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ABSTRACT BOOK

ORAL PRESENTATIONS IN PARALLEL SESSIONS

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O1.1

ATRX alterations mediate an immunogenic phenotype and macrophage infiltration in neuroblastoma.

Lorenzi F[1], Gao Q[2], Martins da Costa B[1], Tall J[1], Barker K[1], Gorrini C[1], Hutchinson C[3], Barone G[3], Anderson J[3], George S[1], Chesler L[1]

[1]The Institute of Cancer Research, Division of Clinical Studies, Paediatric Tumour Biology, London, United Kingdom, [2]The Institute of Cancer Research, Cancer Therapeutics Unit, Computational Biology and Chemogenomics, London, United Kingdom, [3]Great Ormond Street Hospital for Children NHS Foundation Trust, London, United Kingdom

Parallel session 1: Genetic defects and dependencies in neuroblastoma, May 15, 2023, 10:45 AM - 11:35 AM

Background
ATRX mutations occur in 11% of neuroblastoma and include in-frame multi-exon deletions, loss-of-function (LoF) and missense mutations. ATRX mutations are mutually exclusive with MYCN amplification and associate with alternative lengthening of telomeres (ALT). ATRX mutant neuroblastoma presents as slow-growing, metastatic disease that is often resistant to chemotherapy. Our aim is to elucidate mechanisms responsible for growth and maintenance of ATRX mutant neuroblastoma.

Methods
CRISPR-Cas9 was used to induce ATRX-LoF mutations in neuroblastoma cell lines. ALT status was determined by c-circle assay. RNA-sequencing was performed to identify deregulated pathways among ATRX-mutated lines, and publicly available RNA-sequencing data of primary neuroblastomas also analysed. Evaluation of neuroblastoma cell-lines by cytokine assay was performed and the tumour microenvironment of xenografts and patient samples was evaluated by histopathological assessment and spatial phenotypic analysis with the Akoya PhenoCycler.

Results
ATRX LoF mediated activation of ALT in NBL-S cells. RNA sequencing identified significant up-regulation of the epithelial-to-mesenchymal transition pathway, reduction of the adrenergic score and up-regulation of several inflammatory pathways, including inflammatory response, interferon gamma and interferon alpha pathways in ATRX-LoF cell lines. Up-regulation of the same pathways in a cohort of patient samples with different types of ATRX mutations was also identified in published patient datasets.
Concordant with this, there was a significant positive correlation between ATRX mutations and the recently identified immunogenic gene signature in both cell lines and patient samples. Subgroup analysis also showed a significant association between the immunogenic score and the presence of an ATRX multi-exon deletion (the most frequent type of ATRX alteration seen in neuroblastoma).
Cytokine array showed secretion of Serpin E1 and CCL2 in ATRX-LoF cell lines, which have both been reported to recruit pro-tumoral macrophages. Spatial phenotypic analysis of paired ATRX wildtype and LoF xenografts confirmed a significantly greater presence of macrophages in ATRX-LoF models. The presence of macrophage infiltration was confirmed in ATRX mutant neuroblastoma patient samples.

Conclusion
Our results suggest that ATRX mutations drive tumour cell changes that result in macrophage recruitment. Tumour-immune microenvironmental changes as a result of ATRX mutations are likely to contribute to the distinct clinical phenotype in this poor outcome group.

Sally L George and Louis Chesler equally contributed to this work.

O1.2

Distinct molecular phenotypes and genetic dependencies within ATRX aberrant neuroblastoma tumors

Van Den Boogaard M[1], van Gerven M[1], Schild L[1], van Arkel J[1], Broeils L[1], Meijs L[1], van Oosterhout R[1], DeMartino J[1], Essing A[1], Koopmans B[1], Ober K[1], Amo-Addae V[1], Eising S[1], van Noesel M[1,2], Drost J[1], Koster J[3], van Hooff S[1], Molenaar J[1,4]

[1]Princess Maxima Center for Pediatric Oncology, Utrecht, Netherlands, [2]Department of Cancer and Imaging, University Medical Center Utrecht, Utrecht, Netherlands, [3]Department of Oncogenomics, Amsterdam UMC, Location UMC, Netherlands, [4]Department of Pharmaceutical Sciences, Utrecht University, Faculty of Science, Netherlands

Parallel session 1: Genetic defects and dependencies in neuroblastoma, May 15, 2023, 10:45 AM - 11:35 AM

Background

The chromatin remodeler ATRX is mutated in 8-10% of high-risk neuroblastomas and patients with ATRX mutated tumors have a poor prognosis. Aberrations in ATRX are strongly associated with Alternative Lengthening of Telomeres. In neuroblastoma multi-exon deletions (MEDs) are the dominant type of ATRX aberration in neuroblastoma. Of these MEDs 75% are predicted to produce in-frame fusion (IFF) proteins, suggesting a potential gain-of-function compared to nonsense mutations. These mutation types could be molecularly very different and might need distinct therapies.

Aims

Identify the molecular phenotypes and genetic dependencies of different ATRX alterations

Methods

Using CRISPR-Cas9 genome editing we created an extensive amount of isogenic ATRX knock-out (KO) and several distinct in-frame MEDs, including the most common MED of exon 2-10, in several neuroblastoma cell line and organoid models. RNA profiles of these models were compared with data from patient-derived ATRX MED cell lines and tumors. Furthermore, we performed CRISPR synthetic lethality screens on an isogenic ATRX KO model, on two patient-derived ATRX MED models and on two ATRX wild-type cell lines.

Results

RNA profiling showed little overlap in differential expressed genes between the isogenic ATRX exon 2-10 MED models and ATRX KO models. Gene set enrichment analysis identified decreased expression of genes related to ribosome biogenesis and metabolic processes in our isogenic ATRX exon 2-10 MED models and in patient-derived ATRX MED cell lines and tumors. Remarkably, our isogenic ATRX KO models showed increased expression of these processes. Similarly, our CRISPR screens identified little overlap in synthetic lethal genes between an isogenic ATRX KO model and two patient-derived ATRX MED models. Interestingly, the ATRX MED models are highly dependent on the ATRX IFF, which implies a gain-of-function. We also identified dependencies on PARP1 and REST, which were both reported as potential therapeutic targets against ATRX MED neuroblastoma cells. Finally, several new synthetic lethal interactions for potential compound interventions were identified among which KDM6A and GSG2.

Conclusion

Our results indicate that different ATRX alterations induce distinct expressions patterns and dependencies, which should be considered for therapy development. Our CRISRP screens indicate some interesting potential targets including ATRX IFF, KDM6A and GSG2.

O1.3

DNA Damage Repair Deficiency Enhances Neuroblastoma Progression and In vivo Sensitivity to PARP Inhibition in Zebrafish

Hayes M[1,2], Cohen-Gogo S[3,4], Layeghifard M[3], Ladumor Y[5], Valencia Sama I[5], Kee L[5], Kaplan D[2,6], Villani A[4,7], Shlien A[3,8], Morgenstern D[4,7], Irwin M[4,5,7]

[1]The Hospital for Sick Children Research Institute, Developmental and Stem Cell Biology, Toronto, Canada, [2]University of Toronto, Department of Molecular Genetics, Toronto, Canada, [3]The Hospital for Sick Children Research Institute, Genetics and Genome Biology, Toronto, Canada, [4]Hospital for Sick Children, Division of Hematology/Oncology, Toronto, Canada, [5]The Hospital for Sick Children Research Institute, Cell Biology, Toronto, Canada, [6]The Hospital for Sick Children Research Institute, Neurosciences and Mental Health, Toronto, Canada, [7]University of Toronto, Department of Pediatrics, Toronto, Canada, [8]Hospital for Sick Children, Department of Pediatric Laboratory Medicine, Toronto, Canada

Parallel session 1: Genetic defects and dependencies in neuroblastoma, May 15, 2023, 10:45 AM - 11:35 AM

Background: Significant progress has been made in identifying mutations and defining biological heterogeneity in neuroblastoma (NB) using high-throughput sequencing approaches. However, the functional roles for many of these specific genetic alterations in NB tumor initiation, growth and metastasis remain to be defined. We recently reported that next-generation sequencing studies of more than 300 patients (348 tumour samples) enrolled in the SickKids' Cancer Sequencing Program (KiCS) revealed significant somatic and inherited genetic variants in genes encoding proteins involved in DNA damage repair (DDR). In our NB cohort, alterations in genes involved in DDR pathways were detected in approximately 30% of patients at diagnosis and relapse, as well as a significant presence of single-substitution signature 3 (SBS3; BRCAness mutational signature). Despite being well studied in many adult cancers, the role for DDR disruption in NB pathogenesis, as well as potential therapeutic implications, remains poorly understood.

Aims: We aimed to investigate the role for DDR-deficiency in NB progression in vivo and define the therapeutic potential of PARP inhibitors in targeted tumor growth inhibition based on NB genetic profiles.

Methods: Using the established zebrafish MYCN transgenic model (Tg(dbh:EGFP-MYCN)), we incorporated patient-relevant loss-of-function mutations in DDR pathway components and visualized tumor onset, growth and metastasis in vivo.

Results: Somatic and germline transmissible CRISPR/Cas9-mediated knock-out of DDR pathway variants, including brca2, atm, palb2, and bard1 were found to increase the penetrance of zebrafish MYCN-induced NB, and enhance metastasis in tumor-burdened animals. Furthermore, DDR-deficient MYCN-induced zebrafish NB were sensitive to the PARP inhibitor Olaparib in combination with Temozolomide (TMZ) treatment, supporting pre-clinical utility for this regimen in DDR-deficient NB.

Conclusions: Taken together, our data support a role for certain DDR pathway variants in promoting NB tumorigenesis and metastasis as well as rationale for targeted PARPi therapy for the treatment of high-risk NB patients with DDR pathway alterations.

O1.4

ALK ligand ALKAL2 potentiates MYCN-driven neuroblastoma in the absence of ALK mutation

Borenäs M[1], Umapathy G[1], Lai W[1], Lind D[1], Witek B[2], Guan J[1], Masudi T[1], Claeys A[3], Chuang T[1], El Wakil A[2], Arefin B[1], Fransson S[4], Koster J[5], Johansson M[6], Gaarder J[4], Van den Eynden J[3], Hallberg B[1], H Palmer R[1]
[1]University of Gothenburg, Department of Medical Biochemistry and Cell Biology, Gothenburg, Sweden, [2]Umeå University, Department of Molecular Biology, Umeå, Sweden, [3]Ghent University, Department of Human Structure and Repair, Ghent, Belgium, [4]University of Gothenburg, Laboratory Medicine, Gothenburg, Sweden, [5]University of Amsterdam, Department of Oncogenomics, Amsterdam, The Netherlands, [6]University of Gothenburg, Clinical Genomics, Gothenburg, Sweden

Parallel session 1: Genetic defects and dependencies in neuroblastoma, May 15, 2023, 10:45 AM - 11:35 AM

Background/Aims
MYCN amplification, located on chromosome 2p, is hallmark of high-risk neuroblastoma. The 2p region is also the home of the ligand to ALK, ALKAL2 as well as ALK itself and is subjected to gain or amplification in 14-30% of the neuroblastoma population. Activating point mutations in ALK are found in 8-10% of primary neuroblastoma and several cases of effective therapeutic use of ALK inhibitors have been described. Here we explore the role of ALKAL2 in 2p-gain neuroblastoma in a preclinical setting, employing mouse models and cell lines, asking whether misexpression of the ALK ligand ALKAL2 is able to drive neuroblastoma in the absence of activating mutations in ALK.

Methods
The ubiquitously expressing Rosa 26 locus was used to introduce the Alkal2 transgene (Rosa26_Alkal2). Tumor penetrance was monitored after Rosa26_Alkal2 mice were crossed with the well-established Th-MYCN neuroblastoma mouse model. Treatment of tumor bearing Rosa26_Alkal2;Th-MYCN mice was initiated with either vehicle or the ALK inhibitor lorlatinib and tumors were subsequently subjected to IHC and RNA-seq analyses. Furthermore, neuroblastoma cell lines were utilized to characterize signaling events induced by ALKAL2 by phosphor-/total proteomics and transcriptomics.

Results
Rosa26_Alkal2;Th-MYCN displayed almost complete penetrance with a survival curve comparable to the survival curve of Alk-F1178S;Th-MYCN mice. Neuroblastoma was confirmed in our mouse models by IHC staining for neuroblastoma markers as well as comparison of the Rosa26_Alkal2;Th-MYCN tumor transcriptome with transcriptomes of human cancers. Significantly, cell lines derived from Alk-F1178S;Th-MYCN tumors as well as Alk-F1178S;Th-MYCN tumors were sensitive to ALK-inhibition.

Conclusions
Our preclinical data suggests that misregulated ALK ligand expression can drive neuroblastoma development through activation of ALK (Borenas et al., 2021). Thus, ALK inhibitors may benefit neuroblastoma patients with misregulated ALK ligand expression, such as neuroblastoma patients with 2p-gain. This is supported by a recent case report where a patient with 2p-gain and an ALKAL2 variant exhibited sustained response to entrectinib (ALK and TRKA inhibitor) (Treis et al., 2022).

O2.1

Outcomes for patients aged 12-18 months with metastatic MYCN non-amplified neuroblastoma and unfavorable biologic features ('Mixed Biology Toddlers')

Taylor M[1,2], Kao P[3], Park J[4], Irwin M[5], Applebaum M[6], Pinto N[1,2], London W[3], Cash W[7]

[1]University of Washington, Department of Pediatrics, Division of Hematology/Oncology, Seattle, United States, [2]Ben Towne Center for Childhood Cancer Research, Seattle Children's Research Institute, Seattle, United States, [3]Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Harvard Medical School, Boston, United States, [4]Department of Oncology, St. Jude Children's Research Hospital, Memphis, United States, [5]Department of Pediatrics, Hospital for Sick Children, Toronto, Canada, [6]Department of Pediatrics, University of Chicago, Chicago, United States, [7]Department of Pediatrics, Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Emory University School of Medicine, Atlanta, United States

Parallel sessions 2: Treatment strategies for low risk NB, May 15, 2023, 10:45 AM - 11:35 AM

Background: Risk-stratification is a central paradigm in neuroblastoma, facilitating improved outcomes through selective therapy intensification or de-escalation. For patients with rare phenotypes, there are limited data to guide treatment. Toddlers (age 365-<547 days) with metastatic disease that is MYCN non-amplified (MYCN-NA) but with at least one unfavorable biologic feature including either unfavorable histology, diploid DNA index, or segmental chromosome aberration ('mixed biology toddlers') are not treated uniformly by international consortia and have been excluded from some clinical trials. A deeper understanding of outcomes and prognostic factors is necessary to inform optimal treatment of these patients.

Aims: Using the International Neuroblastoma Risk Group (INRG) Data Commons, we aimed to 1) describe the event-free survival (EFS) and overall survival (OS) for newly diagnosed toddlers with mixed biology tumors, 2) to compare outcomes for toddlers with mixed biology tumors versus those with all favorable biologic features, and 3) to describe the outcome for other clinically-defined subgroups, including by mitotis-karyorrhexis index (MKI), differentiation, lactate dehydrogenase (LDH), ferritin, sex, and race.

Methods: Kaplan-Meier curves of EFS and OS were generated for each of the cohorts and compared using a log rank test. Hazard ratios with 95% confidence intervals were calculated using Cox proportional hazard regression models to describe the prognostic strength of histology, ploidy, MKI, grade, LDH, ferritin, sex, and race.

Results: We identified 441 toddlers with metastatic, MYCN-NA disease with known survival data, including 156 with at least one unfavorable biologic feature and 26 with all favorable biologic features. The 5-year EFS and OS (± standard error) for the entire cohort were 76±2.1% and 81±1.96%, respectively. The 5-year EFS for mixed biology toddlers was 74±3.7% vs. 88±6.3% for those with all favorable biologic features (p=0.12); 5-year OS was 78±3.5% vs. 100% (p=0.01). Toddlers with MYCN-NA metastatic disease and the following features had lower OS (p<0.05) compared to other subgroups: unfavorable histology, diploid, int/high MKI, 1p LOH, and LDH ≥1400 U/L.

Conclusion: Toddlers with mixed biology had lower OS compared to toddlers with favorable biology metastatic disease. Future analyses will incorporate the effect of treatment to re-evaluate which subgroups warrant inclusion in HRNB clinical trials.

O2.2

Short term motor outcomes for patients with spinal canal invasion in neuroblastoma – a SIOPEN prospective study

Ash S[1], Fragola M[2], Sorrentino S[3], Plantaz D[4], Schiff I[4], Hero B[5], Simon T[5], Kachanov D[6], Shamanskaya T[6], Kraal K[7], Littooij A[7], Wieczoreck A[8], Balwierz W[8], Laureys G[9], Trager C[10], Sertorio F[11], Beck-Popovic M[12], De Bernardi B[3], Haupt R[13], Trahair T[14]

[1]Joan and Sanford Weill Paediatric Haematology Oncology and Bone Marrow Transplantation Division, Ruth Rappaport Children's Hospital, Rambam health care campus, Haifa, Israel, [2]Scientific Directorate, IRCCS Istituto Giannina Gaslini, Genova, Italy, [3]Paediatric Oncology Unit, IRCCS Istituto Giannina Gaslini, Genova, Italy, [4]Department de Paediatrics, Hôpital Couple Enfants, CHU Grenoble, France, [5]Department of Paediatric Haematology and Oncology, Children's Hospital, University of Cologne, Cologne, Germany, [6]Department of Clinical Oncology, Dmitry Rogachev National Medical Research Center of Paediatric Haematology, Oncology and Immunology, Moscow, Russian Federation, [7]Princess Màxima Centre for Paediatric Oncology, Utrecht, The Netherlands, [8]Paediatric Oncology and Haematology Department, Institute of Paediatrics, Jagiellonian University, Krakow, Poland, [9]Department of Paediatric Haematology-Oncology, Prinses Elisabeth Kinderziekenhuis, University Hospital, Gent, Belgium, [10]Women`s and Childrens Health, Karolinska Institutet, Stockholm, Sweden, [11]Radiology Unit, IRCCS Istituto Giannina Gaslini, Genova, Italy, [12]Centre Hospitalier Universitaire Vaudois, Unité d'Hémato-oncologie pédiatrique, Lausanne, Switzerland, [13]DOPO Clinic, Department of Paediatric Haematology and Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy, [14]Kids Cancer Center, Sydney Children's Hospital; School of Women's & Children's Health; Children's Cancer Institute, Lowy Cancer Research Centre, University of New South Wales, Sydney, Australia

Parallel sessions 2: Treatment strategies for low risk NB, May 15, 2023, 10:45 AM - 11:35 AM

Background: Patients with neuroblastoma and spinal canal invasion (NB-SCI) have excellent survival but are at risk of functional sequelae, of which motor deficit (MD) is the most frequent.
Aim: Describe the short-term motor outcome following NB-SCI.
Methods: First international SIOPEN prospective registry collecting presenting and follow-up data at standardized timepoints on patients with NB-SCI. Front line treatment approach was decided by the local institutions.
Results: Between 2014-2022, 221 evaluable patients (median age 11 months) were registered from 16 countries. At least one symptom was present in 147 (67%) patients, which was mild in 20%, moderate in 32%, severe in 48%. MD was present in 116 (52%) patients which was mild in 12%, moderate in 14%, and severe in 26%.
Initial therapy included chemotherapy (n=159; 72%), neurosurgery (n=41; 18%), extraspinal tumour resection (n=13, 6%), wait-and-see (n=7; 3%) or radiotherapy (n=1; 1%). Symptomatic patients were more likely to undergo neurosurgery (n=34; 23%) than asymptomatic ones (n=7; 9%, p=0.014). Patients with moderate/severe symptoms were more likely to receive neurosurgery (32/118, 27%) than mildly symptomatic ones (2/29, 7%, p=0.041). Further treatment was delivered to 54 patients (24%) which was driven by stage and histo-biological features in 45 (83%), and by persistent or worsening symptoms in 9 (17%). It included neurosurgery (n=14), chemotherapy (n=32), radiotherapy (n=1) or extraspinal tumour resection (n=7). Follow-up at 72 hours, 1, 2, 4 weeks and 2 months after diagnosis documented a progressive MD improvement in most cases, however it worsened or appeared de novo in a few. At the 2 month endpoint, among symptomatic patients MD had disappeared in 34 (29%), improved in 41 (36%), remained unchanged in 35 (30%) and worsened in 6 (5%). The MD prevalence was 39% (n=86) which was mild in 20%, moderate in 9%, and severe in 10% of patients.
Conclusion: This prospective study documents that MD is present at diagnosis in 52% of NB-SCI patients which reduces to 39% after 2 months. Improvement may be slow but also worsening may appear.
A multivariable approach will evaluate the impact on clinical outcome based on risk factors at diagnosis and during follow-up, adjusted for treatment decisions.

