to White patients. Adjustment for risk-group abrogated the differences in EFS and OS for Black patients in Cohort 1. In Cohort 2, significantly worse OS was identified for Black patients (P=0.028) and a trend towards worse OS (P=0.056) was identified for Hispanic patients after adjusting for risk group. No differences in EFS were observed in these populations compared to White patients. No differences in EFS or OS by race/ethnicity were observed for low-risk or intermediate-risk patients in either cohort. Among high-risk patients assigned to receive dinutuximab between 2001-2014 (n=919), Black and Hispanic patients had significantly worse OS compared to White patients (P=0.006 and P=0.02, respectively); EFS was not significantly different. Among high-risk patients diagnosed during this period who did not receive dinutuximab, EFS/OS did not significantly differ for Black or Hispanic patients compared to White patients.

Conclusion:
Racial and ethnic disparities among patients with neuroblastoma have persisted despite improvements in outcome with current era therapy. The benefit of immunotherapy for high-risk patients differs by race/ethnicity, with inferior survival for Hispanic and Black patients. Additional studies to elucidate the mechanisms underlying these differences are warranted.

P272

Modeling strategies for survival in Neuroblastoma: focus on long-term survival prediction in stage 4 patients >1 year treated within SIOPEN-HR-NBL1.

Poetschger U[1], Valteau-Couanet D[2], Elliot M[3], Luksch R[4], Cañete A[5], Ash S[6], Laureys G[7], Balwierz W[8], Papadakis V[9], Glogova E[1], Taschner-Mandl S[1], Arnardottir H[1], Kurzmann P[1], Ruud E[10], Kogner P[11], Malis J[12], Schroeder H[13], Owen C[14], Chan G[15], Trohair T[16], Schleiermacher G[17], Beck Popovic M[18], Ladenstein R[1]
[1]Children's Cancer Research Institute, Vienna, Austria, [2]Institut Gustave Roussy, Villejuif, France, [3]Leeds Teaching Hospital Trust, Department of Paediatric Oncology and Haematology , Leeds, United Kingdom, [4]Fondazione IRCCS Istituto Nazionale dei Tumori, S.C. Pediatria, Milan, Italy, [5]Hospital La Fe, Unidad de Oncohematologia Pediatrica , Valencia, Spain, [6]Schneider Children´s Medical Center of Israel, Dept. Paediatric Haematology/Oncology, Petah Tikva, Israel, [7]University Hospital Gent, Paediatric Haematology and Oncology, Gent, Belgium, [8]Jagiellonian University Medical College, Polish-American Institute of Paediatrics, Kraków, Poland, [9]Agia Sofia Children's Hospital, Department of Pediatric Hematology- Oncology, Athens, Greece, [10]Rikshospitalet Oslo, Department of Paediatrics, Oslo, Norway, [11]Karolinska Hospital, Astrid Lindgren Children's Hospital,Childhood Cancer Research Unit, Stockholm, Sweden, [12]University Hospital Motol, Dept. of Paediatric Oncology, Prague, Czech Republic, [13] University Hospital of Aarhus, Department of Paediatrics,, Aarhus, Denmark, [14]Children's Health Ireland, Dublin, Ireland, [15]University of Hong Kong, Hong Kong, Hong Kong, [16]Sydney Children's Hospital , Kids Cancer Centre, University of Tampere, Randwick, Australia, [17]Institut Curie, Laboratory of Translational Research in Pediatric Oncology – INSERMU830, Paris, France, [18]University Hospital (CHUV), Dept. of Paediatrics, Lausanne, Switzerland

Rapid Fire session 2B, May 18, 2023, 12:05 PM - 12:20 PM

Background
Standard survival models do not specifically address the primary interest in long-term outcome.

Aim
A novel statistical approach, the pseudo-value regression (PVR), is introduced that directly models survival probabilities at a pre-specified long-term time.

Methods
Prediction-models are almost exclusively derived from Cox-regressions or less frequently from tree-based approaches (survival-trees, random-forests). While Cox-regression models covariate-effects on hazards by assuming that hazard ratios are constant in time (proportional hazards), the PVR directly models survival probabilities at a given time without this strong assumption. Pros and cons of various approaches are illustrated with data from 2210 stage 4 patients older than 1 year. Evaluated predictors are age, MYCN-amplification (MNA), LDH and metastatic compartments. Primary endpoint is 10-year overall survival (OS) described by Kaplan-Meier estimates (KME) [95% confidence-intervals] and model-based estimates.

Results
10-year OS is 46[44-48]% (median observation-time of 8.2 years). In 223 patients younger than 1.5 years and 1987 patients older than 1.5 years the KME of 10-year OS are 53[45-60]% and 38[35-40]%, respectively. Non-proportional hazards are observed and consequently, the HR of 1.2 [0.99-1.5, p=0.071] and the predicted 10-year OS of 45% and 38% of a Cox-model are not valid. A cumulative HR (cHR) of 1.5 [1.2-1.8, p<0.001] was estimated with PVR and 10-years survival predictions correctly coincide with the KME . For 797 patients with MNA the KME for 10-year OS was 39[35-42]% and 39[36-42]% for 1413 without MNA. Again, due to non-proportional hazards, the estimated HR of 1.3 [1.1-1.4, p<0.001] is influenced by the

transient early disadvantage of MNA and the predicted OS of 42% and 33% of the Cox-model are not meaningful. The cHR of PVR is 1 [0.9-1.2, p=0.776] with 10-year survival-predictions of 39% in both groups what correctly corresponds to the KME. Prediction models evaluating all risk-factors are explored using various approaches. Tree-based approaches as well as Cox-models tend to over- and underestimate the impact of MYCN and age,respectively. PVR provides valid estimates and long-term predictions even in difficult non-proportional hazard situations.

Conclusion

PVR is a novel statistical approach directly modeling long-term outcome with a clear interpretation and should become part of standard statistical modelling.

P274

GANGLIONEUROMAS IN CHILDHOOD: A SINGLE-CENTER EXPERIENCE WITH 70 CASES

Aydin Goker E[1], Yalcin B[2], Karnak I[3], Orhan D[4], Haliloglu M[5], Ekinci S[3], Oguz B[5], Aydin B[2], Kurucu N[2], Varan A[2], Kutluk T[2]

[1]Hacettepe University Faculty of Medicine Department of Pediatrics, Ankara, Turkey, [2]Hacettepe University Faculty of Medicine Department of Pediatric Oncology, Ankara, Turkey, [3]Hacettepe University Faculty of Medicine Department of Pediatric Surgery, Ankara, Turkey, [4]Hacettepe University Faculty of Medicine Department of Pathology, Ankara, Turkey, [5]Hacettepe University Faculty of Medicine Department of Radiology, Ankara, Turkey

Background and Aims: Ganglioneuromas (GNs) are rare benign peripheral neuroblastic tumors (PNTs). The primary treatment modality is surgical resection. We aimed to review our institutional experience with childhood GNs. Methods: Hospital files of the children with PNTs between January 1995 and December 2021 were reviewed, and cases with histopathological diagnoses of GN were identified. Data concerning demographic characteristics, clinical, laboratory, and radiological findings, image-defined risk factors (IDRF), INRG stages, diagnostic and surgical, histopathological findings, and overall outcomes were recorded. Results: Of 668 cases procedures with PNTs, 70 (10.4%) had GNs. The median age was 7.4 years (range, 2.6-15.7 years; 16/70 <5 years) (females/males, 41/29). Common presenting complaints were abdominal pain and cough; 33/70 cases (47.1%) were diagnosed incidentally. Primary tumor sites were the abdomen in 41/70, the thorax in 25/70, the neck in 3 cases, and the pelvis in one. Urinary homovanillic acid (HVA) and vanillylmandelic acid (VMA) were elevated in 8/63 cases. The median tumor size was 6.5 cm (range, 1.4-17). Fifty cases (71.4%) with no IDRF were staged as INRG-L1; 20 cases with IDRF(s) (15 single, five >1) were staged as INRG-L2. Complete and partial tumor resections were performed in 58/70 and 6/70 cases, respectively (6 had no resection). The overall complication rate was 17.1% (11/64) (Horner syndrome 3, renal atrophy 2, leg hypoesthesia 2). At a median follow-up of 9 years (range, 0-27), 5 cases were lost to follow-up; 65 were alive. One patient with a gross tumor residue underwent total resection due to tumor progression 13 years after the initial surgery. Eleven other cases with gross residual tumors experienced no tumor progression in the follow-up. Conclusions: GNs are benign PNTs, and most are free of IDRFs. Even with incomplete or partial resection, long-term outcomes are excellent. A multidisciplinary approach is necessary, and the decision on tumor resection should be made meticulously for each case.

P275

Second Malignant Neoplasms in Neuroblastoma Patients: clinical data of Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology, Immunology

Utalieva D[1], Kachanov D[1], Moiseenko R[1], Grachev N[1], Vorozhtsov I[1], Babaskina N[1], Shamanskaya T[1]
[1]*Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology and Immunology, Moscow, Russian Federation*

Background: Intensification of therapy in intermediate/high risk patients with neuroblastoma (NB) resulted in improved overall survival. However, the survivors have a high incidence of long-term side effects of therapy. The most formidable complications are second malignant neoplasms (SMN) with a frequency of 1.2% (M.A. Applebaum, 2015).

Aim: to analyze the incidence of SMN in a cohort of Russian patients with intermediate/high risk NB.
Methods: The study included 176 patients with NB of the intermediate/ high risk group, diagnosed for the period 01.2012-12.2019, who completed specific treatment according to the modified German NB-2004 protocol at Dmitry Rogachev National Medical Research Center of Pediatric Hematology, Oncology, Immunology. High-dose preparative regimens included carboplatin/etoposide/melphalan (CEM) (till June 2013) and treosulfan/melphalan (TreoMel) (since July 2013). Since July 2014 patients with clear MIBG-positive residual primary tumor and/or metastases prior to hematopoietic stem cell transplantation (HSCT) received 131I-MIBG-therapy. Median follow-up was 58.7 months (range 16-124.8).
Results: Of the 176 patients, three cases (1.7%) developed a SMN. The types of SMNs included papillary thyroid carcinoma (n=2), acute myeloid leukemia (AML) (n=1). Age at diagnosis of NB was 39, 52, 55 months.

AML was diagnosed in a boy three month after completion of therapy for NB for the intermediate risk group and relapse therapy, which included 6 courses of topotecan/temozolomide, HSCT (TreoMel), radiotherapy to the tumor recurrence area localized in the retroperitoneal space (21 Gy), metronomic therapy (vinblastine/celecoxib/cyclophosphamide/etoposide). The patient underwent specific therapy for secondary AML. The follow-up period is 25 months.

Two papillary thyroid cancers (pT1bN0M0 and pT2N1bM0) were detected in patients with relapses of high-risk abdominal NB diagnosed 10 и 16 months after completion of therapy. One patient received first-line 131I-MIBG therapy and was diagnosed with primary hypothyroidism 12 months later. Both patients received high-dose regimens in the first line (TreoMel). Two patients underwent radical surgery for thyroid carcinoma. The patients are alive, the follow-up period is 3,8 and 10,7 months, respectively.

Conclusions: The intensive multimodal treatment strategy currently used to treat intermediate/ high risk NB can be associated with an increased risk of SMN. Comprehensive follow-up of these survivors is essential.

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Clinicopathological Implications of Ganglioneuroblastoma Intermixed Detected at Diagnosis

Modak S[1], Nezami B[2], Iglesias Cardenas F[1], Judy S[2], Sirintrapun S[1], Gopalan A[2], Chen Y[2], Al-Ahmadie H[2], Fine S[2], Reuter V[2], Tickoo S[2]

[1]Memorial Sloan Kettering Cancer Center, Department of Pediatrics, New York, United States, [2]Memorial Sloan Kettering Cancer Center, Department of Pathology, New York, United States

Background: Ganglioneuroblastoma Intermixed (GNBI) is regarded as a tumor with "favorable" histology by International Neuroblastoma Pathology Classification (INPC) system that incorporates histology, mitosis-karyorrhexis index (MKI) and age at diagnosis. On the other hand, the International Neuroblastoma Risk Group (INRG) pretreatment classification schema stratifies patients into low, intermediate, and high-risk using patient age, clinical stage, histology, 11q aberrations and MYCN amplification. The diagnosis of GNBI is typically made on examination of the resected tumor sample. We studied GNBI noted at diagnosis to evaluate its correlation with INRG staging and other clinicopathological features.

Methods: In this retrospective study, cases of GNBI at diagnosis seen between 1995 and 2021 were identified and confirmed histologically. Data collected included clinical, radiological, pathological, cytogenetic and targeted exome sequencing information. INRG staging was performed.

Results: Of the 15827 neuroblastoma specimens from 2805 patients, GNBI was noted in 237 patients. Of these, 53 had the initial pathological diagnosis of GNBI and had a median follow up of 3.5 (range 0.2-14) years. 41/53 patients (77%) presented with locoregional disease (16 staged as L1 and 25 as L2); none relapsed (median follow up 58 (range 5-275) months. Twelve patients (23%) had metastatic disease at presentation; 6 (50%) relapsed, and 2 died of disease. MYCN amplification was detected in 2 (4%) patients, both with metastatic disease. Recurrent cytogenetic abnormalities were present in 6/29 tumors tested (3 locoregional and 3 metastatic). Non-recurrent somatic gene mutations were present in 10/24 (42%) tumors. Adverse molecular findings were significantly higher in number ($p<0.05$) in metastatic (10/12, 83%) compared to locoregional GNBI (4/41, 10%). For patients with localized GNBI, biopsy and resections samples were reviewed in 18 patients: 17 (94%) had GNBI diagnosed in both samples.

Conclusions: Localized GNBI at diagnosis has an excellent long-term clinical outcome and can be managed without cytotoxic therapy. In patients with localized GNBI, a biopsy sample is adequate to make the diagnosis of GNBI. However, when GNBI at diagnosis is associated with metastasis, prognosis is poorer. Adverse molecular and cytogenetic findings may play a role in the biology behavior of metastatic GNBI.

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Successful endoscopic approach for peripheral neuroblastic tumor -Single center experience-

Kawakubo N[1], Maniwa J[1], Fukuta A[1], Souzaki R[1], Obata S[1], Nagata K[1], Matsuura T[1], Tajiri T[1]
[1]*Department of Pediatric Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan*

Background) Recently, reports about endoscopic approach for neuroblastoma, ganglioneuroblastoma, and ganglioneuroma (peripheral neuroblastic tumor; PNT) is increasing.
Aim)In this study, we aim to clarify the safe indication of endoscopic surgery for PNT , especially considering the preoperative Image Defined Risk Factor (IDRF).
Patients and Methods) Pediatric patients who underwent endoscopic surgery for PNT in our institution from 2013 to 2022 were included. IDRF was analyzed using preoperative Computed Tomography (CT). Diagnosis, complications, mortality, and prognosis were retrospectively analyzed.

Results) During the above period, 24 patients underwent endoscopic surgery for PNT(median age 2, range 1month-15years). Diagnosis was neuroblastoma (11 cases, 45.8%), ganglioneuroma(10 cases, 41.6%) and ganglioneuroblastoma (3 cases, 12.5%). International Neuroblastoma Staging Group was as follows ; 5 cases of stage M, 9 cases of stage L2, and 10 cases of stage L1. About the tumor site, there were 18 cases of adrenal tumor, 5 cases of mediastinal tumor, and 1 case of retroperitoneal tumor, respectively.
Regarding IDRF, IDRF was positive in 8 cases (6 cases contacted to renal artery, and 2 case compressed to principal bronchi). Complete resection was accomplished in 20 cases (neuroblastoma 9 cases , ganglinoneuroma 9 cases, ganglioneuroblastoma 2 cases). Contrarily, 4 cases was incompletely resected (neuroblastoma 2 cases, ganglioneuroma 1 case, and ganglioneruoblastoma 1 case).
Complete resection was accomplished in 13 of 16 IDRF negative cases (81.2%), and 7 of 8 IDRF positive (87.5%) cases. Details of 7 cases of IDRF positive tumor which was completely resected were as follows; 5 cases contacted to renal artery, and 2 cases compressed to principal bronchi.
In short-term complication, there was 1 case of chylous ascites, and 1 case of atelectasis. Regarding long-term complication, there was 1 case of scoliosis, and 1 case of horner syndrome. All patients alived without recurrence during the follow-up period.

Conclusion) At least, the CT finding of 'contact to renal artery' seems not to be the indicator of incomplete resection and perioperative complication.
Endoscopic approach to peripheral neuroblastic tumor in pediatric patients is feasible with good prognosis and acceptable quality of life if we select the patient strictly.

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Localised unresectable Neuroblastoma: a report of the Italian neuroblastoma registry

Fragola M[1], Sorrentino S[2], Mazzocco K[3], Viscardi E[4], Podda M[5], Bianchi M[6], De Ioris M[7], Di Cataldo A[8], Bertolini P[9], Mura R[10], De Marco E[11], Coccia P[12], Conte M[2], Garaventa A[2]

[1]Scientific Directorate, IRCCS Istituto Giannina Gaslini, Genova, Italy, [2]Pediatric Oncology Unit, IRCCS Istituto Giannina Gaslini, Genova, Italy, [3]Department of Pathology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [4]Pediatric Oncology Unit, Azienda Ospedaliera di Padova, Padova, Italy, [5]Pediatric Oncology Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy, [6]Pediatric Oncology Unit, AOU Città della Salute e della scienza di Torino Presidio Infantile Regina Margherita, Torino, Italy, [7]Pediatric Oncology Unit, IRCCS Ospedale Pediatrico Bambino Gesù, Roma, Italy, [8]Pediatric Oncology Unit, AOU Policlinico Vittorio Emanuele, Catania, Italy, [9]Department of Pediatric Oncology, Azienda Ospedaliero Universitaria di Parma, Parma, Italy, [10]Pediatric Oncology Unit, Ospedale Microcitemico di Cagliari, Cagliari, Italy, [11]Paediatric Oncology and Haematology Department, Ospedale Santa Chiara, Azienda Ospedaliero Universitaria Pisana, Pisa, Italy, [12]Division of Pediatric Hematology and Oncology, Azienda Ospedali Riuniti G. Salesi, Ancona, Italy

Background: Stage L2 identifies a localized neuroblastoma(NB) with one or more image-defined risk factors (IDRFs). It is a dishomogenous group of patients with a good prognosis for patients with MYCN not amplified tumour .

Aim: Evaluate overall survival (OS) and disease free survival (DFS) and to define a risk score of children diagnosed with L2 NB based on clinical factors present at diagnosis.
Patients and methods: Between January 1991 and May 2017, 578 patients with a malignant PNT stage L2 was enrolled in the Italian NB Registry. Of these, 132 were excluded because enrolled into ongoing SIOPEN protocols or for benign histology or not enough data. Considering the multivariate regression model, to each prognostic factor was assigned a risk score and 3 groups have been defined based on median score: low, intermediate and high risk.

Results: 446 evaluable patients were stratified into 4 periods of diagnosis, based on the treatment protocols (1991-2000,2001-2006,2007-2012,2013-2017). These groups differed in a lower percentage of incomplete surgery and higher percentage of biopsy, a higher percentage of MycN amplification and pathological LDH, and an increased percentage of patients treated with radiotherapy and high doses of chemotherapy with blood stem cells transplant in the last period.
In total we registered 97 dead, 12(2.7%) due to treatments related toxicity. The 5 years-OS was 82.6% and DFS 73.9% . Survivals are better in patients <12 months, with neck and pelvis tumours, with pathological HVA/VMA, normal LDH and ferritin, unamplified MycN, or a maximum surgery more than a biopsy.Cox's multivariable regression analysis showed better outcomes in patients <12 months, LDH abnormal and ferritin normal.
For each patients was calculated the risk score(range 0-4.6, median 1.4) and based on this is estimated 5-years-OS that was: 100% for patients with no risk factors, 96.9% for intermediate risk and 75.9% for high risk.

Conclusion: We have define a risk score based on 3 characteristic at diagnosis (LDH, age, ferritin) that correlate to the OS and DFS.It could results useful to define prognosis and treatment based on clinical factors for patients with undefined biological data. The correlation with genetic prognosis needs to be verified.

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Altered gut Microbiome composition in infants and children with Neuroblastoma

Valles-Colomer M[1], Armanini F[1], Pinto F[1], Garaventa A[2], Amoroso L[2], Segata N[1], Ponzoni M[3], Corrias M[3]
[1]Department CIBIO, University of Trento, Trento, Italy, [2]Oncology Unit, IRCCS Istituto Giannina Gaslini, Genova, Italy, [3]Laboratory of Experimental therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy

Background. In several types of adult cancer, gut microbiome influence in insurgence, progression and response to therapy has been documented. The gut microbiome is shaped in infancy by type of delivery and feeding. Due to change in diet, at around two years of age, the microbiome stabilizes with limited changes during healthy life. Neuroblastoma (NB) prognosis significantly associates with age at diagnosis, with a cut-off of 18-24 months.

Aims. To evaluate the gut microbiome composition of infants and children with NB, together with that of their mothers in comparison to that of healthy subjects to verify whether specific microbiome features are associated with NB insurgence and prognosis.

Methods. The study was approved by the Regional Ethics Committee. Stool samples were collected in DNA/RNA shield faecal collection tubes (Zymoresearch) from infants and children diagnosed with NB (N=62) at the IRCCS Istituto Giannina Gaslini from May 2019 to June 2022, from their mothers (N=59) and from healthy infants and children (N=94) and their mothers (N=59) after informed consent was signed. DNA extraction was performed with the DNeasy PowerSoil Pro kit (Qiagen) and shotgun sequencing libraries were prepared with the NexteraXT DNA library preparation kit (Illumina) run on the Illumina NovaSeq 600 platform. Data was pre-processed with a custom pipeline (https://github.com/SegataLab/preprocessing). Species-level microbiome profiles were obtained with MetaPhlAn4.0.3 (Blanco-Miguez et al. 2022), and statistical analysis was performed in R.

Results. Overall microbiome composition in patients with NB differed from that in healthy controls (R2=2%, P=0.001), being characterized by lower observed richness (r=-0.2, P=0.02) and lower relative abundance of the probiotic species Bifidobacterium bifidum (r=-0.4, Padj=1.6e-3), with the two groups being similar based on age, type of delivery, and feeding mode (P>0.05). In contrast, microbiome composition of mothers of patients did not differ significantly from mothers of controls (P>0.05).

Conclusion. Infants and children with NB display an altered microbiome taxonomic composition when compared to healthy controls of similar age. Follow-up analyses will investigate these differences in further detail.

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The extent of resection does not alter the outcome in patients with Ganglioneuroma and Ganglioneuroblastoma Intermixed.

Qureshi S[1], Prasad M[1], Chinnaswamy G[1], Ramadwar M[1]
[1]*TATA MEMORIAL CENTRE. , MUMBAI, India*

Background
Ganglioneuroma (GN) and Ganglioneuroblastoma intermixed (GNBI) are on the benign end of the neuroblastic tumor spectrum. They have a better prognosis when compared to Ganglioneuroblastoma nodular (GNBN) and Neuroblastoma (NB). Children may require resection of symptomatic tumors; however, there seems to be a debate on the completeness of resection because of surgical complications.

Aims
To study the association of completeness of resection and outcome

Methods
Clinical characteristics and outcome data of GN and GNBI were collected from a prospectively maintained institutional database between 2005 to 2022. After surgery, the extent of residual disease was confirmed with operative notes and postoperative imaging. The patients were divided into two groups; no residual disease or < 2cm residual and > 2cm, respectively.

Results
GN or GNBI was observed in 49 children. The median age of presentation was 5.1 years (range-1.7 to 17 years). There were 20 (40.8%) males and 29 (59.2%) females. MIBG scan was performed in 29 patients, showing avidity in the primary in 59%. Neoadjuvant chemotherapy was given to seven children due to the presence of image-defined risk factors. However, all had stable disease or marginal increase in disease following chemotherapy. Complete surgical resection was performed in 57% of patients. There was no mortality, and the postoperative complications were less than Clavein Dindo Grade II. 78% of patients were in group I and 24.1% in Group II. The median duration of follow-up is 51 months (range- 7 to 153 months). Progression of disease was noted only in one patient in group I with a residual tumor less than 2 cm. It was salvaged with surgery and radiotherapy.

Conclusion
The outcome of surgery in GN and GNBI is excellent. Incomplete resection may not alter the outcome of GN and GNBI in children, irrespective of the extent of the residual tumor. Chemotherapy does not decrease the size of the tumor or aid in the resection of GN and GNBI

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Associations of gut microbiota and GD2/GD3 vaccine response in pediatric patients with high-risk neuroblastoma

Miltiadous O[1], Nguyen C[2], Cheung I[1], Kushner B[1], Mauguen A[3], Waters N[2], Dai A[2], Kivovich V[1], Littmann E[2], Harford L[2], Raj S[4], Peled J[4,5], Markey K[4,5,6], Andrlova H[2], Ragupathi G[3], Roberts S[7], Pamer E[8], Burgos da Silva M[2], Cheung N[1], Modak S[1], van den Brink M[2,4,5]

[1]Department of Pediatrics, Memorial Sloan Kettering Cancer Center, New York, United States, [2]Department of Immunology, Sloan Kettering Institute, New York, United States, [3]Department of Epidemiology and Biostatistics, Memorial Sloan Kettering Cancer Center, New York, United States, [4]Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, United States, [5]Adult Bone Marrow Transplantation Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, United States, [6]Department of Medicine, University of Washington, Seattle, United States, [7]Division of Pediatric Hematology and Oncology, Knight Cancer Institute, Doernbecher Children's Hospital, Oregon Health & Science University, Portland, United States, [8]Duchossois Family Institute, University of Chicago, Chicago, United States

Background: A GD2/GD3 cancer vaccine that incorporates oral beta-glucan as an adjuvant, tested in phase I and II trials (Clinical Trials.gov NCT00911560) in patients with high-risk neuroblastoma (HR-NB), elicited high anti-GD2 IgG1 titers which correlated with patient survival (Cheung et al. JCO 2021, JAMA Oncology 2022). Interestingly, evidence is beginning to emerge that gut microbiome can modify responses to cancer immunotherapies and immunizations.

Aim: To evaluate if the gut microbiome composition is associated with responses to the GD2/GD3 vaccine.

Methods: 16s rRNA gene sequencing was performed on 345 stool samples from 139 patients with HR-NB receiving the GD2/GD3 vaccine. We used linear discriminant analysis effect size (LefSE) for initial comparisons of microbiome composition between patients with high and low anti-GD2 titers in patients using baseline (+/-15 days) stool samples and then tested the importance of the associations between microbiome and titer response using the MaAslin2 multivariable model. For correlation with outcomes, LefSE was used initially, and taxa were then tested for associations with progression-free survival (PFS) in a Cox regression model.

Results: High abundance of taxa in the Clostridia class (including Blautia and Intestinibacter) in baseline stool samples was associated with higher anti-GD2 IgG1 titers. In a multivariate model adjusting on prognostic clinical parameters (including number of prior relapses and glucan use during priming), Intestinibacter had the strongest correlation with high titers (FDR=0.14). Several species within this genus are known to produce short-chain fatty acids, which are microbial fermentation products with anti-inflammatory properties while enhancing B/plasma cell responses to vaccines. After adjusting for the number of prior relapses, anti-GD2 IgG titers and glucan timing, high Clostridia abundance was associated with better PFS (HR=0.40; 95%CI 0.18-0.92, p=0.03). Interestingly, exposure to moderate/high perturbation antibiotics (to which many Clostridia are sensitive) ±1 month from the start of the vaccine was associated with worse PFS (HR 1.71, 95% CI .93-3.14) though p=0.08.

Conclusions: Patients with HR-NB with higher fecal abundance of Clostridia mounted greater antibody response to GD2/GD3 vaccine and had favorable PFS. The immunobiology behind this association as well as the impact of gut microbiome modulation in improving clinical outcomes deserves further investigation.

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RATIONAL FOR IRRADIATION OF PERSISTING OLIGO-SKELETAL METASTASES TO IMPROVE SURVIVAL OF METASTATIC NEUROBLASTOMA PATIENTS WITH A POOR RESPONSE TO CHEMOTHERAPY?

Rossillon L[1], Edeline V[2], Agrigoroaie L[2], **Berlanga P[1]**, Pasqualini C[1], Dufour C[1]
[1]Department of Childhood and Adolescent Cancer Gustave Roussy Cancer Campus, Villejuif, France, [2]Department of Imaging Roussy Cancer Campus, Villejuif, France

Background
Most High-Risk Neuroblastoma are metastatic at diagnosis with positive skeletal uptakes on MIBG scintigraphy. Persistent skeletal uptakes at the end of induction correlate with a poor outcome. Radiotherapy of residual bone metastases may improve outcome of these patients.

Aims
To investigate if there is a rational for a prospective randomized study evaluating the impact of radiotherapy of oligo residual bone metastatic sites with the aim of improving survival.

Methods
Patients over one year at diagnosis with a stage M neuroblastoma treated between 2000 and 2020 at Gustave Roussy were screened Patients were selected by a positive MIBG scan at diagnosis and persistent skeletal metastases after high-dose chemotherapy (HDC). Patient's disease, treatments and outcome were retrospectively collected. MIBG scans were reviewed by two nuclear physicians.

Results
30/201 patients (15%) had persistent skeletal uptakes post HDC. Of note, 49/201 (24%) had a progressive disease (PD) before post-HDC evaluation.

2/30 patients relapsed during maintenance. 4 patients had a negative MIBG scan at the end of treatment and had persistent response (PR) with a median follow-up (FU) of 8 years [2-12].

Out of 24 patients with persistent skeletal uptakes at the end of the treatment, 17 had a PD within a median time of 14 months [2-43], 6 had a stable disease with a median FU of 8 years [4-15] and 1 had negatived his MIBG scan and did not relapse [FU: 6 years].

Persistent metastatic sites at the end of treatment represent 23% of the metastatic sites identified at time of progression when 85% of sites of progression were present at diagnosis.

Treatment on this long period was heterogeneous and patients with PD had a higher SIOPEN score at each evaluation and received a lower treatment intensity.

Conclusions
Our study underlines that radiotherapy of persisting oligo-skeletal metastases would be beneficial to a minority of patients. Recurrence mainly occurred in disease sites present at diagnosis that cleared with chemotherapy. On-therapy control of the disease is the main issue. In addition, persisting MIBG sites cannot be by itself a criterion to include patients in innovative treatment study. A larger retrospective study is needed .

P285

Low-Dose Intravenous Ketamine for Primary Pain Control During Naxitamab and Dinutuximab Anti-GD2 Immunotherapy Treatment

Silber R[1], Modak S[1], Fernandez N[1], Hahn E[1], Szabo J[1], Greenfield D[1], Record B[1]
[1]*Memorial Sloan Kettering Cancer Center, New York, United States*

Background
Naxitamab and dinutuximab are anti-GD2 monoclonal antibody immunotherapies approved for treatment of neuroblastoma (NB). Both are associated with severe pain, typically managed with IV opioids. Ketamine, an NMDA receptor antagonist, is useful for the management of neuropathic pain at subanesthetic doses. An outpatient pilot program was started at Memorial Sloan Kettering Cancer Center (MSKCC) where low dose intravenous ketamine (LDIVK) (0.5mg/kg/h or less), was used to treat refractory pain during naxitamab. LDIVK was used for all patients receiving dinutuximab inpatient with opioids as needed.

Aims
To describe a single center experience using LDIVK as primary pain management, evaluate the opioid sparing effects with LDIVK, and determine the frequency of AEs related to LDIVK during anti-GD2 immunotherapy.

Methods
A retrospective chart review gathered data on patients who received anti-GD2 immunotherapy and LDIVK. For patients who received naxitamab, pain outcomes were compared between the last cycle where LDIVK was not used to the cycle with LDIVK. Outcomes included amount of opioids required, vital signs, fluid boluses, and AEs. Statistical analysis was determined using a t-test. For those receiving dinutuximab, the number of patients requiring the addition of a continuous opioid infusion was collected.

Results
53 patients met inclusion criteria for this study. Ten received LDIVK during outpatient naxitamab and 43 received LDIVK during inpatient dinutuximab administration. There were no LDIVK related AEs (death, seizure, loss of consciousness, respiratory depression, dissociation, sialorrhea, tachycardia, or hypertension). For patients receiving LDIVK during naxitamab, there was a statistically significant reduction in opioid used between cycles (hydromorphone dose equivalent of 0.02mg/kg/cycle with LDIVK vs. 0.04mg/kg/cycle without, p=0.04). Only 8 patients (18.6%) treated with dinutuximab and LDIVK required a continuous infusion of opioids. All patients in both groups requested continuing LDIVK during subsequent cycles.

Conclusion
LDIVK was safely and successfully used as the primary analgesic in a multimodal pain regimen for patients being treated with anti-GD2 therapy in both the inpatient and outpatient settings. The use of LDIVK had an opioid sparing effect. A prospective investigation of LDIVK for treatment of anti-GD2 antibody related pain is recommended to better characterize impact on pain experience and treatment.

P286

Focal nodular hyperplasia in children after treatment of neuroblastoma: A report of 84 cases

Paulsen V[1], Simon T[1], Körber F[2], Butzer S[1], Decarolis B[1], Plum G[1], Rosswog C[1,3,4], Schmidt M[5], Fischer M[1,3,4], Eggert A[6], Lode H[7], Berthold F[1], Hero B[1]

[1]Department of Pediatric Oncology and Hematology, University Children's Hospital of Cologne, Medical Faculty, University of Cologne, Cologne, Germany, [2]Division of Pediatric Radiology, Department of Radiology, University Hospital of Cologne, Medical Faculty, University of Cologne, Cologne, Germany, [3]Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Medical Faculty, University of Cologne, Köln, Germany, [4]Center for Molecular Medicine Cologne (CMMC), Medical Faculty, University of Cologne, Köln, Germany, [5]Department of Nuclear Medicine, University Hospital of Cologne, Medical Faculty, University of Cologne, Cologne, Germany, [6]Department of Pediatric Oncology and Hematology, Charité - Universitätsmedizin Berlin, Campus Virchow-Klinikum, Berlin, Germany, [7]Department of Pediatric Oncology and Hematology, University Medicine Greifswald, Greifswald,

Background:
Focal nodular hyperplasia (FNH) of the liver has been reported as late effect after chemotherapy of childhood malignancies including neuroblastoma. The discrimination between FNH and hepatic relapse is often difficult, especially in case of progressive lesions.

Aim:
We were interested in the radiological findings and the course of FNH in patients previously diagnosed with neuroblastoma.

Methods:
Data of patients registered in the German neuroblastoma trials between 1990–2016 were retrospectively searched for FNH reported by the treating institutions. The clinical and radiological documentation was reviewed at diagnosis of the hepatic lesions and during further follow-up.

Results:
Out of 2174 patients followed for more than 12 months after treatment start for neuroblastoma, FNH was reported in 84 patients (3.9%). Most patients were previously treated for high-risk neuroblastoma including high-dose chemotherapy with stem cell rescue (59/84, 70.2%), but nine patients received only up to four cycles of chemotherapy. A hepatic veno-occlusive disease (VOD) was previously reported in nine patients. FNH was detected 12-258 months after the diagnosis of neuroblastoma (median 71 months). The median age at diagnosis of FNH was 111 months (21-326 months). The patients presented with a median of 2 (1-7) lesions, 39/80 (48.8%) patients showed only one single lesion. All lesions were found during routine follow-up (MRI 52/83, 62.6%; ultrasound 27/83, 32.5%). None of the patients was symptomatic. In 13 of 36 patients with concurrent MRI and ultrasound, lesions detected by MRI could not be verified by ultrasound. FNH was confirmed histologically in nine and by contrast-enhanced ultrasound in 19 patients. No patient subsequently developed progressive liver metastases confirming the benign characteristics of the lesions. In 53 patients, follow-up of FNH over at least 24 months was available. During this time, the lesions remained stable in 19 (35.8%) and were progressive in size and/or number in 34 patients (64.2%). In six of the patients with progressive lesions, a stabilisation with further follow up was reported

Conclusion:

FNH is often observed during follow up after cytotoxic treatment for neuroblastoma. FNH might be missed by standard ultrasound. Progression of FNH is common and does not necessarily indicate progression of neuroblastoma.

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Pulmonary complications in paediatric high risk neuroblastoma: A single centre experience

Sri Haran S[1], Omer N[1], Soosay Raj T[1], Thomas R[2], Nicholls W[1]
[1]Queensland Children's hospital Oncology Service Group, Brisbane , Australia, [2]Queensland Children's hospital, Respiratory Department, Brisbane , Australia

Background: Children with high-risk (HR) neuroblastoma are exposed to multimodal therapy with high incidence of toxicities. In our experience, acute and chronic pulmonary complications seem more frequent since tandem high-dose chemotherapy (HDT). It may result in impaired patient outcome due to respiratory toxicity itself, but also due to treatment delay or interruption and neuroblastoma relapse and progression.

Method: This is a retrospective chart review of HR neuroblastoma patients who underwent either single or tandem HDT treated between January 2012 to December 2022 at the Queensland Children's Hospital, Brisbane, and who developed severe respiratory complications. Medical records were reviewed for neuroblastoma therapy received, co-morbidities, underlying respiratory symptoms, admissions for respiratory symptoms, timing, management and investigations of respiratory complications, and outcome of patients were analysed.

Results: There were a total of 38 patients with HR neuroblastoma who underwent single or tandem HDT between January 2012 to December 2022. Nine (23%) patients suffered pulmonary complications. There were 20/38 (52%) patients treated with Busulfan/Melphalan(Bu/Mel) HDT with 3/20 (15%) pulmonary complications. These complications occurred on average 14 months post therapy. Eighteen patients were planned to receive tandem HDT, 4 of them only underwent Cyclophosphamide/Thiotepa (Cy/TT) due to toxicity. Three of these 4 patients had pulmonary complications. One developed severe pulmonary veno-occlusive disease (PVOD) 37 days post-HDT with significant pulmonary hypertension and had progression of disease while on treatment for PVOD. Two had proven pulmonary fibrosis on biopsy and died due to type 2 respiratory failure (11 and 40 months post conditioning). Of the 14/38 who underwent tandem HDT, 1 patient developed biopsy-proven pulmonary fibrosis 4 months post HDT and 2 patients had adenovirus pneumonitis - one died due to progressive respiratory failure on a background of interstitial lung disease 41 months post HDT, the other had acute infection day 18 post first Cy/TT HDT, completed all high-risk treatment and remains alive.

Conclusion: Severe pulmonary complication appears to occur most frequently post HDT with Cy/TT compared to Bu/Mel and have an increase risk of progressive/relapsed disease while treatment is halted for treatment of pulmonary complications.

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Long-term results of combined polychemotherapy, MIBGI131 in vivo purging, ABMT and allogeneic dendritic cell vaccine (DCVs) for high-risk neuroblastomas (HRNBs)

Halley N[2], Prandini A[1,2], Vince C[1,2], Brumatti M[1,2], Pereira P[1,2], Novak E[1,3,4], Gimenez T[1,3,4], Siqueira S[1], Bendit I[5], Weltman E[2], Ricardi L[1], Plaza R[1], Maluf P[1], de Almeida M[1], Garcia J[1,2], Dutra A[1], Gomes A[1], Mafra A[1], Pereira A, França C[1], Borba C[1], Zamperlini G[1,2], Minami K[1], Fernandes J[1,2], Barreto J[1], Barbuto T[1], Forsait S[1], Fernandes R[1], Batista G[1], Fonseca M, Cristofani L[1], Odone Filho V[1,2]

[1]Instituto Tratamento Cancer Infantil -ITACI/ Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil, [2]Hospital Israelita Albert Einstein, São Paulo, Brazil, [3]Laboratório de Investigação Médica (LIM36), Instituto da Criança, HC/FMUSP, São Paulo, Brazil, [4]Fundação Pró-Sangue Hemocentro de São Paulo, São paulo, Brazil, [5]Laboratorio de Biologia Molecular - Universidadade de São Paulo (USP), São Paulo, Brazil, [6]ACKNOWLEDGMENTS This work was supported by grants from Associação Beneficente Stella Demarco, Fundação Criança, and SH Formas., ,

Background

HRNBs continue representing an enormous difficulty. Stem-cell transplants and anti-GD2 are significant steps in their treatment road. Not all necessary resources are easily available for institutions throughout the world. To stablish an effective program, considering all these difficulties, is a true challenge.

Aims

To provide a feasible and useful program for Brazilian institutions, considering the disparity of resources existing for the country and within the country.

Methods

From 2000-2017, 439 children with NB were referred to ITACI. 150/439(34%) had been previously treated. 357/439(81.30%) were suitable for analysis.

This analysis (as of 10/18/2022) encloses 74/357(20.7%) children, age >18 months at diagnosis, admitted after 2001, without any previous management, stages 4 or 3 with regional node involvement and/or amplified MYCN. They fulfilled ABMT criteria, representing 62% of children admitted for this purpose.

Treatment: i=induction polychemotherapy with cycles of: A)Topotecan/Cyclophosphamide; B)Vincristine/Cyclophosphamide/Adriamycin/ Carboplatin/Etoposide (2001-2010: A-A-B-B-B-B; after 2010: A-A-B-B-A-A-B-B); ii=delayed surgery; iii=Peripheral Blood Stem Cell Collection (PBSCC), after previous exposure to therapeutic MIBGI131(10 mci/kg, as previously defined); iv= ABMT (conditioning regimen until 2012: Carboplatin/Etoposide/Melphalan; Busulfan/Melphalan afterwards); v=individualized radiotherapy; vi=Cis-Retinoic Acid and; vii=DCVs.

Response criteria: at PBSCC the children must have: absence of any sign of progressive disease (PD) and documented bone marrow (BM) involvement (9 BM aspirations/2 biopsies & negative immunohistochemical study), normal lactate dehydrogenase levels and PD absence in images (TC/MRI/PET CT/PET MRI/Tc/MIBGI123). Any sign of PD or persistence of documented marrow disease at PBSCC determined additional treatment before new collection attempt.

Results

74 children were transplanted. Results as of October/2022: for children whose initial attempt of PBSCC was possible (58/74, 78%), PFS is: 58.32±6.69%, favorably comparing to the ones (16/74, 22%) who had chemo additions to original program: 18.75±9.76%, p:0.0028.

Conclusions

This program determines satisfactory long-term results, with use in Brazil is restricted to few institutions. Although each component's role is unestablished, DCVs will be maintained for future propositions. Addition of anti-GD2 is the key point of these new possibilities, despite its elevated cost. An ongoing study was

designed including a single cycle of Beta-Dinutuximab as in vivo purging. Initial two patients have just completed their entire program.

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131I-MIBG Therapy for Patients with Neuroblastoma in Singapore

Soh S[1], Loke K[2], Tong A[2], Tan E[1]
[1]Paediatric Haematology/Oncology Service, KK Women's and Children's Hospital, Singapore, Singapore, [2]Department of Nuclear Medicine and Molecular Imaging, Singapore General Hospital, Singapore, Singapore

Background
Patients with high-risk neuroblastoma have suboptimal survival outcomes despite intensive multimodality therapies. Radionuclide therapy using 131I-mIBG delivers targeted radiation to neuroblastoma. This has been shown to be efficacious in relapsed refractory neuroblastoma, and has been used as a salvage option.

Methods
We report our experience with setting up and treating neuroblastoma patients with 131I-mIBG therapy at the SingHealth Group of healthcare facilities in Singapore.

Results
An mIBG therapy workgroup was formed in 2014, consisting of nuclear medicine physicians, radiation safety officers, nuclear medicine nurses, paediatric oncologists, paediatric oncology nurses, anaesthesists and intensivists, pharmacists, dieticians, childlife specialists and medical social workers. From 2014 to 2022, 6 infusions of 131I-mIBG (range 12mCi/kg to 15mCi/kg) were delivered to 5 patients (age 5 to 37 years old) with relapsed /refractory neuroblastoma. The treatment was well tolerated. None of the patients were given sedation or anxiolytic medication during the mIBG therapy. All were given stem cell rescue post-mIBG therapy, and all received additional salvage treatment modalities (conventional salvage chemotherapy, chemo-immunotherapy, haploidentical transplant, peptide receptor radionuclide therapy). All the patients eventually succumbed to disease progression.

Conclusion
131I-mIBG therapy is feasible but the set-up and treatment delivery is logistically challenging especially in low-volume centers. 131-mIBG therapy remains a treatment that is not widely or readily available. Centralization of resources and comprehensive standard operating procedures would be helpful to develop experience and expertise and ensure continued competency and safety.

P290

Interim analysis of the UK relapsed high risk neuroblastoma study

Basta N[1], Herd F[2], Carruthers V[3], Hawley J[3], Gabriel A[4], Allinson L[4], Fong F[4], Bown N[5], Goodman A[5], Pearson A[6], Moreno L[7], Elliott M[8], Gaze M[9], Morgenstern D[10], Feltbower R[11], Stiller C[12], Whiteman S[3], Makin G[13], Gray J[14], Wheeler K[15], Blanco E[15], Ng A[16], Howell L[17], Johnston R[18], Mark C[18], Yeomanson D[19], King D[19], Morley-Jacob C[20], Barone G[21], Howe K[21], Morland B[22], Vaidya S[23], Angelini P[23], Dandapani M[24], Alomari Y[24], Murphey D[25], McNally R[1], Tweddle D[3,4]

[1]Population Health Sciences Institute, Newcastle University, Newcastle Upon Tyne, United Kingdom, [2]Department of Paediatric Oncology, Royal Aberdeen Children's Hospital, Aberdeen, United Kingdom, [3]Department of Paediatric Oncology, The Great North Children's Hospital, Newcastle Upon Tyne, United Kingdom, [4]Wolfson Childhood Cancer Research Centre, Institute of Translational & Clinical Medicine, Newcastle University, Newcastle Upon Tyne, United Kingdom, [5]Institute of Genetic Medicine, Newcastle University, , Newcastle Upon Tyne, United Kingdom, [6]Children and Young People's Unit, The Royal Marsden NHS Foundation Trust and Division of Clinical Studies and Cancer Therapeutics, The Institute of Cancer Research (Retired), Sutton , United Kingdom , [7]Paediatric Haematology & Oncology, Vall d'Hebron Hospital,, Barcelona , Spain , [8]Paediatric Oncology and Haematology Department, Leeds Teaching Hospitals NHS Trust, Leeds , United Kingdom, [9]University College London Hospitals NHS Foundation Trust, London, United Kingdom, [10]Division of Paediatric Haematology/Oncology, The Hospital for Sick Children, Toronto, Canada, [11]Leeds Institute for Data Analytics, School of Medicine, University of Leeds, Leeds, United Kingdom , [12]National Cancer Registration and Analysis Service, NHS Digital, England, United Kingdom , [13]Department of Paediatric Oncology, Royal Manchester Children's Hospital, Manchester, United Kingdom, [14]Department of Paediatric Oncology, University Hospital Southampton, Southampton , United Kingdom, [15]Department of Paediatric Oncology, Oxford Children's Hospital, Oxford, United Kingdom, [16]Department of Paediatric Oncology, Bristol Royal Hospital for Children, Bristol , United Kingdom, [17]Department of Paediatric Oncology, Alder Hey Children's Hospital, , Liverpool, United Kingdom , [18]Department of Paediatric Oncology, Royal Belfast Hospital for Sick Children, , Belfast , United Kingdom , [19]Department of Paediatric Oncology, Sheffield Children's NHS Foundation Trust, Sheffield , United Kingdom , [20]Department of Paediatric Oncology, Children's Hospital for Wales, University Hospital, Cardiff , United Kingdom , [21]Department of Paediatric Oncology, Great Ormond Street Hospital for Children NHS Foundation Trust, London , United Kingdom , [22]Department of Oncology/Haematology, Birmingham Women's and Children's NHS Foundation Trust , Birmingham, United Kingdom , [23]Department of Paediatric Oncology, The Royal Marsden Hospital NHS Foundation, Marsden , United Kingdom , [24]Department of Paediatric Oncology, Nottingham Children's Hospital, Nottingham University Hospitals , Nottingham , United Kingdom , [25]Department of Paediatric Oncology, Royal Hospital for Children, NHS Greater Glasgow and Clyde, Glasgow , United Kingdom

Background: Despite advances in neuroblastoma treatment, relapse still occurs in 50% of high risk (HRNB) cases and in most cure is no longer possible. Some clinical and genetic factors associated with length of survival following relapse have been identified. However, other factors may be important in predicting response to Phase I and II treatments given at relapse.

Aims: To investigate clinical and genetic factors associated with neuroblastoma relapse and length of survival following relapse.
Methods: Retrospective study of relapsed HRNB from 2000-2021 (aged 0-40y) from UK paediatric oncology treatment centres. Relapse was defined as recurrence or progression following initial response (including partial) to any neuroblastoma therapy.

Results: 211 cases of relapsed HRNB were identified from 15 centres. At diagnosis, 94% were INSS stage 4 with a median age at diagnosis of 3.4 years (interquartile range (IQR) 2-4.5), 85/189 (45%) MYCN amplified (MNA), 54/101 (53%) 1p loss, 30/75 (40%) 11q deletion, and 71/116 (65%) 17q gain. Median progression free survival (PFS) from diagnosis to relapse was 14 months (IQR 10-23 months). Median post relapse overall survival (PROS) was 8 months (IQR 3–18); for MNA disease 5 months (IQR 2-11) and MYCN non-

amplified (NMNA) 13 months (IQR 7-27). 5-year PROS was 12% (95% CI 8-18%); 10% (95% CI 5-18%) for MNA vs 16% (95% CI 10-24%) for NMNA (P<0.001). There was no significant difference in PROS for 1p or 11q deleted cases or 17q gain vs non-deleted or gained. 5-year PROS was 14% (95% CI 9-22%) for cases relapsing after 2010 compared to 10% (95% CI 5-17%) before 2010 (P=0.045). For cases from 2000-2010, 5-year PROS for MNA cases was 11% (95% CI 3-23%) and 12% (95% CI 4-25%) for NMNA (Log rank test P=0.01). For cases from 2011-2021, 5-year PROS for MNA cases was 9% (95% CI 3-20%) vs 18% (95% CI 10-29%) for NMNA (Log rank P<0.001). The most frequent treatment regimens given at first relapse included: Temozolomide/Irinotecan (21%), Topotecan-Vincristine-Doxorubicin (TVD) (16%), Topotecan/cyclophosphamide (10%), Temozolomide (7%); 17% of patients were entered in phase II trials.

Conclusion: The 5-year survival for relapsed HR patients has improved for cases relapsing after 2010.

P292

Neuroblastoma Disease Response Tool: Implementation of a longitudinal disease assessment tool in neuroblastoma to calculate response.

Granger M[1], Brentlinger A[1], Sims J[2]
[1]Cook Children's Medical Center, Hematology & Oncology, Fort Worth , United States, [2]Cook Children's Medical Center, Research Administration Office, Fort Worth, United States

Background:
A key endpoint to clinical research is assessment of disease response. Historically, pediatric oncology research protocols define disease response criteria, but lack the ability to easily track the response over time throughout the protocol therapy. As early phase clinical trials become more complex, it becomes more difficult and time consuming to retrospectively review lesion assessments and specifics needed to determine response. Neuroblastoma disease assessment is particularly complex due to the variation of response among primary and metastatic lesions. Target soft tissue lesions may be generally discussed in radiology reports but not reported with precise or accurate measurements. There can also be discordance between different imaging modalities and also which lesions are reported. Over time with varying interpretations from radiologists, tracking target versus non-target lesions can be ambiguous regarding differences in which lesions are measured and even the order in which the three planes are reported.

Aims:
To ease and ensure accuracy in the longitudinal assessment of the patient's disease response to protocol therapy using protocol defined response criteria.

Methods:
A tool was created to track neuroblastoma disease sites, in particular, soft tissue lesions in parallel comparison while determining response at individual disease evaluation. The tool contains all data needed for response criteria and subsequent case report forms. This form is used collaboratively among research team members to collect pertinent disease details and allows real time multidisciplinary assessment to obtain the most accurate measurement of disease. The principal investigator then reviews all response evaluations for completeness prior to submission.

Results:
Longitudinal real-time disease assessment using the Neuroblastoma Disease Response Tool increases efficiency and accuracy in determining disease response in patients with neuroblastoma on early phase clinical trials which results in greater protocol compliance and data quality.

Conclusion:
Implementation of a standardized tool is beneficial for disease assessments and determination of response that will increase accuracy of reported assessments and response criteria across clinical trials.

P293

Incidence of Adrenal Insufficiency in Children with Neuroblastoma During and/or Shortly After Therapy

Patterson K[2], Horvath K[2], Metzger G[2], Bourgeois T[2], Scruggs M[1], Stanek J[1], Minneci P[2], Aldrink J[2], Streby K[1]
[1]Nationwide Children's Hospital/The Ohio State University Department of Hematology/Oncology/BMT, Columbus, United States of America, [2]Nationwide Children's Hospital/The Ohio State University Department of Pediatric Surgery, Columbus, United States of America

BACKGROUND
Patients with neuroblastoma (NBL) are at increased risk of toxicities due to aggressive, multi-modal therapy, particularly those with high-risk neuroblastoma (HRNBL). The incidence of adrenal insufficiency (AI) during therapy has not been reported.

AIMS
Our primary aim was to determine the incidence of AI among patients with HRNBL and non-HRNBL at our institution and nationally.

METHODS
A retrospective cohort study was performed at our institution including patients with NBL from 1998 to 2021. Demographic and clinical characteristics were evaluated in association with AI incidence. Summary statistics were computed and bivariate analyses were conducted using Pearson chi-squared tests, Fisher's exact tests, and Wilcoxon tests. Cox hazard regression models were used to examine the effects of gender and age at neuroblastoma diagnosis on time to AI diagnosis. A review of the Pediatric Health Information Systems (PHIS) database from 2010 to 2021 was performed to estimate the national incidence of AI in HRNBL.

RESULTS
Among the institutional cohort of 93 HRNBL patients, 13 developed AI (14%). The adrenal gland was the primary tumor site in 61.3%. Two patients developed AI within 6 months and the latest case was detected at 218 months from time of HRNBL diagnosis. Though not statistically significant, survival curves demonstrate that females had a higher probability of developing AI compared to males (22.2% vs 6.25%; log-rank Chi-square = 1.9310, P = 0.1646). When adjusting for gender, each year increase in age at diagnosis suggests a 96% decreased risk of developing AI (P = 0.6755). Among the institutional cohort of 99 non-HRBL patients, 3 developed AI (3%).
From the PHIS database an AI incidence of 5.2% was identified amongst patients with HRNBL (95% confidence interval for this prevalence: 4.75-5.70%).

CONCLUSION
We report a 5-14% incidence of AI in children with HRNBL and a 3% incidence in children with non-HR NBL. Larger multi-institutional studies are needed to validate these findings and determine which patients are at particular risk of developing AI during or after treatment.

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Skeletal muscle metastases in neuroblastoma share common progenitors with primary tumor and biologically resemble stage MS disease

Modak S[1], Fong C[1], Di Giannatale A[2], Gundem G[3], Li S[1], Roberts S[1], Basu E[1], Price A[4], Cheung N[1]
[1] Memorial Sloan Kettering Cancer Center, Department of Pediatrics, New York, United States, [2]Weill Cornell Medical Center, Department of Pediatrics, New York, United States, [3]Memorial Sloan Kettering Cancer Center, Department of Epidemiology and Biostatistics, New York, United States, [4]Memorial Sloan Kettering Cancer Center, Department of Radiology, New York, United States

Background:
While subcutaneous metastases are often observed with stage MS neuroblastoma, an entity that usually resolves spontaneously, skeletal muscle metastases (SMM) have been rarely described and little is known about their natural history.

Aim:
The purpose of this study was to investigate the significance of SMM in neuroblastoma.

Methods:
After IRB approval, a retrospective review of patients with neuroblastoma and associated SMM was performed.

Results:
Sixteen patients with neuroblastoma SMM, detected at diagnosis at a median age of 3.9 (0.1-15.6) months, were identified. All had metastases at other sites and 14/16 (88%) had ≥ 2 SMM in disparate muscle groups. One, thirteen, and two patients had low, intermediate, and high-risk disease respectively. Fourteen tumors had favorable histology without MYCN amplification, 2 were MYCN-amplified. Most SMM (78%; n=11/14 evaluated) were MIBG-avid. Only 1 patient (with MYCN-non-amplified neuroblastoma) had disease progression. All patients survive at median follow-up of 32.7 (3.9-305.5) months post-diagnosis. Management and biological markers (histology and chromosomal aberrations) were not prognostic. Genomic analysis of 3 matched primary and SMM lesions suggested that both primary and metastatic tumors arose from the same progenitor. SMM completely resolved in 10 patients by 12 months post-diagnosis. Of 4 patients managed with watchful observation alone without any cytotoxic therapy, 3 maintain complete remission with SMM resolving by 5, 13, and 21 months post-diagnosis respectively.