O2.3

MYCN amplification in INSS stage 1 tumors: is there a need for treatment?

Fiedler C[1,2], Theißen J[1,3,4], Fischer M[1,3,4], Borkhardt A[5], Christiansen H[6], Classen C[7], Gruhn B[8], Klingebiel T[9], Leipold A[10], Pekrun A[11], Reinhard H[12], Eggert A[13], Lode H[14], Berthold F[1], Simon T[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]Department of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, [3]Department of Experimental Pediatric Oncology, University Children's Hospital of Cologne, Medical Faculty, University of Cologne, Center for Molecular Medicine Cologne (CMMC), Medical Faculty, University of Cologne, Germany, [4]Center for Molecular Medicine Cologne (CMMC), Medical Faculty, University of Cologne, Cologne, Germany, [5]Department of Pediatric Oncology, Hematology and Clinical Immunology, Medical Faculty, Heinrich Heine University Düsseldorf, Düsseldorf, Germany, [6]Department of Pediatric Oncology and Hematology, University of Leipzig, Leipzig, , [7]Division of Pediatric Oncology, Hematology and Palliative Medicine Section, Department of Pediatrics and Adolescent Medicine, University Medicine Rostock, Rostock, Germany, [8]Department of Pediatrics, Jena University Hospital, Jena, Germany, [9]Department for Children and Adolescents Medicine, University Hospital Frankfurt, Frankfurt, , [10]Pediatric Oncology, Children's Hospital Karlsruhe, Karlsruhe, Germany, [11]Department of Pediatric Hematology and Oncology, Hospital Bremen-Mitte, Bremen, Germany, [12]Department of Pediatric Hematology and Oncology, Asklepios Hospital Sankt Augustin, St.Augustin, Germany, [13]Department of Pediatric Hematology and Oncology, Charité -Universitätsmedizin Berlin, Berlin, Germany, [14]Department of Pediatric Oncology and Hematology, University Medicine Greifswald, Greifswald, Germany

Parallel sessions 2: Treatment strategies for low risk NB, May 15, 2023, 10:45 AM - 11:35 AM

Background: Amplification of the MYCN-oncogene (NMA) is associated with poor prognosis, even in localized neuroblastoma, and qualifies for intensive treatment. However, high-risk treatment in completely resected unilateral tumours without locoregional metastases (INSS stage 1) is discussed controversially.
Aim: We were interested in the course of disease in patients with INSS stage 1 completely resected tumors with MYCN amplification.
Methods:
Patients with MYCN-amplified INSS stage 1 registered in the German Neuroblastoma Trials between 1990 and 2016 were included. Besides complete resection, no further treatment was scheduled between 1990 and 1995, but starting in 1995, MYCN amplification qualified for the high-risk arm of the respective trial in INSS stage 1.
Results: Among 3,763 neuroblastoma patients, MYCN was assessed in 3,356, and amplified in 604 patients (18%). Of those, only twelve patients (0,4%) had INSS stage 1. After tumour resection, five patients underwent systemic treatment, while seven patients received no further treatment.
Of the five patients with systemic frontline treatment, four patients were treated according to the high-risk arms of the respective trials including high-dose chemotherapy, one patient received only four cycles chemotherapy. None of those patients experienced a relapse (last follow up 17 months to 23 years after initial diagnosis).
Of the seven patients without systemic frontline treatment, six patients experienced a relapse, which was locoregional in four and combined in two patients. Of note, four of six relapses occurred within the first six months after resection. For relapse, all six patients received high-risk treatment, including high-dose chemotherapy in three, mIBG-therapy in one, retinoic acid treatment in one and immunotherapy in three patients. One patient died from subsequent progression. The other patients are alive without having experienced further relapses 13 to 20 years after diagnosis.
Conclusion: INSS stage 1 NMA patients who underwent post-operative observation are at very high risk of relapse. However, we found similar long-term outcome between patients who underwent pre-emptive high-risk therapy and those who received treatment after progression. Therefore, close monitoring without systemic treatment seems possible in this special subgroup given the good outcome after high-risk treatment at relapse.

O2.4

Polyclonal lymphoid expansion drives paraneoplastic autoimmunity in neuroblastoma Associated with Opsoclonus Myoclonus Ataxia Syndrome (OMAS)

Rosenberg M[1], Greenstein E[2], Buchkovich M[3], Peres A[4], Santoni-Rugiu E[5], Yang L[6], Mikl M[7], Vaksman Z[8], Gibbs D[9], Reshef D[2], Salovin A[10], Irwin M[12], Naranjo A[13], Ulitsky I[11], de Alarcon P[14], Matthay K[15], Weigman V[3], Yaari G[4], Panzer J[10], Friedman N[2], Maris J[16]

[1]Hebrew University of Jerusalem, Givat Ram; Department of Genetics, Jerusalem, Israel, [2]Weizmann Institute of Science, Department of Immunology, Rehovot, Israel, [3]Q2 Solutions, Morrisville, North Carolina, USA, [4]Bar Ilan University, Faculty of Engineering, Ramat Gan, Israel, [5]Rigshospitalet, Department of Pathology, University Hospital , Copenhagen, Danmark, [6]Pacific Northwest Research Institute, Seattle, Washington, USA, [7]University of Haifa, Department of Human Biology, Haifa, Israel, [8]New York Genome Center, New York, New York, USA, [9]Insitute for Systems Biology, Seattle, Washington, USA, [10]Children's Hospital of Philadelphia, Division of Neurology, Department of Pediatrics, Philadelphia, Pennsylvania, USA, [11]Weizmann Institute of Science, Department of Biological Regulation, Rehovot, Israel, [12]Hospital for Sick Children, Department of Pediatrics and Division of Hematology-Oncology, , Toronto, Ontario, Canada, [13]Department of Biostatistics, University of Florida, Children's Oncology Group Statistics & Data Center, Gainesville, Florida, USA, [14]University of Illinois School of Medicine, Department of Pediatric Hematology/Oncology, Peoria, Illinois, USA, [15]UCSF School of Medicine, Department of Pediatrics, San Francisco, California, USA, [16]Children's Hospital of Philadelphia, Department of Pediatrics and Division of Oncology, Philadelphia, PA, USA

Parallel sessions 2: Treatment strategies for low risk NB, May 15, 2023, 10:45 AM - 11:35 AM

Background: OMAS is a paraneoplastic disorder affecting about 2% of children with neuroblastoma. It is associated with excellent tumor-related outcomes but devastating neurological sequelae impacting cerebellar and brain stem functions.

Aim: To discover the molecular basis of OMAS and its adaptive immunological recognition of neuroblastoma as a window into novel therapeutic strategies.

Methods: We retrospectively procured all primary tumor samples (N=39) available from the COG ANBL00P3 clinical trial testing the efficacy of IVIg in neuroblastoma patients with OMAS (de Alarcon, 2018) and 13 low- and 13 high-risk tumor samples from neuroblastoma patients without OMAS as a comparator. All samples were subjected to immunohistochemical analysis, bulk RNA sequencing (Illumina RNA Access), and TCRbeta and IgH repertoire analyses using the Adaptive Immunoseq platform. Finally, we carried out an association study to identify HLA alleles enriched in neuroblastoma patients with OMAS compared to neuroblastoma patients without OMAS symptoms.

Results. OMAS associated tumors exhibited higher expression of cell type and activation markers of both T and B lymphocytes compared to control neuroblastomas. Differential expression analysis revealed upregulated memory B cell-, Th17-, and T cell activation networks in OMAS, while a developmental/proliferative network and an extracellular matrix network were reduced in OMAS. Histologically, more OMAS-associated tumors than controls were enriched for tertiary lymphoid structures, sites of T:B cell interaction which are positive prognostic indicators in many adults cancers. However, unexpectedly, we observed striking diversity and reduced clonal expansions in both T and B cell repertoires in OMAS compared to controls. We confirm and extend prior observations of MHC Class II alleles significantly associated with OMAS (HLA-DRB*01:01, HLA-DOB*01:01), but found no MHC Class I alleles enriched, implicating CD4+ T cells and/or B cells in OMAS neuropathology and tumor immunity.

Conclusions: The absence of dominant clones of either T or B cells in OMAS tumors suggests no single antigen as a driver of the anti-tumor immune response. Instead, polyclonal B cells essential for OMAS neuroimmunity, localized to TLS with activated T cells, and enriched MHC Class II allele expression, are critical parameters of the OMAS immune process underlying both tumor restriction and brain neuropathology.

O3.1

Single-cell analysis of heterogeneity in a neuroblastoma relapse model in vivo

Westerhout E[1], Stroeken P[1], Hoyng L[2], Lakeman A[1], Volckmann R[2], Chan A[1], Wolthuis R[1], Koster J[2], Versteeg R[3], van Nes J[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

Parallel Sessions 3: Plasticity in neuroblastoma and normal development, May 15, 2023, 3:30 PM - 4:20 PM

Background
Most high-risk neuroblastoma respond to therapy by complete clinical remission, but frequently relapse as therapy resistant disease. Both genetic and non-genetic mechanisms have been proposed to contribute to relapse development. Neuroblastoma displays intra-tumor heterogeneity and includes a majority of lineage-committed adrenergic (ADRN) tumor cells and a minor population of immature mesenchymal (MES) tumor cells. MES-type cells are more resistant to chemotherapy, suggesting a role in relapse development. Recently, we showed in a neuroblastoma in vivo model that relapses can be delayed by inclusion of the MES-specific TRAIL in addition to current treatment modalities.

Aims
First, to characterize phenotype(s) and dependency-pathways of drug-resistant tumor cells that exist during relapse development by single-cell RNA sequencing. And secondly, to identify candidate drug targets to test in combination with the primary treatment in the neuroblastoma relapse model.

Methods
SK-N-SH-GFP+ xenografts were treated with a six-weeks Lorlatinib treatment. Discontinuation of treatment led to tumor regrowth over the course of several weeks. Single, viable and GFP-positive tumor cells were isolated from the relapses, used for single-cell mRNA sequencing (10xGenomics technology) and analyzed on the R2 genomics analysis and visualization platform (http://r2.amc.nl/).

Results
We successfully generated four different single-cell RNA libraries from three relapsed tumors. We obtained RNA sequencing data from 8363 single cells, with a median of 11077 reads per cell. Bio-informatic processing and UMAP analysis revealed several clusters. Interestingly, we found that these clusters of cells were distinct in their expression of mesenchymal (MES) and adrenergic (ADRN) gene signatures. We confirmed the tumor cell identity by mapping the expressed reads of the GFP transgene. In addition, we confirmed the SK-N-SH identity of the MES-type cluster through identification of SK-N-SH specific SNVs in the expressed reads. Through differential gene expression analysis and gene signature analysis we were able to detect a range of cell types in the relapsed tumors and new candidates for specific surface markers or targeted inhibition.

Conclusion
We conclude that our in vivo experimental set-up is functional for the analysis of relapse development and can identify divergent neuroblastoma cell types in vivo through single cell RNA-sequencing.

O3.2

Epigenetically manifested regulatory networks in three major neuroblastoma subtypes

Henrich K[1], Okonechnikov K[2], Wehrmann L[1], Jansky S[1], Westermann F[1]
[1]Hopp-Children's Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Germany, [2]Hopp-Children's Cancer Center (KiTZ) & German Cancer Research Center (DKFZ), Pediatric Neurooncology, Heidelberg, Germany

Parallel Sessions 3: Plasticity in neuroblastoma and normal development, May 15, 2023, 3:30 PM - 4:20 PM

Background
In high-risk neuroblastoma known genetic aberrations, like amplification of MYCN, only partially explain its aggressive tumor behavior, and evidence is accumulating that epigenetic deregulation plays a prominent role in neuroblastoma pathogenesis. The role of DNA methylation in neuroblastoma biology has been mainly addressed by low-resolution methods, which only partially allowed in-depth investigation of regulatory mechanisms including altered transcription factor binding profiles.

Aims
Elucidate the regulatory and developmental role of DNA methylation in neuroblastoma via an integrated genome-wide approach.

Methods
Whole-genome bisulfite sequencing (WGBS), RNAseq and Chromatin ChIPseq were performed in MYCN-amplified, TERT-rearranged, ALT-positive and low-risk primary neuroblastomas (n=51) as well as in drug-demethylated MYCN-amplified SK-N-Be(2)C cells. A validation cohort (n=207) was assessed by DNA methylation arrays and RNAseq. DNA methylation-dependent redistribution of MYCN binding was monitored by MYCN ChIPseq. Candidate regulatory methylated DNA elements were investigated by CRISPR/Cas9-TET1 fusion proteins allowing targeted demethylation.

Results
Dimension reduction on WGBS data identified three epigenetically defined groups being characterized by low-risk disease, amplified MYCN or presence of telomere maintenance mechanisms (TMMs, including TERT and ALT), respectively. Integration of differentially methylated regions with associated transcriptional programs and chromatin states revealed subtype-specific regulatory networks. Low-risk tumors were characterized by a significant hypomethylation and upregulation of genes that are specifically expressed in normal neuroblasts of the developing adrenal medulla. These genes include EBF1, encoding a repressor of TERT expression, whose positive autoregulatory binding site is hypermethylated in high-risk neuroblastomas. In TMM-positive tumors, DNA methylation-regulated overexpression of the STAT3 stabilizer ZNF467 is in line with significantly higher STAT3 target gene activity in this tumor cohort. For MYCN-amplified neuroblastoma, demethylation experiments suggested a substantial impact of DNA methylation on MYCN DNA binding distribution. We identified a subgroup of genes that are protected from activating MYCN-binding via hypermethylation, including the pro-ferroptotic hypoxia-inducible factor-2alpha encoding gene EPAS1.

Conclusion
Our integrative investigation of DNA methylation at single nucleotide level identifies three major neuroblastoma subtypes, suggests an impact of their developmental origin and sheds light on their epigenetically manifested regulatory networks, which will aid identification of therapeutic intervention targets.

O3.3

Single-cell clonal heterogeneity and cell-state analysis reveals genomic evolution and developmental cell-state transitions in neuroblastoma

Otte J[1], Olsen T[1], Mei S[2], Kameneva P[3,8], Embaie B[1], Gao T[2], Björklund Å[4], Johansson A[4], Sundström E[5], Martinsson T[6], Fransson S[6], Stenman J[1], Cheng H[7], Zachariadis V[7], Enge M[7], Johnsen J[1], Kogner P[1], Adameyko I[3,8], Kharchenko P[2,9], Baryawno N[1]

[1]Childhood Cancer Research Unit, Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden, [2]Department of Biomedical Informatics, Harvard Medical School, Boston, MA, USA, [3]Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden, [4]National Bioinformatics Infrastructure Sweden, SciLifeLab, Uppsala University, Uppsala, Sweden, [5]Division of Neurogeriatrics, Department of Neurobiology, Care Sciences and Society, Karolinska Institutet, Stockholm, Sweden, [6]Department of Laboratory Medicine, The Sahlgrenska Academy, University of Gothenburg, Sahlgrenska University Hospital, Gothenburg, Sweden, [7]Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden, [8]Department of Molecular Neurosciences, Center for Brain Research, Medical University Vienna, Vienna, Austria, [9]Harvard Stem Cell Institute, Cambridge, MA, USA

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Background
A great challenge in the understanding and treatment of neuroblastoma is clinical heterogeneity which is also reflected by its molecular biology and genetics. Besides well-known clinically relevant genetic aberrations such as MYCN amplification, 11p deletion or 17q gain, the extent of the genetic and cell-state intra-tumoral heterogeneity at the single-cell level is still unknown.
Recent insights by comprehensive single-cell transcriptomic studies of human fetal tissues by us and others have extended our knowledge of cellular identities in the developing adrenal gland and neuroblastoma.

Aims
Based on a deeper understanding of normal human fetal cell-states, we aimed to define the heterogeneity of neuroblastoma to understand genetic events that contribute to tumor plasticity and treatment resistance, a major cause of death in neuroblastoma.

Methods
We sequenced 21 human neuroblastoma samples by single-cell RNA-sequencing (10x Genomics and Nuc-Seq) and profiled more than 80.000 cells. To identify genetic copy number variations from our RNA data we applied Numbat, a recently developed algorithm to resolve the clonal architecture and evolutionary relationships between distinct subclones. We further investigated the subclonal structure of six additional neuroblastoma samples by DNTR-Seq, a new multi-omics sequencing method that allows us to jointly analyze the whole genome and transcriptome from single cells sorted for specific markers.

Results
We revealed a complex clonal structure of neuroblastoma showing that genomic evolution is accompanied by cell-state transitions mimicking developmental trajectories. Next to a heterogeneous adrenergic cell population of sympathoblast-like and chromaffin-like cells, we further identified malignant Schwann cell precursor (SCP)-like cells. We found evidence that malignant SCP-like cells can act as an ancestral clone in neuroblastoma development, and we identified one genetic aberration, a gain of Chr 17, as the first malignant hit initially present only in pre-malignant SCP-like cells. We detected a multi-clonal structure of adrenergic subclones and, interestingly, revealed switching cell-states between different subclones.

Conclusions
We identified an unexpected heterogeneity and plasticity in human neuroblastoma, reflected in a unique clonal architecture, that might be relevant for therapeutic resistance and relapse. Our data suggest that, in some samples, the newly identified malignant SCP-like cells might be the cell-of-origin.