Conclusion:
Children with neuroblastoma SMM have an excellent prognosis, with a clinical course suggestive of stage MS disease. Based on these results, the initial management of infants with non-MYCN-amplified NB with SMM could be watchful observation, which could eliminate or reduce exposure to genotoxic therapy.

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INCIDENCE, SEVERITY, AND DURATION OF SINUSOIDAL OBSTRUCTION SYNDROME IN HIGH-RISK NEUROBLASTOMA: CONTRIBUTORS, MANAGEMENT, AND OUTCOMES IN A MODERN MULTI-INSTITUTIONAL COHORT

Lucas J[1], Braunstein S[2], Liu K[5], Darji H[6], Li Y[6], Sunkara A[6], Kang G[6], Devine C[5], Burghen E[11], Cross S[13], Orlina L[5], Zaslowe-Dude C[5], Goldsby R[3], Dvorak C[14], Tolbert V[3], Krasin M[1], Marcus K[5], DuBois S[4], Shusterman S[4], Diller L[4], Lehmann L[4], Matthay K[3], Margossian S[4], Haas-Kogan D[5], Federico S[8], Santana V[8], Cunningham L[12], Furman W[8], Triplett B[12]

[1]Department of Radiation Oncology, St. Jude Children's Research Hospital, Memphis, United States, [2]Department of Radiation Oncology, UCSF Benioff Children's Hospitals, San Francisco, United States, [3]Pediatric Hematology-Oncology, UCSF Benioff Children's Hospitals, San Francisco, United States, [4]Department of Pediatric Hematology-Oncology, Massachusetts General Hospital, Boston, United States, [5]Department of Radiation Oncology, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, United States, [6]Department of Biostatistics, St Jude Children's Research Hospital, Memphis, United States, [7]Department of Diagnostic Imaging, St Jude Children's Research Hospital, Memphis, United States, [8]Department of Oncology, Solid Tumor Division, St Jude Children's Research Hospital, Memphis, United States, [9]Department of Pathology, St Jude Children's Research Hospital, Memphis, United States, [10]Department of Pediatrics, St Jude Children's Research Hospital, Memphis, United States, [11]Division of Nursing Research, St Jude Children's Research Hospital, Memphis, United States, [12]Department of Bone Marrow Transplantation and Cellular Therapy, St. Jude Children's Research Hospital, Memphis, United States, [13]Department of Clinical Pharmacy and Translational Science, The University of Tennessee Health Sciences Center, Memphis, United States, [14]Pediatric Allergy, Immunology and Bone Marrow Transplant Division, UCSF Benioff Children's Hospitals, San Franscisco, United States

Background: Sinusoidal Obstruction Syndrome (SOS) is a significant complication of conditioning chemotherapy administered prior to autologous stem cell transplant in the treatment of High-Risk Neuroblastoma.

Aims: The primary objectives were to evaluate the incidence, timing, morbidity and predictors of SOS and its impact on Event Free and Overall Survival.

Methods: A multicenter retrospective investigation of patients (age <18 years) with INSS stage IIB, III or IV High Risk Neuroblastoma was conducted on 219 patients treated from 1996 to 2018 at three high volume institutions. Patients received Induction chemotherapy, surgery, conditioning chemotherapy followed by single or tandem transplant, consolidative radiotherapy and maintenance therapy. An illness death model was constructed to evaluate predictors and rates of transitions between an immediate post-transplant, SOS or progression/death state using univariate and multivariate multi-state regression models.

Results: Of the 208 patients eligible for inclusion, 30 (14.4%) experienced SOS during treatment. The median time to SOS following transplant was 16 days (IQR 13-23), with only 6.7% of cases occurring following radiotherapy. SOS resulted in a median treatment delay of 42 days (IQR 28-52). The busulfan/melphalan (BuMel) conditioning regimen (HR 2.72 95% CI 1.03 – 7.19, p=0.044) contributed to the increased hazard of SOS in the final multivariate multi-state model of SOS after adjusting for other relevant treatment exposures. While SOS was associated with decreased survival, the effect was lost following correction for treatment, and patient related factors in the multi-state model.

Conclusions: Use of BuMel conditioning was the dominant risk factor for SOS and was independent of radiotherapy. While SOS varied in the morbidity profile, appropriate management at high volume treatment centers resulted in the lack of a decrement in overall survival.

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Long-term Follow-up of the Children's Oncology Group (COG) Intermediate-risk Phase III ANBL0531 Study Using Response- and Biology-Based Therapy

Barr E[1], Naranjo A[2], Twist C[3], Tenney S[2], Schmidt M[4], London W[5], Gastier-Foster J[6], Adkins E[7], Mattei P[8], Handler M[9], Matthay K[10], Park J[11], Maris J[12], Desai A[13], Cohn S[13]

[1]Texas Tech University Health Sciences, Pediatrics, Lubbock, United States, [2]University of Florida Children's Oncology Group Statistics & Data Center, Gainesville, United states, [3]Roswell Park Comprehensive Cancer Center, Pediatrics, Buffalo, United States, [4]University of Illinois at Chicago, Pediatrics, Chicago, United States, [5]Harvard Medical School, Pediatrics, Boston, United States, [6]Baylor College of Medicine, Pediatrics, Houston, United States, [7]Palmetto Health- USC Medical Group, Surgery, Columbia, United States, [8]Children's Hospital of Philadelphia, Surgery, Philadelphia, United States, [9]Children's Hospital of Colorado, Neurosurgery, Aurora, United States, [10]University of California, Pediatrics, San Francisco , United States, [11]Seattle Children's Hospital, Pediatrics, Seattle, United States, [12]Perelman School of Medicine at the University of Pennsylvania, Pediatrics, Philidelphia, United States, [13]University of Chicago, Pediatrics, Chicago, United States

Rapid Fire session 1B, May 16, 2023, 11:30 AM - 11:45 AM

Background: The COG ANBL0531 intermediate-risk (IR) trial demonstrated excellent survival with reduced therapy for subsets of patients using a biology- and response-based algorithm.

Aim: To determine if the excellent 3-year event-free survival (EFS) and overall survival (OS) of patients on ANBL0531 is maintained over time.

Methods: Infants and children who met COG criteria for IR neuroblastoma were eligible. Treatment was based on clinical and biological prognostic markers. EFS and OS were estimated by the Kaplan-Meier method from the time of enrollment.

Results: Between 2007 and 2011, 404 evaluable patients were enrolled. The 10-year EFS and OS rates were 82.0% (95% CI, 77.2 to 86.9%) and 94.7% (95% CI, 91.8 to 97.5%), respectively. International Neuroblastoma Staging System (INSS) stage 4 patients (n=133) had inferior OS compared to non-stage 4 patients (n=271) (p=0.02). Infants with stage 4 tumors with one or more unfavorable biologic features (n=47) had significantly inferior EFS compared to those with favorable biology (n=61) (p=0.02); although OS was not significantly different (p=0.08). Significantly inferior EFS was observed among patients with 11q loss of heterozygosity (LOH) (n=26) compared to those without 11qLOH (n=314, p=0.03). OS did not differ (p=0.09). Of patients with known tumor 11qLOH status, 11qLOH was more frequently observed in stage 4 patients (16%; 18/114) compared to other stages (4%; 8/187) (p<0.0001). 10-year EFS and OS for stage 4 patients with 11qLOH (n=18) were 65.5% (95% CI, 34.7-96.3%) and 88.2% (95% CI, 68.4-100%), respectively compared to 80% (95% CI, 70.0-90.0%) and 90.4% (95% CI, 83.0-97.8%) for those without 11qLOH (n=96) (EFS p=0.14; OS p=0.77). Among all patients with tumors harboring 11qLOH, metastatic relapse occurred in 5/16 (31.3%) stage 4 infants and 1 of 2 infants with stage 4S disease. Local relapse was found in 1/5 of patients with stage 3 disease and with 11qLOH.

Conclusions: The treatment algorithm utilized in the ANBL0531 trial resulted in excellent long-term survival. Partial response or better as the treatment endpoint for patients with biologically favorable, non-metastatic neuroblastoma was associated with excellent outcome. More effective treatments are needed for patients harboring 11qLOH and infants with unfavorable biology stage 4 disease.

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Predictors of Differential Outcomes According to Response to Induction Chemotherapy in High-Risk Neuroblastoma

Sokol E[1], Naranjo A[2], LaBarre B[2], Pinto N[3], Kreissman S[4], Granger M[5], Park J[3], Bagatell R[6], DuBois S[7]
[1]Department of Pediatrics, Division of Hematology, Oncology, Neuro-Oncology and Stem Cell Transplant, Ann & Robert H. Lurie Children's Hospital of Chicago , Chicago, United States, [2]Children's Oncology Group Statistics and Data Center, Department of Biostatistics, University of Florida, Gainesville, United States, [3]Department of Pediatrics, Seattle Children's Hospital and University of Washington School of Medicine, Seattle, United States, [4]Department of Pediatrics, Duke University School of Medicine, Durham, United States, [5]Cook Children's Health System, Fort Worth, United States, [6]Department of Pediatrics, Children's Hospital of Philadelphia and Perelman School of Medicine, University of Pennsylvania, Philadelphia, United States, [7]Dana-Farber/Boston Children's Cancer and Blood Disorders Center and Harvard Medical School, Boston, United States

Rapid Fire session 1B, May 16, 2023, 11:30 AM - 11:45 AM

Background: Response to induction chemotherapy has been shown to predict outcome in patients with high-risk neuroblastoma, with those achieving a complete response (CR) having superior outcomes. Little is known about what factors impact survival within groups of patients with favorable and unfavorable end-induction response. We evaluated whether conventional prognostic factors remain prognostic in subsets of patients defined by response to induction.

Aims: We aimed to identify cohorts of patients with differential outcomes to induction chemotherapy that may ultimately be used to alter the standard treatment paradigm.

Methods: Patients from four COG high risk trials (A3973, ANBL02P1, ANBL0532, and ANBL12P1) were included. End-induction response was determined according to the 1993 International Neuroblastoma Response Criteria (INRC). Patients were categorized as having end-induction responses of CR, partial response (PR) or better, less than PR without progressive disease (PD), and PD. Univariate Cox models calculated OS hazard ratios for clinical and biological variables in subsets defined by each response category.

Results: 1,244 patients were included. Among all patients, age >5 years, INSS stage 4 disease, adrenal primary site and unfavorable histology by INPC were significantly associated with inferior OS. Among patients who achieved an end-induction CR, stage 4 disease was the only factor that remained significantly associated with worse OS. Among those who achieved end-induction PR or better, age >5 years, stage 4 disease and unfavorable histology remained significantly associated with inferior OS. For those with less than PR but without PD, adrenal primary site, MYCN amplification and 1p LOH were significantly associated with inferior OS. For those with PD during or at end-induction, MYCN amplification and 1p LOH were significantly associated with worse OS, but older age was associated with better OS.

Conclusions: Specific prognostic factors in neuroblastoma are associated with differential survival in groups defined by response to induction. Age, stage, and histology appear to be associated with OS for patients with more favorable response to induction, whereas MYCN and 1p LOH play a greater role in patients with unfavorable response to induction. These data can help to further define prognosis for patients with variable responses to induction.

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Response-Adapted Consolidation Therapy Strategy for Patients With Metastatic High-Risk Neuroblastoma: Results From the SMC NB-2014 Study

Seo E[1,2], Lee J[1], Cho H[1], Ju H[1], Yoo K[1], Sung K[1]

[1]Department of Pediatrics, Samsung Medical Center, Gangnam-gu, South Korea, [2]Samsung Advanced Institute for Health Sciences & Technology, Sungkyunkwan University, Gangnam-gu, South Korea

Rapid Fire session 1B, May 16, 2023, 11:30 AM - 11:45 AM

Background: Previously, our prospective trial demonstrated that incorporation of 131I-metaiodobenzylguanidine (131I-MIBG) treatment into tandem high-dose chemotherapy and autologous stem cell transplantation (HDCT/auto-SCT) resulted in satisfying outcomes in high-risk neuroblastoma. However, more optimized treatment strategies still are needed.

Aims: The NB-2014 study was a nonrandomized, prospective trial that examined survival outcomes among patients with high-risk neuroblastoma compared with a historical control group from the NB-2009 study. Methods: All patients received nine cycles of induction chemotherapy, followed by assignment to tandem HDCT/auto-SCT and 131I-MIBG treatment based on their response to the chemotherapy in metastatic sites. Patients with a complete resolution of MIBG uptake at metastatic sites after induction chemotherapy were eligible for reduced first HDCT/auto-SCT, defined as 20% dose reduction in HDCT. After first HDCT/auto-SCT, patients who had a complete resolution of MIBG at metastatic sites were assigned to receive 12 mCi/kg of 131I-MIBG treatment while patients with persistent MIBG uptake at metastatic sites were assigned to receive 18 mCi/kg of 131I-MIBG treatment.

Results: Of 57 patients who commenced treatment, 66.7% achieved complete resolution of MIBG uptake at metastatic sites following induction chemotherapy, while 24.6% patients still had MIBG uptake at metastatic sites after first HDCT/auto-SCT. The 3-year event-free survival (EFS) and overall survival rates were 63.2% ± 6.4% and 82.3% ± 5.1%. The 3-year EFS for patients with negative MIBG uptake at metastatic sites after induction chemotherapy in the NB-2014 was not inferior to that of patients in the NB-2009 (55.7% ± 8.5% vs. 61.3% ± 8.8%, P = 0.770). The 3-year EFS of patients with persistent MIBG uptake after induction chemotherapy in the NB-2014 was higher than that of patients in the NB-2009, though not statistically significant (64.7% ± 11.6% vs. 50.0% ± 14.4%, P = 0.721). Compared to the NB-2009, patients in the NB-2014 experienced significantly lower rates of grade 3-4 toxicities including veno-occlusive disease (P = 0.046) and toxic death (P = 0.033).

Conclusion: Our results suggest that response-adaptive consolidation therapy based on chemotherapy response at metastatic sites facilitates better treatment tailoring and seems promising for patients with metastatic high-risk neuroblastoma.

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Surgical Outcome of A Nationwide Phase II Study of Delayed Local Treatment for Children with High-risk Neuroblastoma (JN-H-11)

Yoneda A[1,2,3], Shichino H[3], Hishiki T[3], Matsumoto K[3], Ohira M[3], Kamijo T[3], Kuroda T[3], Soejima T[3], Nakazawa A[3], Takimoto T[3], Yokota I[3], Teramukai S[3], Takahashi H[3], Fukushima T[3], Hara J[3], Kaneko M[3], Ikeda H[3], Tajiri T[3], Mugishima H[3], Nakagawara A[3]

[1]Department of Surgical Oncology, Children's Cancer Center, National Center for Child Health and Development, Tokyo, Japan, [2]Department of Pediatric Surgical Oncology, National Cancer Center Hospital, Tokyo, Japan, [3]The Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG), Kyoto, Japan

Rapid Fire session 2B, May 18, 2023, 12:05 PM - 12:20 PM

Background: As most of the recurrences in high-risk neuroblastoma occur in distant metastatic lesions, systemic disease control seems to be the key strategy for achieving better survival. Based on this hypothesis, the JNBSG conducted JN-H-11, a nationwide phase II study of delayed local treatment for children with high-risk neuroblastoma.

Aims: To investigate the feasibility of delayed local treatment, particularly of surgery after high-dose chemotherapy, we evaluated the surgical outcome of JN-H-11.

Methods: Seventy-five patients with newly diagnosed high-risk neuroblastoma patients were enrolled. Induction chemotherapy consisted of cisplatin (100 mg/m²), pirarubicin (40 mg/m²), vincristine (1.5 mg/m²), and cyclophosphamide (1,200 mg/m² in the 1st course and 2,400 mg/m² in the 2nd-5th course). After 5 courses of induction chemotherapy, all patients were immediately followed by high-dose chemotherapy with melphalan (200 mg/m²), etoposide (800 mg/m²) and carboplatin (1600 mg/m²). Local tumor extirpation was performed within 100 days after the date of hematopoietic stem cell infusion. Finally, irradiation (19.8 Gy) was administered.

Results: Of 75 patients were enrolled, 64 were evaluable (median age, 2.0 years; stage 4, n=56; MYCN amplification, n=21). Thirty-seven patients completed the protocol treatment. The estimated 3-year progression-free survival rate and overall survival rate (95%CI) were 44.4% (31.8%-56.3%) and 80.7% (68.5%-88.5%), respectively. Surgery rather than a biopsy was performed safely in 52 patients. One patient underwent surgery after three courses of induction chemotherapy at the discretion of the local institution. Median period from stem cell transfusion to surgery was 51.5 (28-161) days. Surgery was not perform within 100 days from the date of stem cell transfusion because of delayed bone marrow recovery in two patients. Complete resection was achieved in 28 patients (53.8%): ≥90% tumor resection, n=10 (19.2%), 50–90% tumor resection n=7 (13.4%); and <50% tumor resection n=6 (11.5%). The extent of resection (100%, ≥90% and <90%) was not related to survivals in this study. Nephrectomy was reported in 3 patients (5.8%). Major surgical complications were observed in 13 patients (25.0%). No surgery-related deaths were reported.

Conclusion: Surgery after high-dose chemotherapy appears to be safe and feasible. Local treatment could be postponed until after high-dose chemotherapy in patients with high-risk neuroblastoma.

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Late disappearance of minimal residual disease after KIR-ligand mismatched allogeneic CBT suggests KIR mismatched NK cell activity to Neuroblastoma

Alahmadi R[1], Nishio N[1,2], Yamashita D[1], Sajiki D, Maemura R[1], Tsumura Y[1], Yamamori A[1], Wakamatsu M[1], Narita K[1], Kataoka S[1], Narita A[1], Muramatsu H[1], Takahashi Y[1]

[1]Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2]Center for Advanced Medicine and Clinical Research, Nagoya University Hospital, Nagoya, Japan

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Background: Killer cell immunoglobulin-like receptor (KIR)/HLA genotypes predictive of missing KIR-ligands are reported to have a favorable result after autologous hematopoietic stem cell transplantation in patients with high-risk neuroblastoma (NB). KIR-ligand mismatched allogeneic cord blood transplantation (CBT), which we previously described as a consolidation therapy, decreased recurrence and increased survival in children with initial high-risk metastatic neuroblastoma (ANR 2018). We hypothesized that KIR-ligand mismatched CBT would reduce relapse due to the graft versus tumor effect.

Aims: We assessed the effectiveness of minimal residual disease (MRD) measurement in bone marrow (BM) to monitor the response to KIR-ligand mismatched CBT.

Methods: From July 2015 to January 2021, 49 patients with high-risk metastatic neuroblastoma received KIR-ligand mismatched CBT. The study comprised 14 of them who had BM-MRD results that were positive before beginning CBT. To detect MRD, PHOX2B and tyrosine hydroxylase (TH) expression were employed as NB-specific markers and assessed by real-time quantitative PCR in BM aspirates before and after CBT. The BM samples were collected from both sides of the posterior iliac crest. MRD was determined as positive when either PHOX2B or TH was detectable.

Results: Median age at diagnosis and CBT was 4.1 and 5.8, respectively. Disease status at CBT was a partial response in all 14 patients. The type of KIR-ligand mismatch was donor C1/C2 to recipient C1/C1 in 12 patients, Bw4 mismatch in 1 patient and A11 mismatch in 1 patient. Follow up period of the living patients from CBT was 1.6-7.3 years (median 3.7 years). MRD in BM became negative at least once in 12 patients (86%) after CBT. Among them, BM-MRD was returned to positive in five patients followed by disease progression. On the other hand, the continuous disappearance of BM-MRD has been observed at a median of 9.4 months (range of 3.0-20.7 months) after CBT in seven patients who remained progression-free at a median of 2.8 years (range, 1.6–7.3 years) after CBT.

Conclusions: The late disappearance of BM-MRD and prolonged relapse-free survival following KIR-ligand mismatched CBT indicates KIR mismatch NK cells activity to neuroblastoma and reduced BM relapse.

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Naxitamab-Based Chemoimmunotherapy for Resistant High-Risk Neuroblastoma: Results of "HITS" Phase II Study (NCT03189706)

Modak S[1], Kushner B[1], Mauguen A[2], Castañeda A[3], Varo A[3], Gorostegui M[3], Muñoz J[3], Santa-Maria V[3], Basu E[1], Iglesias Cardenas F[1], Pandit-Taskar N[4], Cheung N[1], Mora J[3]

[1]Memorial Sloan Kettering Cancer Center, Department of Pediatrics, New York, United States, [2]Memorial Sloan Kettering Cancer Center, Department of Epidemiology and Biostatistics, New York, United States, [3]Hospital Sant Joan de Deu, Barcelona, Spain, [4]Memorial Sloan Kettering Cancer Center, Department of Radiology, New York, United States

Background: Chemoresistant disease is an obstacle for cure of high-risk neuroblastoma (HR-NB). Anti-GD2 monoclonal antibodies (MoAb) dinutuximab and naxitamab are FDA-approved to consolidate remission and for chemorefractory osteomedullary HR-NB, but responses in progressive disease (PD) are rare. We investigated the combination of Humanized anti-GD2 MoAb naxitamab (Hu3F8), Irinotecan, Temozolomide and Sargramostim (GMCSF) in a phase II "HITS" protocol against resistant HR-NB.

Methods: Patients with evaluable or measurable chemoresistant HR-NB were treated at Memorial Sloan Kettering (MSK) on protocol and at Hospital Sant Joan de Déu (HJSD) per protocol on compassionate basis. Prior anti-GD2 MoAb or irinotecan/temozolomide (IT) therapy was permitted. Each cycle, administered 3-5 weeks apart, comprised irinotecan 50 mg/m2/day intravenously (IV) plus temozolomide 150 mg/m2/day IV or orally (days 1-5); naxitamab 2.25 mg/kg/day IV, days 2,4,8 and10, and GMCSF 250 mg/m2/day subcutaneously, days 6-10. Toxicity was measured by CTCAE v4.0 and responses by International Neuroblastoma Response Criteria.

Results: Of 90 heavily prior-treated patients (38 at MSK evaluated on trial, 52 at HJSD), 8 had HR-NB refractory to induction chemotherapy while 82 had up to 6 prior relapses (median=1). 503 cycles (median 5/patient) were administered. Toxicities included myelosuppression and diarrhea expected with IT, pain and hypertension expected with naxitamab, plus febrile neutropenia in 4%. No other >grade 2 unexpected toxicities occurred; treatment was outpatient. Primary endpoint was reached in the phase II trial: CR+PR INRC response = 30.6%, lower boundary = 20.4%. In the entire cohort, best responses were CR (26%), PR (11%), mixed response (9%), stable disease (27%) and PD (27%). Objective responses (OR) were noted in 64%, with soft tissue (48%) and skeleton (66%) with CR in BM in 57%. OR occurred in MYCN-amplified (25%), refractory (100%) and relapsed (61%) HR-NB; and patients who had previously received I/T (64%) or naxitamab (68%). In patients who had previously received dinutuximab/IT, HITS achieved an OR rate of 42% (5/12). Human anti-human antibody did not develop in any patient (n=50).

Conclusions: Naxitamab-based chemoimmunotherapy was safe without immunogenicity. It was effective against chemoresistant HR-NB i even in patients with multiple prior relapses, and in patients who previously received anti-GD2 MoAbs and/or IT.

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ALK inhibitors for therapy of adult-onset neuroblastoma

Stiefel J[1], Kushner B[1], Iglesias Cardenas F[1], Roberts S[1,2], Basu E[1], Modak S[1]
[1]Memorial Sloan Kettering Cancer Center, New York, United States, [2]Doernbecher Children's Hospital, Portland, United States

Background: Adult-onset neuroblastoma (AON) differs significantly in biology and clinical behavior from childhood-onset disease. AON poses therapeutic challenges since tolerance of intensive multimodality therapies that are standard-of-care for pediatric neuroblastoma is poor. Further, adults are typically ineligible for therapeutic trials. AON is enriched for somatic mutations (Suzuki etal. 2018) including ALK, deemed to be an oncogenic driver in NB. ALK inhibitors (ALKi) therefore, have the potential to be of therapeutic benefit in AON.

Aim: To review and report on the use of ALKi in AON.
Methods: A single-center retrospective review of patients receiving ALKi (2012-2022) was performed. Response was evaluated using International Neuroblastoma Response Criteria.

Results: Fifteen patients with ALK-mutated AON (8, 4, 2 and 1 with mutations at F1174, R1275, F1245 and other sites respectively) were treated with FDA-approved ALKi (14/15 without concomitant anti-NB therapy) starting at a median age of 34 (16-71) years. All had previously received chemotherapy, immunotherapy, and/or radiotherapy. Indications for ALKi included primary refractory unresectable L2 (n=2) or metastatic disease (n=6), or relapsed/progressive disease (PD) (n=7). Initial ALKi was lorlatinib, crizotinib and alectinib in 7,5 and 3 patients respectively; 7 received multiple ALKis due to PD/intolerability of one agent. All patients experienced ≥grade 2 adverse events (AEs): crizotinib and alectinib were associated primarily with gastrointestinal AEs, lorlatinib with neurological AEs, weight gain and hyperlipidemia. Dose-reduction or discontinuation of ALKi was needed in 2/5, 1/1, 1/3 and 6/13 patients receiving crizotinib, ceritinib, alectinib and lorlatinib respectively. No responses were observed with crizotinib (n=5 evaluable), ceritinib, alectinib or brigatinib (n=1 each). Of the 13 patients treated with lorlatinib (including 5 treated with other ALKis), 4,5 and 4 patients had complete or partial response (major response rate 69%), and stable disease respectively. Responses were noted in all disease compartments; complete metabolic response (negative FDG-PET) was seen in two L2 patients. 10/13 patients remain progression-free at a median of 19 (6-50) months on lorlatinib. Three (all receiving dose-reduced therapy) had PD. Median survival from start of first ALKi was 43±26 months.

Conclusion: ALKis, particularly lorlatinib are effective treatment options for therapeutically- challenging AON. However, AEs necessitating dose-reduction are common.

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A Randomised Phase II Trial of Radiotherapy Dose Escalation, Facilitated by Intensity Modulated Arc Radiotherapy Techniques, in High-Risk Neuroblastoma

Gains J[1], Patel A[2], Chang Y[1], Mandeville H[3], Smyth G[3], Stacey C[1], Talbot J[3], Wheatley K[2], Gaze M[1]
[1]Department of Oncology, University College London Hospitals NHS Foundation Trust, London, United Kingdom, [2]Cancer Research UK Clinical Trials Unit, University of Birmingham, Birmingham, United Kingdom, [3]Department of Radiotherapy, The Royal Marsden NHS Foundation Trust, London, United Kingdom

BACKGROUND
Radiotherapy forms part of the standard treatment for high-risk neuroblastoma, but there is little randomised trial evidence to prove its value or to define optimal treatment parameters. Only one prior randomised trial (1991), evaluated radiotherapy. Older techniques compromise the protocol dose to respect adjacent organ at risk (OAR) tolerance. Intensity modulated arc radiotherapy (IMAT) is a more sophisticated technique, which may allow better sparing of OAR, and enable dose escalation.

AIMS
This national multicentre randomised Phase 2 trial aimed to establish whether IMAT could facilitate dose escalation to the planning target volume in high-risk neuroblastoma compared to conventional radiotherapy techniques (CRT). Secondary objectives included determining the dose that 80% of patients received and the proportions able to receive the allocated dose with IMAT and conventional radiotherapy.

METHODS
Patients with high-risk neuroblastoma receiving radical radiotherapy to the primary tumour site were randomised to receive either standard dose (21 Gray (Gy) in 14 fractions) or escalated dose (36 Gy in 24 fractions). Patients were dual planned using both anterior posterior parallel opposed pair CRT and IMAT techniques. Prospective radiotherapy quality assurance (RTQA) of targets and organ at risk delineations and dosimetric plans were undertaken. Centres selected the optimal technique, IMAT or CRT, based on analysis of dosimetric parameters, with this then confirmed as part of the RTQA process. This feasibility trial was not powered to detect improvement in outcome with dose escalation.

RESULTS
Between 2017 and 2020, 50 patients were randomised and dual planned. IMAT was judged more favourable. In all patients randomised to receive 36 Gy, IMAT permitted delivery of the full dose to the target volume, sometimes with minor compromise to protect OAR, whereas compromise of total dose would have been required with CRT.

CONCLUSION
IMAT facilitates safe dose escalation to 36 Gy in patients with high-risk neuroblastoma receiving treatment of the primary tumour site and should be considered the technique of choice for photon radiotherapy where available. The value of dose escalation in patients with residual disease following surgery is being formally evaluated in the current SIOPEN prospective phase III randomised trial (ClinicalTrials.gov Identifier: NCT04221035).

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Alpha-1 adrenergic antagonists sensitize neuroblastoma to differentiation by isotretinoin

Broso F[2], Gatto P[1], Sidarovich V[1], Adami V[1], Ambrosini C[1], De Sanctis V[5], Bertorelli R[5], Destefanis E[1], Zaccheroni E[1], Sebastiani E[1], Tebaldi T[3,4], Quattrone A[1]

[1]Laboratory of Translational Genomics, Department of Cellular, Computational and Integrative Biology,University of Trento, Trento , Italia , [2]High-Throughput Screening Core Facility, Department of Cellular, Computational and Integrative Biology, University of Trento, Trento , Italia , [3]Laboratory of RNA and Disease Data Science, Department of Cellular, Computational and Integrative Biology, University of Trento, Trento, Italia, [4]Section of Hematology, Yale Cancer Center and Department of Internal Medicine, Yale University School of Medicine, New Haven, United States, [5]Next Generation Sequencing Core Facility LaBSSAH, Department of Cellular, Computational and Integrative Biology, University of Trento, Trento, Italia

Background. Despite the aggressive high-risk neuroblastoma (NB) treatment protocol, the 5-year overall survival probability is approximately 50%. The protocol includes isotretinoin (13cRA), which is used in the post-consolidation phase as an antiproliferative and prodifferentiative agent to minimize residual disease and prevent relapse.

Aims. We aimed to identify a drug that could be combined with 13cRA to increase its activity.

Experimental Method. High-throughput small-molecule screening was used to select 13cRA sensitizing compounds. Bulk and single-cell transcriptome analyses and phenotypic readouts of wild-type and gene knockout cell lines were followed by xenotransplantation and treatment in mice.

Results. We identified isorhamnetin (ISR) as a synergistic compound with 13cRA in inhibiting up to 80% of NB cell viability. This synergism is accompanied by a marked increase in adrenergic receptor α1B (ADRA1B) gene expression. Either gene knockout of ADRA1B or specific blockage of its protein product by α1 adrenergic antagonists leads to sensitization of NB cells to 13cRA in terms of reduction of cell viability and promotion of neural differentiation, thus mimicking ISR action. Further screening of AR ligands confirmed that blockage of α1-AR is the best way to sensitize NB cells to 13cRA. Administration of doxazosin, a safe ADRA1 antagonist used in pediatric patients, with 13cRA in NB-xenografted mice, exerted a marked control of tumor growth. Simultaneously, each drug alone was ineffective.

Conclusions. We identified ADRA1B as a novel pharmacological target for NB. We propose the addition of α1-AR antagonists in the post-consolidation therapy of NB to more efficiently control the residual disease.

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Single-agent activity of the anti-GD2 antibody dinutuximab beta given as long-term continuous infusion in relapsed and refractory neuroblastoma (APN311-304).

Lode H[1], Ehlert K[1], Huber S[1], Siebert N[1], Troschke-Meurer S[1], Zumpe M[1], Loibner H[2], Ladenstein R[3]
[1]Pediatric Hematology and Oncology, University Medicine Greifswald, Greifswald, Germany, [2]HL Bioscience Research GmbH, Vienna, Austria, [3]St. Anna Children's Hospital and Department of Paediatrics, Medical University Vienna, Vienna, Austria

Background
Severe pain is a common side effect associated with short-term infusions of anti-GD2 antibodies in combination with cytokines and was reduced by the long-term infusison (LTI) strategy.

Aim
Investigate efficacy and tolerability of a single-agent DB-LTI in patients with primary refractory or relapsed stage 4 neuroblastoma in a phase 2 study.

Methods
Patients with evaluable disease were enrolled. Previous anti-GD2 antibody therapies were allowed, if anti-DB antibody could not be detected. Patients received 5 cycles of DB-LTI (100 mg/m2/10 days). The primary endpoint was objective INRC response 24 weeks after the end of cycle 5. Secondary endpoints included safety, morphine use, best response and duration of response, pharmacodynamics and pharmacokinetics, 3-year progression-free (PFS) and overall survival (OS).

Results
38 patients had evaluable response. The objective response rate 24 weeks after the end of cycle 5 was 26% (10/38) (CR/PR) (p=0.0034) and 32% (12/38) (CR/PR/MR), with a higher response rate in refractory vs. relapsed patients (50% vs 25%; p=0.0159). The best response rate was 53% (20/53). Of 14 patients with bone marrow involvement, 13 patients responded (93%) (12 CR, 1 PR). The median duration of response (CR/PR) was 238 days (95% CI [108-290d]). The 3-year probablility of PFS was 31% (95% CI [0.17–0.47]) and of OS was 66% (95% CI [0.47–0.79]). Survival was higher for patients with refractory compared to relapsed disease (PFS 47% vs 19%, OS 93% vs 50%; p=0.015). Grade 3 treatment emergent adverse events (TEAE) with a frequency >10% were inflammation (20%), gastrointestinal disorders (12.5%) and pyrexia (10%). The following grade 3&4 TEAEs were not observed: pain, capillary leak, neurotoxicity. The morphine usage decreased from cycle 2 to 5 (C2 95%; C3 12%; C4 0%; C5 0%). DB peak serum concentration in cycle 1 was 10.8±0.7 µg/ml and patients showed 7- and 11-fold increase of antibody-dependent cell-mediated (ADCC) and complement-dependent cytotoxicity (CDC), respectively. HACA were found in 24% of patients. There was no association between exposure to DB or HACA and response rate or survival in this cohort.

Conclusion
Single-agent treatment with DB-LTI is effective and highly tolerable and in relapsed/refractory high risk neuroblastoma.

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Sustained complete response of neuroblastoma with metronomic chemotherapy: A Case Series

Sun Y[1], Li Y[3], Ma N[3], Zhang J[2,3]

[1]Department of Pediatrics, The Forth Affiliated Hospital of China Medical University, shenyang, China, [2]Department of Pediatrics, Shengjing Hospital of China Medical University, shenyang, China, [3]Diagnosis and Treatment Center of Neuroblastoma, Northeast International Hospital, shenyang, China

Background: Neuroblastoma (NB) is the most common pediatric extra-cranial solid tumor.
Aims: The aim of this study was to retrospectively describe the clinical findings of infant NB patients of sustained complete remission with metronomic chemotherapy treatment.
Methods: This was a retrospective study including patients under the age of 12 months with neuroblastoma from two centers in Shenyang, China (The Forth Affiliated Hospital of China Medical University and Weikang Hospital).

Results: Fifteen infants with NB were identified during the study period (male =7 and female =8). 9(60%) patients were classified as having high-risk disease, and 6 (40%) as having very low-risk disease. According to the INSS, 8 (53.3%) patients had stage 4 disease and 7 (46.7%) had stage 4S disease. The most common metastatic sites included bone marrow (n=7, 46.7%), liver (n=6, 40%), skull bone (n=3, 20%), distant lymph node (n=3, 20.0%), and soft tissue (n=1, 6.7%). Of the total number of patients enrolled in this study, only 1 (6.7%) underwent surgical excision one side of the primary tumor. 6 (40%) underwent biopsied primary tumors. 9 (60) were diagnosed by bone marrow aspirates. Only 1 patient died, and the major cause of death is disease progression. 14 patients was sustained complete remission.

Conclusion: Infants with neuroblastoma achieve a reasonable clinical outcome when treated with metronomic chemotherapy in our institution. Such information will provide a basis for a selective regimen to this rare disease.

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BEACON2: a SIOPEN-ITCC platform multi-arm multi-stage trial for relapsed neuroblastoma.

Moreno L[1], Weston R[2], Owens C[3], Rubio A[4], Berlanga P[5], Bird N[6], Laidler J[2], Firth C[2], Valteau-Couanet D[5], Beck-Popovic M[7], Kearns P[2], Pearson A[8], Gates S[2], Gray J[9]

[1]Division of Pediatric Hematology and Oncology. Vall d'Hebron Barcelona Hospital Campus, Barcelona, Spain, [2]Cancer Research UK Clinical Trials Unit, University of Birmingham, Birmingham, United Kingdom, [3]Children's Hospital Ireland, Dublin, Ireland, [4]Hospital Universitario Niño Jesus, Madrid, Spain, [5]Institut Gustave Roussy, Villejuif, France, [6]Solving Kids Cancer UK, London, United Kingdom, [7]CHUV, Lausanne, Switzerland, [8]ACCELERATE, Brussels, Belgium, [9]University of Southampton, Southampton, United Kingdom

Background

Outcomes for relapsed neuroblastoma remain dismal. Despite the incorporation of novel regimens developed by cooperative groups internationally, the majority of patients still relapse, new therapies and an efficient approach for their evaluation are needed.

Aims

Results from single arm trials or small randomized trials are challenging to implement into clinical practice. Platform multi-arm multi-stage trials are an efficient tool to evaluate novel combinations in rare diseases such as relapsed neuroblastoma and bring more effective therapies into clinical care.

Methods

A multi-arm multi-stage phase 1-2-3 trial design is proposed. The starting trial design is: Tier 1 (Phase 2-3 component), patients with relapsed neuroblastoma with adequate performance, wash-out periods and organ function, will be randomized 1:1 to arm A (bevacizumab, irinotecan and temozolomide) or B (dinutuximab beta, irinotecan and temozolomide). The trial Phase 2 component will comprise an interim analysis after 40 patients per arm and complete evaluation after 75 patients per arm have been recruited. A phase 3 component including up to 112 patients per arm could be incorporated. Primary endpoint will be progression-free survival.

Tier 2 (Phase 1 component) will include dose confirmation cohorts for novel regimens in patients that are not eligible for Tier 1. Arm C will be the initial Tier 2 cohort and will include dinutuximab beta, bevacizumab, irinotecan and temozolomide.

As the trial progresses, novel arms will be incorporated into Tier 2 and Tier 1. Biomarker studies embedded in the trial will improve our understanding of relapsed neuroblastoma biology and mechanisms of resistance, and identify predictive and prognostic biomarkers.

Results

This Fight Kids Cancer-funded clinical trial sponsored by the University of Birmingham is currently in set up. The trial will run through an international SIOPEN-ITCC network of national coordinators in 15 countries and 60+ sites across the globe. Trial predicted timelines are to enroll the first patient by mid 2023.

Conclusion

The BEACON2 trial will serve to evaluate novel combination therapies in relapsed neuroblastoma, identifying novel regimens that can improve outcomes for patients in relapse or be brought forward into frontline.

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Toxicity of Anti-GD2 Antibody Immunotherapy after KIR Ligand-mismatched Allogeneic Stem Cell Transplantation in Patients with High-risk Neuroblastoma

Nishio N[1,2], Kataoka S[1], Alahmadi R[1], Yamashita D[1], Sajiki D[1], Maemura R[1], Tsumura Y[1], Yamamori A[1], Wakamatsu M[1], Narita K[1], Narita A[1], Muramatsu H[1], Takahashi Y[1]
[1]Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2]Department of Advanced Medicine and Clinical Research, Nagoya University Hospital, Nagoya, Japan

Background: Dinutuximab (DIN) is a recombinant chimeric monoclonal antibody that targets disialoganglioside (GD2). DIN was approved in Japan in 2021 for patients with neuroblastoma following completion of high-dose chemotherapy in combination with granulocyte colony-stimulating factor (G-CSF) and interleukin-2 teceleukin. DIN toxicity has been described after autologous stem cell transplantation, but it is unknown after KIR ligand-mismatched allogeneic stem cell transplantation.
Aims: To assess the toxicity of DIN after KIR ligand-mismatched allogeneic stem cell transplantation in patients with high-risk neuroblastoma.

Methods: This study included seven patients with high-risk neuroblastoma who received at least one course of DIN treatment after allogeneic stem cell transplantation between October 2021 and December 2022. The treatment protocol included 17.5 mg/m2 of DIN for 4 days during each of six consecutive 28-day cycles. Patients were also given G-CSF during cycles 1, 3, and 5 as well as interleukin-2 during cycles 2, 4, and 6. The toxicity of DIN treatment in these patients was studied retrospectively.

Results: The median age was 6 years (range: 5–8). All of the patients had previously received KIR ligand-mismatched allogeneic cord blood transplants. Three patients had primary disease, while four had relapsed disease. Seven patients received a total of 17 courses of DIN treatment. DIN treatment was discontinued in three patients due to disease relapse. During cycle 1, common grade 2 or higher adverse events included fever in seven cases, pain in seven cases, and liver dysfunction in five cases, through these adverse events improved during subsequent courses. During the course of DIN treatment, no patient developed graft versus host disease. However, two of the seven patients experienced serious adverse events and were forced to discontinue DIN treatment. Defibrotide was given to one patient who developed thrombotic microangiopathy (TMA) after cycle 1 of DIN treatment and met the diagnostic criteria for hepatic sinusoidal obstruction syndrome (SOS). After cycle 3 of DIN treatment, the other patient developed renal dysfunction.

Conclusion: When DIN therapy is administered after KIR ligand-mismatched allogeneic stem cell transplantation in high-risk neuroblastoma patients, the risk of TMA/SOS and renal dysfunction should be considered.

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Real-world outcomes of High-risk Neuroblastoma from a tertiary care centre in India

Gollamudi V[1], Parambil B[1], Prasad M[1], Khanna N[1], Laskar S[1], Patil V[1], Baheti A[1], Chichra A[1], Shah S[1], Manjali J[1], Ramadwar M[1], Qureshi S[1], Patil R[2], Sharma R[2], Kini P[2], Manglani M[2], Keerthivasagam S[1], Chinnaswamy G[1]
[1]Tata Memorial Hospital, Homi Bhabha National Institute, Mumbai, India, [2]MCGM-Comprehensive Thalassemia Care, Pediatric Hemato-Oncology and BMT Centre, Mumbai, India

Background and Aim:
Outcomes of High-risk neuroblastoma(HR-NBL) in LMIC are adversely affected by toxicities, non-availability of (or delay in) Autologous-stem cell transplant(Auto-SCT) and immunotherapy. Herein, we study the clinical profile and outcomes of HR-NBL treated with intensive protocol without immunotherapy in a resource constrained setting.

Methods:
Treatment-naive children with biopsy-proven HR-NBL from July 2018-December 2020 were retrospectively analysed. INRG stratification was used based on MIBG scan, FDG-PETCT scan, bone-marrow studies, N-Myc/SCA reports. Rapid COJEC induction and surgery of primary after achieving metastatic Complete Response/Very Good Partial Response(mCR/VGPR) was followed by Auto-SCT and radiotherapy(RT). If end-induction bone marrow was involved, 2 cycles of TVD(topotecan,vincristine,doxorubicin) were administered. Oral maintenance consisting cis-retinoic acid, cyclophosphamide, etoposide and celecoxib was administered post-RT. Survival was analysed by log rank test and prognostic factors by cox proportional-Hazard model using SPSS(Version-26). Auto-SCT was performed by establishing a collaborative network between pediatric oncology centres.

Results:
Among 64 eligible patients, median age was 39months(16-128months) with suprarenal primary in majority[42(65.6%)]. Molecular data was available for 43 of whom 17(39.5%) had N-Myc amplification. All but one were metastatic at presentation with bone-marrow, bone, lymph-nodes, lung metastases seen in 47(73%), 26(46%), 3(4.6%), 2(3.1%), respectively. At the end of Rapid COJEC induction, mCR/mVGPR was achieved in 39(60.9%).There were 6(9.4%) toxic-deaths, 5(7.8%) progression, 1(1.5%) palliation due to poor tolerance. Half(7/14) of those who received TVD attained mCR. Out of 42 who underwent surgery, 31 achieved gross total resection. Thirty-eight(59.4%) underwent Auto-SCT(toxic-death post Auto-SCT-1). At a median follow-up of 29months(95%CI:9.0-20.99), 3year EFS/OS of the entire cohort was 24±7.2% and 25.2±5.8%, and of those who completed the multi-modality treatment was 32.7±11.1% and 36.6±8.7% respectively.On multivariate analysis, PET response (in mCR/mVGPR) [HR:2.9(1.01-8.53,p=0.04)] and gross total surgical resection [HR:3.1(1.03-9.88,p=0.04)] were prognostic.

Conclusions:
Outcomes of HR-NBL even with a strategy including Auto-SCT is suboptimal in LMICs. Subtotal tumour resection and inadequate metastatic response had a poor outcome despite intensive therapy. Wider availability of immunotherapy at affordable cost would be the way forward in the improving the HR-NBL outcomes in LMICs.

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Mixed response after radiopharmaceutical therapy with 177Lu-DOTATATE in a refractory high-risk neuroblastoma patient – early results from the LuDO-N Trial

Park S[1], Fransson S[2], Djos A[2], Fagman H[2], Sjögren H[3], Nilsson J[4], Wessman S[5], Georgantzi K[6,7], Strömgren J[8], Herold N[6,7], Kogner P[6,7], Karlsson K[1], Martinsson T[2], Stenman J[6,7]

[1]Department of Oncology-Pathology, Karolinska Institute, Stockholm, Sweden, [2]Department of Laboratory Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, [3]Clinical Genetics and Genomics, Sahlgrenska University Hospital, Gothenburg, Sweden, [4]Department of Molecular Medicine and Surgery, Karolinska University Hospital, Stockholm, Sweden, [5]Department of Pathology, Karolinska University Hospital, Stockholm, Sweden, [6]Department of Women's and Children's Health, Karolinska Institute, Stockholm, Sweden, [7]Astrid Lindgren Children's Hospital, Karolinska University Hospital, Stockholm, Sweden, [8]Department of Pediatric Radiology, Karolinska University Hospital, Stockholm, Sweden

Background

A 21 months old, previously healthy female was diagnosed with high-risk neuroblastoma with widespread skeletal and bone marrow metastasis. Induction therapy was initiated according to the SIOPEN/HR-NBL1 protocol followed by 4 courses of topotecan/vincristine/doxorubicin (TVD) and three courses of temozolomide/irinotecan (TEMIRI) due to persistent bone marrow involvement. This was followed by radioisotope (^{131}I-mIBG) therapy with concomitant topotecan and stem-cell rescue prior to high-dose chemotherapy (busulfan-melphalan) with stem-cell rescue. Due to progression of the distal femoral metastasis, the patient was screened for inclusion in the LuDO-N Trial.

Methods

^{68}Ga-DOTATOC PET/CT imaging was performed to confirm trial inclusion criteria as well as at 1 and 2,5 months after end of treatment with ^{177}Lu-DOTATATE. Dosimetry was performed by 4 serial SPECT/CT scans during days 1-7 after each of the two treatment cycles. Copy number profiling and post-hoc analysis with SSTR-2 immunohistochemistry (IHC) was performed on the primary tumor and the distal femoral metastasis that eventually progressed.

Results

Treatment with ^{177}Lu-DOTATATE was well tolerated. ^{68}Ga-DOTATOC PET/CT imaging showed uptake in the primary tumor and at multiple metastatic lesions. The uptake of ^{177}Lu-DOTATATE resulted in the delivery of 54 Gy of radiation to the primary tumor, but only 2 Gy to the distal femoral metastasis. Response evaluation at 1 month showed a stable disease with clinical improvement. At 2.5 months, however, the patient developed a pathological fracture at the metastatic site in the distal femur. IHC of the metastatic tumor tissue showed overall weak SSTR-2 expression. Sequencing revealed a KIAA1549::BRAF fusion, that was not present in the primary tumor at diagnosis. A request for compassionate use of with pan-RAF inhibitor tovorafenib was filed, however the disease progression was rapid, and the patient died of the disease before treatment could be initiated.

Conclusion

We report a patient with refractory high-risk neuroblastoma, presenting an early response to ^{177}Lu-DOTATATE at 1 month followed by a mixed response and local progression of the distal femoral metastasis at 2.5 months. Further analysis of the progressed metastatic tissue revealed poor SSTR-2 expression and a

novel KIAA1549::BRAF fusion acquisition, possibly contributing to the resistance to the radiopharmaceutical therapy ^{177}Lu-DOTATATE.

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A postinduction Curie Score of more than 2 in MYCN-nonamplified tumor treated by JN-H-15 was not associated with poor prognosis

Matsumoto K[1,10], Nozawa K[2,10], Shichino H[3,10], Yoneda A[1,10], Soejima T[4,10], Okita H[5,10], Takimoto T[1,10], Yokota I[6,10], Kamijo T[7,10], Nakagawara A[8,10], Tajiri T[9,10]

[1]Children's Cancer Center, National Center for Child Health and Development, Tokyo, Japan, [2]Radiology, Kanagawa Children's Medical Center, Kanagawa, Japan, [3]Pediatrics, National Center for Global Health and Medicine, Tokyo, Japan, [4]Radiology, Hyogo Prefectural Kobe Children's Hospital, Hyogo, Japan, [5]Pathology, Keio University Hospital, Tokyo, Japan, [6]Biostatistics, Graduate School of Medicine, Hokkaido University, Hokkaido, Japan, [7]Saitama Cancer Center, Saitama, Japan, [8]SAGA Heavy Ion Medical Accelerator in Tosu, Saga, Japan, [9]Pediatric Surgery, Kyushu University Hospital, Fukuoka, Japan, [10]Neuroblastoma Committee of Japan Children's Cancer Group, , Japan

Background: Clearance of MIBG avidity after induction therapy is reported to be associated with good event-free survival, therefore, strategy for high-risk neuroblastoma patients aims to get rid of MIBG avidity as much as possible before high-dose therapy. JN-H-15 is a unique Japanese protocol that is characterized by "delayed local treatment", two courses of ICE regimens and busulfan-melphalan for high-dose chemotherapy.

Aim: The aim of this study was to reveal the MIBG reactivity after induction in JN-H-15 protocol and to validate SIOPEN or COG reports.

Methods: Fifty-nine patients with stage M tumors in JN-H-15 protocol were examined. Curie Score (CS) was evaluated retrospectively in patients with 123I-MIBG scans obtained at diagnosis and after induction therapies (n=42).

Results: The 3-year progression-free survival (PFS) for the 42 patients evaluated was 51.9±7.8%, with no significant difference of 3-y PFS between patients with MYCN-amplified tumors (60.0±12.6%, n=15) and with MYCN-nonamplified tumors (45.3±9.9%, n=26). There exist no significant difference between patients with a postinduction CS of 2 or less and more than 2 (3-y EFS, 54.8±8.9% (CS<2, n=31) vs. 36.0±16.1% (CS>=2, n=10), p=0.530).

For patients with MYCN-amplified tumor, a nearly significant outcome difference existed by postinduction CS, with a 3-y PFS of 69.2±12.8% (CS<2, n=13) versus 0.0±0.0% (CS>=2, n=2) (P=0.0546). Conversely, for patients with MYCN-nonamplified tumor, there exist no difference by postinduction CS, with a 3-y PFS of 44.4±11.7% (CS<2, n=18) versus 46.9±18.7% (CS>=2, n=8) (P=0.6211).

Conclusion: Despite of shortness of observation time and lower numbers of enrolled patients in JN-H-15, a postinduction CS of more than 2 in patients with MYCN -nonamplified tumor was not associated with poor progression-free survival. The Japanese style of induction chemotherapy and delayed local treatment strategy might produce different outcome as of postinduction CS from SIOPEN or COG reports. We might need to change our strategy of Japanese protocols according to status of the tumor.

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KIR ligand-incompatible allogeneic cord blood transplantation for relapsed stage 4 neuroblastoma in a multicenter trial

Kataoka S[1], Nishio N[1], Arakawa Y[2], Kakuda H[3], Tamura S[4], Terui K[5], Hamada S[6], Karakawa S[7], Takahashi Y[1]
[1]Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2]Department of Hematology/Oncology, Saitama Children's Medical Center, Saitama, Japam, [3]Department of Hematology/Oncology, Chiba Children's Hospital, Chiba, Japan, [4]Department of Pediatrics, Kyoto City Hospital, Kyoto, Japan, [5]Department of Pediatrics, Hirosaki University Graduate School of Medicine, Hirosaki, Japan, [6]Department of Pediatrics, University of the Ryukyus Hospital, Okinawa, Japan, [7]Department of Pediatrics, Hiroshima University Graduate School of Biomedical and Health Sciences, Hiroshima, Japan

Background: There is only a 3% 5-year overall survival rate after recurrence of stage 4 neuroblastoma, and there are few cases of long-term survival. In the setting of autologous transplantation for patients with high-risk neuroblastoma, it has been reported that the survival rate was significantly higher in patients with natural killer (NK) cells missing any killer immunoglobulin-like receptor (KIR) ligands than in patients with matching all KIR ligands.

Aims: We conducted a multicenter clinical study of KIR ligand-incompatible allogeneic cord blood transplantation (CBT) to test the antitumor effect of NK cells.
Methods: From 2010 to 2020, 22 patients with recurrent stage 4 neuroblastoma who received KIR ligand-incompatible allogeneic CBT were included. They were 16 boys and 6 girls, with a median age of 6.7 years (range: 3.4–10.8 years) at the time of transplantation. Before CBT, 14 patients achieved the second or third complete response; the remaining 8 had residual diseases. For CBT, a conditioning regimen consisting of fludarabine, melphalan, and low-dose total body irradiation was mainly used.

Results: The median follow-up period after CBT was 3.7 years, and the 5-year overall survival rate was 57.3% ± 21.6%, with a 5-year progression-free survival rate of 45.5% ± 20.8%. Moreover, 9 patients were alive with no disease progression, 2 were alive with disease, and the remaining 11 died. Seven deaths were caused by neuroblastoma progression, three by transplant-related mortality, and one by second malignancy. In order to determine the disease status during CBT, we compared the second response group (n = 13) who achieved the second complete response at CBT and the residual disease group (n = 9) who had residual disease or second recurrence at CBT. The 3-year cumulative relapse rate in the second response group was significantly lower than in the residual disease group (23.1% vs. 66.7%, p = 0.04). Six long-term survivors without disease progression were found more than 5 years after CBT.

Conclusions: KIR ligand-incompatible allogeneic CBT as immunotherapy could be a salvage treatment option for relapsed neuroblastoma. However, this treatment should only be considered after the patient has achieved complete response prior to CBT.

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Development of combinations of ABTL0812 to inform the first in child phase I trial in children with relapsed/refractory neuroblastoma

Perez M[1], Valero L[1,2], Boloix A[1], Molero-Valenzuela A[1], Soriano A[1], Alfón J[3], Pérez-Montoyo H[3], Yeste-Velasco M[3], Domènech C[3], Roma J[1], Lizcano J[4,5], Tweddle D[6], Moreno L[1,2], Segura M[1]

[1] Vall Hebron Research Institute (VHIR), Grup of Childhood Cancer & Blood Disorders, Barcelona, España, [2]Hospital Vall d'Hebron. Division of Pediatric Onco-Hematology, Barcelona, España, [3]Parc Tecnològic del Vallès, Ability Pharmaceuticals, SL, Cerdanyola, España, [4]Universitat Autònoma de Barcelona (UAB),Departament de Bioquímica i Biologia Molecular, Unitat de Medicina, and Institut de Neurociències, Cerdanyola, España, [5]Vall Hebron Research Institute (VHIR), Protein Kinases in Cancer Research, Barcelona, España, [6]The Medical School, University of Newcastle, Newcastle upon Tyne, Cancer Research Unit, Newcastle, United Kingdom

Background

Childhood cancer is the first cause of death due to disease in children. New drugs are needed to increase cure rates.

ABTL0812 is a first-in-class oral targeted anticancer compound that produces autophagy-mediated cytotoxicity selectively in cancer cells resulting in cancer cell death. Our preclinical data indicated that ABTL0812 was effective as a single agent and can potentiate the antitumor activity of chemotherapies in neuroblastoma (NB).

Aims

The main objective of this work is to develop and validate an in vitro and in vivo model to demonstrate the added value of combining ABTL0812 with immunotherapeutics (nivolumab, dinutuximab beta, bevacizumab and conventional chemotherapeutics to inform the combination arms of the "first- in-child" clinical trial.

Methods

Cell viability assays and western blot analyses have been used to evaluate the response of TH-MYCN isogenic neuroblastoma cell lines to ABTL0812.