O3.4

Single-cell RNA sequencing identifies differences between paired samples of primary tumour and bone marrow metastasis

Hochheuser C[1,2], Boltjes A[1], Keller K[1], van Hooff S[1], Tol S[3], Voermans C[2], Molenaar J[1], Timmerman I[1,2], Tytgat L[1,2]

[1]Princess Maxima Centre for Pediatric Oncology, Utrecht, Netherlands, [2]Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, Netherlands, [3]Department of Research Facilities, Sanquin Research and Landsteiner Laboratory, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, Netherlands

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Background & Aims
Neuroblastoma often presents with metastases at diagnosis, of which 90% are localized in the bone marrow (BM). Those disseminated tumour cells are believed to contribute to over 40% of relapses in high-risk disease. In this study we aim to define characteristics of metastatic cells in the BM (BM-NBL) compared to cells in primary tumour (PT) to allow more specific targeting and prevention of relapse.
Methods
We applied single-cell RNA-sequencing (CEL-seq2) to a cohort of 7 patients with matched PT- and BM-samples at diagnosis. BM cells were single-cell index-sorted, allowing us to link the transcriptional profile to cell surface expression of a panel of 12 markers.
Results
Comparative gene expression analysis among tumour cells revealed enrichment of MAPK signalling in the PT of 6/7 patients, while cell cycle-related genes were enriched in BM-NBL of all patients. Indeed, for all seven patients the percentage of cycling cells (as defined by a high G2M- and S-phase score) was higher in BM than in PT. Interestingly, for five patients the cell populations with high cycling activity were less 'mesenchymal', based on published gene signatures. On phenotypic level, we observed a strong positive correlation between BM-NBL GD2 surface expression and both the cell cycle status and adrenergic cell state. When analysing copy number variations (inferCNV), genetic heterogeneity between PT and BM was observed in 6/7 patients. One patient's PT displayed striking heterogeneity for chr9 copy number (normal vs deletion), with no chr9 deletion in BM-NBL cells. Comparing the two PT populations, gene sets of TNFα- and TGFβ-signalling and those related to migration and invasiveness were enriched in the cells without the deletion, suggesting an increased metastatic potential to colonize the BM.
Conclusion & Outlook
We found more fast-cycling cells in BM-NBL cells than in the PT, which were less mesenchymal in the majority of patients. Next, we aim to identify surface markers of novel tumour subpopulations in the BM which could serve as additional therapeutic targets or biomarkers for minimal residual disease. Such knowledge is essential for targeting BM-NBL more efficiently in the future and lower the relapse rate in high-risk patients.

O4.1

Neuroblastoma develops in early fetal development and its evolutionary duration predicts outcome

Körber V[1], Stainczyk S[2,3], Kurilov R[4], Henrich K[2,3], Hero B[5], Brors B[4], Westermann F[2,3], Höfer T[1]
[1]Division of Theoretical Systems Biology, German Cancer Research Centre, Heidelberg, Germany, [2]Hopp Children's Cancer Center (KiTZ), Heidelberg, Germany, [3]Division of Neuroblastoma Genomics, German Cancer Research Centre, Heidelberg, Germany, [4]Division of Applied Bioinformatics, German Cancer Research Centre, Heidelberg, Germany, [5]Department of Pediatric Oncology and Hematology, University Children's Hospital of Cologne, Cologne, Heidelberg

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Background
Neuroblastoma shows very diverse outcomes from spontaneous regression, requiring light or no treatment, to high-risk disease that remains fatal for ~50% of patients. Despite several established criteria, among them the age at diagnosis, the molecular nature of chromosomal gains and losses, and acquisition of telomere maintenance, accurate risk stratification of patients into observation and treatment groups currently remains a formidable challenge.

Aims
We asked whether understanding when neuroblastomas originate in development and how they evolve genetically will shed light on disease severity and outcome.

Methods
We quantified the somatic evolution of neuroblastomas by deep whole-genome sequencing (WGS), molecular clock analysis and population-genetics modeling in a comprehensive cohort covering all subtypes. To this end, we inferred the evolutionary dynamics of neuroblastomas across the clinical spectrum of this cancer from the variant allele frequency distribution of somatic single-nucleotide variants (SSNVs) and related the rate of SSNV acquisition to real time by factoring in the age at diagnosis. To scrutinize the significance of our findings, we employed a discovery-validation cohort design.

Results
We found that tumors across the entire clinical spectrum begin to develop via aberrant mitoses as early as in the first trimester of pregnancy. Neuroblastomas with favorable prognosis cease to evolve early, whereas aggressive neuroblastomas show prolonged evolution during which they acquire telomere maintenance mechanisms. The initial aneuploidization events condition subsequent evolution, with aggressive neuroblastomas exhibiting early genomic instability. We found in the discovery cohort (N=100), and validated in an independent cohort (N=86), that the duration of evolution is an accurate predictor of outcome.

Conclusion
Our results suggest that the duration of early evolution is an accurate predictor of outcome. The duration of evolution is directly accessible from WGS data at custom coverage of 30x and may hence be tested in clinical trials with reasonable effort.

O4.2

SIOPEN BIOPORTAL: An international registry linked to a virtual biobank for patients with peripheral neuroblastic tumours

Devi-marulkar P[1], Sansone C[1], Beiske K[2], Burchill S[3], Canete A[4], Eggert A[5], Garaventa A[6], Hayn D[7], Fragola M[6], Ladenstein R[8], Moreno L[9], D.J. Pearson A[10], Schreier G[7], Angelini P[11], Simon T[12], Knox L[13], Taschner-Mandl S[14], G. A. M. Tytgat L[15], A. Tweddle D[16], Valteau-Couanet D[17], Beck-Popovic M[18], Schleiermacher G[1]

[1]Institut Curie, Paris, France, [2]Department of Pathology, Oslo University Hospital, and Medical Faculty, University of Oslo, Oslo, Norway, [3]Leeds Institute of Medical Research, St James University Hospital, Leeds, UK, [4]Pediatric Oncology Unit, Hospital Universitario y Politecnico La Fe, Valencia, Spain, [5]Department of Pediatric Oncology and Hematology, Charité University Medicine, Berlin, Germany, [6]Paediatric Oncology Unit and Epidemiology and Biostatistics Unit, IRCCS Istituto Giannina Gaslini, Genova, Italy, [7]AIT Austrian Institute of Technology GmbH, Graz, Austria, [8]Paediatric Haematology/Oncology, St Anna Kinderspital and St Anna Kinderkrebforschung, Vienna, Austria, [9]Division of Pediatric Hematology and Oncology, Hospital Universitari Vall d'Hebron, Barcelona, Spain, [10]Division of Clinical Studies and Cancer Therapeutics, The Institute of Cancer Research, Sutton, SM2 5NG, UK, [11]Children and Young People's Unit, Royal Marsden NHS Foundation Trust, London, UK, [12]Department of Pediatric Oncology and Hematology, University Hospital , Cologne, Germany, [13]Solving Kids' Cancer UK, London, UK, [14]St Anna Children's Cancer Research Institute, Vienna, Austria, [15]Princess Máxima Center for Pediatric Oncology, Utrecht University, 3584 CS, Utrecht, The Netherlands, [16]Wolfson Childhood Cancer Research Centre, Newcastle Centre for Cancer, Translational & Clinical Research Institute, Newcastle University, Newcastle upon Tyne, UK, [17]Department of Paediatric and Adolescent Oncology, Institut Gustave Roussy, Viellejuif, France, [18]Unit of Pediatric Hematology-Oncology, University Hospital CHUV, Lausanne, Switzerland

Parallel Sessions 4: Clinical data and sample repositories, May 15, 2023, 3:30 PM - 4:20 PM

Background: Although Neuroblastoma (NB) is the most common extra-cranial solid tumor in childhood, it is a rare disease. Clinical, epidemiological information and tissue samples collected on NB patients are limited and often unavailable for researchers. Linking patient data and biological sample data is crucial for clinical-biological research. To date, country-specific and international databases/biobanks exists but with limited data modules and accessibilities. However, it lacks a pan-European registry or biobank for NB patients. The BIOPORTAL is a prospective non-therapeutic multicenter study to develop an international registry linked with virtual biobank for all NB patients within countries of the European Society for Pediatric Oncology Neuroblastoma Group (SIOPEN) network.

Aims: To provide the necessary framework for regulatory compliance with consent, data reuse, and collection of biological samples for international research purposes. Post-hoc hypotheses based on data generated will allow research to improve understanding of NB origins, better defining prognostic subgroups, predicting treatment and accelerating development of novel therapies.

Results: The registry will focus on core clinical data (clinical & epidemiological) collection and information on bio-banking of patient samples. Prospective patient enrolment will be conducted at ~250 sites located across SIOPEN countries. Enrolment of ~600 patients/ year estimated over 5-years, with 10 years follow up. A "Consortium Agreement" between SIOPEN and national representatives of participating countries provides the legal framework. Case report forms align with the International Neuroblastoma Risk Group to map patient treatment from diagnosis to follow up. Data entry is built on pseudonymization according to the European patient identifier (EUPID) concept. This ensures separation of clinical data and hashed identifiers. Data linkage from different contexts (i.e. trial, national registry) is ensured via EUPID in a privacy preserving fully GDPR-compliant manner. Soon after signing the consortium agreement with participating countries, we plan to open the study in France in early 2023.

Conclusion: The BIOPORTAL is a new international framework to streamline collection and reuse of data across the SIOPEN network. This overall structure will enable interaction with international databases. The BIOPORTAL experience may serve as a blueprint for other pediatric cancer networks to build international research infrastructures and foster clinical and translational initiatives

O4.3

Systematic review of studies identifying clinical and biological markers of poor survival in high-risk neuroblastoma patients at diagnosis:

INRG-BORNEO project

Vilaplana Blanes A[1], Li A[2], Morell M[3], Kao P[4], Rubio A[5], Morgenstern D[6], Bautista F[7], Irwin M[6], Schleiermacher G[8], Moreno L[1,3], London W[4]

[1]Vall d'Hebron Research Institute, Barcelona, Spain, [2]University of Michigan, Michigan, United States, [3]Vall d'Hebron University Hospital, Barcelona, Spain, [4]Dana Farber / Boston Children's Cancer Center, Boston, United States, [5]Niño Jesús Hospital, Madrid, Spain, [6]Hospital for Sick Children, Toronto, Canada, [7]Princess Maxima Center for Pediatric Oncology, Utrecht, Netherlands, [8]Institut Curie , Paris, France

Parallel Sessions 4: Clinical data and sample repositories, May 15, 2023, 3:30 PM - 4:20 PM

Background

Outcome for children with high-risk neuroblastoma (HRNBL) remains poor. New prognostic biomarkers have been reported, including clinical, imaging, genomic or expression characteristics; none have had head-to-head comparisons of prognostic strength comparing new and existing biomarkers. Identifying and validating prognostic factors upfront would enable individualization of treatment, facilitate earlier assignment to experimental therapies for HRNBL patients with the worst outcome, or could support reduced treatment intensity in HRNBL patients who have better outcome.

Aims

This systematic review (PROSPERO registry CRD42018116666) and meta-analysis aims to identify published biomarkers (at diagnosis) that are the most strongly prognostic, according to progression-free, event-free and/or overall survival. Our objective is to use these biomarkers to inform improvements to risk stratification for patients with HRNBL.

Methods

Systematic review was conducted following PRISMA guidelines. Literature search was conducted in PubMed for articles, published in English language from 1Jan1995-31Dec2020, that examined prognostic biomarkers in HRNBL (per internationally accepted definition).

Article inclusion criteria: (i) biomarkers at diagnosis; (ii) data presented for survival hazard ratios (HR), odds ratios (OR), relative risk (RR), or point estimates of survival probability; (iii) analytic cohort was only HRNBL patients OR data were presented separately for HRNBL; and, (iv) the HRNBL sample size was ≥50. Publication quality was evaluated using the QUIPS score (range: 6-18; low scores indicate highest risk of bias).

Results

Of 5,830 papers identified, 59 publications met inclusion criteria. Among those, 14 publications investigated clinical biomarkers, 29 tumor genomic biomarkers, 13 gene expression biomarkers, and 18 protein biomarkers. Type of risk measurement was RR for 2, OR for 3, HR for 29, and HR not provided but could be calculated for 37. Median sample size of the publications was 180.5 (range=58-2,623). The median QUIPS score was 15 (range=9-18).

Conclusion

This is the first systematic review of prognostic biomarkers focused on HRNBL. Based on prognostic impact, statistical strength and quality, up to 10 biomarkers will be selected for further study. Patient-level data will be requested from the publication authors, and incorporated into the INRG Data Commons. A formal head-to-head statistical comparison of biomarker prognostic strength may reveal ways to improve HRNBL risk stratification.

O4.4

Building a REDCap on FHIR Tool to Abstract Neuroblastoma Data from Electronic Health Records (EHRs): A Proof-of-Concept Study

Furner B[1], Cheng A[2], Desai A[1], Benedetti D[4], Friedman D[4], Wyatt K[3], Watkins M[1], Volchenboum S[1], Cohn S[1]
[1]Department of Pediatrics, Section of Hematology/Oncology, The University of Chicago, Chicago, USA, [2]Department of Biomedical Informatics, Vanderbilt University Medical Center, Nashville, USA, [3]Department of Pediatric Hematology/Oncology, Roger Maris Cancer Center, Sanford Health, Fargo, USA, [4]Department of Pediatrics, Division of Hematology/Oncology, Vanderbilt University Medical Center, Nashville, USA

Parallel Sessions 4: Clinical data and sample repositories, May 15, 2023, 3:30 PM - 4:20 PM

Background
The International Neuroblastoma Risk Group (INRG) Data Commons (INRGdc) is part of the Pediatric Cancer Data Commons at the University of Chicago (UChicago). Information on >24,000 neuroblastoma patients enrolled on cooperative-group studies is housed in the INRGdc and available to the research community. Seminal studies have been conducted using these data. However, the lack of real-world electronic health record (EHR) data, particularly treatment data is limiting. To mitigate this, we have developed a method using REDCap's FHIR Clinical Data Interoperability Services (CDIS) module to extract EHR-derived up-front cancer treatment data on neuroblastoma patients. Our long-term goal is to enrich treatment data for patients in the INRGdc from all sites' EHRs.

Aims
To conduct a proof-of-concept using CDIS to deploy EHR-to-REDCap transfer of up-front cancer treatment data (chemotherapy and immunotherapy) from neuroblastoma patients enrolled on ANBL00B1 and assess accuracy and completeness of data extracted from the EHRs at the UChicago and Vanderbilt University (VUMC) Medical Centers.

Methods
Patients enrolled on ANBL00B1 were added to CDIS-enabled REDCap projects at UChicago and VUMC, which were configured to pull medication orders from Epic. Software was developed to extract medications-of-interest through the REDCap API and de-identify these data for later submission to the INRGdc. CDIS output was assessed at UChicago against a 'gold standard' data set pulled from the clinical research data warehouse with records matched using medication labels and age at medication order.

Results
Data extracted through CDIS achieved high accuracy and completeness, when compared against a gold standard. Of 1859 treatment orders in the gold standard data set of 41 patients, 1845 (>99%) were correctly extracted from CDIS, with all 14 of the missing orders originating from a single participant.

Conclusion
With an eye to downstream implementation ease, a key design principle in this pilot study was to leverage widely-adopted technologies (e.g., FHIR, REDCap). Preliminary results demonstrate the feasibility of abstracting neuroblastoma patient treatment data from EHRs using CDIS. Future work will explore other treatment data and additional sites. Enriching the INRGdc with EHR data will transform neuroblastoma research and enable new discoveries unavailable in the existing data set.

O5.1

Maternal high fat diet accelerates neuroblastoma tumorigenesis

Cheung B[1,2], Mittra R[1], Chung N[1], Raipuria M[1], Joseph M[1], Wang K[1], Jayatilleke N[1], Carter D[1,3], Yeo K[4], Zhu S[4], Marshall G[1,5]

[1]Children's Cancer Institute Australia for Medical Research, Lowy Cancer Research Centre, UNSW Sydney, NSW,, , Australia, [2]School of Clinical Medicine, Faculty of Medicine & Health, UNSW Sydney, Australia, , , [3]School of Biomedical Engineering, University of Technology Sydney, Australia, , , [4]Department of Biochemistry and Molecular Biology, Cancer Center and Center for Individualized Medicine, Mayo Clinic, Rochester, MN, 55902, USA, , , [5]Kids Cancer Centre, Sydney Children's Hospital, Randwick, NSW, Australia, ,

Parallel session 5: Metabolomics, May 16, 2023, 10:40 AM - 11:30 AM

Background: Neuroblastoma (NB) begins in embryonal neuroblasts indicating an aetiological relationship between NB tumorigenesis and embryonal environment. Some studies have suggested that maternal obesity and high birth weight are risk factors for childhood cancer. High birth weight has been associated with an increased risk of NB. However, little is known about embryonal environmental factors which might initiate embryonal cancer.

Aims: To administer high fat diet (HFD) pre- and post-conception to neuroblastoma-bearing TH-MYCN mice, and, identify candidate pathways causing the effects of HFD on neuroblastoma tumorigenesis.

Methods: A HFD was given to maternal TH-MYCN transgenic mice which induced maternal obesity and high birth weight. The effect of HFD on the onset of NB tumorigenesis was also evaluated in MYCN-driven zebrafish NB model. For the mechanism studies, we used comparative RNA-sequencing of ganglia and tumour tissues from offspring born to HFD or control diet fed mothers to map the transcriptional landscape.

Results: TH-MYCN hemizygous female mice were placed on HFD (43% kcal from fat) or a control diet (9.3% kcal from fat) from four weeks prior to mating, during pregnancy, and through lactation. The homozygous offspring from the HFD group showed an 24% reduction in tumour latency and an 24% reduction in tumour-free survival, as well as increased neuroblast hyperplasia in coeliac ganglia in 2-week-old TH-MYCN homozygous offspring. A similar result was found in a MYCN-driven zebrafish NB model: 45% of zebrafish fed with HFD, in comparison with only 14% of zebrafish fed with low fat diet, developed a tumour. Gene set enrichment analysis of ganglia and tumour bulk mRNA revealed that the genes positively regulated by HFD were mostly significantly associated with oxidative phosphorylation (OXPHOS) and fatty acid (FA) metabolism. Importantly, we showed that the in vitro treatment of MYCN-amplified human NB cells with a FA metabolism inhibitor, Fatostatin, reduced cell proliferation which had been induced by treatment with fatty acids. Fatostatin was also synergistic with chemotherapeutic drugs vincristine and doxorubicin in reducing cell viability of human MYCN-amplified NB cell lines. Experiments are ongoing to identify the molecular mechanism through which HFD-activated OXPHOS and FA metabolism and to develop therapeutic strategies.