In vivo xenografts were established by subcutaneous injection of the TH-MYCN isogenic cell lines in sv/129 immunocompetent mice; tumor growth, tumor volume, expression of atf4, trib3 were analyzed after treatment with ABTL0812.

Results

ABTL0812 treatment reduced the viability of TH-MYCN isogenic cell lines, increased the expression of ATF4, cleavage of caspase-3 and PARP. While PD-1 is expressed in the analyzed cell lines, the enzyme of the GD2-synthesis pathway is barely detectable in these cell lines.

In vivo experiments injecting 3261 cells in the flank have showed that these cells can grow and form tumors. ABTL0812 treatment reduced the growth of the xenografted 3261 cell lines. Molecular analyses showed increased expression of the autophagy-related biomarkers atf4 and trib3, demonstrating on-target effects.

Conclusions

- The response of the mouse neuroblastoma cell lines to ABTL0812 is similar to the one observed in human neuroblastoma cell lines in vitro and in vivo.
- These cell lines can be used to test the combination of ABTL0812 and anti-PD1.
- G2 synthesis pathway is altered in the tested mouse neuroblastoma cell lines, and therefore it should be reconstituted to test the combination of ABTL0812 with anti-GD2 immunotherapy.

•In vitro and in vivo combination experiments are ongoing and will inform the design of the phase I clinical trial combination arms

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Poor radiotherapy worsens overall survival for patients with high-risk neuroblastoma: report from the SIOPEN High-Risk Neuroblastoma Study 1 radiotherapy committee.

Kelly S[1,2,3], Pötschger U[4,5], Arnardottir H[5], Boterberg T[6], Dieckmann K[5,7], Ladenstein R[5], Malinova B[8], Magelssen H[9], Mandeville H[10], Gaze M[11]

[1]SIOP Europe, QUARTET Project, Brussels, Belgium, [2]EORTC Headquarters, Radiotherapy Quality Assurance, Brussels, Belgium, [3]Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium, [4]Department for Studies and Statistics and Integrated Research, Children's Cancer Research Institute, Vienna, Austria, [5]Children's Cancer Research Institute, St Anna Kinderkrebsforschung, Medical University of Vienna, Vienna, Austria, [6]Department of Radiation Oncology, Ghent University Hospital, Ghent, Belgium, [7]Department of Radiation Oncology, Medical University of Vienna, Vienna, Austria, [8]University Hospital Motol, Department of Radiation Oncology, Prague, Czechia, [9]Oslo University Hospital, Department of Oncology, Oslo, Norway, [10]The Royal Marsden Hospital and Institute of Cancer Research, Sutton, United Kingdom, [11]Department of Oncology, University College London Hospitals NHS Foundation Trust, London, United Kingdom

Background

For patients with high-risk metastatic and MYCN-amplified localised neuroblastoma, radiotherapy contributes to local control and constitutes an important component of a multi-modality treatment regime. The SIOPEN High-Risk Neuroblastoma Study 1 protocol (HRNBL1/SIOPEN, EUDRACT 2006-001489-17) defined that all included patients were to receive 21 Gy in 1.5 Gy fractions to the pre-operative extension at the primary tumour site. The quality of radiotherapy treatment may directly impact local control, therefore a retrospective radiotherapy quality assurance (RTQA) assessment was performed and analysed against survival outcomes for all patients who received radiotherapy.

Aims

The purpose of this work was firstly to perform a plan quality evaluation of all available radiotherapy treatment planning data, and secondly to investigate if treatment quality correlated with survival outcomes.

Methods

Radiotherapy data were submitted via the trial data centre, SIOPEN R-NET, or QUARTET platform. At least two members of the radiotherapy committee performed a RTQA review which evaluated plan parameters including prescription and fractionation, delineations and dosimetry for target and normal tissue structures, as well as treatment technique and overall dosimetry, against protocol requirements. Plans were rated as per protocol, justified, or unjustified deviation and whether there was any potential for harm. A statistical analysis for correlation between the RTQA outcome and overall survival (OS) was performed.

Results

2034 patients received radiotherapy, of which 242 (13.5%) had sufficient data for RTQA evaluation and 236 for statistical analysis. 112 (47%) cases were per protocol with 62 justified (26%) and 62 unjustified deviations (26%). Cases indicated with a potential for harm (n=63, 27%) mainly fell within the unjustified deviation category and fared significantly worse in terms of 5-year OS compared with per protocol cases; 0.34±0.08, 0.63±0.05 respectively (p=0.029). The RTQA cohort is representative of all patients who received radiotherapy.

Conclusion

Despite recommendations for RTQA review within the HR-NBL1/SIOPEN protocol, submission of complete radiotherapy data was lacking. Protocol deviations were demonstrated in the majority of cases. Cases with an RTQA outcome including potential for harm demonstrate worse OS, correlation with local control is currently being analysed. Prospective RTQA is recommended for high-risk neuroblastoma to standardise justified deviations and minimise unjustified deviations.

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Characterizing Response to Repeated Episodes of Chemoimmunotherapy in Patients with Relapsed and Refractory Neuroblastoma

Onyeama S[1], Prorok R[1], Frazer B[1], Marachelian A[1,2]
[1]Children's Hospital Los Angeles, Pediatric Hematology Oncology, Los Angeles, California, United States, [2]University of Southern California, Department of Pediatrics, Los Angeles, California, United States

Background:
Temozolomide, Irinotecan and Dinutuximab (T/I/D) is a mainstay in treatment of relapsed and refractory neuroblastoma (RR-NB). Alternative chemoimmunotherapy (CI): Cyclophosphamide, Topotecan and Dinutuximab (C/T/D) is utilized with limited data describing response. Understanding how RR-NB responds to repeated CI could guide therapeutic decision-making.

Aim:
Describe response to repeated administration of CI in patients with RR disease

Methods:
Retrospective study at Children's Hospital Los Angeles to evaluate patients who received 2 or more treatment episodes (TEs) of CI for RR-NB.

Results:
Nineteen patients were identified. Fourteen patients received T/I/D for at least 2 TEs (Group 1) and 10 patients received C/T/D following T/I/D for a subsequent TE (Group 2); Five patients fell into both Groups 1 and 2. For all TEs, median number of cycles was 4.5 (range 1-18).

For the first TE in Group 1, best overall response (BOR) was: 5 complete response (CR), 1 partial response (PR), 3 minor response (MR), 2 stable disease (SD), 1 progressive disease (PD) and 2 not evaluable. For the second TE with T/I/D, BOR rate of PR/CR was 57% and of PR/CR/MR was 71%. For patients who had PR/CR in first TE, BOR of PR/CR was 100% for subsequent TEs with T/I/D.

For the first TE in Group 2, BOR was: 3 CR, 1 PR, 2 MR and 4 SD. Three patients with initial response to T/I/D had PD in later cycles. BOR rate of CR/PR for C/T/D following T/I/D was 50%. For patients who had BOR of PR/CR in prior episodes of T/I/D, subsequent BOR of PR/CR when treated with C/T/D was 75%. For patients with prior BOR of MR or SD with T/I/D, 33% had BOR of PR/CR and 50% had BOR of SD when given C/T/D.

Conclusion:
Treatment with repeat CI results in BOR of CR/PR/MR for most patients with SD or better in first TE. For both T/I/D and C/T/D, having a BOR of CR/PR may be predictive of response in subsequent TEs. The use of C/T/D may result in response despite PD on T/I/D. Future studies to investigate the predictive potential of prior response to CI are needed.

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Differentiation of residual neuroblastoma manifestations in patients with neuroblastoma Stage 4S

Hero B[1], Berthold F[1], Körber F[2], Butzer S[1], Decarolis B[1], Paulsen V[1], Plum G[1], Rosswog C[1,3,4], Theißen J[1,3,4], Lode H[5], Eggert A[6], Fischer M[1,3,4], Simon T[1]

[1]Department of Pediatric Oncology and Hematology, University Children's Hospital of Cologne, Medical Faculty, University of Cologne, Cologne, Germany, [2]Division of Pediatric Radiology, Department of Radiology, University Hospital of Cologne, Medical Faculty, University of Cologne, Cologne, Germany, [3]Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Medical Faculty, University of Cologne, Cologne, Germany, [4]Center for Molecular Medicine Cologne (CMMC), Medical Faculty, University of Cologne, Cologne, Germany, [5]Department of Pediatric Oncology and Hematology, University Medicine Greifswald, Greifswald, , [6]Department of Pediatric Hematology and Oncology, Charité -Universitätsmedizin Berlin, Berlin,

Background:
Neuroblastoma stage 4S is known for spontaneous regression, but differentiation of neuroblastoma manifestations has also been reported.

Aims:
We were interested in the outcome of patients with neuroblastoma stage 4S with special attention to differentiation of neuroblastoma manifestations.

Methods:
Patients with stage 4S presenting with MYCN-non-amplified tumors and diagnosed between 1997 and 2016 were included. For event-free-survival, progression to stage 4 metastatic pattern, progression after the first year of life, secondary malignancy and death of any cause were considered, but not progression of primary or liver, skin, or soft tissue metastases during the first year of life typically known for stage 4S.

Results:
A total of 253 patients were included in the analysis. Median follow up was 9.1 years (range: 0.13-22 years). The patients presented at a median of 3.0 months of age (range 0-11.7 months) with metastases in the bone marrow (68%), liver (75%) and/or skin (11%). Chemotherapy (median 2, range 1-10 cycles) was only given to 118/253 patients (47%). Ninety-seven patients underwent resection of the primary. Of interest, the primary regressed completely in 45 out of 57 patients (79%) never treated with chemotherapy or resected. For the whole cohort, 5-year-EFS was 0.82±0.03 and 5-year-OS 0.93±0.02. Eleven patients (4.4%) died from early fatal progression of liver metastases. One patient each developed a second malignancy, died from chemotherapy or died from other causes.
Progression to stage 4 was observed in 22 patients (8.7%) in median 11.2 months after first diagnosis (range 1.5–83 months). All 22 patients received chemotherapy. Nine patients died.
After the first year of life, progression of primary (n=8) or metastases (n=3, skin or testes) was observed (4.4%) 8.3-51.6 months after diagnosis (median 14.1 months). Only two patients received chemotherapy for progression. Histologic assessment at progression revealed differentiation to ganglioneuroma/ganglioneuroblastoma intermixed in 4/11 patients. Similarly, differentiation was observed in seven out of 15 patients where a residual primary was resected beyond the first year of life.

Conclusions:
This analysis confirms the excellent prognosis of neuroblastoma stage 4S and the potential for regression. Besides progression, differentiation seems to be a regular feature in this subgroup of patients.

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EVALUATION OF PALBOCICLIB IN COMBINATION WITH TOPOTECAN AND CYCLOPHOSPHAMIDE IN PEDIATRIC PATIENTS WITH RECURRENT OR REFRACTORY NEUROBLASTOMA

Laetsch T[1], Navid F[2], Weigel B[4], Fox E[5], Hawkins D[6], Muscal J[7], Juszczak-Kosela K[8], Zhang Z[9], Macy M[9]
[1]Division of Oncology, Children's Hospital of Philadelphia, and Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA, United States, [2]Children's Hospital Los Angeles, Los Angeles, CA, United States, [3]Department of Pediatrics, University of Minnesota, Minneapolis, MN, United States, [4]St. Jude Children's Research Hospital, Memphis, TN, United States, [5]Seattle Children's Hospital, University of Washington, Seattle, WA, United States, [6]Pfizer Inc, New York, NY, United States, [7]Pfizer Inc, Warsaw, Poland, [8]Pfizer Inc, San Diego, CA, United States, [9]Children's Hospital Colorado, Aurora, CO, United States

Background:
Palbociclib is an orally administered, highly selective, reversible, small molecule cyclin-dependent kinases 4/6 (CDK4/6) inhibitor. Inhibition of CDK4/6 reduced growth of neuroblastoma cell lines. The phase 1 portion of the study, conducted and funded by Pfizer in collaboration with the Children's Oncology Group, established the recommended phase 2 dose (RP2D) of palbociclib with topotecan and cyclophosphamide (TOPO/CTX) in children with solid tumors. Per protocol, if 2 or more patients from the phase 1 study show a confirmed objective response (OR) in a specific tumor type, an open-label, non-randomized tumor-specific cohort (TSC) may be opened.

Aims:
To evaluate the preliminary antitumor activity of palbociclib with TOPO/CTX in the treatment of patients with recurrent/refractory neuroblastoma (neuroblastoma TSC) using a modified Simon's 2-stage optimal design.

Methods:
Up to 21 patients with recurrent/refractory neuroblastoma will receive palbociclib with TOPO/CTX. The primary efficacy endpoint is OR, as assessed by investigator using International Neuroblastoma Response Criteria (INRC). Secondary efficacy endpoints include duration of response, progression-free survival, and overall survival.

Key eligibility criteria include: recurrent/refractory neuroblastoma, measurable disease as defined by INRC, aged ≥2 and <21 years at the time of study entry, and adequate blood counts, organ function, and performance status. Patients may have received prior TOPO and/or CTX, provided their disease did not progress on this regimen. Prior treatment with a CDK4/6 inhibitor is exclusionary. Patients will receive palbociclib at the RP2D of 75 mg/m² orally once daily on days 1–14 of each 21-day treatment cycle. CTX (250 mg/m²) followed by TOPO (0.75 mg/m²) will be administered intravenously once daily on days 1–5. Concurrent radiotherapy or surgery for local control will be permitted. Treatment will continue until disease progression, patient and/or legal guardian refusal, unacceptable toxicity, or up to 24 months of treatment, whichever occurs first.

Results:
The protocol criteria for opening an open-label, non-randomized TSC have been met for recurrent/refractory neuroblastoma.

Conclusion:
Enrollment in the neuroblastoma TSC was initiated in December 2022 (NCT03709680).

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Combination of Carboplatin and Etoposide in Patients with Relapsed or Pro-gressing Neuroblastoma

Butzer S[1], Hero B[1], Fischer M[1,2,3], Vieth S[4], Kratz C[5], Berthold F[1], Lode H[6], Eggert A[7], Simon T[1]
[1]Department of Pediatric Oncology and Hematology, University Children's Hospital of Cologne, Medical Faculty, University of Cologne, Cologne, Germany, [2]Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Medical Faculty, University of Cologne, Cologne, Germany, [3]Center for Molecular Medicine Cologne (CMMC), Medical Faculty, University of Cologne, Cologne, Germany, [4]Department of Pediatric Oncology and Hematology, University Hospital Schleswig-Holstein, Kiel, Germany, [5]Department of Pediatric Haematology and Oncology, Hannover Medical School, Hannover, Germany, [6]Department of Pediatric Hematology and Oncology, University Medicine Greifswald, Greifswald, Germany, [7]Department of Pediatric Oncology and Hematology, Charité-Universitätsmedizin Berlin, Berlin, Germany

Background
Despite therapeutic advances, outcome following relapse/progression of patients with neuroblastoma (NB) remains poor. We retrospectively analyzed patients with relapsed/progressing NB treated with a combination of carboplatin and etoposide (CE).

Methods
Patients were included if registered in the prospective national NB trials NB97 and NB2004 and treated with CE (standard dose carboplatin 300 mg/m²/d, etoposide 300 mg/m²/d for 2-3 days) for relapse/progression between 1997 and 2019. Patients concurrently receiving other cytotoxic agents were excluded. We correlated first and best response with clinical features and MYCN status, and analysed time-to-progression and post-relapse overall survival (OS).

Results
A total of 38 patients with 43 relapses were identified. Twenty-seven relapses (71.1%) were INRG high-risk (HR) NB (INSS stage 4 and ≥18 months of age or MYCN amplification at first diagnosis). The remaining eleven episodes (28.9%) either presented with metastatic relapse/progression (n=4, 10.5%) or localized relapse/progression after previous intermediate-risk therapy (n=7, 18.4%). All patients had been treated with platin-derivates and etoposide in first-line treatment.
Among 43 relapses, CE was given at first relapse in 19 (44.2%), at second relapse in 10 (23.3%), and at third or more relapse in 14 (32.6%). A median of two CE cycles was applied (1-10 cycles). In 29 episodes, early response was evaluated after 1-2 CE cycles and revealed complete remission (CR) or very good partial response (VGPR) in one, partial response (PR) in six, stable disease (SD) in sev-en, and progressive disease (PD) in 14 patients. Best response was assessed in 36 episodes after a median of 2 CE cycles (range 1 – 10): CR or VGPR was observed in four, PR in 11, SD in five, and PD in 16 patients. Median time-to-progression was 2.6 months.
First and best response was not associated with number of previous events, HR disease at diagnosis or age at relapse. Patients without MYCN amplification tended to have a better response than patients with MYCN amplification (SD or better in 12/16 vs. 7/17, p=0.08). In the entire cohort, 3-year-post-relapse OS was 0.19±0.06.

Conclusion
Treatment with CE was effective in heavily treated patients with relapsed/progressing NB. This combination therapy warrants further evaluation.

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Matched External Control Analysis of Survival in High-Risk Neuroblastoma (HRNB) Patients Receiving DFMO Maintenance: MYCN Status Impact

Saulnier Sholler G[1], Ferguson W[3], Bergendahl G[1], Clinch T[4], Lorenzi E[5], Berry D[5], Oesterheld J[1], Brown V[6], Eslin D[7], Hanson D[8], Harrod V[9], Isakoff M[10], MItchell D[11], Roberts W[13], Wada R[12], Zage P[13], Kraveka J[2]
[1]Levine Children's Hospital, Atrium Health, Dept of Pediatrics, Charlotte, United States, [2]Medical University of South Carolina, Hollings Cancer Center, Division of Pediatric Hematology-Oncology, Charleston, United States, [3]Cardinal Glennon Children's Hospital, Dept of Pediatrics, St. Louis, United States, [4]USWorldMed, , United States, [5]Berry Consultants, Houston, United States, [6]Penn State Health Children's Hospital and Penn State College of Medicine, Dept of Pediatrics, Hershey, United States, [7]St Joseph's Children's Hospital, Dept of Pediatrics, Tampa, United States, [8]Joseph M. Sanzari Children's Hospital, Hackensack University, Dept of Pediatrics, Hackensack, United States, [9]Dell Children's Medical Center, University of Texas Dell Medical School, Dept of Pediatrics, Austin, United States, [10]University of Connecticut School of Medicine, Dept of Pediatrics, Hartford, United States, [11]Helen DeVos Children's Hospital, Michigan State University, Dept of Pediatrics, Grand Rapids, United States, [12]University of Hawaii, Dept of Pediatrics, Honalulu, United States, [13]Rady Children's Hospital, Dept of Pediatrics, San Diego, United States

Background: MYCN-amplification is a genetic marker of risk in neuroblastoma at diagnosis. DFMO inhibits ornithine decarboxylase and decreases MYCN in neuroblastoma. We reported the risk of relapse for patients in remission after upfront immunotherapy treated with DFMO on NMTRC003B compared to matched control (NO-DFMO) patients from the COG trial, ANBL0032 as decreased by approximately 50%. We used an exact match on MYCN status (not amplified/amplified) between DFMO and NO-DFMO patients. Aims: We evaluated the impact of MYCN status on survival post immunotherapy and the effect of DFMO in MYCN amplified and non-amplified subpopulations.

Methods: NMTRC003b enrolled N=141 HRNB patients in remission post completion of immunotherapy (2012–2016) for DFMO treatment (2 years; 750 ±250 mg/m2 BID) and follow-up (≤7 years). ANBL0032 enrolled N=1328 HRNB patients (2001–2015) assigned to dinutuximab treatment and follow up (≤10 years). Selection rules identified like-groups of DFMO treated and NO-DFMO control patients eligible for matching. Propensity-Score Matching balanced cohorts on demographic and risk characteristics (1:3 ratio for treated:control), including MYCN as a covariate. Subgroup analyses used all available patients meeting selection rules with non-missing MYCN.

Results: N=92 treated and N=852 control patients met selection rules with complete covariate data for analysis in n=91 and n=516, respectively. Removing MCYN as an exact match and treating it like the other 10 covariates demonstrated an improved hazard ratio of 0.42 [95% CI 0.24, 0.73] p=0.0024 vs. 0.48 [95% CI 0.27, 0.85], p=0.0114. Subgroup analysis of the MYCN non-amplified patients treated with DFMO relative to NO-DFMO show an EFS of 78.4% and 67.8% HR 0.60 [95% CI 0.33, 1.12] and OS of 86.3% and 79.7% HR 0.57 [95% CI 0.26,1.25] respectively. Analysis of the MYCN amplified patients treated with DFMO relative to NO-DFMO show an EFS of 90% and 75.2% HR 0.37 [95% CI 0.14, 1.02] and OS of 97.5% and 84.1%, HR 0.13 [95% CI 0.02,0.92] respectively.

Conclusion: Both MYCN amplified and non-amplified patients treated with DFMO showed improvement in EFS and OS. Therefore, patients in remission post-immunotherapy, regardless of MYCN status, treated with DFMO have a decreased risk of relapse compared to non-treated matched control patients.

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Sensitivity Analyses of Event Free and Overall Survival in High-Risk Neuroblastoma Patients Receiving DFMO Maintenance with Matched External Controls

Saulnier Sholler G[1], Ferguson W[3], Bergendahl G[1], Clinch T[4], Lorenzi E[5], Berry D[5], Oesterheld J[1], Brown V[6], Eslin D[7], Hanson D[8], Harrod V[9], Isakoff M[10], Mitchell D[11], Roberts W[13], Wada R[12], Zage P[13], Kraveka J[2]

[1]Levine Children's Hospital, Atrium Health, Division of Pediatric Hematology-Oncology, Charlotte, United States, [2]Medical University of South Carolina, Hollings Cancer Center, Division of Pediatric Hematology-Oncology, Charleston, United States, [3]Cardinal Glennon Children's Hospital, St. Louis, United States, [4]USWorldMed, , United States, [5]Berry Consultantsq, Houston, United States, [6]Penn State Health Children's Hospital and Penn State College of Medicine, Dept of Pediatrics, Hershey, United States, [7]St Joseph's Children's Hospital, Dept of Pediatrics, Tampa, United States, [8]Joseph M. Sanzari Children's Hospital, Hackensack University, Dept of Pediatrics, Hackensack, United States, [9]Dell Children's Medical Center, University of Texas Dell Medical School, Dept of Pediatrics, Austin, United States, [10]University of Connecticut School of Medicine, Dept of Pediatrics, Hartford, United States, [11]Helen DeVos Children's Hospital, Michigan State University, Dept of Pediatrics, Grand Rapids , United States, [12]University of Hawaii, Dept of Pediatrics, Honalulu, United States, [13]Rady Children's Hospital, Dept of Pediatrics, San Diego, United States

Background: Long-term survival in HRNB remains challenging, with relapse as the primary cause of mortality. We reported previously that the risk of relapse for HRNB patients in remission after upfront dinutuximab treated with DFMO on NMTRC003B was approximately half that of matched control patients from the COG trial, ANBL0032.

Aims: We further interrogate these survival outcomes using multiple sensitivity analyses.

Methods: Our phase 2 trial NMTRC003B enrolled N=141 HRNB patients in remission post completion of disease treatment (2012–2016) for continuous DFMO treatment (≤2 years; 750 ±250 mg/m2 BID). ANBL0032 enrolled N=1328 HRNB patients (2001–2015) assigned to Dinutuximab. Selection rules identified like-groups of patients eligible for matching, covariates of prognostic importance, and matching algorithm. Propensity-score matching (PSM) balanced cohorts on risk characteristics (a 1:3 ratio for treated:control). Inferential analyses compared EFS and OS. Sensitivity analyses including modifying the PSM ratio to 1:2 and 1:1 (single best match), removing patients with a partial response (PR) or with events <123 days post immunotherapy from the control group, and evaluation of a contemporaneous population.

Results: N=92 treated patients and N=852 control patients met selection criteria with complete covariate data for analysis in n=91 and n=516, respectively. EFS and OS findings were consistent across all sensitivity analyses and reproducibly confirmed the statistically significant EFS and OS improvements with DFMO treatment pre- and post-PSM. PSM ratio to 1:2 and 1:1 confirmed improved EFS: HR 0.39 [0.22, 0.70], p-value=0.0016), HR 0.39 [0.21, 0.74], p-value=0.0038) and OS: HR 0.0.26 [0.12, 0.57], p-value=0.00009), HR 0.22 [0.10, 0.52], p-value=0.0005). Excluding NO-DFMO group patients with PR at end-of-immunotherapy, removal of events <123 days, or contemporaneous comparison all significantly improved EFS: HR 0.49 [0.28, 0.85], p-value=0.012), HR 0.54 [0.30, 0.98], p-value=0.044), HR 0.57 [0.33, 1.00], p-value=0.050) respectively as well as OS: HR 0.44 [0.20, 0.87], p-value=0.0208), HR 0.43 [0.19, 0.96], p-value=0.0395), HR 0.39 [0.18, 0.84], p-value=0.0319).

Conclusion: Multiple sensitivity analyses consistently confirmed that HNRB patients in remission after standard upfront therapy treated with DFMO have approximately half the risk of relapse compared to matched control patients, further supporting DFMO as maintenance treatment for HRNB.

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Tolerability and Safety of Eflornithine (DFMO) in High-Risk Neuroblastoma (HRNB) Patients Treated with DFMO Maintenance Therapy

Kraveka J[1], Ferguson W[3], Clinch T[4], Bergendahl G[2], Oesterheld J[2], Brown V[8], Eslin D[7], Wada R[5], Roberts W[9], Isakoff M[6], Harrod V[10], Mitchell D[11], Hanson D[12], Zage P[9], Saulnier Sholler G[2]

[1]Medical University of South Carolina, MUSC Shawn Jenkins Children's Hospital, Hollings Cancer Center , Charleston, United States, [2]Levine Children's Hospital, Atrium Health, Charlotte , United States, [3]Saint Louis University School of Medicine & Cardinal Glennon Children's Medical Center , St Louis, United States, [4]US WorldMeds, Louisviile, United States, [5]University of Hawaii, Honolulu, United States, [6]University of Connecticut School of Medicine, Connecticut Children's Medical Center , Hartford, United States, [7]Bayless Children's Cancer Institute, St. Joseph's Children's Hospital, Tampa, United States, [8]Penn State Milton S. Hershey Medical Center and Children's Hospital, Hershey, United States, [9]University of California San Diego, Peckham Center for Cancer and Blood Disorders, Rady Children's Hospital, San Diego, United States, [10]Dell Children's Medical Center, University of Texas Dell Medical School, Austin, United States, [11]Helen DeVos Children's Hospital, Michigan State University, Grand Rapids, United States, [12]Joseph M. Sanzari Children's Hospital, Hackensack University Medical Center, Hackensack, United States

Background

The polyamine and ornithine decarboxylase inhibitor DFMO has been studied as a chemopreventative agent in several cancers including neuroblastoma. It has been employed as a maintenance therapy for HRNB patients in remission in Beat Childhood Cancer Research Consortium (BCC) Trials.

Aims

We report on the tolerability/safety profile of DFMO in HRNB patients in remission

Methods:

Complete case safety data from two open-label prospective, pediatric Phase II studies (NMTRC003B and NMTRC014) of HNRB patients in remission after upfront or relapse treatment who were treated with DFMO (DFMO 750 ± 250 mg/m2 BID for up to 2 years with a 5-year follow-up) were pooled for evaluation of DFMO tolerability/safety. Adverse events (AEs) were graded according to the Common Terminology Criteria for Adverse Events (CTCAE).

Results

311 subjects (n=52 on NMTRC003B and n= 259 on NMTRC014) with a mean DFMO exposure of 1.5 years, were included in the analysis. DFMO was well-tolerated with 4.8% (n=15) discontinuing treatment due to AEs; 10.6% (n=33) requiring temporary dose interruptions; and 4.8% (n=15) requiring dose reductions. Hypoacusis was the most common reason for dose modification (interruption or reduction). Thirty-five subjects (11.3%) experienced hypoacusis with 12 (3.9%) subjects requiring dose modification; the hypoacusis resolved in the majority 67% (8/12) upon temporary interruption of DFMO dosing and resuming treatment at a lower or original dose. Four patients (1.3%) discontinued treatment due to hearing loss. The other most common AEs were elevations in liver functions, vomiting, diarrhea, anemia, and neutropenia. Most AEs occurred within the first 6 months of treatment and resolved with continued treatment and/or dose adjustment. Serious adverse events (SAEs) occurred in 16.7% (n=52) of patients with the most common being dehydration, vomiting, pneumonia, and hypoglycemia. The majority (88%) of these SAEs were unrelated to DFMO. No AEs resulted in death. There was no impact on QT/ECG values, and no new or unexpected risks associated with SAEs were identified.

Conclusion

DFMO was well-tolerated with few discontinuations overall. Hypoacusis was managed by DFMO interruption and/or dose reduction The DFMO tolerability/safety profile supports its use as long-term maintenance treatment for HRNB.

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Canadian Relapse/Refractory High-Risk Neuroblastoma (CANOE) Registry

Tibout P[1], Deyell R[2], Johnston D[3], Portwine C[4], Zorzi A[5], Wheaton L[6], Goodyear L[7], Martin G[8], Wilson B[9], Desai S[9], Cuvelier G[10], Stapleton C[10], Crooks B[11], Teira P[12], Vairy S[13], Mitchell D[14], Cohen-Gogo S[1], Morgenstern D[1]
[1]The Hospital for Sick Children, Department of of Pediatrics, Division of Hematology/Oncology, Toronto, Canada, [2]University of British Columbia, British Columbia Children's Hospital and Research Institute, Division of Pediatric Hematology/Oncology/BMT, Vancouver, Canada, [3]Children's Hospital of Eastern Ontario, Division of Hematology/Oncology, Ottawa, Canada, [4]McMaster University, Department of Pediatrics, Division of Hematology/Oncology, Hamilton, Canada, [5]Western University, Division of Haematology/Oncology, London, Canada, [6]Kingston Health Sciences Centre, Division of Hematology/Oncology, Kingston, Canada, [7]Janeway Children's Health and Rehabilitation Centre, Section of Pediatric Hematology/Oncology, St-John's, Canada, [8]Jim Pattison Children's Hospital, Departments of Pediatric, Hematology & Oncology, Saskatoon, Canada, [9]University of Alberta, Department of Pediatrics, Edmonton, Canada, [10]University of Manitoba, CancerCare Manitoba, Section of Pediatric Hematology/Oncology/BMT, Winnipeg, Canada, [11]IWK Health Centre, Division of Hematology-Oncology, Halifax, Canada, [12]University of Montreal, Hospital Sainte-Justine, Hematology-Oncology department, Montreal, Canada, [13]CIUSSS de l'Estrie - CHUS, Department of Pediatrics, Division of Pediatric Hematology-Oncology , Sherbrooke, Canada, [14]McGill University Health Centre, Montreal Children's Hospital, Division of Hematology and Oncology, Montreal, Canada

Background
Although the literature includes several reports of long-term outcomes for patients with relapse/refractory (RR) high-risk neuroblastoma, these data are generally from patients treated prior to the introduction of immunotherapy into upfront treatment and without use of contemporary strategies for managing RR disease. There is therefore a need for contemporary data on the outcomes of these patients.

Aim
To enhance understanding of the longitudinal management and outcomes of patients with relapse or refractory high-risk neuroblastoma and allow for contemporary progression-free and overall survival estimates for these patients to be determined.

Methods
The CANOE Registry is designed to collect patient, disease, treatment and outcome data obtained through routine clinical, laboratory and radiological assessments. Data collected will include details of initial neuroblastoma diagnosis, relapse/refractory/primary progressive status, subsequent therapies and overall neuroblastoma response and survival status. Data will be collected using electronic case report forms via REDCap, a validated computing environment. Statistical analysis of the data derived from the Registry will be undertaken using Excel, PRISM, SAS or other relevant software. Individual statistical planning will be done as necessary for specific analyses.

Results
With approximately 80 new diagnoses of neuroblastoma annually in Canada, of which approximately 40 would have high-risk disease, the number of patients with RR disease is expected to be 20-25 per year. This represents a manageable cohort from which to collect data, but also a sufficient number over time to build a cohort that will be useful for future research. Twelve Canadian paediatric oncology centres have agreed to participate in the registry, and the data collection for retrospective patients is underway.

Conclusion
A multicentre registry for relapsed/refractory high-risk neuroblastoma is feasible and provides a unique opportunity to obtain near-population level data on patients with RR high-risk neuroblastoma

P323

Oral Venetoclax plus cyclophosphamide and topotecan in heavily pre-treated, relapsed metastatic neuroblastoma: a single center case series

De Ioris M[1], Del Bufalo F[1], Fabozzi F[1], Del Baldo G[1], Villani M[2], Cefalo M[1], Garganese M[2], Stracuzzi A[3], Tangari F[4], Greco A[4], Toma P[2], Mastronuzzi A[1], Alaggio R[3], Locatelli F[1,4]

[1]Pediatric Hematology and Oncology IRCCS Ospedale Pediatrico Bambino Gesù , Rome, Italy, [2]Imaging IRCCS Ospedale Pediatrico Bambino Gesù , Rome, Italy, [3]Pathology IRCCS Ospedale Pediatrico Bambino Gesù , Rome, Italy, [4]Catholic University of the Sacred Heart, Rome, Italy

Backgrounds:The prognosis of relapsed/refractory(R/R) neuroblastoma (NB) is dismal, prompting the need for new therapeutic strategies. The BCL-2 family of proteins controls the apoptosis pathway and is often overexpressed in NB cells. Venetoclax (VEN) is a highly selective, potent, orally bioavailable, BCL-2 inhibitor small-molecule that showed a synergistic effect with cyclophosphamide and topotecan (Cy-Topo) in murine NB models.

Aim: To evaluate the feasibility of VEN plus Cy-Topo in children with RR NB.
Methods: Heavily pre-treated patients affected by relapsed NB were treated with VEN plus Cy-Topo based on a 28-day schedule: on week 1, VEN was started at 200-mg adult-equivalent dose, increased to 400-mg adult-equivalent dose on day 2, if tolerated, and was then administered daily throughout the entire cycle. In week 2, Cy was administered at the dose of 250 mg/m2/day for 5 days, concurrently with Topo, at the dose of 0,75 mg/m2/day for 5 days. VEN was discontinued only in case of severe neutropenia (absolute neutrophil count, ANC, <500/mmc) and re-started upon resolution. Hematologic criteria to restart a subsequent cycle were ANC >500/mmc and platelet count >50000/mmc. BCL-2 expression in immunochemistry (IHC) on tumor samples at relapse and the BCL-2 gene status were evaluated in all patients.

Results: Four patients, who had previously received >3 lines of treatment, were treated with VEN plus Cy-Topo in an outpatient setting. The main toxicity was hematological, with grade 4 neutropenia and thrombocytopenia occurring in all courses and leading to transient VEN discontinuation. Grade 3 oral mucositis was observed in 1/8 courses. No other grade 2 to 4 toxicities were observed. BCL-2 was expressed in all tumors, while no molecular abnormalities in the BCL-2 genes were detected. In terms of response, a stable disease(SD) was observed in all patients, without any progression during the study period. MIBG SIOPEN skeleton score improved in 3/4 cases.

Conclusion:VEN plus Cy-Topo is well tolerated, the major toxicity being hematological. A VEN pulsed schedule may improve the hematological tolerability. The SD observed are encouraging and response may be improved by testing the schedule in less pretreated patients and correlate with the degree of BCL-2 expression in IHC.

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Individualized treatment plans for patients with the Recurrent/Refractory Neuroblastoma (R/R NB): experience from the Children Hospital, Anna Meyer, Florence (Italy).

Carra F[1], Cuzzubbo D[1], Trevisan F[1], Peri M[2], Trambusti I[2], Scala L[3], Ponzoni M[4], Pastorino F[4], Garaventa A[8], Amoroso L[8], Simontacchi G[5], Tintori V[1], Perrone A[6], Olianti C[7], Tondo A[1]

[1]Department of Pediatric Oncology and Hematology, Meyer Children's Hospital IRCCS, Florence, Italy, [2]Meyer Children's Hospital IRCCS, University of Florence, Florence, Italy, [3]Department of Hospital Pharmacy, Meyer Children's Hospital IRCCS, Florence, Italy, [4]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genova, italy, [5]Radiation Oncology Unit, Careggi University Hospital, Florence, Italy, [6]Department of Radiology, Meyer Children's Hospital IRCCS, Florence, Italy, [7]Nuclear Medicine Unit, Careggi University Hospital, Florence, Italy, [8]IRCCS Istituto Giannina Gaslini, Genova, Italy

Background: The management of patients with R/R NB depends on the timing and nature of the relapse, prior therapy, tumor biology and patient's health.
Case1: male, 16 years, late relapsed neuroblastoma (previous treatment HR-NB01, maintenance therapy without immunotherapy) at the left elbow and multiple skeletal metastases. CT-guided tumor biopsy was performed: Neuroblastoma, MYCN non-amplified. WES: mutation c.A484G in the PSMC2 gene, targeted by Bortezomib. We performed a re-induction therapy (conventional chemotherapy, Bortezomib), a consolidation strategy with myeloablative chemotherapy with autologous stem-cell rescue, radiation and maintenance therapy with anti-GD2, allowing a complete remission. A second systemic relapse was diagnosed after 8 months. A personalized third-line treatment, based on previous molecular target and systemic radiopharmaceutical 131I-MIBG, was proposed to achieve a complete remission. A haploidentical transplantation will be considered in combination with anti-GD2 whether the quality of life is preserved.
Case2: female, 8 years, refractory neuroblastoma with poor response to induction chemotherapy, eligible for entry into VERITAS Randomization. After 3 courses of Temozolamide-Irinotecan, partial response was achieved. Peripheral blood stem-cell rescue was not available, then surgery was performed before the intensified consolidation chemotherapy. The imaging evaluation showed a progressive disease. The patient quit the clinical trial, a tailored second-line treatment was performed. After 5 courses of Temozolamide-Irinotecan a stable disease was reached. The personalized treatment provides: therapeutic 131I-MIBG, myeloablative chemotherapy (Bu-Mel) or a haploidentical transplantation with anti-GD2 whether a complete remission is achieved.
Case3: male, 6 years, Ukrainian war refugee, R/R neuroblastoma (uncertain medical history). CT-guided tumor biopsy was performed: Neuroblastoma, MYCN non-amplified. Mute genomic profile. He underwent 3 courses of conventional chemotherapy (Temozolamide-Irinotecan) achieving stable disease. After 3 further courses of Temozolamide-Irinotecan, a progressive disease was observed. The combination of Temozolamide-Irinotecan and anti-GD2 was planned. A therapeutic 131I-MIBG could be a chance to reach a long remission period.

Conclusion: The choice of personalized treatment in R/R NB patients includes: biopsy to identify molecular targets followed by rescue strategy with standard combination chemotherapy; tailored therapy with precision medicine programs; stem-cell rescue for the myeloablative therapy; organ and performance status. The quality of life must be contemplated in the overall individualized treatment plan.

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INCIDENCE AND 5-YEAR SURVIVAL OF PEDIATRIC NEUROBLASTOMA IN THE UNITED STATES

Campbell K[1], Siegel D[2], Dai S[3], Heczey A[4,6], Lupo P[4,5,6], Schraw J[4,5,6], Thompson T[2], Scheurer M[4,5,6], Foster J[4,6]
[1]Division of Hematology-Oncology and Bone Marrow Transplantation, Children's Mercy Hospitals and Clinics, Kansas City, Missouri, United States, [2]Division of Cancer Prevention and Control, National Center for Chronic Disease Prevention and Health Promotion, Centers for Disease Control and Prevention, Atalanta, United States , [3]Cyberdata Technologies, Inc., Herndon, United states , [4]Division of Hematology-Oncology, Department of Pediatrics, Baylor College of Medicine, Houston, Texas, United States , [5]Center for Epidemiology and Population Health, Department of Pediatrics, Baylor College of Medicine, , United States, [6]Cancer and Hematology Centers, Texas Children's Hospital, Houston, Texas, United States, [7]Department of Pathology and Immunology, Baylor College of Medicine, Houston, Texas, United States, [8]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas, United States

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Background:
Neuroblastoma (NB) is the most common extracranial pediatric solid tumor. Prior studies demonstrated increasing incidence of neuroblastoma in children and improved overall survival over time in all age groups; however these covered less than thirty percent of the U.S. population.

Aims:
In this study, we aimed to characterize the incidence and 5-year survival of children with neuroblastoma stratified by demographic and clinical factors based on the comprehensive data from United States Cancer Statistics (USCS) and the National Program of Cancer Registries (NPCR).

Methods:
We analyzed the incidence of neuroblastoma per million persons from USCS (2003–2019) and survival data from NPCR (2001–2018) for patients aged <20 years covering 99.1% and 87.5% of the U.S population, respectively. Analyses included first primary and malignant tumors only. We assessed incidence trends by calculating average annual percent change (AAPC) using joinpoint regression. Differences in relative survival by general demographic groups (age, sex, race/ethnicity), year of diagnosis, and disease stage were estimated comparing non-overlapping confidence intervals.

Results:
We identified 11,543 primary neuroblastoma cases in USCS during 2003–2019. Age-adjusted incidence was 8.3 per million persons (95% confidence interval [CI]: 8.2, 8.5) with an AAPC of 0.4% (95% CI: -0.1, 0.9). Cases represented males in 52.7%, age <12 months in 34.0%, and distant disease at diagnosis in 46.9%. Five-year relative survival from the NPCR dataset (n=10,676) was 79.7% (95% CI: 78.8, 80.5). Patients aged <12 months had higher 5-year relative survival (92.5%; 95% CI: 91.5, 93.3) compared to patients aged 1–4 years (73.4%; 95% CI: 72.1, 74.7), 5–9 years (71.3%; 95% CI: 68.3, 74.0), 10–14 years (73.4%; 95% CI: 67.9, 78.2) and 15–19 years (66.7%; 95% CI: 56.7, 74.9). Five-year survival was higher for female patients (81.6%; 95% CI: 80.4, 82.7) compared to male patients (78.0%; 95% CI: 76.8, 79.2), and was higher for non-Hispanic White patients (80.7%; 95% CI: 79.7, 81.7) or Hispanic patients (80.8%; 95% CI, 78.8, 82.7) compared to non-Hispanic Black patients (72.6%; 95% CI: 70.0, 75.1). Five-year relative survival was lower for cases diagnosed during 2001–2009 (76.8%; 95% CI: 75.6, 78.0) than during 2010–2018 (83.2%; 95% CI: 82.0, 84.3). Five-year

relative survival was lower for distant stage (64.4%; 95% CI: 60.3, 65.8) than regional (93.3%; 95% CI: 92.1, 94.3) or localized stage (96.9%; 95% CI: 96.1, 97.6).

Conclusion:
Neuroblastoma incidence was stable between 2003 and 2019. Differences in relative survival exist by sex, age, race/ethnicity, and stage; patients who were female, older, non-Hispanic Black, or with distant stage had worse survival. Five-year survival improved over time. Future studies could seek to identify the factors affecting survival and evaluate interventions to reduce disparities in survival.

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Whole transcriptome profiling of liquid biopsies from tumour xenografted mouse models enables specific monitoring of tumour-derived extracellular RNA

Deleu J[1,2], Vermeirssen V[1,2,3,4], Morlion A[1,2], Everaert C[1,2], De Wilde J[2,5], Anckaert J[1,2], Durinck K[2,6], Nuytens J[1,2], Rishfi M[2,6], Speleman F[2,6], Van Droogenbroeck H[1,2], Verniers K[1,2], Baietti M[7,8], Albersen M[9], Leucci E[7,8], Post E[10,11], G. Best M[10,11], Van Maerken T[1,2,12], De Wilde B[1,2,13], Vandesompele J[1,2], Decock A[1,2]

[1]OncoRNALab, Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [2]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium, [3]CBIGR, Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [4]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium, [5]Department of Pathology, Ghent University Hospital, Ghent, Belgium, [6]PPOL, Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [7]Laboratory for RNA Cancer Biology, Department of Oncology, KU Leuven, Leuven, Belgium, [8]TRACE, Leuven Cancer Institute, KU Leuven, Leuven, Belgium, [9]Laboratory of Experimental Urology, Department of Development and Regeneration, KU Leuven, Department of Urology, University Hospital Leuven, Leuven, Belgium, [10]Amsterdam UMC Location Vrije Universiteit Amsterdam, Department of Neurosurgery, Amsterdam, the Netherlands, [11]Cancer Center Amsterdam, Brain Tumor Center and Liquid Biopsy Center, Amsterdam, the Netherlands, [12]Department of Laboratory Medicine, AZ Groeninge, Kortrijk, Belgium, [13]Department of Paediatric Haematology Oncology and Stem Cell Transplantation, Ghent University Hospital, Ghent, Belgium

Rapid Fire session 2B, May 18, 2023, 12:05 PM - 12:20 PM

While cell-free DNA (cfDNA) is widely being investigated, free circulating RNA (extracellular RNA, exRNA) has the potential to improve cancer therapy response monitoring and detection due to its dynamic nature. However, it remains unclear in which blood subcompartment tumour-derived exRNAs primarily reside. We developed a host-xenograft deconvolution framework, exRNAxeno, with mapping strategies to either a combined human-mouse reference genome or both species genomes in parallel, applicable to exRNA sequencing data from liquid biopsies of human xenograft mouse models. The tool enables to distinguish (human) tumoural RNA from (murine) host RNA, to specifically analyse tumour-derived exRNA. We applied the combined pipeline to total exRNA sequencing data from 95 blood-derived liquid biopsy samples from 30 mice, xenografted with 11 different tumours. Tumoural exRNA concentrations are not determined by plasma platelet levels, while host exRNA concentrations increase with platelet content. Furthermore, a large variability in exRNA abundance and transcript content across individual mice is observed. The tumoural gene detectability in plasma is largely correlated with the RNA expression levels in the tumour tissue or cell line. These findings unravel new aspects of tumour-derived exRNA biology in xenograft models and open new avenues to further investigate the role of exRNA in cancer.

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Interference in the Measurement of Metanephrines by Over acidification of Urine samples of Neuroblatoma patients

Tamir R[1]
[1]*Rambam Medical Center, Biochemistry Laboratory, Haifa, Israel*

Background: Quantification of urine metanephrines (metanephrine and normatanephrine) is commonly requested in the diagnosis and monitoring of neuroblatoma patients. For measurement of metanephrines, use of stabilizers such as 6 M Hydrochloric acid (6 M HCL) to reduce degradation is a standard procedure after reception of urine samples by the laboratory. Levels of these analytes are measured by our home-made HPLC method composed of internal standard in each sample. It was noticed that the internal standard on a 3-year-old patient chromatogram, suspected with neuroblastoma, was sporadically reduced due to some interference.

Aims: Our aim was to investigate whether over acidification of urine samples may interfere in the measurement of metanephrines.

Methods: Urine samples were preserved by adding of 6 M HCL and prepared for analysis using a home-made assay involving ion-exchange resins. An internal standard of 4-hydroxy-3-methoxybenzylamine (HMBA), was added to the diluted urine and acidic hydrolysis of conjugated metanephrines was followed by reverse-phase HPLC separation and electrochemical detection. Quantitative analysis was performed using an internal standard addition method. The internal standard was used for calibration by plotting the ratio of the analytes' signal to the internal standard signal as a function of the analyte concentration of the standards. The ratio of the samples was then used to obtain their analyte concentration from the calibration curve.

Results: A urine sample of a 3-year-old child suspected with neuroblastoma, adjusted to PH 1, was tested for urine metanephrines. The patient's sample chromatogram revealed a twofold reduction in the internal standard compared to the internal standard of the calibrators' chromatogram. This interference produced an artifact measurement of metanephrines which were falsely above the normal reference range. The reduction in the internal standard was prevented in an aliquot of the same urine sample, which was adjusted to PH 3, providing accurate measurement. Changing the PH of the urine sample eliminated the interference which falsely increased the estimation of metanehrines.

Conclusions: We can conclude that by careful adjustment of the PH of urine samples for metanephrines measurement, we can avoid false positive results which may occur from over acidification of internal standards.

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The prognostic value of detecting isolated neuroblastoma cells in bone marrow by immunocytochemistry

Assy J[1], Borovkov A[1], Luporsie M[2], Mosseri V[3], Orbach D[1], Michon J[1], Doz F[1,4], Aerts I[1], Bourdeaut F[1], Cardoen L[5], Klijanienko J[6], Cyrta J[6], Gauthier A[6], Schleiermacher G[1,7]

[1]SIREDO Integrated Pediatric Oncology Center, Institut Curie, Paris, France, [2]Department of Nuclear Medicine, Institut Curie, Paris, France, [3]Department of Biostatistics, Institut Curie, Paris, France, [4]Université Paris Cité, Paris, France, [5]Department of Radiology, Institut Curie, Paris, France, [6]Department of Pathology, Institut Curie, Paris, France, [7]SiRIC RTOP « Recherche Translationelle en Oncologie Pédiatrique », Translational Research Department and U830 INSERM, Institut Curie, Paris, France

Background:
In neuroblastoma, 40% of patients have metastatic disease at diagnosis, 70% of whom have bone marrow (BM) involvement.
BM evaluation (BME) is crucial for correct staging, definition of a risk-adapted treatment strategy, and follow up. The gold standard of BME is cytology/morphology (CM), which consists of cytological examination of BM aspirates and morphological examination + immunohistochemistry (IHC) of BM trephines. Other techniques include BM immunocytochemistry (ICC), flow cytometry, and automatic immunofluorescence plus FISH.
In this study, we sought to evaluate the sensitivity, specificity and prognostic impact of ICC BME as compared to the standard techniques.

Methods :
We studied all patients with peripheral neuroblastic tumors diagnosed and treated at Institut Curie between 2000 and 2020, with available data on BME. BM was studied by CM for initial staging according to INRG criteria and ICC by antiGD2 antibody.

Results :
Among 390 patients (214 boys/176 girls; mean age 40 months, range 0-330 months; INRG stage L1 55,L2 114,M 178,Ms 32, localised disease 11; median FU 100 months, range 2–250 months), 1095 BM samples were analysed by cytology/morphology +IHC (CM+ in case of positivity of any of these analyses) and/or by ICC (ICC+ according to previously published criteria).

At diagnosis, 134 were CM+ICC+, 213 CM-ICC-, 17 CM+ICC-, and 26 CM-ICC+ (x2-test, $p<0.0001$). Among the 26 CM-ICC+ patients, 12 had metastatic and 14 had localised disease according to clinical/radiological criteria.

During treatment/follow-up, after induction or first line chemotherapy, 23 remained CM+ICC+, 337 were CM-ICC-, 12 CM+ICC-, and 18 CM-ICC+ (none of whom were CM-ICC- at diagnosis).

In this cohort, 5-year overall survival (OS) of patients with CM-ICC+ was not statistically different from CM-ICC- (CM+ICC+: 47± 4.6%; CM-ICC-: 71±2.4%; CM+ICC-: 44% ±12%, CM-ICC+: 78± 8.5%; CM-ICC+ vs. CM-ICC-, P=0.3).

Among the 180 patients with clinically localised disease, isolated ICC positivity was not associated with a statistically significant worse 5-year OS (92% versus 100%).

Conclusion :
The isolated presence of NB cells in BM detected by ICC (anti-GD2) is not associated with a poorer prognosis in this study. Further biological analyses, including single-cell RNAseq, will determine the role of disseminating tumor cells in BM.

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Optimization of neuroblastoma xenograft models for the analysis of tumoral cell-free RNA in murine blood plasma

Van Droogenbroeck H[1,2], Deleu J[1,2], Nuytens J[1,2], Verniers K[1,2], Yigit N[1,2], Speleman F[2,3], Durinck K[2,3], Rishfi M[2,3], de Carvalho Nunes C[2,3], Mus L[2,3,6], De Wilde J[2,4], Van Maerken T[1,2,5], De Wilde B[1,2,6], Vandesompele J[1,2]
[1]OncoRNALab, Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [2]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium, [3]Pediatric Precision Oncology Lab (PPOL), Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [4]Department of Pathology, Ghent University Hospital, Ghent, Belgium, [5]Department of Laboratory Medicine, AZ Groeninge, Kortrijk, Belgium, [6]Department of Paediatric Haematology Oncology and Stem Cell Transplantation, Ghent University Hospital, Ghent, Belgium

Background: Minimally invasive liquid biopsies, such as blood plasma, are becoming increasingly important in the diagnosis and treatment follow-up of patients with neuroblastoma, as they might contain biomarkers such as cell-free DNA (cfDNA) and the less explored cell-free RNA (cfRNA). To study treatment-induced alterations of tumoral cfRNA in mice, a high shedding rate into the circulation is desired. However, it remains unclear which factors determine the shedding of tumoral RNA into the circulation.

Aims: We evaluated whether the injected cell line, its injection site and the tumor size influence the amount of circulating tumoral cfRNA. To that end, mouse xenografts frequently used in neuroblastoma research were generated, and the fraction of circulating tumoral cfRNA was compared between these models.

Methods: Different neuroblastoma cell lines were injected subcutaneously in the right flank of mice. Tumor sizes were followed up twice weekly and cardiac punctures were performed to evaluate the tumoral cfRNA fractions. Distinguishing the tumor-derived circulating cfRNA response to treatment from the host-derived response is challenging in patient plasma. This distinction can more easily be made using mice models with a human tumor xenograft, in which human cfRNA is originating from the tumor, while murine cfRNA is host-derived. Next, we compared orthotopic and subcutaneous injection of the cell line with highest shedding into the circulation, to evaluate the added value of a more labor-intensive orthotopic injection.

Results: When looking into the results of subcutaneous mouse models, tumoral cfRNA levels in the circulation are highly dependent on the injected cell line. The size of the tumor at sacrifice is also a determining factor, with no further increase in circulating tumoral cfRNA for tumors larger than 1500 mm^3. Orthotopic injection has the potential to increase the amount of circulating tumoral cfRNA compared to subcutaneous injection.

Conclusion: The injected cell line, the site of injection and the tumor size at sacrifice determine the amount of circulating tumoral cfRNA and should therefore carefully be selected to perform experiments aiming at RNA biomarker discovery.

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Multiplexing of genomic targets for high-risk neuroblastoma MRD diagnostics using mediator probe PCR chemistry

Schröer A[1], Winkler A[1], Fillies M[1], Lehnert M[2,3], Kranig C[1], Deubzer H[1,4,5,6,7], Eggert A[1,5,6,7], Schulte J[1,5,6,7,8], Szymansky A[1], Eckert C[1]

[1]Department of Pediatric Hematology, Oncology and Stem Cell Transplantation, Charité – Universitätsmedizin Berlin, Berlin, Germany, [2]Hahn-Schickard-Gesellschaft für angewandte Forschung e.V., Freiburg, Germany, [3]Laboratory for MEMS Applications, IMTEK - Department of Microsystems Engineering, University of Freiburg, Freiburg, Germany, [4]Experimental and Clinical Research Center (ECRC) of the Charité and Max-Delbrück-Center for Molecular Medicine (MDC) in the Helmholtz Association, Berlin, Germany, [5]German Cancer Consortium (DKTK), Berlin, Germany, [6]German Cancer Research Center (DKFZ), Heidelberg, Germany, [7]Berlin Institute of Health (BIH), Berlin, Germany, [8]Department of General Pediatrics, Hematology/Oncology, University Children´s Hospital Tuebingen, Tuebingen, Germany

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Background: High-risk neuroblastoma can harbor a variety of molecular features, such as an amplification of the MYCN oncogene, rearrangements of the TERT locus or loss-of-function mutations of ATRX. Even though most of the pediatric patients with a high-risk neuroblastoma achieve remission, the majority still has a minimal residual disease (MRD) that can cause relapse despite intensive therapy. We developed a new approach to detect MRD cells with high sensitivity in bone marrow aspirates taken during treatment. Our multiplex assay makes use of a novel mediator probe PCR (MP-PCR), a quantitative PCR chemistry based on label-free patient specific molecular probes and standardized universal fluorogenic reporters, which was originally developed for MRD detection of genomic targets in acute lymphoblastic leukemia.

Aims: To capture neuroblastoma tumor heterogeneity and clonal evolution during treatment, we utilized up to four patient-specific, genetic alterations as MRD markers in one assay by MP-PCR multiplexing. We monitored the level of MRD represented by neuroblastoma clones in bone marrow samples collected over the course of therapy and in case of a relapse.

Methods: Our cohort consists of 10 patients with high-risk neuroblastoma and their respective initial and if available relapse tumors, as well as 102 longitudinally corresponding bone marrow aspirations. As quantitative PCR we employed a Rotor-Gene Q device using a colorimetric 4-plex MP-PCR assay.