O5.2

Ferroptosis, a novel liability of high-risk neuroblastoma

Garbowicz K[1,2], Kreth S[1,2], Henrich K[1,2], Bechmann N[3], Peitzsch M[3], Poschet G[4], Klinke G[4], Westermann F[1,2]
[1]Hopp Children´s Cancer Center (KiTZ), Heidelberg, Germany, [2]Division of Neuroblastoma Genomics, German Cancer Research Center (DKFZ), Heidelberg, Germany, [3]Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus Dresden, Technische Universität Dresden, Dresden, Germany, [4]Metabolomics Core Technology Platform, University of Heidelberg, Heidelberg, Germany

Parallel session 5: Metabolomics, May 16, 2023, 10:40 AM - 11:30 AM

Background / introduction
Aberrant MYCN oncogene expression determines a subset of highly aggressive neuroblastomas (NB) with poor clinical outcome, mainly due to resistance to conventional treatment. Recent findings have established a novel functional link between oncogenic MYCN and ferroptosis – a regulated, iron-dependent form of cell death.

Aims
Our aim is to develop a precise mechanistic model of cysteine usage in ferroptosis sensitive/resistant cells and to validate this novel metabolic vulnerability in various preclinical models.

Methods / materials
To enhance our understanding of MYCN-mediated metabolic reprogramming in NB, we tested a large group of NB cell lines for their sensitivity to cystine deprivation and ferroptosis-inducing agents. The acquired area under the curve values from drug treatments were correlated to base-line gene expression data to identify mechanisms that could drive ferroptosis sensitivity. To identify patients that could benefit from ferroptosis induction, a linear regression model was used to predict tumor sensitivity to ferroptosis induction based on data obtained from the cell lines. Moreover, extensive metabolic profiling of cellular models was performed to better understand the cellular changes driven by MYCN, and findings were validated using cellular models.

Results
Titration experiment show that MYCN-amplified NB cells are strongly addicted to cyst(e)ine supply, whereas cells lacking MYCN expression are largely resistant to limiting cystine supply. By correlating gene expression to cell lines sensitivity to ferroptosis induction, we identified coenzyme Q10-related pathways as an important resistance mechanism which is downregulated in MYCN-amplified tumors. Moreover, MYCN-amplified primary tumors were predicted to be more sensitive to ferroptosis induction, with several ferroptosis inhibitory pathway being downregulated in this tumor group. Flux experiments using stable isotope labelled amino acids revealed striking differences in distribution and flux into different metabolic processes when comparing MYCN-amplified and non-amplified cell types.

Summary / conclusion
In summary, our data show that ferroptosis execution emerges as a novel tumor suppressor function that needs to be inactivated during tumor formation and malignant progression. MYCN-amplified cells are highly sensitive to ferroptosis induction, disclosing a yet unknown metabolic vulnerability that can be exploited therapeutically.

O5.3

Reprogramming the neuroblastoma epigenome with mitochondrial uncoupler to promote differentiation therapy

Jiang H[1], Tiche S[1], He B[1], Forgo B[1], Li A[1], Shimada H[1], Chiu B[1], Ye J[1]
[1]*Stanford University, Stanford, United States*

Parallel session 5: Metabolomics, May 16, 2023, 10:40 AM - 11:30 AM

Background: Retinoic acid (RA)-based differentiation therapy has been successfully applied in the treatment of multiple types of cancer, including neuroblastoma (NB). RA treatment induces clear neuronal differentiation morphology, associated with proliferation arrest in most NB cell lines. Despite the significant response observed in vitro, no significant therapeutic responses were observed in clinical trials testing RA as a single agent in NB patients. Clinical trials showed that RA significantly increased event-free survival in patients who took myeloablative therapy followed by autologous bone marrow transplantation (ABMT) or intensive chemotherapy. However, even in the ABMT+RA group, over 40% of the patients did not respond to RA treatment. These data suggest that it is necessary to develop combination therapeutic strategies to overcome RA-resistance.

Recently, we discovered that treatment with a mitochondrial uncoupler (MU), niclosamide ethanolamine (NEN), increased NAD+/NADH and the α-ketoglutarate (α-KG)/2-hydroxyglutarate (2-HG) ratio in NB cells. Consequently, NEN treatment induced promoter CpG island demethylation and epigenetic landscape remodeling, activating the neural differentiation program(Jiang et al., 2022). Because it was reported DNA methyltransferase inhibitor can reduce RA-resistance (Almeida et al., 2017; Westerlund et al., 2017) in NB, we hypothesize that MU treatment can be combined with RA to eliminate RA-resistance and promote differentiation therapy.

Methods: In this study, mitochondrial uncouplers were combined with 13-cis-RA to treat NB cells. Cellular metabolites levels and metabolic flux were measured using LC-MS. NB differentiation morphology changes were quantified with neurite length measure. Gene expression changes were determined with RNA-seq, and the DNA methylation profile changes were determined with EPIC methylation array.

Results: MU treatment promotes demethylation in RA-signaling pathway genes. MU+RA combination treatment activates RA-signaling pathway gene expression, and increases neurites length and differentiation marker expression in NB cells. The result of Chou-Talalay analysis demonstrates that MU+RA inhibits NB cell proliferation synergistically.

Conclusions: Together, these results indicates that successful differentiation therapy requires both MU (which remodels the epigenome) and differentiation agent to activate the differentiation program. Because both agents have low toxicity and are FDA-approved, this combination therapeutic strategy has a good potential in clinical translation for NB treatment.

O5.4

Optimising polyamine inhibition therapy combined with standard-of-care chemotherapy and anti-GD2 immunotherapy through preclinical modelling in Th-MYCN mice

Murray J[1], Pandher R[1], Xiao L[1], Alfred S[1], Brand J[1], Mosmann E[1], Kusuma F[1], Kearns A[1], Somers K[1], Park J[7], Marshall L[4,8], Gorrini C[4], Chesler L[4], Hogarty M[5,6], Pearson A[4], Burns M[3], Fletcher J[1], Ziegler D[1,2], Norris M[1], Haber M[1]

[1]Children's Cancer Institute, Sydney, Australia, [2]Kids Cancer Centre, Sydney Children's Hospital, Randwick, Australia, [3]Aminex Therapeutics Inc, Kirkland, USA, [4]The Institute of Cancer Research, London, Greater London, United Kingdom, [5]Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, USA, [6]Department of Pediatrics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, USA, [7]Seattle Children's Hospital, Fred Hutchinson Cancer Research Center, University of Washington, Seattle, USA, [8]Children and Young People's Unit, The Royal Marsden Hospital , London, United Kingdom, [9]Division of Clinical Studies, Institute of Cancer Research, Royal Marsden Hospital, Sutton, United Kingdom

Parallel session 5: Metabolomics, May 16, 2023, 10:40 AM - 11:30 AM

Background: Polyamines are essential cations frequently upregulated in tumours. A randomized Ph2 trial of DFMO, an irreversible polyamine synthesis inhibitor, added to chemotherapy and anti-GD2 immunotherapy is ongoing for relapsed neuroblastoma (NCT03794349). In preclinical models, we showed that DFMO efficacy is enhanced by adding the polyamine transport inhibitor AMXT1501 (Gamble, Sci Transl Med, 2019). DFMO/AMXT1501 is currently in an adult clinical trial (NCT05500508), with an international DFMO/AMXT1501/chemotherapy/anti-GD2 trial for neuroblastoma planned. Preclinical modelling of polyamine-targeted therapeutics in Th-MYCN mice has been invaluable for supporting clinical trial design but has yet to include anti-GD2 immunotherapy.

Aims: To use the Th-MYCN model to optimize DFMO/AMXT1501 therapy combined with temozolomide/irinotecan (TEM/IRI) or cyclophosphamide/topotecan (CYCLO/TOPO), and 14G2a antibody.

Methods: Escalating doses of DFMO and AMXT1501 were studied (n=10/group), with or without TEM/IRI or CYCLO/TOPO. 14G2a regimen was also titrated toward human-relevant pharmacokinetic exposures to establish suitable conditions for further combination therapies. Targeted tumour metabolite profiling was carried out by LC-MS.

Results: In the absence of chemo-immunotherapy, highest-dose DFMO (1.5%) and AMXT1501 (2.5 mg/kg/d) resulted in greatest efficacy without increased toxicity. Median mouse survival was 2-fold greater compared with lower dose DFMO/AMXT1501 combinations. The data suggested that maximising DFMO dose is critical. Similar improved efficacy was observed in combination with TEM/IRI or CYCLO/TOPO. Higher DFMO dose, but not AMXT1501, correlated with lower intra-tumoral putrescine levels. Combining 14G2a antibody (100ug twice weekly, 18 weeks; McNerney, Oncoimmunology, 2022) with either CYCLO/TOPO (single 5-day chemo cycle) or DFMO/AMXT1501 (1.5%/2.5 mg/kg/d) significantly increased survival compared with either treatment alone. Combining all 5 drugs resulted in 100% survival at 1 year, with minimal toxicity. Identification of clinically relevant backbones for polyamine inhibition optimisation, which targeted human-relevant 14G2a pharmacokinetics whilst allowing demonstration of the benefit of adding polyamine depletion to chemo-immunotherapy, required substantial dose reductions of 14G2a antibody (≤25µg, 2 doses) and TEM/IRI or CYCLO/TOPO (single 2-day chemo cycle).

Conclusions: Our study has increased the utility of the Th-MYCN mouse model for preclinical modelling of polyamine inhibition, and additional investigational treatments, by pharmacokinetics-driven optimization of anti-GD2 therapy with standard-of-care chemotherapy backbones. This approach will support improved optimization for future clinical trials.

O6.1

Growth retardation in long-term high-risk neuroblastoma survivors treated with high-dose chemotherapy

Haghiri S[1], Karamouza E[2,3], Fayech C[1], Thebault E[1,4], Allard J[1], Thomas-Teinturier C[5,6], Dufour C[1], Bolle S[7], Haddy N[6], de Vathaire F[6], Valteau-Couanet D[1], Le Teuff G[2,3], Fresneau B[1,6]

[1]Gustave Roussy, Université Paris-Saclay, Department of Children and Adolescents Oncology, Villejuif, F-94805, France, [2]Gustave Roussy, Université Paris-Saclay, Biostatistics and Epidemiology, Villejuif, France, [3]INSERM, Université Paris-Saclay, Oncostat U1018, Labeled Ligue Contre le Cancer, Villejuif, France, [4]Oscar Lambret Center, Pediatric Oncology, Lille, France, [5]Bicêtre Hospital, Pediatric Endocrinology and diabetology, Le Kremlin-Bicêtre, France, [6]Université Paris-Saclay, Université Paris-Sud, UVSQ, CESP, Cancer and Radiation Team, Villejuif, F-94805, France, [7]Gustave Roussy, Université Paris-Saclay, Radiation Oncology Department, Villejuif, France

Parallel session 6: Clinical cohort studies, May 16, 2023, 10:40 AM - 11:30 AM

Background:
High-risk neuroblastoma (HRNB) survivors treated with high-dose chemotherapy followed by autologous stem cell rescue (HDC-ASCR) experience multiple late effects, including endocrine disorders and gonadal endocrine dysfunction.

Aims:
We evaluated growth profile after ASCR by using height-for-age (HFA) Z-scores, in a large cohort of 145 five-year disease-free survivors treated for HRNB with HDC-ASCR from 1980-2012 at Gustave Roussy.

Methods:
Association of clinical and therapeutic risk factors with growth curves were evaluated using linear mixed models for repeated measures data.

Results:
Heights were available for 138/145 HRNB survivors. Sex-ratio M/F=1.12, median age at diagnosis=2.5y (range=0-13.3), median HFA Z-score at diagnosis=0.41 (-2.6-3.5). With a median follow-up of 14.1 years (range=3.3-27), HFA Z-score at last follow-up had decreased, with a median HFA Z-score of -0.92 (-3.6-2.2) at a median age of 18.1y (5.5-33.6). Growth curve analysis showed progressively decreasing of HFA after ASCR, with no recovery after 15y, resulting in a median HFA Z-score variation of -0.6 (-3.1-2.1) after 15 years post-ASCR.
Pre-existing small height and young age at HDC were important predicting factors for growth alteration (estimate=0.73+/-0.06, p<0.0001; 0.12+/-0.002, p<0.0001, respectively). Gonadal insufficiency had a negative impact (-0.27 +/-0.05, p<0.0001), whereas hormonal replacement therapy (HRT) was beneficial (0.14 +/-0.06, p=0.02). After adjustment for gonadal endocrine dysfunction, HDC regimen was not significantly associated with HFA Z-score, whereas negative effects were observed with doxorubicin (cumulative doses ≥250mg/m²) and cisplatin (≥400mg/m²), (-0.92 +/-0.18, p<0.0001 and -0.38 +/-0.14, p=0.006, respectively). Spinal radiotherapy doses were not associated with growth impairment (p=0.46).

Conclusion:
In conclusion, we demonstrate for the first time in a large cohort of HRNB survivors a frequent growth alteration after ASCR, worsening over time, mainly related to gonadal dysfunction. HRT was beneficial, supporting adequate hormonal substitution. The effect of high cumulative doses of doxorubicin and cisplatin before HDC have to be explored.

O6.2

High Risk Neuroblastoma (HR-NB) with MNA and age <18months: Results from the HR-NBL1/SIOPEN trial

Ladenstein R[1], Poetschger U[2], Schleiermacher G[3], Valteau-Couanet D[4], Ash S[5], Beck-Popovic M[6], Balwierz W[7], Beiske K[8], Bician P[9], Boterberg T[10], Burchill S[11], Chan G[12], Cesen Mazic M[13], Elliott M[14], Kogner P[15], Lode H[16], Forjaz de Lacerda A[17], Garami M[18], Laureys G[19], Luksch R[20], Malis J[21], Owens C[22], Papadakis V[23], Ruud E[24], Sarnacki S[25], Schroeder H[26], Trahair T[27], Vettenranta K[28], Vujic D[29], Taschner-Mandl S[30], Canete A[31]

[1]St Anna Children's Hospital Department of Paediatrics, Medical University of Vienna, Vienna, Austria and Children's Cancer Research Institute (CCRI), St. Anna Kinderkrebsforschung GmbH, Department for Studies and Statistics and Integrated Research, Vienna, Austria, [2]Children's Cancer Research Institute (CCRI), St. Anna Kinderkrebsforschung GmbH, Department for Studies and Statistics and Integrated Research;, Vienna, Austria, [3]Institut Curie, SIREDO Pediatric Oncology Center, Paris, France, [4]Institut Gustave Roussy, Children and Adolescent Oncology Department, Paris-Sud University, Paris, France, [5]Schneider Children's Medical Center of Israel, Sackler Faculty of Medicine Tel Aviv University, Petach, Tikvah, Israel, [6]University Hospital Lausanne, Paediatric Hematology Oncology Unit, Department Women-Mother-Child, Lausanne, Switzerland, [7]Oncology, Jagiellonian University Medical College, Paediatric Haematology Oncology, Krakow, Poland, [8]Rikshospitalet, Dept. of Pathology, Oslo, Norway, [9]University Children´s Hospital, Clinic of Pediatric Oncology and Hematology, Banska Bystrica, Slovakia, [10]Ghent University Hospital, Department of Radiation Oncology, Ghent, Belgium, [11]St James's University Hospital, Leeds Institute of Medical Research, Leeds, UK, [12]Queen Mary Hospital, The University of Hong Kong, Department of Pediatrics & Adolescent Medicine , Hong Kong, Hong Kong, [13]University Children's Hospital Ljubljana, Department of children oncology and haematology, Ljubljana, Slovenia, [14]Leeds Children's Hospital, Leeds General Infirmary, Leeds, UK, [15]Karolinska Institutet, Childhood Cancer Research Unit, Department of Women's and Children's Health, Stockholm, Sweden, [16]University Medicine Greifswald,Pediatric Hematology and Oncology, Greifswald, Germany, [17]Instituto Portugues De Oncologia De Francisco Gentil, Dept. of Paediatrics, Lisboa, Portugal, [18]Semmelweis University, Division of Paediatric Oncology, Budapest, Hungary, [19]Ghent University Hospital, Department of Pediatric Hematology, Oncology and Stem Cell Transplant, Ghent, Belgium, [20]Fondazione IRCCS Istituto Nazionale dei Tumori, S.C. Pediatria Oncologia, Milan, Italy, [21]University Hospital Motol, Dept. of Paediatric Oncology, Prague, Czech Republic, [22]Children's Health Ireland, Paediatric Haematology Oncology, Dublin, Ireland, [23]Agia Sofia Children's Hospital, Department of Pediatric Hematology-Oncology, Athens, Greece, [24]Rikshospitalet, Dept. of Paediatrics, Oslo, Norway, [25]Hôpital Universitaire Necker-Enfants Malades, Université de Paris Cité, Department of Pediatric Surgery, Paris, France, [26]University Hospital of Aarhus, 26. Department of Paediatrics, Aarhus, Denmark, [27]Sydney Children's Hospital, Kids Cancer Centre, Randwick, Australia, [28]University of Tampere, Department of Pediatrics, Hospital for Children and Adolescents Univ. of Helsinki, Helsinki, Finland, [29]Mother and Child Healthcare Institute of Serbia "Dr. Vukan Cupic", , Belgrade, Serbia, [30]Children's Cancer Research Institute (CCRI), St. Anna Kinderkrebsforschung GmbH, Department for Tumour Biology, Vienna, Austria, [31]Hospital Universitario y Politecnico La Fe, Paediatric Haematology Oncology, Valencia, Spain

Parallel session 6: Clinical cohort studies, May 16, 2023, 10:40 AM - 11:30 AM

Background

The INES 99.4 trial (JCO 2009,27(7):1014-9) for MYCN amplified (MNA) infants using 6 courses of CBDCA-Vp16/CADO showed early progressions or non-response in 30%. Two-year overall survival (OS) was 30% (SE, 0.08) with median survival of 12 months.

Aims

To report risk factors and outcomes for patients <18 months with MNA neuroblastoma treated on HR-NBL1/SIOPEN.

Methods

From 2006-2022, patients <18 months with MNA INSS stages >1 were eligible. All infants received Rapid Cojec induction, eventually 2 TVDs, Busulfan-Melphalan as high-dose treatment and could receive

Dinutuximab- beta immunotherapy. Toddlers (12-18 months) followed the HR-NBL1/SIOPEN respective eligibility criteria.

Results

Median age at diagnosis was 1.2 years in 414 patients (median follow up 7 years): 56% were male; 18.6% had localised tumours, 6.8% stage 4S and 75% stage 4. The predominant primary tumour site was abdominal (85%). Rapid Cojec was used in 85%, BUMEL in 89% and 26% received Dinutuximab-beta. Multiple metastatic compartments were reported in 82% of stage 4. Five-year event-free (5y-EFS) and overall survival (5y-OS) was 0.46±0.03 and 0.51±0.03. Stage 2,3&4S patients had a better 5y-EFS of 0.64±0.03 ($p<0.005$) (5y-OS:0.66±0.06), compared to stage 4 with 0.41±0.03 (OS:0.46±0.03). Infants' 5y-EFS was superior with 0.53±0.04 (5y-OS:0.57±0.04) ($p=0.015$) over the toddlers with 5y-EFS 0.42±0.03 (5y-OS:0.46±0.03). Patients with bone marrow (BM) and skeletal metastases ± other sites (MS) did worse ($p< 0.005$) with a 5y-EFS of 0.31±0.04 (5y-OS:0.36±0.04) whilst other metastatic combinations revealed outcomes above 0.50 ±0.03. LDH (2x>normal) predicted a worse 5y-EFS of 0.43±0.03 (5y-OS:0.46±0.03) ($p< 0.001$) versus normal LDH (5y-EFS 0.71±0.07; 5y-OS:0.80±0.06). EFS multivariable analysis at diagnosis (MVA) showed independent significance for stage 4 (p-value 0.0139; HR: 1.608) and LDH (p-value 0.0042; HR: 2,237), but not for age. In stage 4 patients MVA on EFS found LDH (p-value 0.0098; HR: 2,567) and BM/skeleton involvement (p-value 0.0024; HR: 1,693) as independent risk predictors. In maintenance Dinutuximab-beta had a major impact on 5y-EFS in stage 4 patients with 0.69±0.05(5y-OS:0.71±0.05) vs. 0.41±0-07 (0.46±0.07) for those treated with 13-cis retinoic acid ($p=0.002$ & $p=0.001$), but not on others.