Results: We created patient-specific multiplex MP-PCR assays for 10 patients with neuroblastoma to assess genomic breakpoints and single-nucleotide variations (SNVs) of up to four MRD targets resulting in a high sensitivity and quantification depth ranging from 10e-2 up to 10e-6 according to EuroMRD-adapted guidelines. We measured the MRD levels using 21 MYCN amplicon breakpoints and eight breakpoints on other chromosomes, including TERT and NF2. Furthermore, we quantitatively detected five SNVs, located within ALK, NBAS, PIK3CB, BRCA1 and BRCA2.

Conclusion: Our results demonstrate proof-of-concept for the assessment of multiplex MP-PCR for detection of MRD levels based on different targets for high-risk neuroblastoma, capturing tumor heterogeneity and reducing the amount of sample needed. An early and sensitive detection and quantification of MRD during treatment could be a technique to adapt therapy according to the patients´ current MRD-level.

P331

Longitudinal cell-free DNA analysis in patients with high-risk neuroblastoma is suitable for early relapse and actionable target detection

Lodrini M[1,2,3], Thole-Kliesch T[1], Graef J[4], Wünschel J[1,2,3], Astrahantseff K[1], Sprüssel A[1], Grimaldi M[1,2,3], Peitz C[1,2,3], Linke R[1,2,3], Hollander J[1,2,3], Tiburtius D[1,5,6], Künkele-Langer A[1,7], Fuchs J[8], Szymansky A[1], Eckert C[1], Amthauer H[4], Schulte J[1,5,6,7], Hundsdörfer P[1,9], Hero B[10], Simon T[10], Eggert A[1,5,6,7], Deubzer H[1,2,3,5,6,7]
[1]Charité - Universitätsmedizin Berlin, Department of Pediatric Hematology and Oncology, Berlin, Germany, [2]Experimental and Clinical Research Center (ECRC) of the Charité and the Max-Delbrück-Center for Molecular Medicine (MDC) in the Helmholtz Association, Berlin, Germany, [3]Max-Delbrück-Center for Molecular Medicine (MDC) in the Helmholtz Association, Berlin, Germany, [4]Charité - Universitätsmedizin Berlin, Department of Nuclear Medicine, Berlin, Germany, [5]German Cancer Consortium (DKTK), partner site Berlin, Berlin, Germany, [6]German Cancer Research Center (DKFZ), Heidelberg, Germany, [7]Berliner Institut für Gesundheitsforschung (BIH), Berlin, Germany, [8]University Children´s Hospital, Eberhard-Karls- University Tuebingen, Department of Pediatric Surgery and Paediatric Urology, Tuebingen, Germany, [9]Helios Klinikum Berlin Buch, Department of Pediatric Oncology, Berlin, Germany, [10]Children's Hospital, University of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany

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Background
The invasive nature of surgical biopsies most often prevents their sequential application to monitor disease. Single biopsies also fail to reflect cancer dynamics, intratumor heterogeneity and drug sensitivities that most likely change during clonal evolution and under the selective pressure of therapy.

Aims
Characterization and analysis of cell-free tumor DNA (cfDNA) in biofluids from patients with neuroblastoma may better support disease monitoring and provide molecular information for clinical decision-making toward personalized medicine.

Methods/Materials
We characterized and analyzed cfDNA size distribution and concentration in blood, bone marrow, cerebrospinal fluid and urine from 84 infants and children with low-, intermediate or high-risk neuroblastoma using the Agilent 4200 TapeStation System. Droplet digital PCR (ddPCR) assays assessing MYCN and ALK copy numbers and ALKF1174L and ALKR1275Q hotspot mutations were applied to longitudinally collected patient samples during or after first-line therapy from 31 patients with high-risk neuroblastoma. Cell-free DNA was purified before ddPCR, and fragmentation of tumor-derived genomic DNA was achieved by direct enzymatic digestion in the ddPCR reaction mixture. Total cfDNA levels and detection of markers were compared with data derived from clinical routine diagnostics including histopathological analyses of tumor samples, bone marrow cytology/immunocytology and imaging in patients (MIBG and MRI scans).

Results
Analysis of DNA electropherograms revealed five different major profiles and characteristic DNA size distribution patterns for each of the biofluids. A favorable response to treatment was associated with a rapid decrease in blood-based cfDNA concentration in patients with high-risk neuroblastoma. Targeted

cfDNA diagnostics using ddPCR proved superior for early relapse detection to all current diagnostics in two patients. Marker analysis in cfDNA indicated intratumor heterogeneity for MYCN amplifcations and druggable ALK alterations that were not detected in matched tumor tissue in 17 patients from our cohort. We provided proof of concept for molecular target detection in cerebrospinal fluid samples from patients with isolated central nervous system relapse.

Conclusion
Our results indicate that tumor-specific alterations can be identified and monitored during disease course using liquid biopsies from patients with neuroblastoma. Surveillance of cfDNA warrants further prospective validation and exploitation for diagnostic purposes.

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5-hydroxymethylcytosine in cell-free DNA as a potential biomarker of therapy response in a phase 3 high-risk neuroblastoma clinical trial

Moore K[1], Chennakesavalu M[1], Chlenski A[1], Lu M[1], Naranjo A[2], Klega K[3], Cohn S[1], Crompton B[3], Bagatell R[4], Dubois S[3], He C[1], Applebaum M[1]

[1]University of Chicago, Chicago, United States, [2]University of Florida, Gainesville, United States, [3]Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, United States, [4]Children's Hospital of Philadelphia, Philadelphia, United States

Background: We previously reported that cell free DNA (cfDNA) 5hmC profiles correlated with disease burden and outcome in patients with neuroblastoma. Importantly, we also found that a cfDNA 5hmC derived biomarker can distinguish patients with superior response to treatment from those at high risk for relapse. We are now prospectively analyzing cfDNA 5hmC profiles in serial blood samples collected on patients enrolled on the COG ANBL1531 Phase 3 study.

Methods: cfDNA is extracted from plasma and 20% of the sample is used for 5-hmC analysis. For 5-hmC libraries, cfDNA is ligated with sequencing adapters and subjected to glucosylation and biotinylation. Biotin-labeled fragments are pulled down using streptavidin beads and PCR amplified for sequencing. Reads per gene are used for downstream analysis using DESeq2 and additional methods comparable for RNAseq sequencing. Neuroblastoma signature enrichment scores are assessed for each sample with Gene Set Variation Analysis (GSVA) which returns values from -1 to 1, with more positive values indicating greater enrichment of the neuroblastoma signature.

Results: As ANBL1531 is ongoing, demographic, response, and outcome data are not available for this cohort. To date, 1,342 cfDNA samples have been received at the University of Chicago. While creation of sequencing libraries is ongoing, 5hmC profiles have been generated for samples at diagnosis (n=171) and pre-cycle 2 (n=204). All samples have had adequate DNA quantity and quality for library generation. In the subset of analyzed samples, neuroblastoma signatures scores at diagnosis had a median GSVA score of 0.3 (range -0.66 to 0.68) while the median prior to cycle 2 was -0.56 (range -0.76 to 0.56). Planned analyses upon completion of the trial and report of results include modeling to predict response to induction therapy, evaluation of signature scores in end induction samples to quantify response to therapy, and determination of molecular pathways that are enriched in relapsed samples.

Conclusion: 5hmC marks correlate with actively transcribed genes allowing for evaluation of neuroblastoma signatures in cfDNA. This methodology is complementary to copy number, mutational, and other methods of detecting circulating neuroblastoma material. The clinical impact of these changes will be evaluated at the conclusion of the trial.

P333

LC-MS/MS detection of circulating GD2 ganglioside in plasma samples from neuroblastoma patients and age-matched healthy children. Diagnostic and prognostic evaluation.

Morini M[1], Barco S[2], Cafaro A[2], Pigliasco F[2], Ardito M[1], Segalerba D[1], Conte M[3], Garaventa A[3], Ponzoni M[1], Cangemi G[2], Corrias M[1]

[1]Laboratory of Experimental therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genoa, Italy, [2]Central Laboratory of Analyses, IRCCS Istituto Giannina Gaslini, Genoa, Italy, [3]Oncology Unit, IRCCS Istituto Giannina Gaslini, Genoa, Italy

Background. GD2 is shed into circulation from neuroblastoma (NB) cells and the C18 and C20 GD2 lipoforms can be simultaneously measured by a validated LC-MS/MS method.

Aims. To evaluate the diagnostic and prognostic power of circulating GD2 levels in children with NB, treated according to SIOPEN trial, as compared to age-matched controls.

Methods. Fifty µL of plasma from 60 children with NB (15 stage L1, 15 stage L2, 25 stage M and 4 stage MS with a median age of 28.5 months (range 1.2-109 months) and from 60 age-matched healthy children were subjected to liquid-liquid extraction and LC-MS/MS analysis. Nineteen samples from the 25 stage M patients were also collected at post induction therapy (PIT).

Results. C18 and C20 GD2 concentrations were significantly higher in children with NB than in age-matched controls (P<0.0001). C18 and C20 median concentrations in NB samples were 155.3 (range 5-1390) and 0.87 (range 0.06-10.6) nM, respectively. C18 and C20 values significantly correlated to each other, particularly in NB patients (Spearman r=0.96 vs. r=0.6 in controls) and C18/C20 ratio was similar in both NB and controls (range 37.5-421.4 vs. 36.4-473). C18 and C20 values were no significantly different in male and female or in infants and children, both in NB and controls.
At NB onset, GD2 concentration was significantly higher in stage M patients (M>MS>L2>L1), in MYCN amplified patients (P=0.008) and in patients who died (P=0.0038).
As for the diagnostic potential, ROC analysis showed a cut-point of 30.5 and 0.4 nM for C18 and C20, respectively, able to discriminate with high specificity and sensitivity NB from controls. Conversely, ROC analysis performed to evaluate the GD2 prognostic potential failed to find prognostic cut-points, independently from stage or MYCN amplification.
C18 and C20 concentrations at PIT were strongly reduced in all stage M patients, but the absolute values or the delta between onset and PIT levels were unable to predict survival.

Conclusion. Measurement of circulating GD2 by LC-MS/MS lacks independent prognostic power, but it is a cost-effective powerful diagnostic tool,which can be performed by microsampling and, thus, can be easily applied to the clinical setting.

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Preliminary digital study of vitronectin and cilengitide related to neuroblast behavior in silk fibroin 3D hydrogel cultures

Vieco-Martí I[1,2], Granados-Aparici S[2], López-Carrasco A[1,2], Navarro S[1,2], Noguera R[1,2]
[1] Centro de Investigación Biomédica en Red de Cáncer (CIBERONC)), Madrid, Spain, [2]Department of Pathology, School of Medical, University of Valencia-INCLIVA, Valencia, Spain

Background: Vitronectin (VN) is an anchoring glycoprotein highly expressed in high-risk neuroblastoma (NB), which may confer stiffness to the microenvironment promoting neuroblasts malignancy. Cilengitide (CLG) is a penta-peptide, which may block the VN-cell interaction. Silk-fibroin (SF) enables the building of hydrogels (HG) with enzymatically-linked molecules.

Aims: Search for VN-related molecular mechanotherapy developing digital analysis tools.

Methods: High-risk NB cell line SK-N-BE(2) was cultured up to 14 and 21 days in casted 4%wt scaffolds of gelatin-tyramine (25%) and SF (75%). In models with VN we added 400µg/mL. HGs were kept at 37ºC with media changed every two-three days, with or without addition of CLG (100µM). HGs were paraffin-embedded, and 3-micron slices were stained with hematoxylin-eosin and immunohistochemistry against VN, Ki67 and BCL2. We digitized the slides (20x) and analyzed them with custom scripts in QuPath. HGs were also sequenced with our own designed NGS-mechanopanel. We used R for statistics and graphics.

Results: SK-N-BE(2) cells grew as cell cluster fashion in all HGs, secreting VN. We focused on n=1102 cell clusters with area range of 1600µm2-40000µm2 (20-500 cells). In VN-conjugated HGs, clusters (n=526) were bigger, expressed higher ki67 and BCL2, had lower values of solidity (more cluster protrusions) than those clusters growing in noVN-conjugated HGs (n=402). For VN-conjugated HGs clusters, the distance to small clusters (<400 µm2~5 cells) is less than in noVN-conjugted. However, noVN-conjugted HGs clusters had an evident coronary VN secretion compared to their counterparts. Regarding CLG effect, treated noVN-conjugated HG clusters (n=89) had slightly smaller size and more solidity than controls of the same condition (n=85). Interestingly, in noVN-conjugted HGs treated with CLG, the VN was present mainly inside the clusters, whereas the controls kept the coronary secretion. All described effects were higher at day 21. We also observed mutations in mechanotransduction related-genes COL11A1, UVSSA, DOCK8, MUC16.

Conclusion: Our results support that VN triggers aggressive behavior in NB cells. Changes in the morphology and different VN location would be related to the loss of cell-mechanosensing of the HG after CLG treatment. These HG models and digital analysis can be adapted for the study of other proteins and drugs.

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Biotensegral studies in neuroblastoma 3D in vitro models reveal potential therapeutic improvements

Monferrer E[1,2], Vieco-Martí I[1,2], Navarro S[1,2], Noguera R[1,2]
[1] Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain, [2]Department of Pathology, School of Medical, University of Valencia-INCLIVA, Valencia, Spain

Background: Mechanical forces transmitted within the tumor microenvironment trigger several tumor adaptations known as biotensegrity, involving cell-surrounding crosstalk and metabolic reprogramming. Our previous results pinpointed that extracellular matrix features such as high expression of the anchoring glycoprotein vitronectin (VN), in combination with an increased stiffness lead to poor neuroblastoma patient's outcome. Adjustable 3D in vitro neuroblastoma models including extracellular matrix features driving neuroblastoma aggressiveness has been previously reported.

Aims: To identify novel diagnostic and therapeutic strategies related to mechanotransduction-driven adaptations using two different in vitro models.

Methods: Gelatin/Alginate (GelMA/AlgMA) and Vitronectin/Polyethilene glycol (VN/PEG) hydrogels were fabricated using different AlgMA or PEG proportions to regulate model stiffness. SK-N-BE(2) or SH-SY5Y neuroblastic cells were cultured in the absence or presence of SW10 Schwann cells within GelMA/AlgMA hydrogels up to 84 days. SK-N-BE(2) cells were cultured in VN/PEG hydrogels up to 7 days. VN expression was evaluated in all conditions; VN secretion (ELISA) was evaluated in VN/PEG models; PKM2 and MCT1 expressions were evaluated to assess glycolysis status. All staining quantifications were made in digital hydrogel images and were based on the presence, distribution and intensity of the staining within experimental conditions.

Results: VN dynamics were influenced by a synergistic effect of model features. Hydrogels rapidly promoted high cytoplasmic and pericellular VN expression in all designed systems, which gradually decreased over time. Cell cluster size favored VN synthesis, and SW10 incorporation initially evened VN expression towards low intensity in GelMA/AlgMA models, but favored high VN expression in long term cocultures. Neuroblastoma cell genetic background and hydrogel stiffness further influenced VN synthesis depending on the system specifications. Synthesized VN escaped from VN/PEG hydrogels and was detected in cell culture medium. PKM2 was robustly expressed in almost all GelMA/AlgMA hydrogel conditions. MCT1 expression in GelMA/AlgMA hydrogels mostly depended on SW10 presence in SK-N-BE(2) cultures, but on stiffness in SH-SY5Y ones.

Conclusion: VN secretion in both in vitro 3D neuroblastoma models suggests that mechanical pathways involving that glycoprotein could be therapeutically targeted, and PKM2 and MCT1 synthesis indicate aberrant glycolysis in neuroblastoma cells and their susceptibility to be treated with metabolism-related drugs.

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Cancer stem cell evaluation on 3D in vitro models has translational impact for neuroblastoma risk classification

Monferrer E[1,2], Vieco-Martí I[1,2], Cañete A[3], Navarro S[1,2], Noguera R[1,2]

[1] Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain, [2]Department of Pathology, School of Medical, University of Valencia-INCLIVA, Valencia, , [3]Pediatric Oncology Unit, University and Polytechnic Hospital La Fe, Valencia, Spain

Background: Cancer cells typically present stem cell-like phenotypes, which allow them to become invasive, proliferative, and potentially immortal. Neuroblastoma differentiation status is a well-known parameter for high-risk preclinical stratification. We previously confirmed that OCT4 stem cell marker expression correlate with neuroblastoma aggressiveness and poor survival, but it has not been validated to distinguish between undifferentiated and poorly differentiated neuroblastoma. 3D in vitro models are preliminary platforms for biomarker evaluation.

Aims: To identify stem cell markers to potentially improve neuroblastoma risk pretreatment classification using a 3D in vitro model.

Methods: Gelatin/Alginate (GelMA/AlgMA) hydrogels were bioprinted using different AlgMA proportions to emulate different microenvironment stiffness. SK-N-BE(2) or SH-SY5Y neuroblastic cells were incorporated without or with 10% SW10 Schwann cells during hydrogel fabrication, and were cultured up to 84 days. Cell differentiation status was quantitatively analyzed in digital hydrogel stained sections (anti-OCT4, anti-SOX2, anti-CD133, anti-CD105, anti-PTBP1, anti-PTBP2 and anti-PSD95 antibodies) applying Pannoramic Viewer software modules. Additionally, obtained results pinpointed the potential of PTBP1 and PTBP2, which were subsequently evaluated in 53 high-risk neuroblastoma human samples as previously did in GelMA/AlgMA models.

Results: SK-N-BE(2) cells exhibited OCT4 and SOX2 opposite expressions, being the former positive in long term cultures and the latter in short/mid term cultures, whereas PTBP1 and PTBP2 increased with culture time. SW10 incorporation favored all three OCT4, SOX2 and PTBP2 expression. All SH-SY5Y cultures became almost OCT4 and SOX2 negative, and PTBP2 highly positive. Besides, PTBP1 decreased with stiffness and SW10 presence in SH-SY5Y models. Low/intermediate PSD-95 and negative CD133 and CD105 expression were found in all GelMA/AlgMA hydrogels. Stem cell marker evaluations on neuroblastoma samples evinced PTBP1 expression correlation with tumor aggressiveness and PTBP2 with poorly differentiated neuroblastoma status.

Conclusion: Digital image analysis allows for 3D in vitro model accurate characterization, thus releasing the potential of these constructs as screening platforms to preliminary detect potential cancer stem cells biomarkers with relevant clinical impact for neuroblastoma.

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Clinical Implications of Residual Lesions Assessed Using Multiple Modalities Following High-Dose Chemotherapy as a Prognostic Factor in High-Risk Neuroblastoma Patients

Koh K[1], Yoon S[1], Koh Y[1], Kang A[1], Kang S[1], Kim H[1], Im H[1]
[1]Division of Pediatric Hematology/Oncology, Department of Pediatrics, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea

Background: Multiple techniques are utilized to assess the response and residual disease in neuroblastoma patients. However, the implications of persistent disease following the completion of neuroblastoma treatment have not yet been clarified.

Aims: This study aimed to investigate the clinical significance of residual lesions assessed by multiple modalities following high-dose chemotherapy and autologous stem cell transplantation.

Methods: We conducted a retrospective analysis of 41 consecutive patients with high-risk neuroblastoma who had tandem HDCT and ASCT between 2008 and 2022. Computed tomography (CT), whole-body magnetic resonance imaging (WB-MRI), iodine-121 (123-I) meta-iodobenzylguanidine single-photon emission computed tomography (MIBG-SPECT), Tc-99m bone scan, bone marrow aspiration, and 18F-3,4-dihydroxyphenylalanine positron emission tomography (F-DOPA PET) were the assessment modalities.

Results: The 5-year overall survival and event-free survival rates were 82.7% and 57.8%, respectively. The 5-year cumulative incidence of relapse did not differ statistically between patients with and without residual lesions on MIBG-SPECT (37.5% vs. 27.1%, p = 0.493). In addition, the 5-year cumulative incidence for relapse was not different between patients with and without multiple bone lesions on WB-MRI (41.6% vs. 28.7%, p = 0.093), between patients with and without positive lesions on Tc-99m bone scan (50.0% vs. 29.9%, p = 0.221), between patients with and without measurable lesions in CT (47.5% vs. 38.8%, p = 0.671), between patients with and without persistent bone marrow disease (37.5% vs. 28.8%, p = 0.078), between patients with and without positive tyrosine hydroxylase transcripts by PCR in bone marrow (50.0% vs. 42.9%, p = 0.661), and between patients with and without positive lesions on F-DOPA PET (40.4% vs. 28.4%, p = 0.923).

Conclusion: This study showed that residual lesions on a single modality are insufficient for long-term neuroblastoma surveillance. This study suggests that persistently positive imaging or bone marrow aspiration results at the end of treatment may not always indicate an active illness, but may instead indicate a stable disease rather than a refractory disease.

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Cell-free RNA from plasma in patients with neuroblastoma: exploring the technical and clinical potential

Lak N[1,2], Seijger A[3], van Zogchel L[1,2], Gelineau N[1,2], Javadi A[2], Zappeij-Kannegieter L[2], Bongiovanni L[3,4], Andriessen A[3], Stutterheim J[1], van der Schoot C[2], de Bruin A[3], Tytgat G[1,2]
[1]Princess Maxima Center for Pediatric Oncology, Utrecht, Netherlands, [2]Sanquin Research, Amsterdam, Netherlands, [3]Faculty of Veterinary Medicine, Utrecht, Netherlands, [4]Faculty of Veterinary Medicine, Teramo, Italy

Background: Liquid biopsies, e.g. molecular analysis of circulating tumor-derived nucleic acids in blood, offer a minimally invasive diagnostic modality paired to high sensitivity. Cell-free RNA (cfRNA) is released by all cells in the body, especially by cancer cells. cfRNA circulates in blood, packed in extracellular vesicles (EV) or in proteins.

Aim: We studied the feasibility to analyze cfRNA from plasma using several multiplex droplet digital PCR (ddPCR) assays in patients with neuroblastoma. Moreover, we investigated whether cfRNA from plasma is concentrated in EV and how this compares to cell-free DNA (cfDNA) from plasma.

Method: We developed a neuroblastoma-specific gene panel (PHOX2B, TH, CHRNA3) and a cell cycle regulation panel (E2F1, CDC6, ATAD2, H2AFZ, MCM2, DHFR). Platelet-poor plasma samples from 40 healthy controls and 40 patients with neuroblastoma (10 patients with localized and 30 with metastatic disease) were collected. Furthermore, platelets from 4 healthy controls were collected. All plasma and platelet samples were analyzed using both cfRNA panels. EV from plasma were isolated using size exclusion chromatography (SEC) and the EV-derived cfRNA was tested using both cfRNA panels. The EV-derived cfDNA was analyzed using ddPCR for methylated RASSF1A (RASSF1A-M) and ACTB.

Results: The neuroblastoma-specific markers had no background in cfRNA from healthy controls and were only present in plasma from patients with metastatic disease (14/30). The cell cycle markers had a background expression in cfRNA and platelets from healthy controls. We established a correction formula for the presence of platelets in plasma and employed this to set a threshold for positivity. Applying this threshold to the patient samples, DHFR was most often positive, only in patients with metastatic disease (14/30). The other markers did not yield more positive samples. Both neuroblastoma-specific and cell cycle markers were concentrated in the EV-enriched SEC fractions, whereas cfDNA was concentrated in the protein-enriched SEC fractions.
Conclusion: cfRNA from plasma and EV can be analyzed using multiplex ddPCR assays. We present an approach to correct for contamination by platelets. Both neuroblastoma-specific and cell cycle markers have higher expression in cfRNA from patients with metastatic disease. cfRNA is an interesting novel liquid biopsy-based target to explore.

P339

Mapping distinct tumor subpopulations in human neuroblastoma using scRNA-seq and novel spatial omics

Tsea I[1], Olsen T[1], Otte J[1], Mei S[2], Embaie B[1], Kogner P[1], Johnsen J[1], Baryawno N[1], Fard S[1]
[1]Karolinska Institutet, Women and Children's Health, Solna, Sweden, [2]Harvard Medical School, Department of Biomedical Informatics, Boston, United States of America

Background: Neuroblastoma (NB) is the most common and deadly infant malignancy, accounting for approximately 15% of pediatric cancer-related deaths. Despite intensive multimodal therapy, survival in the high-risk group is still less than 50%. Hence, the urgent need to identify heterogeneity in NB emergence as well as the subpopulations of tumour cells displaying therapy resistance.

Method: In our study, we have used single-cell RNA sequencing to provide a detailed description of the cellular and genetic diversity within human NB. We have profiled 17 NB samples from 15 patients representing a broad spectrum of disease characteristics and have generated a database of ~70,000 single cells. This is followed by spatial multi-omics using a combined multiplex single-molecule DNA and RNA-FISH approach on NB patient biopsies.

Results: Unbiased clustering revealed thirteen main cell types spanning tumor cells, stromal cells, and immune cells. Among the tumor population, we found two clusters expressing known mesenchymal (PRRX1, LEPR, PDGFRA, DCN) and adrenergic (TH, DBH) genes, indicative of mesenchymal and adrenergic tumor origin, respectively. These two putative tumor populations were connected by a “bridge” population that expressed key neural crest and Schwann lineage markers (SOX10, S100B) and was annotated as Schwann Cell Precursors –like (SCPs). Transcriptional profiling of the three distinct tumor populations revealed a continuous transition to both the MES and ADR lineages via the SCP-like population which harboured malignant aberrations. To further define the three tumor cell-states and investigate a possible transition from malignant SCPs to malignant MES and/or ADR cells, we are in the proses of implementing a single-molecule DNA and RNA-FISH approach. This method allows us to simultaneously, detect and validate abnormal DNA allelic expression and study RNA signatures through which we distinguish tumor subpopulations from normal stroma cells.

Conclusion: We conclude that there are three subpopulations of malignant NB tumour cells, and are in the proses of investigating possible links between them. Increasing our understanding of the interactions between SCPs and their downstream tumor subpopulations may provide novel information aiding in the strategy for therapeutic targeting of resistance tumor cell types in high-risk NB.

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Liquid Biopsy Identifies Divergent Epigenomic Pathways with Actionable Targets in High-Risk Neuroblastomas

Trinidad E[1], Vidal E[1], Coronado E[1], Esteve-Codina A[2], Castel V[1,3], Cañete A[1,3], Gut M[2], Heath S[2], Font de Mora J[1]

[1]Cellular and Molecular Biology lab, Translational Research in Cancer Group, Health Research Institute Hospital La Fe, Valencia, España, [2]National Center for Genomic Analysis – Centre for Genomic Regulation (CNAG-CRG), Barcelona Institute of Science and Technology, Universitat Pompeu Fabra, Barcelona, Spain, [3]Pediatric Oncology Unit, La Fe University Hospital, Valencia, Spain

Background & Aims: Blood-derived liquid biopsy represents a promising clinical tool for improving treatment of neuroblastoma because it reflects the precise disease status at diagnosis and throughout progression. Analysis of the methylome of tumor cell-free DNA (cfDNA) has emerged as a powerful non-invasive technique for cancer subtyping and prognosis. Although several epigenetic biomarkers have already been studied in situ, only RASSF1A has shown some value to predict prognosis in blood-derived plasma samples, and still, the potential epigenomic value of cfDNA has not yet been demonstrated in neuroblastoma.

Methods: We analyzed cfDNA whole methylome sequencing at diagnosis in a cohort of 64 high-risk neuroblastoma patients using enzymatic conversion of unmethylated cysteines (EM-seq) as an alternative to bisulfite conversion. Data were compared with normal reference cohorts, embryo cohorts and neuroblastoma in situ cohorts. Validation cohorts used bisulfite conversion and Infinium Human Methylation 450K BeadChip arrays.

Results: Despite the different techniques and origins of the samples, we demonstrate that information on cfDNA methylation is comparable with in situ cohorts. Based solely on our methylation data, we built a model for epigenomic subtyping into MYCN-amplified or 11q-deleted high-risk neuroblastomas that accurately predict subtype in in situ cohorts. This model reveals a divergent methylome signature between MYCN and 11q, supporting their different epigenetic origin. In addition, RASSF1A hypermethylation is a common event that precedes the divergent methylome signature. Genes targeted by the methylated CpGs in the model are differentially expressed between 11q-deleted or MYCN-amplified tumor subtypes and orchestrate cellular processes including signaling, neuronal differentiation and immune activity. Eight differentially methylated CpGs showed prognostic value in 11q-deleted neuroblastomas, and two of them, CSF1R and CCR7 are actionable targets that could be exploited therapeutically.

Conclusions: Our findings support the use of liquid biopsy followed by EM-seq to assess the cfDNA methylome of cancer patients as a source of biomarkers, some with potential prognostic and therapeutic value, and strengthen the use of liquid biopsy for disease stratification and follow-up.