Conclusion

In HR-NBL1/SIOPEN outcomes improved with almost half of patients alive at 5 years and only few late events.

O6.3

Improved outcome for patients with alternative lengthening of telomeres (ALT) neuroblastoma randomized to tandem myeloablative therapy on COG ANBL0532

Koneru B[1], Burrow T[1], McCoy K[1], Nance J[1], Naranjo A[2], Zhang F[2], Irwin M[3], Park J[4], Reynolds C[1]
[1]Cancer Center and Department of Pediatrics, Texas Tech University Health Sciences Center School of Medicine, Lubbock, Texas, United States, [2]Children's Oncology Group Statistics and Data Center, University of Florida, Gainesville, FL, United States, [3]Department of Pediatrics, Hospital for Sick Children, University of Toronto, Toronto, ON, Canada, [4]Department of Oncology, St. Jude Children's Research Hospital , Memphis, TN, United States

Parallel session 6: Clinical cohort studies, May 16, 2023, 10:40 AM - 11:30 AM

Background: High-risk neuroblastoma (HRNB) patients with alternative lengthening of telomeres (ALT) tumors (~23% of patients) have lower overall survival (OS) compared to other HRNB patients. HRNB patients enrolled in COG ANBL0532 randomized to tandem autologous stem cell transplant (ASCT) had a superior event-free survival (EFS) compared to those randomized to single ASCT. We sought to determine if ALT HRNB patients on ANBL0532 benefited from tandem ASCT.
Methods: We determined the presence of telomere maintenance mechanisms (TMM) in 204 primary tumors from ANBL0532 patients. Telomere maintenance mechanism was defined as per Cancer Res 80:2663, 2020; patients designated as TERT+ had high TERT mRNA expressing tumors and as ALT if tumors were positive for the telomeric DNA C-circle assay (CCA) or ultrabright telomere foci (UTF). Due to non-proportional hazards, survival comparisons were performed using the Improved Two-Stage Procedure (J Stat Comput Simul. 2017; 87:1877).
Results: TMM status was: ALT n=48 (23.5%), TERT+ n=140 (68.6%), TMM negative n=16 (7.8%). Patients with TERT+ tumors more frequently progressed early, but 8-year OS was superior for TERT+ compared to ALT (58.1±4.6% vs 41.6±8.2%; p=0.0007), while 8-year OS for TMM negative was 67.7±12.2%. The 8-year OS for TERT+ patients randomized to single (n=67) vs tandem (n=59) ASCT was 56.9±6.7% vs 71.8±6.5% (p=0.32) and for ALT patients randomized to single (n=20) vs tandem (n=19) ASCT was 26.9±11.5% vs 61.1±13.5% (p=0.011). For patients on ANBL0532 who received dinutuximab post-consolidation therapy, the 8-year OS for TERT+ patients who underwent single (n=52) vs tandem (n=43) ASCT was 53.5±7.6% vs 75.8±7.8% (p=0.10) while for ALT patients who underwent single (n=13) vs tandem (n=10) ASCT it was 23.1±14.3% vs 77.8±16.4% (p=0.022).
Conclusions: This is the first study of TMM for neuroblastoma patients treated on a single prospective clinical trial. As previously reported, patients with ALT HRNB have inferior OS relative to TERT+ HRNB. As expected, TMM negative HRNB patients had apparently higher OS than ALT or TERT+ but small numbers preclude formal analyses. OS was significantly improved in ALT HRNB by tandem ASCT. These data support use of tandem myeloablative therapy for patients with ALT neuroblastoma until effective targeted therapies become available.

O6.4

A Multicenter Cooperative Group Study of Survivors of High-Risk Neuroblastoma: The Late Effects after High-Risk Neuroblastoma (LEAHRN) Study (COG ALTE15N2)

Diller L[1], London W[1], Bardwell J[2], Kao P[1], Nathan P[3], Landier W[6], Moustafi-Moab S[5], Brinkman T[4], Henderson T[2]

[1]Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Harvard Medical School, Boston, United States, [2]University of Chicago, Chicago, United States, [3]The Hospital for Sick Children, Toronto, Canada, [4]St. Jude Children's Research Hospital, Memphis, United States, [5]Children's Hospital of Philadelphia, Philadelphia, United States, [6]University of Alabama, Birmingham, United States

Parallel session 6: Clinical cohort studies, May 16, 2023, 10:40 AM - 11:30 AM

Background
Aggressive therapies for high-risk neuroblastoma (HRNBL) have resulted in increased survival, but late effects have not been well-characterized.

Patients and Methods
Comprehensive cross-sectional evaluation of eligible subjects (survivors of HRNBL enrolled on the COG Neuroblastoma Biology Study after 01/2000 and ≥5 years from original diagnosis) included medical record abstraction and physical/diagnostic/neurobehavioral assessments. Descriptive, univariate and multivariable analyses identified extent of and risk factors for late toxicity.

Results
375 eligible subjects from 88 COG centers enrolled. Median age at neuroblastoma was 2.5 years (range: 0.2-15.8) and at enrollment was 12.0 years (range: 5.0-24.0). All subjects received chemotherapy plus other therapies; 94% received cisplatin (median dose:398mg/m2). Other exposures included anti-GD2 antibody therapy (64%;231/363); radiation (95%;355/373); isotretinoin (91%;336/368); MIBG-therapy (7%;27/368); single (79%;288/363) or tandem transplant (20%;72/360); Busulfan/Melphalan (BuMel) (18%;66/363), Carboplatin/Etoposide/Melphalan (CEM) (73%;273/375); Thiotepa/Cytoxan (19%;70/375) and total body irradiation (5%;17/363).

Severe ototoxicity (requiring hearing aids) was observed in 58% of 372 subjects. Specific toxicity counts included hypothyroidism (17%;62/369), pulmonary hypertension (1%;4/365); congestive heart failure (9/373), restrictive lung disease (RLD) (Total Lung Capacity <70% predicted) (8%;17/207), hypertension (6%;22/373) and elevated creatinine (7%;24/365). Growth failure (height Z-score <-1.7) was observed in 121/361(34%). 15/375(4%) developed a secondary malignancy.

Adjusting for sex, follow up time, age and race, there were no observed differences in risk of second cancer, severe ototoxicity, growth failure, nor RLD for survivors who received BuMel vs. CEM, nor comparing those who did or did not receive anti-GD2 antibody therapy. Severe ototoxicity risk was not elevated with use of carboplatin during transplant for any category of cisplatin induction dose. Comparing subjects who received CEM as a component of tandem transplant (vs. single CEM), there was increased risk of growth failure [OR (95%CI)=4.2(2.1, 8.4);p<0.0001], but not of RLD. Exposure to isotretinoin was not associated with increased risk of growth failure. Further multivariable analyses will be presented, exploring risks for late toxicity

associated with conditioning regimens, radiation therapy, surgical approaches, and acute and chronic complications of therapy.

Conclusions
The burden of late toxicity in survivors of HRNBL is significant. Anti-GD2 immunotherapy and isotretinoin do not appear to be associated with SMN, ototoxicity, growth failure, or RLD.

O7.1

SOX11 is a mediator of core regulatory circuit robustness: upregulation of SWI/SNF components and recruitment of transcriptional enhancer NONO

Louwagie A[1,2], Decaesteker B[1,2], Bekaert S[1,2], De Vloed F[1,2], Sanders E[1,2], Berezovskaya A[3], Van Haver S[2,4], Pieters T[1,2], Van Vlierberghe P[1,2], Roberts S[4], Ek S[5], Versteeg R[6], Koster J[6], van Nes J[6], Zimmerman M[3], De Preter K[1,2], Bek J[1,2], Speleman F[1,2]

[1]Ghent University, Department of Biomolecular Medicine, Belgium, [2]Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [3]Dana-Farber Cancer Institute, Department of Pediatric Oncology, USA, [4]Memorial Sloan Kettering Cancer Center, Department of Pediatrics, USA, [5]Lund University, Department of Immunotechnology, Sweden, [6]Academic Medical Center, Department of Oncogenomics, The Netherlands

Parallel session 7: Genetic defects and dependencies in neuroblastoma, May 16, 2023, 10:40 AM - 11:30 AM

Background: The SOXC family of transcription factors are multifunctional regulators of neurodevelopment. Family member SOX11 is highly expressed in neuroblastoma (NB) tumors and is a lineage-specific gene dependency in this cell type. We previously identified the SOX11 locus as a target for recurrent focal copy number alterations in MYCN-amplified tumors. Functional analysis revealed a role for SOX11 in NB cell proliferation and survival. Further studies demonstrated that SOX11-regulated transcription is essential for NB gene expression control, cytoskeletal morphology, and neurodevelopment.

Aims: We sought to map SOX11 DNA binding sites and the SOX11-interactome in NB to understand the role of SOX11 in regulating the adrenergic core regulatory circuit (CRC) and facilitating tumorigenesis.

Methods/materials: We mapped SOX11 genome occupancy using CUT&RUN in SOX11-expressing adrenergic NB cell lines and the mesenchymal NB cell line SH-EP with SOX11 over-expression. The SOX11-interactome was established through chromatin immunoprecipitation combined with mass spectrometry (RIME).

Results: SOX11 directly binds the promoter regions and transcriptionally regulates 10 SWI/SNF core components, including SMARCC1, SMARCA4/BRG1 and ARID1A. Additionally, epigenetic regulators, including histone deacetylase HDAC2, PRC1 component CBX2, chromatin-modifying enzyme KDM1A/LSD1 and pioneer factor c-MYB, are directly regulated by SOX11. SOX11 DNA binding data also unequivocally establishes SOX11 as integral part of the CRC in adrenergic neuroblastoma. Next, interactome mapping revealed enrichment of proteins involved in alternative mRNA splicing, including spliceosome-associated proteins such as splicing factor SRSF2 and RNA binding proteins RBMX and RBM4. SOX11 additionally interacts with paraspeckle proteins NONO, SFPQ and PSCP1. Using publicly available gene dependency and expression data (DepMap and R2), NONO was identified as a genetic dependency with high expression associated with poor survival in NB. NONO is a multifunctional protein with various roles in gene regulation, pre-mRNA processing and splicing, which is implicated in genome maintenance and undergoes phase separation at DNA damage foci. Of particular interest, NONO enhances mRNA processing of super-enhancer-associated genes and CRC members GATA2 and HAND2 genes in NB.

Conclusion: Based on our genomic mapping and interactome data, we conclude that SOX11 is a central mediator of core regulatory circuit robustness through interaction with SWI/SNF components and recruitment of transcriptional regulator NONO.

O7.2

Interplay between LDB1 and core transcription factors instructs enhancer-promoter interactions and determines cell-fate in neuroblastoma

Liu Z[1], Hong J[1], Li S[2], Zhang X[1], Kang J[1], Xu M[1], Sun M[1], Lei H[1], Shern J[1], Hannenhalli S[2], Thiele C[1]
[1]Pediatric Oncology Branch, National Cancer Institute, Bethesda, United States, [2]Cancer Data Science Laboratory, National Cancer Institute, Bethesda, United States

Parallel session 7: Genetic defects and dependencies in neuroblastoma, May 16, 2023, 10:40 AM - 11:30 AM

Background: Cell type-specific activation of enhancers determines cell-fate. The noradrenergic neuroblastoma (NB) phenotype is determined by MYCN and core regulatory circuitry (CRC) transcription factors (TFs) such as GATA3, HAND2 and ISL1. However, the mechanisms by which MYCN/CRC TFs-bound enhancers loop to distal target genes to regulate gene expression and specify NB cell identity is unknown.

Aims: Investigate how MYCN/CRC TFs regulate the expression of their distal target genes.

Methods: To investigate enhancer-promoter interactions in NB, we performed RNA sequencing, chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq), high throughput chromosome conformation capture (HiC), and chromosome conformation capture coupled with immunoprecipitation (HiChIP) experiments.

Results: HiC and HiChIP studies in IMR32 and KCNR cells demonstrate extensive chromatin looping of MYCN and CRC TF-bound enhancers with distal genes. Interactome assays revealed that chromatin looping factor, LDB1, interacts with MYCN and CRC TFs. ChIP-seq results indicated genome-wide co-localization of LDB1 and MYCN/CRC TFs, with LDB1 binding to DNA assisted by CRC TF ISL1 and GATA3 in both IMR32 and BE(2)C cells. Depmap portal gene expression analysis indicated that LDB1 is more highly expressed in NB compared to other cancers ($p<0.0001$). The depmap cancer dependency analysis revealed that LDB1 is selectively essential in NB ($p<0.0001$). Consistently, LDB1 knockdown using siRNAs in IMR32, BE(2)C and KCNR NB cells resulted in a 20-30% decrease of cell proliferation ($p<0.01$) as shown by the IncuCyte cell confluence assay. Transcriptomic profiling in IMR32 and KCNR cells revealed that high LDB1 is associated with a neural stem cell like transcriptional program, whereas LDB1 knockdown resulted in a negative enrichment of noradrenergic signature genes and a positive enrichment of genes expressed in mature neurons. HiChIP for H3K27ac in IMR32 cells indicates that LDB1 colocalizes with MYCN/CRC TFs but not CTCF and cohesin at interconnected enhancers. Importantly, the depletion of LDB1 results in a disruption of the chromatin looping and a rewiring of promoter-to-enhancer interactions of MYCN/CRC TF-regulated genes.

Conclusion: Our study demonstrates that LDB1 cooperates with MYCN/CRC TFs to instruct enhancer-promoter interactions and regulate the transcriptional program responsible for the maintenance of the noradrenergic NB phenotype.

O7.3

Regulatory non-coding somatic mutations as drivers of neuroblastoma

Tirelli M[1,2], Montella A[2,3], Lasorsa V[2], Capasso M[2,3], Iolascon A[2,3]
[1]European School of Molecular Medicine, Università degli Studi di Milano, Milan, Italy, [2]CEINGE Biotecnologie avanzate Franco Salvatore, Naples, Italy, [3]Università degli Studi di Napoli Federico II, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Naples, Italy

Parallel session 7: Genetic defects and dependencies in neuroblastoma, May 16, 2023, 10:40 AM - 11:30 AM

Background
Emerging evidence suggest that non-coding somatic variants in cancer can contribute to disease, mainly by residing in functional regulatory regions and ultimately affecting gene expression networks. However, their interpretation is challenging. Whole genome-sequencing (WGS) data from 151 neuroblastoma (NB) primary tumors identified two clusters of non-coding Single Nucleotide Variants (SNVs) in two distinct NB specifically active cis-Regulatory Elements (cREs), denominated CTTNBP2-cRE and MCF2L-cRE based on their predicted target genes, respectively. Decreased expression of these genes in RNA-seq data from NB patients correlated with poor survival and unfavorable NB prognostic markers.

Aims
We aim to investigate the pathogenic effects of SNVs falling in cREs, suggesting that they can cause regulatory network deregulation and affect target gene expression in NB tumorigenesis.

Methods
Luciferase assays assessed alterations in cREs activity. Motif analysis (R package motifbreakR) investigated Transcription Factors (TF) binding motifs alterations. siRNA silencing experiments evaluated TFs and target genes correlation, and ChIP-qPCR assays evaluated TFs binding differences to mutated cREs. MTT and invasion assays with target gene silencing assessed cell proliferation and migratory capability.

Results
Luciferase assays showed that chr7:117513318:G>C (CTTNBP2_V1), chr13:114058179:T>C (MCF2L_V1) and chr13:114058184:G>A (MCF2L_V2) caused a lower activity of their respective cREs, possibly due to binding affinity alterations of TFs acting as activators or repressors, determining decreased expression of downstream genes. Alternatively, chr7:117513582:G>T (CTTNBP2_V2) increased cRE transcriptional activity. Motif analysis predicted TFs whose binding motifs were affected by SNVs; we selected STAT3 for CTTNBP2_V1 and MCF2L_V1, and SIN3A for CTTNBP2_V2, based on their role as activator and repressor, respectively. ChIP-qPCR experiments confirmed the predicted binding alterations.
Correlations between the identified TFs and CTTNBP2 and MCF2L were confirmed by TFs silencing experiments. MTT and invasion assays in NB cells showed that lower CTTNBP2 and lower MCF2L levels contributed to cell growth and greater migratory behavior, suggesting their possible role as oncosuppressors in NB.

Conclusion
Our results show that the presence of SNVs in cREs modulates the binding capacity of TFs, thus altering transcription of their target genes. We suggest a potential role of these TFs in NB development and pathogenic effects of SNVs falling in their cREs on gene function.

O7.4

Parallel sequencing of extrachromosomal circular DNA and mRNA in single neuroblastoma cells

Chamorro Gonzalez M[1], Conrad T[2], Stöber M[2], Xu R[1], Giurgiu M[1], Kasack K[3], Brückner L[4], van Leen E[1], Rodriguez-Fos E[1], Helmsauer K[1], Dorado Garcia H[1], Bei Y[1], Schmelz K[1], Sauer S[2], Eggert A[1,5,6], Schulte J[1,5,6], Schwarz R[2,7,8], Haase K[1,6], Koche R[9], Henssen A[1,4,5,6]

[1]Department of Pediatric Oncology/Hematology, Charité-Universitätsmedizin , Berlin, Germany, [2]Berlin Institute for Medical Systems Biology, Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany, [3]Fraunhofer Institute for Cell Therapy and Immunology Bioanalytics and Bioprocesses IZI-BB, Postdam, Germany, [4]Experimental and Clinical Research Center (ECRC) of the MDC and Charité Berlin, Berlin, Germany, [5]Berlin Institute of Health, 10178 , Berlin, Germany, [6]German Cancer Consortium (DKTK), partner site Berlin, and German Cancer Research Center (DKFZ), Heidelberg, Germany, [7]Berlin Institute for the Foundations of Learning and Data (BIFOLD), Berlin, Germany, [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]Center for Epigenetics Research, Memorial Sloan Kettering Cancer Center, New York, USA

Parallel session 7: Genetic defects and dependencies in neuroblastoma, May 16, 2023, 10:40 AM - 11:30 AM

Extrachromosomal DNA (ecDNA) amplification is a common event in cancer associated with worse patient outcome. In neuroblastoma, ecDNA is identified in 30% of patients. One key feature of ecDNA is its ability to randomly segregate during mitosis, which promotes rapid intercellular heterogeneity allowing tumors to rapidly evolve and scape therapy. Therefore, understanding how ecDNA contributes to intercellular heterogeneity in cancer remains crucial. However, methods for an unbiased characterization of ecDNA in single cells are lacking. We developed scEC&T-seq (single cell extrachromosomal circular DNA and transcriptomic sequencing), a method for parallel sequencing and detection of extrachromosomal circular DNA and full-length mRNA in single cells. We demonstrate the ability of our method to isolate and detect ecDNA in single neuroblastoma cells. Besides MYCN, other neuroblastoma-associated genes, including CDK4 and MDM2, were identified on ecDNA. Our method was able to capture and recapitulate the structural complexity of ecDNAs in single neuroblastoma cells, and the matching transcriptomic data allowed us to identify fusion transcripts resulting from the rearranged extrachromosomal structures. More interestingly, we were able to characterize tumor subpopulations based on intercellular differences in ecDNA structure, presumably resulting from the on-going evolution of the initial ecDNA structure in neuroblastoma primary tumors. Additionally, we observed that intercellular differences in ecDNAs' content can drive differences in oncogene transcription levels in single neuroblastoma cells. Besides ecDNA, our method also characterized a large number of smaller circular DNAs per single cell, which are also abundantly identified in both healthy and malignant tissues, but their function in cancer is still unknown. We observed that whereas ecDNAs are clonally present in most cancer cells, only a very small fraction of small circular DNAs is recurrently identified in single cells indicating yet unknown prerequisites for maintenance and propagation. We envision that by integrating extrachromosomal circular DNA and mRNA sequencing, our method will be useful to investigate the impact of intercellular heterogeneity in extrachromosomal circular DNA on tumor evolution, as well as to interrogate its function in other biological and pathological processes.

O8.1

Drug sensitivity profiling of 3D neuroblastoma cultures in the pediatric precision oncology program INFORM

Gatzweiler C[1,2,3,4], Peterziel H[1,2,3], ElHarouni D[1,5,6,7], Herter S[1,2,3,5], Gerloff X[1,2,3,8], Jamaladdin N[1,2,3,5], Kreth S[1,9], Najafi S[1,2,3], Blattner-Johnson M[1,10], Jones D[1,10], Milde T[1,2,3,11], Westermann F[1,9], Witt O[1,2,3,11], Oehme I[1,2,3]

[1]Hopp Children's Cancer Center Heidelberg (KiTZ), Heidelberg, Germany, [2]Clinical Cooperation Unit Pediatric Oncology, German Cancer Research Center (DKFZ) and German Cancer Consortium (DKTK), Heidelberg, Germany, [3]National Center for Tumor Diseases (NCT), Heidelberg, Germany, [4]Faculty of Medicine, Heidelberg University, Heidelberg, Germany, [5]Bioinformatics and Omics Data Analytics, German Cancer Research Center (DKFZ), Heidelberg, Germany, [6]Faculty of Biosciences, Heidelberg University, Heidelberg, Germany, [7]Division of Pediatric Neurooncology, German Cancer Research Center (DKFZ) and German Cancer Consortium (DKTK), Heidelberg, Germany, [8]Faculty of Mathematics and Computer Science, Heidelberg University, Heidelberg, Germany, [9]Division of Neuroblastoma Genomics, German Cancer Research Center, Heidelberg, Germany, [10]Division of Pediatric Glioma Research, German Cancer Research Center (DKFZ) and German Cancer Consortium (DKTK), Heidelberg, Germany, [11]Department of Pediatric Oncology, Hematology, Immunology and Pulmonology, Heidelberg University Hospital, Heidelberg, Germany

Parallel session 8: Precision therapy: Novel targets, May 16, 2023, 4:00 PM - 4:50 PM

Background: The survival rate among children with relapsed neuroblastomas continues to be poor, and thus, novel personalized medicine approaches tailored to each tumor are being developed. Current pediatric precision oncology platforms, such as the INFORM (INdividualized Therapy FOr Relapsed Malignancies in Childhood) program, suggest that molecular profiling of tumor tissue should be complemented with functional assays.
Aim: Our aim was to apply functional ex vivo drug sensitivity profiling to neuroblastoma samples obtained through INFORM using freshly patient-derived tumor cells, mouse PDX-derived tumor cells and organoid-like cultures. Our second aim was to establish corresponding zebrafish PDX (zPDX) models for in vivo validation.
Methods: We used INFORM patient-derived multicellular fresh-tissue spheroid neuroblastoma cultures for ex vivo drug screening. To assess drug effects, a metabolic activity readout was performed after 72 h of exposure to a library of > 75 drugs. We compared these initial drug sensitivity results obtained with the fresh tissue cultures with the results obtained with the corresponding long-term organoid-like cultures (LTC) and with neuroblastoma cells isolated from the mouse PDX tumors derived from the respective fresh tissue material. Moreover, we applied a corresponding zPDX model for further in vivo hit validation and testing of an appropriate combinational treatment.
Results: Fresh tissue drug sensitivity profiling was feasible within a turnaround time of three weeks and demonstrated a strong effectiveness of BCL2 inhibitors in all screened neuroblastoma samples. One sample highly sensitive to 4/5 ALK inhibitors in the library was revealed to have an ALK p.R1275Q mutation. Another sample harboring a PIK3CA p.H1047R mutation demonstrated high sensitivity toward the PI3K inhibitor alpelisib. The direct comparison of the sensitivities of fresh tissue, mouse PDX-derived and LTC cultures revealed a large overlap (21/25 drugs) for all three culture models. Seven drug hits and combinations were further tested in vivo and identified two treatment options inducing partial response in up to 30% of the zPDX avatars.
Conclusions: Our results provide evidence that functional ex vivo drug sensitivity profiling is a promising tool in precision oncology that can be combined with rapid in vivo zPDX drug screening to find novel treatment options for relapsed neuroblastomas.

O8.2

Interactive online repository demonstrating drug sensitivity associated with genomic and transcriptomic profiles of patient-derived neuroblastoma organoids and classical cell lines

Langenberg K[1], van Hooff S[1], Koopmans B[1], Strijker J[1], Kholosy W[1], Ober K[1], Wijnen M[1], van Noesel M[1], de Krijger R[1], Eising S[1], Koster J[3], Dolman E[1,4], Molenaar J[1,2]

[1]Princess Máxima Center, Utrecht, The Netherlands, [2]Department of pharmaceutical sciences, Utrecht University, Utrecht, The Netherlands, [3]Department of Oncogenomics, Cancer Center Amsterdam, Amsterdam UMC, Amsterdam, The Netherlands, [4]ZERO Childhood Cancer , Sydney, Australia

Parallel session 8: Precision therapy: Novel targets, May 16, 2023, 4:00 PM - 4:50 PM

Background
Despite druggable events in 80% of neuroblastoma patients within The Princess Máxima Center individualized THERapies program, clinical uptake of treatment recommendations has been low, and individual clinical impact remains hard to predict, stressing the need for a method integrating genomics and transcriptomics with functional approaches into therapeutic decision making.

Aims
To establish an online repository integrating genomics and transcriptomics with high-throughput drug screening (HTS) of classical cell neuroblastoma lines and novel generated neuroblastoma patient derived organoids (NBL-PDOs) to improve identification of molecularly matched therapies and support clinical uptake.

Methods
Nineteen cell lines were included as well as fourteen NBL-PDOs. Cell lines, NBL-PDOs and their parental tumors were characterized utilizing (lc)WGS, WES and RNAseq. Cells were exposed to a 196-compound library, and viability was assessed using CellTiterGlo.
Molecular and HTS results were transferred to the R2 platform (https://hgserver2.amc.nl/cgi-bin/r2/main.cgi?option=about_dscope) where a comprehensive suite of visualizations and analysis options were implemented.

Results
A dynamic, online available user-friendly repository of neuroblastoma cell lines and NBL-PDOs with detailed genetic and pharmacological annotation was established.
A powerful reference set of cell lines was incorporated, reflecting distinct known pharmacologic vulnerabilities, including drug-resistance of TP53-inactivated cell lines to idasanutlin; ALK-inhibitor sensitivity in ALK-activated lines, and sensitivity of IMR32 harboring ATM deletion to PARP- and CHEK1-inhibitors.
NBL-PDOs retained molecular features of the parental tumor. HTS reflected established drug sensitivities and identified additional therapeutic vulnerabilities in vitro, for example a striking venetoclax sensitivity correlated to mesenchymal signature. Finally, we explored personalized drug sensitivities within iTHER, demonstrating HTS can support genomic and transcriptomic results, thereby strengthening the rationale for clinical uptake. Furthermore, in vitro insensitivity may avoid ineffective treatments. Lastly, novel treatment options may be identified, as indicated by sensitivity to ponatinib correlating with overexpression of RET or KIT.

Conclusion

We established a dynamic publicly available dataset with detailed genomic, transcriptomic, and pharmacological annotation of classical neuroblastoma cell lines as well as NBL-PDOs, representing the heterogeneous landscape of neuroblastoma. We anticipate that in vitro drug screening will be complementary to genomic-guided precision medicine by supporting clinical decision making, thereby improving prognosis for all neuroblastoma patients in the future.

O8.3

Drugging the ATR-CHK1-RRM2 replicative stress signalling axis in neuroblastoma: exploring deeper mechanistic insights and novel drugging options

Nelen I[1,2], Poon E[3], Depestel L[1,2], Nunes C[1,2], Loontiens S[1,2], de Wilde B[2,4], Goossens S[1,2], Chesler L[3], Speleman F[1,2], Durinck K[1,2]

[1]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium, [2]Cancer Research Institute Ghent, Ghent, Belgium, [3]Division of Clinial Studies and Cancer Therapeutics, The Institute of Cancer Research, London, UK, [4]Department of Pediatric Hematology, Oncology and Stem Cell Transplantation, Ghent University Hospital, Ghent, Belgium

Parallel session 8: Precision therapy: Novel targets, May 16, 2023, 4:00 PM - 4:50 PM

Background: We recently identified RRM2 as a dosage sensitive co-driver of MYCN-driven NB formation. RRM2 is the rate-limiting component of the ribonucleotide reductase (RNR) enzyme that supplies cells with dNTPs for DNA replication and repair. RRM2 is tightly regulated throughout the cell cycle by the ATR-CHK1 signaling pathway and is a critical regulator in suppression of replicative stress (Nunes et al., 2022).

Aim: We aimed to (1) further investigate the mechanistic basis of RRM2 dependence in high-risk NB, (2) expand in vivo drugging in an immune-competent TH-MYCN allograft model and (3) broaden the drug portfolio to explore novel potent and safe drug combinations to target ATR-CHK1 dependency.

Methods: Immunoblotting for replicative stress markers was performed in NB cell lines and tumors dissected from MYCN single versus MYCN/RRM2 double transgenic zebrafish lines. The combination of triapine (3AP, RRM2 inhibitor) and prexasertib (CHK1 inhibitor) was tested in a TH-MYCN immune-competent allograft mouse model. The more selective CHK1 inhibitor SRA737 and the new RNR inhibitor TAS1553 were included in the preclinical in vitro evaluation pipeline to assess synergistic inhibition with ATR-CHK1-DNA damage inhibitors.

Results: Forced RRM2 expression in NB cells in vitro and in MYCN/RRM2 double transgenic zebrafish revealed quantitative reduction in replicative stress markers. A one-month scheme of combined 3AP/prexasertib drugging resulted in a rapid, complete and durable regression (>150 days after drug release) in the TH-MYCN immune-competent allograft mouse model. CHK1 inhibitor SRA737 revealed strong synergism with 3AP in a panel of MYCN-amplified and non-amplified cell lines. In contrast to prexasertib, SRA737 is presumed to have no detectable effect on CHK2 activity. Currently, the highly potent and selective small-molecule inhibitor TAS1553, targeting protein-protein interaction between RRM1 and RRM2, is being tested.

Conclusion: Increased RRM2 levels reduce replicative stress in high-risk NB cells in vitro and in tumors in vivo. Combined RRM2 and CHK1 inhibition in an immune-competent TH-MYCN allograft mouse model causes complete tumor regression and durable response more than 150 days after drug release. Further experiments are ongoing to test SRA737 and TAS1553 as novel compounds for combined RRM2/CHK1 inhibition to mediate RNR-driven sensitization of CHK1 dependency in high-risk NB.

O8.4

Nanoarchitectonics of the M13 phage provides a potent and specific anti-GD2 vector platform for Neuroblastoma Therapy

Zadran S[1], De Rosa P[1], Aloisi S[1], Milazzo G[1], Costantini P[1], Ulfo L[1], Saporetti R[1], Zuccheri G[1], Calvaresi M[1], Danielli A[1], Perini G[1]

[1]*University of Bologna, Department of Pharmacy and Biotechnology, Bologna, Italy*

Parallel session 8: Precision therapy: Novel targets, May 16, 2023, 4:00 PM - 4:50 PM

Background
Disialoganglioside-GD2 is a tumor-associated antigen, over-expressed on the surface of neuroblastoma (NB) cells, that is a well-suited target for immunotherapy.

Aim
Here we propose to adopt an orthogonal nanoarchitectonic approach to genetically and chemically manipulate M13 filamentous bacteriophage for the efficient and selective photodynamic killing of GD2+ NB cells or 3D spheroids. With respect to the anti-GD2 monoclonal antibody, the M13 phage nanocargo displays very low toxicity and a highly tuneable surface that can anchor and deliver thousands of conjugating drugs and/or imaging tags to individual cells.

Methodology
The M13 phage was engineered (M13GD2) to display the 14G2a-based Single chain variable Fragment ScFv (Lv-Hv) as a fusion protein with the C-terminus of the PIII protein of the phage, allowing for the pentavalent display of the ScFv against GD2 at the phage proximal end. The M13GD2 phages, following Atomic Force Microscopy validation, were, conjugated with the CF488 fluorophore to evaluate their selective and firm binding to GD2+ cells. Next, M13GD2 major capsid proteins were conjugated with hundreds of photosensitizers (Oligothiophene: ECB04 or Rose Bangal: RB) without impairing GD2-specific recognition. Photosensitizers, when excited with light, generate high levels of reactive oxygen species that trigger localized cytotoxicity.

Results
We showed that the M13GD2 recombinant phage specifically and only targets GD2+ NB cells. Next, we incubated M13GD2-ECB04 or M13GD2-RB with GD2+ or GD2- cells which were, subsequently, irradiated with ultralow white light. Results show that photodynamic cell death (>95%) occurred only in a GD2+ panel of NB cell lines at picomolar concentrations of ECB04 or RB (the lowest concentration ever recorded in Photodynamic Therapy), whereas no significant death was detected in GD2- cells. As a control, the M13GD2-ECB04 and M13GD2-RB did not display toxicity when not irradiated. Furthermore, modified phages were applied to spheroid models of IMR-32(GD2+) or SK-N-SH(GD2-) cells. In contrast to the anti-GD2 monoclonal antibody, M13GD2 can efficiently penetrate into the IMR-32 (GD2+) spheroids and induce cytotoxicity at nanomolar concentrations. No significant effects were, instead, observed in SK-N-SH (GD2-)-based spheroids.

Conclusions
Our phage vector platform provides an unprecedented, novel, and potent strategy for high drug delivery capability to GD2+ NBs.

O9.1

Single-nuclei transcriptomes from human adrenal gland reveals distinct cellular identities of low and high-risk neuroblastoma tumors

Bedoya O[1], Li W[1], Plescher M[1], Bullova P[1], Pui H[2], Kaucka M[3], Kharchenko P[4,5], Martinsson T[6], Holmberg J[7], Adameyko I[2,8], Deng Q[2], Larsson C[9], Juhlin C[9], Kogner P[10], Schlisio S[1]

[1]Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden, [2]Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden, [3]Max Planck Institute for Evolutionary Biology, Plön, Germany, [4]Department of Biomedical Informatics, Harvard Medical School, Boston, USA, [5]Harvard Stem Cell Institute, Cambridge, USA, [6]Department of Pathology and Genetics, University of Gothenburg, Sahlgrenska University Hospital, Gothenburg, Sweden, [7]Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden, [8]Department of Neuroimmunology, Center for Brain Research, Medical University of Vienna, Vienna, Austria, [9]Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden, [10]Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

Parallel session 9: Plasticity in neuroblastoma and normal development, May 16, 2023, 4:00 PM - 4:50 PM

Background

Childhood neuroblastoma has a remarkable variability in outcome. Age-at-diagnosis is one of the most important prognostic factors, with children younger than 18 months at the time of diagnosis displaying a better prognosis (i.e., low-risk) than children diagnosed at a later age. We aimed at identifying transcriptional and composition differences in cells of high- and low-risk neuroblastomas, and their similarities with developing and post-natal murine and human adrenal glands.

Methods

We sequenced to high-depth, and studied single-cell and single-nuclei transcriptomes of neuroblastoma with different clinical risk groups and stages, including healthy adrenal gland. We compared tumor cell populations with embryonic mouse sympatho-adrenal derivatives, and post-natal human adrenal gland.

Results

We provide evidence that low and high-risk neuroblastoma have different cell identities, representing two disease entities. Low-risk neuroblastoma presents a transcriptome that resembles sympatho- and chromaffin cells, whereas malignant cells enriched in high-risk neuroblastoma resembles a subtype of TRKB+ progenitor population identified in human post-natal gland. Analyses of these populations reveal different gene expression programs for worst and better survival in correlation with age at diagnosis.

Conclusions

Our findings reveal two cellular identities and a composition of human neuroblastoma tumors reflecting clinical heterogeneity and outcome.

O9.2

Trunk neural crest enriched MOXD1 suppresses tumor growth in neuroblastoma

Fredlund E[1,2,3], Andersson S[1,2,3], Hilgert E[4], Álvarez-Hernán G[1,2,3], Monferrer E[5], Karakaya S[1,2,3], Gregor T[1,2,3], Loontiens S[4], Bek J[4], Lecomte E[1,2,3], Magnusson E[1,2,3], Miltenyte E[1,2,3], Cabirol M[1,2,3], Kyknas M[1,2,3], Engström N[1,2,3], Arsenian-Henriksson M[6,7], Hammarlund E[2,3,7], Noguera R[5], Speleman F[4], van Nes J[8], 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]Center for Medical Genetics, Ghent University, Ghent, Belgium, [5]Department of Pathology, Medical School, University of Valencia-INCLIVA Biomedical Health Research Institute, Valencia, Spain, [6]Department of Microbiology, Tumor and Cell Biology (MTC), Biomedicum B7, Karolinska Institute, Stockholm, Sweden, [7]Department of Laboratory Medicine, Lund University, Lund, Sweden, [8]Department of Oncogenomics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Parallel session 9: Plasticity in neuroblastoma and normal development, May 16, 2023, 4:00 PM - 4:50 PM

Background
Neuroblastoma stems from cells of the developing sympathoadrenal lineage of early trunk neural crest (NC). Chromosomal aberrations are frequent in neuroblastoma, including loss of distal 6q, which holds the genomic location for the NC-specific gene MOXD1 (monooxygenase DBH-like 1).

Aims
Investigate the impact of MOXD1 on neuroblastoma and normal trunk NC development.