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Mechanotransduction-related genes are dysregulated in high-risk neuroblastomas

López-Carrasco A[1,2], Ramírez M[3], Fernández A[3], López-Guerrero J[3], Noguera R[1,2]

[1] Centro de Investigación Biomédica en Red de Cáncer(CIBERONC), Valencia, Spain, [2]2 Department of Pathology, School of Medical, University of Valencia-INCLIVA, Valencia, Spain, [3]Laboratory of Molecular Biology, Instituto Valenciano de Oncología (IVO), Valencia, Spain

Background. Tumor cells are submitted to mechanical forces, transformed in chemical signals through mechanotransduction. Our previous results in in vitro and in vivo NB models showed that extracellular matrix stiffness and composition leads to clonal selection of specific segmental chromosomal aberrations.

Aim: Identify altered mechanotransduction-related genes in HR-NB as possible biomarkers.

Material and methods. 77 samples of 52 HR-NB patients were sequenced with our own designed NGS-mechanopanel that comprehends 18 genes (or gene regions) mechanotransduction-related, and 11 essential genes in cancer and especially in NB. We employed the KAPA HyperPlus kit (Roche), and NextSeq 550 sequencer (Illumina) to obtain at least 100x coverage. Gene expression of 48 of the mentioned neuroblastomas was analyzed with the Oncology Biomarker Panel on the HTG EdgeSeq system (HTG Molecular Diagnostics) allowing a simultaneous and quantitative detection of 2549 genes associated with tumor biology by obtaining its fold change (FC).

Results. We identified 6 clonal and subclonal mutations in the mechanotransduction genes COL5A2 (p.G591D, 1 case), PTPN11 (p.E69K and p.E69Q, 2 cases), FGFR1 (p.C551Y and p.R622*, 2 cases), FOXA1 (p.G591D, 1 case), 5 clonal and subclonal mutations in ALK (p.R1275Q, 3 cases and p.F1174L, 2 cases), 1 subclonal mutation in TP53 (p.R175H, 1 case), and 1 deletion in ATRX (p.F201Yfs*2, 1 case). Moreover, we found 279 differentially expressed genes (p-value<0.05) considering clinical and genetic data of the HR-NB. Many of them were mechanotransduction genes, affecting the RAS, RHOA, JAK-STAT and PI3K-AKT pathways, focal adhesions mediated by integrins, extracellular matrix components, and extracellular matrix-cellular receptor interactions. Notably, we detected dysregulation of 5 integrins in tumors with loss of 11q (FC of ITGA8: 3.18, ITGA3: 1.51, ITGB8: -1.81, ITGA9: -1.66, ITGA6: -1.39), downregulation of 4 collagens in HR-NB with gain of 17q (FC of COL11A1: -4,92, COL1A2: -6.8, COL1A1: -4.15, COL5A2: -3.52), and upregulation of the metalloproteinase MMP7 in MYCN-amplified HR-NB (FC: 1.81). PTPN11 and COL5A2 showed both single mutations and impaired expression (FC: -1.36 and -3.52, respectively).

Conclusions. Further studies with larger samples will reveal the possible prognostic or therapeutic value of these genes together with dysregulated integrins, collagens and MMP7 associated with tumor chromosomal profile.

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Plasma 3-O-methyldopa: a promising biomarker that can be measured from dried plasma microsamples using liquid chromatography-tandem mass spectrometry

Barco S[1], Biondi M[1], Cafaro A[1,2], Pigliasco F[1], Morini M[3], Cangelosi D[4], Conte M[5], Garaventa A[5], Cangemi G[1]
[1]Central Laboratory of Analyses, IRCCS Istituto Giannina Gaslini, Genova, Italy, [2]Department of Internal Medicine, University of Genova, Genova, Italy, [3]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [4]Clinical Bioinformatics Unit, IRCCS Istituto Giannina Gaslini, Genova, Italy, [5]Pediatric Oncology Unit, Genova, Italy

BACKGROUND: Catecholamine metabolites are well-known biomarkers of NB. Analytical and pre-analytical challenges contribute to their scarce investigation in NB, specifically in blood, because high amounts of plasma are usually needed for their measurement. We have previously demonstrated that plasma 3-O-methyldopa (3-OMD) is a novel prognostic biomarker of high-risk NB. 3-OMD is the direct metabolite of DOPA produced by the enzyme COMT and found at high concentrations in blood of NB patients with unfavourable NB. High expression of 3-OMD is associated with poor outcome patients with stage M tumor and, among them, it was confirmed as a prognostic factor able to stratify high-risk patients older than 18 months. 3-OMD has never been explored in large prospective studies. The availability of a micromethod would facilitate its investigation.
AIMS: The aim of our study was to develop a novel analytical micro-method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the quantitative determination of 3-OMD from dried plasma spots (DPS) and to analyze 3-OMD in a cohort of patients with localized and metastatic NB.
METHODS: A LC-MS/MS method combined with DPS has been developed for the measurement of 3-OMD starting from 30 µL plasma and validated following current international guideline for bioanalytical method validation. The method has been tested on 60 samples derived from Italian patients with NB (30 with localized and 30 with metastatic NB) and 60 age matched control subjects centralized at Giannina Gaslini Institute.
RESULTS: The method is robust, specific and accurate for the measurement of 3-OMD from very small amounts of dried plasma in a large range of concentrations. 3-OMD in DPS is stable at room temperature for at least two weeks. The analysis of 3-OMD in the new cohort of patients confirms previously reported results.
CONCLUSIONS: The novel method based on DPS and LC-MS/MS offers the advantage of minimizing the volume of plasma and the possibility of storage and delivery at room temperature, thus being potentially adoptable for multi-center studies. The addition of 3-OMD in the panel of catecholamines might be useful for detecting tumors characterized by limited production of downstream metabolites and could offer prognostic added value.

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Prospective multicenter observational study of minimal residual disease detected by 7NB-mRNAs ddPCR assay in high-risk neuroblastoma patients

Nishimura N[1], Ishida T[2], Yokota I[3], Matsumoto K[4], Shichino H[5], Fujisaki H[6], Sarashina T[7], Kamijo T[8], Takimoto T[4], Iehara T[9], Tajiri T[10]

[1]Kobe University, Department of Health Science, Kobe, Japan, [2]Kobe Children's Hospital, Department of Hematology and Oncolgy, Kobe, Japan, [3]Hokkaido University, Department of Biostatics, Sapporo, Japan, [4]National Center for Child Health and Development, Department of Pediatric Oncology, Tokyo, Japan, [5]National Center for Global Health and Medicine, Department of Pediatrics, Tokyo, Japan, [6]Osaka City General Hospital, Department of Pediatrics, Osaka, Japan, [7]Asahikawa Medical University, Department of Pediatrics, Asahikawa, Japan, [8]Saitama Cancer Center, Department of Clinical Research, Saitama, Japan, [9]Kyoto Prefectural University of Medicine, Department of Pediatrics, Kyoto, Japan, [10]Kyushu University of Medicine, Department of Pediatric Surgery, Fukuoka, Japan

Background: Several assays quantitating different sets of neuroblastoma-associated mRNAs (NB-mRNAs) by quantitative PCR (qPCR) or droplet digital PCR (ddPCR) were shown to possess a significant prognostic value for minimal residual disease (MRD) in high-risk neuroblastoma (HR-NB) patients. 7NB-mRNAs ddPCR assay was reported to outperform other qPCR assays by a retrospective in-house observational study. Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG) conducted a prospective multicenter observational study of MRD in HR-NB patients registered in JNBSG clinical trial to assess a prognostic value for MRD in bone marrow (BM-MRD) and peripheral blood (PB-MRD) detected by 7NB-mRNAs ddPCR assay.

Aims: To evaluate the positive and negative predictive values (PPV and NPV) of BM-MRD and PB-MRD detected by 7NB-mRNAs ddPCR assay in a prospective multicenter observational study.

Methods: Between August 2018 and August 2022, 7 HR-NB patients who registered JNBSG clinical trial were enrolled. BM and PB samples were collected as residual samples of standard clinical practice after their completion of JN-H-11/JN-H-15 protocol treatment. 7NB-mRNAs ddPCR assay quantitated the expression of 7 NB-mRNAs (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNAs) and a reference mRNA (HPRT1 mRNA) by a QX200 ddPCR system (Bio-Rad Laboratories) and calculated the level of 7NB-mRNAs (relative copy number) as the weighted sum of 7 relative copy numbers of each NB-mRNA.

Results: A total of 19 BM and 19 PB samples (1-7 BM and 1-7 PB samples per patient) were collected from 7 patients registered at 16-87 (median 33) months after diagnosis. 4/15 BM and 4/15 PB samples were classified as progressive disease (PD)/non-PD samples. BM-MRD and PB-MRD (level of 7NB-mRNAs) ranged 2.0-4,948.8 (median 8.7) and 0.0-52.0 (median 4.6) relative copy number, respectively. BM-MRD and PB-MRD estimated area under curve (AUC) of 0.767 and 0.800, respectively, validating a previous retrospective in-house observational study of BM-MRD (AUC of 0.723). BM-MRD (cut-off value 15) and PB-MRD (cut-off value 13) were estimated to have PPV/NPV of 75%/67% and 100%/73%, respectively.

Conclusion: The present case series have validated a 7NB-mRNAs ddPCR assay and suggested a prognostic value of BM-MRD and PB-MRD.

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COMBINED BLOOD AND BONE MARROW CELL FREE DNA AND DISSEMINATED TUMOR CELL ANALYSIS FOR DISEASE MONITORING AND EARLY RELAPSE DETECTION

Bernkopf M[1,2], Gerber T[1], Repiscak P[1], Bozsaky E[1], Stojanovic I[1,3], Zöscher P[1,2], Glogova E[1], Fiedler S[1,4], Saloberger-Sindhöringer L[1], Rifatbegovic F[1,2], Ambros P[1,2], Ambros I[1,2], Pötschger U[1], Ladenstein R[1], Taschner-Mandl S[1,2]

[1]St. Anna Children's Cancer Research Institute (CCRI), Vienna, Austria, [2]Labdia Labordiagnostik GmbH, Vienna, Austria, [3]TU Wien, Vienna, Austria, [4]St. Anna Children's Hospital, Vienna, Austria

Background

Liquid biopsy (LB) approaches are increasingly applied in cancer diagnostics due to their potential to overcome current challenges in therapy response assessment, minimal residual disease (MRD)-monitoring and early relapse / progressive disease (PD/REL) detection. However in neuroblastoma (NB), tumor heterogeneity and the paucity of recurrent mutations pose hurdles in defining biomarkers for sensitive monitoring in individual patients.

Aims

In this study we combined circulating cell-free (cf)DNA analysis in peripheral blood (PB) and bone marrow (BM) with the detection of disseminated tumor cells (DTC) in BM and assessed their value in evaluating treatment response and detecting relapse.

Methods

509 PB and 315 BM samples from 32 patients with high-risk NB were collected. From 16 patients Next-Generation-Sequencing (NGS, WES) of the tumor or metastasis at diagnosis was performed. BM mononuclear cells were isolated and immunofluorescence-stained for GD2/CD56 positive DTCs using the Automated-Immunofluorescence-Plus-FISH technique (AIPF). CfDNA was isolated from PB and BM plasma and either analyzed with digital droplet (dd)PCR for MYCN, other gene amplifications and ALK hotspot mutations or an NGS custom capture library was prepared and sequenced.

Results

Our study showed a significant reduction of cfDNA markers and similar DTC count kinetics in response to therapy in PB and BM at mid-/end of induction therapy. All three markers were concordantly negative at 80 / 165 evaluation timepoints (TPs) and at 35 equally positive, while in 21 TPs only PB or BM cfDNA was positive and vice versa in 29 TPs MRD was exclusively detected by AIPF analysis. When clinical response evaluation was available, overall response was correlated with response detected in LB. At least one of the LB markers was positive at/before 80% of PD/REL events. Remarkably, in two patients with local PD/REL without BM involvement, PB cfDNA markers were positive six weeks prior clinically confirmed relapse.

Conclusion

The combined analysis of genetic markers and actionable targets in PB and BM plasma cfDNA and DTC enumeration is a highly sensitive approach for monitoring MRD, especially in patients with localized relapse or when clinical evaluation fails to detect PD/REL. Thus, combined LB analysis will be an important component of neuroblastoma diagnostics.

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DETECTION OF MINIMAL RESIDUAL DISEASE (MRD) IN HIGH RISK NEUROBLASTOMA IS ASSOCIATED WITH SURVIVAL OUTCOMES

Gelineau N[1,2], van Zogchel L[1,2], De Carolis B[3], van Wezel E[2], Stutterheim J[1], Zappeij-Kannegieter L[4], Javadi A[4], Schumacher-Kuckelkorn R[3], Simon T[3], Berthold F[3], Fiocco M[1,5,6], van der Schoot E[2], Hero B[3], Tytgat G[1,2]
[1]Princess Máxima Center for pediatric oncology, Research, Utrecht, Netherlands, [2]Sanquin Research and Landsteiner Laboratory of the AMC- University of Amsterdam, Department of Experimental Immunohematology, Amsterdam, Netherlands, [3]Children's Hospital - University of Cologne, Department of Pediatric Hematology and Oncology, Cologne, Germany, [4]Sanquin Research and Landsteiner Laboratory of the AMC- University of Amsterdam, Department of Immunocytology, Amsterdam, Netherlands, [5]Mathematical Institute, Leiden University, Leiden, Netherlands, [6]Leiden University Medical Center, department of Biomedical Data Science, Medical Statistics Section, Leiden, Netherlands

Background and aims: Despite very intense treatment, about 60% of high-rIsk neuroblastoma (NB) patients will suffer from recurrent disease. Specific mRNA detection in bone marrow (BM) by real-time quantitative PCR (qPCR) has been shown to be associated with survival outcome. We now validated in a larger and prospective study, NB-mRNA detection in BM and peripheral blood (PB) in high-risk NB patients, treated in NB2004-HR (GPOH) and NBL2009 (DCOG).
Methods: From 345 patients 723 BM- and 572 PB-samples were collected between 2008 and 2016: at diagnosis, after 2 courses- and end of induction therapy. NB-mRNA detection (PHOX2B, TH, DDC, CHRNA3 and GAP43 or DBH for BM or PB) was compared to BM immunocytology (anti-GD2). Association between BM- and PB-infiltration levels and event-free survival (EFS) and overall survival (OS) was studied.
Results: BM-infiltration >10% by qPCR at diagnosis was prognostic for survival: the adjusted hazard ratio (HR) was 1.82 [95% CI 1.25-2.63] and 2.04 [1.33-3.14] for EFS and OS, respectively. After 2 courses, the adjusted HR for BM-infiltration >1% was 1.79 [1.07-3.00] and 2.39 [1.37-4.15] for EFS and OS respectively. Poor outcome was seen for any post-induction BM-positivity. 5-year EFS and OS (SE) were 27% (±5.2) and 44% (±5.9) for qPCRpositive patients, versus 60% (±6.7) and 66% (±6.6) for qPCRnegative patients ($p<0.0001$). In multivariate Cox regression model, BM-qPCR positivity was associated with EFS and OS, with an HR of 2.10 [1.27-3.49] and 1.76 [1.00-3.08], respectively. The detection of mRNA in PB at diagnosis, after 2 courses- and end of induction therapy, was associated with BM-infiltration levels (rs 0.69, 0.34 and 0.70 respectively). Moreover, in PB diagnostic mRNA-levels was significantly associated with EFS and OS, however to a lesser extent than BM-mRNA. Importantly though, at diagnosis NB-mRNA detection in PB can distinguish survival groups between those patients with >10% BM-infiltration.
Conclusion: This study shows a strong association between BM-infiltration levels determined by qPCR and survival at different time points. At diagnosis, an association between NB-mRNA detection in PB and BM is seen with the level of detection in PB adding to the prediction of survival of patients with an initial BM-infiltration >10%.

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Revival of the catecholamines

Matser Y[1], van Kuilenburg A[2], Tytgat G[1]
[1]Prinses Maxima Centrum for pediatric oncology, Utrecht, Netherlands, [2]Laboratory Genetic Metabolic Diseases, Amsterdam UMC, location University of Amsterdam, Amsterdam, Netherlands

Urinary catecholamine metabolite measurements are part of the diagnostic workup of neuroblastoma. Moreover, catecholamine metabolites are useful for prognostic stratification and may be beneficial for disease monitoring.

Currently both 24-hour urine collection and spot urine sampling and various urinary catecholamine metabolite panels are tested at diagnosis. 24-hour urine collection is well-known for patient discomfort and collection failure rate and the classic metabolites VMA and HVA have a combined diagnostic sensitivity of only 84%. We studied if these disadvantages may be overcome with the use of spot urines and a wider panel of metabolites. First, we showed that spot urine is as reliable as 24-hour urine for the diagnostic use of catecholamine metabolites. Next, we reached a diagnostic sensitivity of 95% with the use of the extended panel of eight catecholamine metabolites including vanillylmandelic acid (VMA), homovanillic acid (HVA), dopamine, 3-methoxytyramine, norepinephrine, epinephrine, metanephrine and normetanephrine.

For the prognostic use of catecholamines metabolites, we showed that elevated levels of 3-methoxytyramine (3MT) are associated with poor survival in patients with any stage of neuroblastoma but also in patient with high-risk neuroblastoma. By comparing gene expression in patients with elevated and non-elevated 3MT levels, we established a 3MT gene signature that correlated with tumor MYC-activity. Future research is needed to investigate the possible relation between catecholamine metabolites and other molecular genetics in the tumor.

By studying catecholamine production in vitro, we established catecholamine profiles that correlate with risk stratification. Furthermore, we established the effect of epithelial to mesenchymal transition, in vitro, and showed that in mesenchymal cells the rate limiting enzymes are downregulated and catecholamine excretion stops. Clinically, in 25% of recurrences, no catecholamine excretion is detected in patients that excreted catecholamines at initial diagnosis.

Finally, VMA and HVA are no longer included in the International Neuroblastoma Risk Group response criteria. However, recent studies described 3MT as a promising new marker for monitoring disease activity. We plan to investigate whether the extended panel of eight catecholamine metabolites is a valuable marker for relapse of disease. Within the SIOPEN Catecholamine working group, we will harmonize the catecholamine metabolite analysis in different centres.

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Bone marrow involvement in patients with high-risk neuroblastoma: a comparison between RTqPCR and [123I]mIBG imaging

Samim A[1,2], Gelineau N[1,3], Elias S[4], de Keizer B[1,2], van Zogchel L[1,3], Blom T[1,3], Zappeij-Kannegieter L[3], Javadi A[3], van der Schoot E[3], van Noesel M[1], Lam M[1,2], Tytgat G[1,3]

[1]Princess Máxima Center for Pediatric Oncology, Research, Utrecht, Netherlands, [2]University Medical Center Utrecht, Department of Radiology and Nuclear Medicine, Utrecht, Netherlands, [3]Sanquin Research, Department of Experimental Immunohematology, Amsterdam, Netherlands, [4]University Medical Center Utrecht, Julius Center for Health Sciences and Primary Care, Utrecht, Netherlands

Background:
Bone marrow (BM) is the most common site of metastasis in patients with neuroblastoma, both at diagnosis and at recurrence. Reverse transcriptase-quantitative PCR (RT-qPCR) using neuroblastoma-specific target genes is a sensitive method for detecting minimal BM-involvement.

Aim:
To compare BM-involvement assessed by RT-qPCR with conventional meta-[^{123}I]iodobenzylguanidine (MIBG) scans in patients with high-risk neuroblastoma.

Methods:
Patients with high-risk neuroblastoma, diagnosed between January 2015 and March 2021, with paired examinations of MIBG imaging and BM sampling (performed within 15 days) at diagnosis and post-induction, were included. SIOPEN scores were collected from MIBG scintigraphy and SPECT-CT (nationally reviewed). BM aspirates from bilateral iliac crests were analyzed by RT-qPCR (adrenergic panel, van Zogchel et al. 2021). Recurrence (progression and relapse) rate was compared for patients with SIOPEN >3 and ≤3 at the end of induction.

Results:
In total, 154 patients with high-risk neuroblastoma were identified (n=7 INSS stage 3, n=145 INSS stage 4) with 142 paired examinations at diagnosis and 70 at post-induction. At diagnosis, BM-involvement was detected by either MIBG scanning or RT-qPCR in 138/142 cases: 107 were MIBG & RT-qPCR positive, 2 only MIBG-positive, and 29 only RT-qPCR-positive. At post-induction, BM-involvement was detected on either MIBG scanning or RT-qPCR in 46/70 cases: 29 were MIBG & RT-qPCR positive, 7 only MIBG-positive, and 10 only RT-qPCR-positive. Interestingly, all nine only-MIBG-positive cases concerned MIBG uptake outside the pelvis. In 27 patients with a post-induction SIOPEN score >3 , 23 were RT-qPCR-positive and 4 RT-qPCR-negative, with 70% and 25% recurrence rate, respectively (p=0.08). In 43 patients with a post-induction SIOPEN score ≤3, 16 were RT-qPCR-positive and 27 RT-qPCR-negative, with 44% and 33% recurrence rate, respectively (p=0.49).

Conclusion:
RT-qPCR detected additional cases of BM-involvement in high-risk patients, both at diagnosis and post-induction. Interestingly, all MIBG-positive, RT-qPCR-negative cases concerned MIBG uptake outside the pelvis. In patients with a post-induction SIOPEN score >3 who were RT-qPCR-negative, only one in four had recurrence, compared to 16/23 patients who were RT-qPCR-positive, although not statistically significant in this small cohort. In future studies, the prognostic relevance of RT-qPCR needs to be assessed in combination with clinical decision-making MIBG scans.

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Wait and see strategy in an adolescent case of bilateral adrenal neuroblastoma.

Tondo A[1], Carra F[1], Conte M[2], Garaventa A[2], Pastorino F[3], Ponzoni M[3], Pezzolo A[2], Mazzocco K[4], Olianti C[5], Perrone A[6], Boggi U[7], Favre C[1]

[1]Department of Pediatric Oncology and Hematology, Meyer Children's Hospital IRCCS, Florence, Italy, [2]IRCCS Istituto Giannina Gaslini, Genova, Italy, [3]Laboratory of Experimental Therapies in Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [4]Department of Pathology, IRCCS Istituto Giannina Gaslini, Genova, italy, [5]Nuclear Medicine Unit, Careggi University Hospital, Florence, Italy, [6]Department of Radiology, Meyer Children's Hospital IRCCS, Florence, Italy, [7]Division of General and Transplant Surgery, University of Pisa, Pisa, Italy

Background: Neuroblastoma occurs rarely in adolescents and ever more in young adults. A more severe prognosis and higher mortality rates have been documented within this population also in localized neuroblastoma.
Case description: Male, 15 years old, with a voluminous left surrenalic mass (cm 17x13x10,5). A CT-guided tumor biopsy was performed: poorly differentiated neuroblastoma, MYCN non-amplified. MIBG-Scintigraphy scan demonstrated uptake only in the left mass. Bilateral bone marrow biopsies revealed no tumor infiltration. WB MRI showed a pathological signal of the right adrenal gland. The patient underwent 3 courses of standard chemotherapy with low dimensional reduction of the tumor. The left adrenal mass was completely resected and also a right tumorectomy was performed. The final pathological evaluation was bilateral adrenal non-metastatic NB of unfavorable histology, MYCN non-amplified. Genomic profile: structural chromosomal abnormalities (19p loss) non-recurrent in neuroblastoma. We chose a close clinical follow-up and imaging surveillance. A complete remission is conserved after 2 years after surgery.
Conclusion: Bilateral adrenal neuroblastoma is very rare. Our case could be considered an L1 NB as a single mass resectable with a second nodular lesion in the contralateral adrenal seat. The 19p loss has been reported in older age NB patients and it correlated with a poor prognosis.
The combination of genetic and radiologic studies, in particular WB MRI with diffusion-weighted sequences, has allowed to develop a tailored treatment plan. We adopted a bilateral adrenal sparing surgery after conventional chemotherapy and a watchful waiting strategy in the absence of life-threatening symptoms.

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Plasma cell-free DNA analysis uncovered tumor heterogeneity and clonal evolution during treatment for relapsed neuroblastoma

Iglesias Cardenas F[1], Kushner B[1], Roberts S[1,2], Basu E[1], Cheung N[1], Modak S[1]
[1]Memorial Sloan Kettering Cancer Center, New York City, United States, [2]OHSU Doernbecher Children's Hospital, Portland, United States

Background: Treatment for relapsed neuroblastoma remains a challenge. Plasma cell-free DNA (cfDNA) has the potential to detect tumor mutations non-invasively and inform neuroblastoma therapy.
Methods: 116 plasma samples were collected from 89 patients with high-risk neuroblastoma (HR-NB) at disease progression after informed consent. CfDNA analysis was done by targeted next-generation sequencing (NGS) using the CLIA-approved Foundation One Liquid assay (324 genes, median exon coverage of 5000X).
Results: 68/89 (76%) patients harbored at least 1 pathogenic genomic alteration in their cfDNA (mean=2, range 0-21). Recurring alterations for the entire cohort included MYCN amplification (n=9 patients), TP53 mutations (n=18), ALK mutations (n=17), RAS-MAPK signaling pathway mutations (KRAS n=3, NRAS n=6, BRAF n=5, PTPN11 n=7, NF1 n=11, FGFR1 n=6); and mutations and/or promotor alterations in genes involved in chromatin remodeling (ATRX n=7 ARID1A, n= 2, TERT n=3 and ATM n= 3). The Comprehensive Tumor Fraction (cTF) was available in 45/89 patients. CTF approximates the fraction of detected circulating tumor DNA (ctDNA). In cfDNA-positive patients, MIBG score correlated with cTF (Spearman's p=0.31, P=0.041). NGS data was available on the primary tumor (prior to relapse) for 54 patients. In 26/54 (48%) patients, we detected mutations unique to the relapse cfDNA sample, and in 11/54 (20%) patients, cfDNA mutations were shared with the prior to relapse tumor sample. Therapy based on cfDNA analysis was instituted in 16 patients (using inhibitors of ALK, MEK in 14, and 2 patients, respectively). 21 patients had sequential cfDNA samples; new mutations were detected as disease progressed, enriched for the RAS-MAPK (n=6) and ALK (G1202R, n=1) pathways among others.
Conclusions: CfDNA analysis detected genomic aberrations in most patients with HR-NB at relapse. Such analysis permits a relatively noninvasive and safe profiling of tumor heterogeneity and clonal evolution, with the potential to guide therapy. cfDNA is a viable alternative for studying mutations when soft tissue is unavailable or if disease is limited to skeletal sites. cfDNA monitoring should be validated in prospective trials for HR-NB.

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Cell cycle dynamics of ASCL1 in neuroblastoma cells

Beckman W[1], Philpott A[1,2]

[1]Wellcome Trust/Medical Research Council Cambridge Stem Cell Institute, University of Cambridge, Cambridge, United Kingdom, [2]Department of Oncology, University of Cambridge, Hutchison/MRC Research Centre, Cambridge Biomedical Campus, Cambridge , United Kingdom

Pro-neural factor ASCL1 exerts dual roles in driving both differentiation and proliferation1. This applies to cancer, where for some tumour types ASCL1 is associated with increased stem-ness and poor prognosis, while in neuroblastoma, we have previously shown that overexpression of ASCL1 is sufficient to drive neuronal differentiation2. Given that ASCL1 activity is partly regulated by CDK mediated multi-site phosphorylation, it seems likely that ASCL1 acts in a cell cycle dependent manner, and that the balance of ASCL1 activity between the phases may influence a cells decision to proliferate or differentiate. Using the FUCCI cell cycle reporter system, we show that the ASCL1 protein level in proliferative BE2C neuroblastoma cells is itself cell cycle dependent. We show that G1 and G2M cells show similar overall levels of accessibility, but that G1 specific ATAC peaks are associated with enhancers, while G2M specific ATAC peaks are associated with promoters, and ASCL1 binding overlaps with these G2M specific accessible regions. We have subsequently performed ChIP-seq and SLAM-seq on cell cycle synchronised BE2C cells, and cells overexpressing degron tagged ASCL1, allowing for its cell cycle dependent degradation, two complementary approaches that reveal how ASCL1 acts throughout the cell cycle. Fully understanding this mechanism during neuroblastoma cell proliferation sets the stage for addressing how ASCL1 functions once the signal to differentiate has been received.

References

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