Methods
Using a novel chick embryo model, MOXD1 was knocked out in trunk NC cells, and normal- and neuroblastoma development was monitored until organs were fully developed. We performed MOXD1 knockout experiments using the chick chorioallantoic membrane assay and zebrafish neuroblastoma models. We overexpressed MOXD1 in neuroblastoma cell lines with no endogenous expression of the protein, performed RNA sequencing, analyzed tumor growth, and various in vitro abilities. We analyzed the impact of MOXD1 in patients using clinical cohorts and a tissue microarray consisting of 50 high-risk neuroblastomas.

Results
MOXD1 expression negatively correlates to advanced stage of neuroblastoma and patient prognosis. Loss of MOXD1 predicted worse overall survival in high-risk patients with an already dismal prognosis. MOXD1 knockout in a zebrafish neuroblastoma model increased tumor penetrance, while overexpression in patient-derived MOXD1 naïve adrenergic (ADRN) neuroblastoma cells repressed tumor formation and prolonged survival in xenograft models. Single nuclei RNA sequencing of primary patient material revealed that cells expressing MOXD1 clustered with undifferentiated neuroblastoma cells, sharing upregulated genes with NC- and neuroblastoma mesenchymal (MES) cells. Sequencing and experimental analyses of neuroblastoma isogenic cell pairs showed that MOXD1 expression discriminates between the MES and ADRN cell populations, with expression restricted to the MES cells and normal NC cells. Single-cell analysis revealed lineage-restricted expression of MOXD1 in Schwann cell precursors during NC-derived organogenesis. Our novel conditional MOXD1-knockout model in chick embryos showed a disorganized adrenal gland-capsule and medulla, and collection of cells in the Organ of Zuckerkandl.

Conclusion
In vivo experiments in zebrafish, chick, and mouse reveal MOXD1 as a novel tumor-suppressor in neuroblastoma. MOXD1 is an independent prognostic marker of patient outcome, and loss of MOXD1 correlates to a survival rate of <20% in high-risk neuroblastoma. MOXD1 displays lineage-restricted expression in normal NC- and neuroblastoma cells, implicating subtype-specific cells-of-origins in neuroblastoma.

O9.3

A harmonized single-cell transcriptomic atlas of human neuroblastoma

Bonine N[1,2,6], Zanzani V[1,3,6,7], Mus L[2,6,8], Vanneste B[4,7], Zwicker C[4,7], Thoné T[4,7], Roelandt S[1,6], Thirant C[5], Kramdi A[5], Janoueix-Lerosey I[5], De Wilde B[2,6,8], Everaert C[1,6], Vermeirssen V[3,7], Scott C[4,7], Speleman F[2,6], De Preter K[1,6]
[1]Laboratory for Translational Oncogenomics and Bioinformatics (TOBI), VIB-UGent Center for Medical Biotechnology, Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [2]Pediatric Precision Oncology Laboratory (PPOL), Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [3]Laboratory for Computational Biology, Integromics and Gene Regulation (CBIGR), Cancer Research Institute Ghent (CRIG), Ghent, Belgium, [4]Laboratory of Myeloid Cell Biology in Tissue Damage and Inflammation, VIB-UGent Center for Inflammation Research, Ghent, Belgium, [5]Inserm U830, Diversity and Plasticity of Childhood Tumors Lab, PSL Research University, Institut Curie Research Centre, Paris, France, [6]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium, [7]Department of Biomedical Molecular Biology, Ghent, Belgium, [8]Department of Pediatric Hematology, Oncology and Stem Cell Transplantation, Ghent University Hospital, Ghent, Belgium

Parallel session 9: Plasticity in neuroblastoma and normal development, May 16, 2023, 4:00 PM - 4:50 PM

Background: Single-cell and single-nucleus RNA sequencing (scRNA-seq or snRNA-seq) are powerful technologies to study the transcriptomic heterogeneity of tumors, including neuroblastoma. Previous studies using scRNA-seq and snRNA-seq were focused on tumoral heterogeneity and aggressiveness in relation to normal developmental and cell-of-origin. While useful, these studies have also raised novel questions.

Aims: At present, a comprehensive overview of these studies and data is lacking. In this study, we present a meta-analysis of published scRNA-seq and snRNA-seq datasets for neuroblastoma patient tumors. We compared wet lab and bioinformatics processing procedures across these studies and combined data to form an integrated transcriptomic atlas of human neuroblastoma tumors.

Methods: We reviewed all published scRNA-seq and snRNA-seq (n=9) studies and collected metadata, including patient information, sample processing details, wet lab protocol, and bioinformatics approaches (quality control, canonical gene marker selection, and cell type annotation). Thereafter, selected studies were combined to generate a cellular atlas, using benchmarked integration tools to correct for technical bias while preserving biological heterogeneity.

Results: Different wet lab protocols and bioinformatics pipelines were applied across the different studies. Most notably, this resulted in a discrepancy in the tumoral composition obtained with scRNA-seq compared to snRNA-seq, with a lack of neuroendocrine cells in scRNA-seq data. Data from more than 50 tumors across various studies (performed on the 10X Genomics platform) were normalized to largely overcome differences in the applied wet lab and bioinformatics approaches. As a result, a harmonized atlas of the transcriptomic landscape of human neuroblastoma tumors was generated. This atlas allows for gaining a more comprehensive view of the heterogeneity of malignant cells and the tumor microenvironment. To illustrate the power of the generated cell atlas as a framework for future single-cell studies, we mapped newly generated scRNA-seq and snRNA-seq data to this reference atlas for cell annotation and observed agreement with manual cell annotation.

Conclusion: Our study provides a comprehensive and harmonized view of the single-cell transcriptomic landscape of neuroblastoma and serves as a valuable reference resource for newly generated scRNA-seq and snRNA-seq data.

O9.4

Single-cell profiling of the neuroblastoma heterogeneity at the bone marrow metastatic niche

Fetahu I[1], Esser-Skala W[2], Rados M[1], Dnyansagar R[2], Sindelar S[2], Rifatbegovic F[1], Bileck A[3,4], Skos L[3], Bozsaky E[1], Lazic D[1], Shaw L[5], Tötzl M[1], Tarlungeanu D[1], Bernkopf M[1], Weninger W[5], Tomazou E[1], Bock C[6,7], Gerner C[3,4], Ladenstein R[1], Farlik M[5], Fortelny N[2], Taschner-Mandl S[1]

[1]St. Anna Children's Cancer Research Institute, Vienna, Österreich, [2]Paris Lodron University of Salzburg, Salzburg, Austria, [3]Department of Analytical Chemistry, Faculty of Chemistry, University of Vienna, Vienna, Austria, [4]Joint Metabolomics Facility, University and Medical University of Vienna, Vienna, Austria, [5]Department of Dermatology, Medical University of Vienna, Vienna, Austria, [6]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria, [7]Center for Medical Statistics, Informatics and Intelligent Systems, Institute of Artificial Intelligence, Medical University of Vienna, Vienna, Austria

Parallel session 9: Plasticity in neuroblastoma and normal development, May 16, 2023, 4:00 PM - 4:50 PM

Background
The bone marrow is the primary site of metastasis and relapse for neuroblastoma (NB). While NB has been molecularly defined at the primary cancer site, the understanding of the cellular diversity underlying its divergent biology and heterogeneous clinical behavior at the metastatic site, especially in subtypes with unfavorable genetics, is still lacking.
Aims
Here, we aimed to investigate the cell-intrinsic traits of NB cells that infiltrate the bone marrow and study the differences in cellular plasticity across NB subtypes in metastatic and primary tumors.
Methods
We performed single-cell transcriptomics (scRNA-seq) of bone marrow aspirates from 16 subjects spanning three major molecular subgroups of neuroblastoma, including patients with MYCN amplification (MNA), ATRX mutations (ATRXmut), and cases that lack these alterations (sporadic), and compared them to age-matched and metastasis-free bone marrow controls.
Results
Our data show that metastatic NB cells are primarily defined by a noradrenergic and, to some extent, also an adrenergic signature, albeit the expression of these markers was variable across patients. However, no NB cells with a mesenchymal or neural crest cell-like signature were discernible. Moreover, comparison of metastatic NB cells to adrenal medulla revealed that metastatic MNA tumor cells primarily consist of cells resembling neuroblasts, cycling neuroblasts, and bridge cells. In contrast, metastatic non-MNA tumor cells were characterized by the presence of late neuroblasts and chromaffin cells. Correlation analysis of gene expression in primary NB and bone marrow metastasis showed that MNA tumors cluster together, irrespective of tumor site, whereas ATRXmut and sporadic subtypes, form another group, further substantiating their divergent biology.
Conclusion
Together, our data show that phenotypic plasticity is conserved upon metastasis and differs in MNA patients compared to ATRXmut and sporadic NB subtypes, the latter two showing more pronounced transcriptional changes in the metastatic niche.

O10.1

Single-cell transcriptomics and epigenomics unravel the role of monocytes at the bone marrow metastatic niche in neuroblastoma

Fetahu I[1], Esser-Skala W[2], Dnyansagar R[2], Rados M[1], Sindelar S[2], Rifatbegovic F[1], Bileck A[3,4], Skos L[3], Bozsaky E[1], Lazic D[1], Shaw L[5], Tötzl M[1], Tarlungeanu D[1], Bernkopf M[1], Weninger W[5], Tomazou E[1], Bock C[6,7], Gerner C[3,4], Ladenstein R[1], Farlik M[5], Fortelny N[2], Taschner-Mandl S[1]

[1]St. Anna Children's Cancer Research Institute, Vienna, Austria, [2]Paris Lodron University of Salzburg, Salzburg, Austria, [3]Department of Analytical Chemistry, Faculty of Chemistry, University of Vienna, Vienna, Austria, [4]Joint Metabolomics Facility, University and Medical University of Vienna, Vienna, Austria, [5]Department of Dermatology, Medical University of Vienna, Vienna, Austria, [6]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria, [7]Center for Medical Statistics, Informatics and Intelligent Systems, Institute of Artificial Intelligence, Medical University of Vienna, Vienna, Austria

Parallel session 10: Tumor-micro environment interactions, May 16, 2023, 4:00 PM - 4:50 PM

Background
The bone marrow (BM) is the third most frequent site of metastasis for solid tumors, creating an unfavorable clinical outcome. It provides a unique microenvironment that promotes the growth of tumors, however, the role of different BM cells, their molecular features, and their interactions with tumor cells are poorly defined. Here, we investigate the BM niche in neuroblastoma (NB). The NB immune microenvironment has been molecularly defined at the primary cancer site, yet, the metastatic site remains to be elucidated.
Aims
In this study, we aimed to (i) investigate interactions between tumor cells and the BM microenvironment, and (ii) unravel metastasis-induced alterations in the BM.
Methods
We performed single-cell transcriptomics (scRNA-seq) and epigenomic profiling (scATAC-seq) of BM aspirates from 11 subjects spanning three major NB subtypes: patients with MYCN amplification (MNA), ATRX mutations (ATRXmut), and cases that lack these alterations (sporadic). NB cases were then compared to five age-matched and metastasis-free BM (controls), followed by in-depth single-cell analyses of tissue diversity and cell-cell interactions.
Results
The interrogation of the BM microenvironment composition in all three NB subtypes compared to controls revealed an enrichment in T- and NK cells, and a depletion of B- and myeloid cells in NB metastases compared to controls. Interactions of tumor cells with the BM microenvironment involve preferred communication with myeloid cells through the MK/LRP1/NCL and the MIF/CD74/CXCR4/CD44 axes. Monocytes present M1 and M2 features indicated by aberrant pro- and anti-inflammatory core TF regulatory loops, pro-differentiation, and reduction of cell cycle genes as well as expression of tumor-promoting factors, such as VEGF and EREG. Integration of scATAC-seq with scRNA-seq links epigenetically regulated myelo-monocytic lineage commitment and polarization with transcriptional changes resulting from external signals provided by and through tumor cells.
Conclusion
NB cells via cell-cell interaction signal to the BM microenvironment, rewiring specifically monocytes, which exhibit M1 and M2 features, marked by activation of pro- and anti-inflammatory programs, and express tumor-promoting factors, reminiscent of tumor-associated macrophages. Our study provides the basis for a therapeutic approach, targeting tumor-to-microenvironment interactions.

O10.2

Cancer-associated fibroblasts drive mesenchymal transition and therapeutic resistance via TGFb/NFkB signaling in neuroblastoma.

Louault K[1], Lee M[1], Blavier L[1], Pawel B[2], Asgharzadeh S[1,2], Declerck Y[1,3]
[1]Children's Hospital Los Angeles, University of Southern California, Pediatrics, Los Angeles, United States, [2]Children's Hospital Los Angeles, University of Southern California, Pathology, Los Angeles, United States, [3]University of Southern California, Biochemistry and Molecular Medicine, Los Angeles, United States

Parallel session 10: Tumor-micro environment interactions, May 16, 2023, 4:00 PM - 4:50 PM

Background
Therapeutic resistance remains a major challenge in cancer. In neuroblastoma (NB), it has recently been shown associated with an adrenergic (ADR) to a mesenchymal (MES) transition (AMT) of NB cells. The AMT mechanisms are not well understood but could involve intrinsic epigenetic mechanisms as well as extrinsic influences through the tumor microenvironment (TME). We have previously reported that cancer-associated fibroblasts (CAF) represent with macrophages the most abundant stromal cells in the TME of NB where they are a source of TGFβ, IL-6, IL-8 and MCP1 and stimulate the expression of TGFβ and IL-6 in NB cells, promoting therapeutic resistance.

Aims
To test the hypothesis that CAF provide an extrinsic mechanism driving AMT in NB.

Methods
NB cells (CHLA-255 and SK-N-BE(2)) and CAF harvested from NB tumors were cocultured and examined for the production of soluble cytokines by ELISA and for signalling pathway activation by western blot (WB). AMT was determined using a combination of phenotypic markers and functional parameters including migration and invasion and sensitivity to Doxorubicin and Cisplatin by CytoGlow analysis.

Results
We first demonstrated that NB cells co-cultured with CAF or CAF conditioned medium undergo AMT with an increase in cell size, a down regulation of PHOX2B, SOX11 and GATA2 and an increase in the expression of VIM, YAP1, SOX9 and FN1. CAF also increased NB cell migration and drug resistance. Interestingly, AMT was inhibited in the presence of the TGFβR1 inhibitor Galunisertib. WB analysis of NB cells revealed an increase in pSMAD2 but also in pNFκB. NFκB activation was prevented upon TAK1 and SMAD2 inhibition demonstrating its TGFβ-dependency. Blocking NFκB nuclear translocation or knocking down p65NFκB in NB cells in the presence of CAF, abolished their effect on AMT, migration and drug resistance.

Conclusions
The data identifies a novel TGFβ/NFκB pathway as an extrinsic mechanism by which CAF drive AMT and drug resistance in NB. Considering that several inhibitors of these pathways are undergoing clinical trials, our data suggests that inhibition of this pathway should be further explored in combination with chemo and immunotherapy in patients with NB.

O10.3

Changes in the bone marrow microenvironment upon neuroblastoma metastasis

Hochheuser C[1,2], Tol S[3], Kuijk C[1], van Zogchel L[2,4], Kleijer M[1], Castenmiller S[1], Wolkers M[1,5], Voermans C[1], Tytgat G[1,2], Timmerman I[1,2]

[1]Department of Hematopoiesis, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, [2]Princess Maxima Centre for Pediatric Oncology, Utrecht, the Netherlands, [3]Department of Research Facilities, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, [4]Department of Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands, [5]Oncode Institute, Utrecht, the Netherlands

Parallel session 10: Tumor-micro environment interactions, May 16, 2023, 4:00 PM - 4:50 PM

Background
Bone marrow (BM) is a preferred metastatic site for several solid tumors. This includes neuroblastoma (NB), for which recurrences in BM are a major obstacle to cure. The microenvironment of the BM appears to possess unique protective features. BM-residing Mesenchymal Stromal Cells (MSCs), for example, have been shown to be instructed by tumor cells to support them. A better understanding of this metastatic niche is therefore of great importance; to identify which (sub)populations support tumor cells in BM and to unravel how we can target the BMcell-tumor dialog to sensitize metastasized tumor cells for therapy.

Aims
Here, we aim to capture metastasis-related changes within the BM microenvironment, with a focus on MSCs, hematopoietic stem and progenitor cells (HSPCs) and T-cells.

Methods
Flow cytometry analysis was performed on fresh BM aspirates of patients with and without metastatic-NB, taken at diagnosis (n=44) or during treatment (n=103 samples of 24 patients). For a subgroup of patients (n=11), MSCs, T-cells and NB-cells were index-sorted from BM and single-cell RNA sequencing was performed (CEL-seq2). Moreover, MSCs and T-cells were isolated and tested for their differentiation and expansion capacity, respectively. The extent of tumor infiltration in BM was determined by qPCR for NB-mRNA.

Results
MSC levels were increased in BM samples with versus without metastasis. No significant differences in HSPC- and T-cell - (helper nor cytotoxic) levels were found. Phenotypically, MSC and T-cell compartments of tumor-infiltrated BM differed from non-infiltrated BM. Functionally, MSCs isolated from the metastatic niche were more prone to differentiate into the osteoblast lineage. BM-infiltrated T-cells were able to efficiently expand ex vivo and respond to autologous tumor digests. In total 4003 MSCs and 3613 T-cells were index-sorted and single cell sequenced. Ongoing analyses are being undertaken to uncover potential transcriptional alterations in BM-cells of the metastatic niche.

Conclusion
We found that the bone marrow microenvironment is altered in the presence of NB tumor cells. These data contribute to the understanding of the interactions between NB-cells and the environment at the metastatic site, which is essential in order to target the tumor cells in BM more effectively.

O10.4

Macrophage infiltration promotes regrowth in MYCN amplified neuroblastoma after chemotherapy

Valind A[1,2], Manouk Verhoeven B[3], Enoksson J[4], Karlsson J[1], Christensson G[1], Mañas A[1], Aaltonen K[1], Jansson C[1], Bexell D[1], Baryawno N[3], Gisselsson D[1,4], Hagerling C[1,4]

[1]Department of Laboratory Medicine, Lund University, Lund, Sweden, [2]Department of Pediatrics, Skåne University Hospital, Lund, Sweden, [3]Department of Women's and Children's Healthy, Karolinska Institute, Stockholm, Sweden, [4]Department of Pathology, Skåne University Hospital, Lund, Sweden

Parallel session 10: Tumor-micro environment interactions, May 16, 2023, 4:00 PM - 4:50 PM

Despite aggressive treatment, the 5-year event-free survival rate for children with high-risk neuroblastoma is <50%. While most high-risk neuroblastoma patients initially respond to treatment, often with complete clinical remission, many eventually relapse with therapy-resistant tumors. Novel therapeutic alternatives that prevent the recurrence of therapy-resistant tumors are urgently needed.

To understand the adaptation of neuroblastoma under therapy, we analyzed the transcriptomic landscape in 46 clinical tumor samples collected before (PRE) or after (POST) treatment from 22 neuroblastoma patients. RNA sequencing revealed that many of the top-upregulated biological processes in POST MYCN amplified (MNA+) tumors compared to PRE MNA+ tumors were immune-related, and there was a significant increase in numerous genes associated with macrophages. The infiltration of macrophages was corroborated by immunohistochemistry and spatial digital protein profiling. Moreover, POST MNA+ tumor cells were more immunogenic compared to PRE MNA+ tumor cells. To find support for the macrophage-induced outgrowth of certain subpopulations of immunogenic tumor cells following treatment, we examined the genetic landscape in multiple clinical PRE and POST tumor samples from nine neuroblastoma patients revealing a significant correlation between an increased amount of copy number aberrations (CNA) and macrophage infiltration in POST MNA+ tumor samples. Using an in vivo neuroblastoma patient-derived xenograft (PDX) chemotherapy model, we further show that inhibition of macrophage recruitment with anti-CSF1R treatment prevents the regrowth of MNA+ tumors following chemotherapy.

Taken together, our work supports a therapeutic strategy for fighting the relapse of MNA+ neuroblastoma by targeting the immune microenvironment.

O11.1

Depletion of CD11b+ myeloid cells augments anti-neuroblastoma immune response induced by the anti-GD2 antibody dinutuximab beta

von Lojewski L[1], Troschke-Meurer S[1], Zumpe M[1], Marx M[1], Lode H[1], Siebert N[1]
[1]*University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany*

Parallel session 11: Immune Therapy, May 16, 2023, 10:00 AM - 10:50 AM

Background / introduction. We recently showed CD11b+ myeloid cell-mediated upregulation of the PD-1/PD-L1 checkpoint on neuroblastoma (NB) cells treated with the chimeric anti-GD2 antibody dinutuximab beta. A blockade of this checkpoint increased anti-tumor efficacy of a GD2-directed treatment.
Aims. Here, we addressed whether the depletion of CD11b+ myeloid regulatory cells augments immunotherapeutic effects of dinutuximab beta against NB.
Methods / materials. Expression of CD11b+ myeloid cell-associated and modulating genes (M-CSF, M-CSFR, GM-CSF, CCL2, TGF-β1, IL-1β, IL-4, IL-6, IL-6R, CXCL8, IL-10, VEGF-A, Arg1, IDO, NOS2 and IFN-γ) was analyzed using RT-PCR. Flow cytometry and immunohistochemistry were used to assess tumor infiltrating leukocytes. Antitumor effects of a GD2-directed treatment with dinutuximab beta (15 mg/kg, days 4-8 after tumor cell inoculation) in combination with depletion of CD11b+ myeloid cells by anti-CD11b Ab (25 mg/kg, days 4, 7, 11, 14 and 18) or 5-Fluorouracil (5-FU; 50 mg/kg, days 4, 11 and 18) were evaluated in a murine syngeneic NB model.
Results. 53% of all leukocytes found in tumor tissue of untreated mice were CD11b+. Analysis of mRNA expression in NB cells used for tumor induction showed high expression of M-CSF, TGF-β1, IL-6, and VEGF-A genes that were further induced in primary tumor tissue. Although analysis of tumors revealed strong mRNA expression of all genes analyzed, the strongest induction was observed for M-CSFR, CCL2, IL-1β, IL-4, IL-6R, CXCL8, Arg1 and NOS2. Compared to controls, application of anti-CD11b antibodies resulted in a reduction of both tumor infiltrating CD11b+ cells and mRNA expression of the genes analyzed. Additionally, CD11b blockade showed delayed tumor growth and prolonged overall survival that could be further improved by either 5-FU treatment or dinutuximab beta. Importantly, the combinatorial immunotherapy with dinutuximab beta and 5-FU showed the strongest anti-tumor effects and superior survival rates.
Summary / conclusion. Depletion of immune suppressive myeloid cells augments anti-neuroblastoma efficacy of a dinutuximab beta-based immunotherapy representing a new effective treatment strategy against GD2-positive cancers.

O11.2

T cell mediated killing of neuroblastoma is inhibited by secreted midkine

Strijker J[1], van Berkum R[1,3], Damen M[2], Wu W[2,3], Molenaar J[1,2], Wienke J[1]
*[1]Princess Máxima Center, Utrecht, The Netherlands, [2]University of Utrecht, Utrecht, The Netherlands, [3]Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), Singapore, Singapore*

Parallel session 11: Immune Therapy, May 16, 2023, 10:00 AM - 10:50 AM

Introduction:
CAR-T cell therapy holds great promise for neuroblastoma, because of its potential for targeted effects and promising results in adult solid cancers. However, neuroblastoma tumour cells can escape T cell mediated killing by, among others, inhibition of T cell activation through secreted immunosuppressive factors. Here, we aimed to identify immunosuppressive factors which are secreted by neuroblastoma tumours and could be targeted to improve CAR-T cell efficacy.

Methods:
Proteins secreted by tumour cells (secretome) were analysed by performing mass spectrometry on conditioned medium from patient derived organoid cultures. The conditioned medium was concentrated using a 3kDa Millipore filters and prepared for LC-MS. Mass spectrometry data were acquired in data-dependent acquisition mode. The immunosuppressive capacity of neuroblastoma organoids was determined by flow cytometry readout of T cell proliferation and activation after incubation with concentrated secretome. To study immunoregulatory interactions in neuroblastoma, single-cell RNA-sequencing (scRNA-seq) data from 25 tumours were analysed using the CEL-seq2 platform. Interactions between tumour and immune cells were predicted using an unbiased ligand-receptor interaction analysis.

Results:
Culturing T cells with concentrated secretome of neuroblastoma organoids resulted in a spectrum of suppression of proliferation of the T cells (0% - 50% normalized suppression). Comparing the secretome from the most immunosuppressive organoids to non-suppression lines identified, among others, midkine in the top 20 most upregulated proteins as measured by LC-MS. ScRNA-seq analysis of neuroblastoma tumours showed a negative correlation between midkine expression and the cytotoxicity of T cells within the tumour. In addition, midkine expression in neuroblastoma tumours was significantly associated with worse survival. Recombinant midkine reduced the activation of FACS-sorted T cells in vitro, which confirms its immunosuppressive effects. These findings points towards midkine negatively influencing the cytotoxicity of T cells and reducing the survival of patients.

Conclusions and future perspective:
Using different unbiased analyses –mass spectrometry of neuroblastoma organoid secretome as well as scRNA-seq analysis of tumours-, we have identified midkine as a potent immunosuppressive factor in neuroblastoma, which could be a promising target for immunotherapy. Currently, we are exploring midkine inhibition as therapeutic strategy to increase T cell mediated killing of neuroblastoma.

O11.3

Allogeneic CAR T cells targeting GD2 for Treatment of Relapsed/Refractory High-Risk Neuroblastoma

del Bufalo F[1], De Ioris M[1], Del Baldo G[1], Fabozzi F[1], Becilli M[1], Rosignoli C[1], Sinibaldi M[1], Bertaina V[1], De Angelis B[1], Quintarelli C[1], Mastronuzzi A[1], Merli P[1], Locatelli F[1,2]

[1]Pediatric Hematology and Oncology IRCCS Ospedale Pediatrico Bambino Gesù, Rome, Italy, [2]Catholic University of the Sacred Heart, Rome, Italy

Parallel session 11: Immune Therapy, May 16, 2023, 10:00 AM - 10:50 AM

Background

Relapsed/refractory high-risk neuroblastoma (r/r HR-NB) is associated with an unsatisfactory long-term survival <10%. Immunotherapy using allogeneic T-cells genetically modified to express a chimeric antigen receptor (CAR) targeting disialoganglioside-2 (GD2) (GD2-ALLO-CART) represents an attractive option.

Aims

We explored the feasibility of GD2-ALLO-CART combined with allogeneic hematopoietic stem cells transplantation (HSCT) in heavily pre-treated patients.

Methods

Children with pluri-relapsed NB, who had failed autologous GD2-CAR T cells at our institution or could not be enrolled in the clinical trial (NCT03373097) for the profound lymphopenia, were infused with third-generation (CD28-4.1bb) GD2-ALLO-CART, whose construct incorporates also the safety switch inducible caspase-9 (iC9), in a hospital exemption setting. All patients received a fludarabine/cyclophosphamide-based lymphodepletion.

Results

Three children, who had failed >3 lines of therapy, were treated between 02/2022 and 11/ 2022. GD2-ALLO-CART (3x106 CAR+cells/kg) were administered after haploidentical HSCT in 2 patients while one child received the cells, generated by a fully HLA-matched family donor, before HSCT. We did not experience any production failure. The toxicities observed were mainly represented by cytokine release syndrome (CRS) and hematological. One patient developing grade 3 CRS and immune-effector cell-associated neurotoxicity syndrome (ICANS) received steroids and the activation of the suicide gene, showing a sharp drop of circulating GD2-ALLO-CART (from 55,6 to 5,4 cells/ul) and a rapid resolution of both toxicities. Grade II-III acute graft-versus-host-disease (GvHD) occurred in all patients, involving mainly skin and, in 2 patients, liver, and resolved rapidly with steroids and extracorporeal photopheresis. GD2-ALLO-CART expanded in vivo, were detectable in peripheral blood by flow-cytometry in all patients, reaching a median peak of 58.5% (range 27.6%-77.6%) of CD3+ cells (192.12/ul, range 142.26/ul -326.91/ul), and persisted long-term, >6 weeks in all patients, despite the GvHD treatment. One patient achieved a complete response, 1 a minor response, with >50% MIBG skeletal score reduction, and the third, treated with a rapidly progressing disease, a stable disease.

Conclusion

Use of GD2-ALLO-CART is feasible and safe in treating pluri-relapsed HR-NB and the suicide gene iC9 increases the safety profile of the approach. Importantly, GD2-ALLO-CART cells are able to induce a promising anti-tumor effect and deserve further evaluation.

O11.4

Library-based Discovery of Peptide-centric CAR T Cells for Neuroblastoma (NB)

Marshall Q[1], Kapoor R[1], Li P[1], Li W[3], Dimitrov D[3], Maris J[1,2], Yarmarkovich M[4,5]
[1]Center for Childhood Cancer Research, Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, United States, [2]Department of Pediatrics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, United States, [3]Center for Antibody Therapeutics, University of Pittsburgh School of Medicine, Pittsburgh, United States, [4]Department of Pathology, New York University Grossman School of Medicine, New York, United States, [5]Perlmutter Cancer Center, New York University Langone Health, New York, United States

Parallel session 11: Immune Therapy, May 16, 2023, 10:00 AM - 10:50 AM

Background: We recently characterized the ligandome of NB and developed peptide-centric-(PC) CAR T cells targeting a peptide derived from the PHOX2B transcription factor recurrently presented on multiple HLA allotypes (Nature, 2021). Despite these results, our upcoming NB clinical trial for our PC-CAR T cell product will be restricted to patients with HLA allotypes presenting the PHOX2B 9mer (~30-45% of the North American population). We have identified additional peptide targets in NB presented on major HLA allotypes and seek to develop a warehouse of PC-CARs that will cover the entire population. Current PC-CAR discovery efforts are often not sensitive enough to identify potent, selective PC-CAR T cells.

Aim: Combination of phage display and primary CAR T cell library screening platforms will dramatically streamline preclinical discovery of PC CAR T cells.

Methods: Phagemids were retrieved from scFv or Vhh phage display libraries ($1X10^{11}$) panned against peptides previously identified from NB ligandomes. Polyclonal receptor libraries were then cloned into a second-generation CAR vector and expressed in primary human T cells. PC-CAR T cells libraries were evaluated polyclonally and on a single cell level using the Berkeley Lights Lightning for avidity, cytotoxicity, and cross reactivity. Clones of interest were recovered and sequenced.

Results: As proof-of-concept, a polyclonal Vhh CAR T cell library targeting a PHOX2B 9mer presented on HLA-A*24:02 demonstrated robust antigen specific binding (60% of transduced cells) with minimal binding to a decoy peptide (11% of transduced cells). This population also demonstrated cross-HLA binding to PHOX2B presented on HLA-A*23:01 as well as a lower frequency (<2%) of binders to the divergent HLA-C*07:02. When evaluated at the single cell level, multiple PC-CAR T cell clones lysed SK-N-AS NB cells without lysis of HLA-matched, non-NB cell lines.

Conclusion: Here, we present a method for high throughput, single-cell screening of polyclonal CAR populations using affinity and functionality-based selection, allowing the identification of rare clones with desired safety and function. We are now applying this method to build a warehouse of population-scale therapies for use in NB. Ongoing work is focused on receptor validation and discovery of CARs targeting additional peptides differentially presented in the NB ligandome.

O12.1

Identification of genetic drivers of relapse in MYCN driven neuroblastoma

Cheng B[1], Farrel A[2], Seo H[3], Morrissy S[3], Maris J[2], Huang M[1]
[1]Children's Hospital Los Angeles, Department of Pediatrics, Los Angeles, United States, [2]Children's Hospital of Philadelphia, Department of Pediatrics, Philadelphia, United States, [3]University of Calgary, Department of Biochemistry and Molecular Biology, Calgary, Canada

Parallel session 12: Stem cell differentiation in vitro at age, May 17, 2023, 10:00 AM - 10:50 AM

Background
Amplification of MYCN has been linked to poor prognosis in neuroblastoma patients. However, it remains unclear how MYCN-amplified tumors are resistant to current standard of care. Because a relapse diagnosis is incurable, obtaining tumor tissue for analysis provides little benefit to the patient and thus, the amount of available relapse tissue to analyze is severely limited. While MYCN is sufficient to drive tumorigenesis in human neural crest cells, it is insufficient for chemotherapy resistance. Therefore, other mutations are required to cooperate with MYCN to promote therapy resistance.

Aims
1) Identify the loss of genes that confer chemotherapy resistance in MYCN driven neuroblastoma.
2) Identify genes which are expressed at lower levels in relapse compared to low risk neuroblastoma.

Methods
Human induced pluripotent stem cells (iPSC) from a healthy adult were transduced with doxycycline-inducible MYCN (DOX-MYCN). iPSC DOX-MYCN cells were differentiated toward trunk neural crest cells and implanted orthotopically into immunocompromised mice. DOX-MYCN tumor lines were derived in vitro and transduced with dCas9-KRAB to enable CRISPR interference (CRISPRi). These cells were then transduced with a whole genome sgRNA library and subsequently treated with etoposide. Genomic DNA of etoposide-resistant MYCN tumor cells were analyzed by deep amplicon sequencing.

Results
Multiple sgRNA targeting INPP4B, SOX6 and EBF1 were identified from the etoposide-resistant tumor cells. Each of these genes were also found to be expressed at lower levels in relapse tumors compared to low risk neuroblastoma tumors. R2 database analysis shows that tumors with lower expression of each of these genes correlates with worse prognosis than tumors with higher expression of these genes.

Conclusion
Our studies implicate three new genes that promote chemotherapy resistance in MYCN driven neuroblastoma tumors and are expressed at lower levels in relapse tumors compared to low risk neuroblastoma. In particular, INPP4B and SOX6 have previously been linked to tumor suppressive functions. Thus, our findings provide clues on how MYCN-amplified neuroblastoma are resistant to chemotherapy.

O12.2

Single-cell transcriptome and epigenome analysis in a stem cell model reveal developmental impact of neuroblastoma-associated chromosomal aberrations

Saldana-Guerrero I[1,2,3], Montano-Gutierrez L[4], Hafemeister C[4], Stavish D[1,2], Shaw L[5], Fetahu I[4], Wenninger-Weinzierl A[4], Sturtzel C[4], Souilhol C[1,2], Tarelli S[1,2], Shoeb M[4], Bernkopf M[4], Bozatzi P[4], Guarini M[6], Bozsaky E[4], Buri M[4], Putz E[4], Andrews P[1], Barbaric I[1,2], Bryant H[3], Distel M[4], Taschner-Mandl S[4], Farlik M[5], Tsakiridis A[1,2], Halbritter F[4]

[1]Centre for Stem Cell Biology, School of Biosciences, The University of Sheffield, Sheffield, United Kingdom, [2]Neuroscience Institute, The University of Sheffield, Sheffield, United Kingdom, [3]Sheffield Institute for Nucleic Acids (SInFoNiA), Department of Oncology and Metabolism, The University of Sheffield, Sheffield, Sheffield, United Kingdom, [4]St. Anna Children's Cancer Research Institute (CCRI), Vienna, Austria, [5]Medical University of Vienna, Department of Dermatology, Vienna, Austria, [6]CeMM Research Center for Molecular Medicine of the Austrian Academy of Science, Vienna, Austria

Parallel session 12: Stem cell differentiation in vitro at age, May 17, 2023, 10:00 AM - 10:50 AM

Background:
Early childhood malignancies are driven by sparse genetic aberrations in oncogenes that often co-occur with large copy number variants (CNVs). The combination of these mutations is thought to transform developmentally pliant embryonic cells to initiate tumorigenesis. However, the mechanistic interactions between CNVs, oncogenes, and differentiation have not been systematically studied due to several obstacles: (i) CNVs cannot be engineered efficiently yet; (ii) transient embryonic progenitors are absent in full-grown tumors; and (iii) inter-species differences in lineage specification limit the applicability of animal models.

Aims:
We sought to investigate the impact of chromosomal aberrations on embryonic tumorigenesis using the embryonal tumor neuroblastoma (NB) as a model.

Methods:
To overcome the aforementioned challenges, we used isogenic human embryonic stem cell (hESC) lines carrying gains of chromosome 17q/1q, which are prevalent in NB. We differentiated these cells toward trunk neural crest (NC) and their sympathoadrenal derivatives, the putative cells-of-origin of NB, and performed single-cell RNA sequencing, epigenome analysis, and cell-biological assays at key differentiation stages.

Results:
We found that CNVs impaired the specification of sympathoadrenal cell types and instead potentiated early Schwann-cell-precursor-like phenotypes. Additional overexpression of the oncogene MYCN (frequently amplified with CNVs in high-risk NB) exacerbated these differentiation defects, enabled tumourigenic cell proliferation, and generated cell states in vitro that transcriptionally resembled NB tumor cells. Finally, we connected these states to a stepwise disruption of gene-regulatory networks centered on developmental transcription factors.

Conclusion:
Together, our results chart a mechanistic route to NB tumorigenesis and provide a general framework for the CNV-driven initiation of embryonal tumors, in which CNVs 'prime' embryonic cells for oncogenic transformation. The tumor-like cells in our model may serve as proxies to experimentally test therapeutic interventions during tumorigenesis.

O12.3

Maintenance of pluripotency in the entire ectoderm enables neural crest formation – insights from normal development to help understand neuroblastoma

Pajanoja C[1], Hsin J[1], Olinger B[1], Schiffmacher A[1], Abrams S, Dapkunas A[1], Zainul Z[1], Doyle A[1], Martin D[1], Kerosuo L[1]

[1]*National Institutes of Health, The National Institute of Deltal and Craniofacial Research, Bethesda, MD, United States*

Parallel session 12: Stem cell differentiation in vitro at age, May 17, 2023, 10:00 AM - 10:50 AM

Background: Neuroblastoma is a prenatally formed neural crest (NC) derived pediatric tumor and it remains unclear when and how during NC development the tumors form. Recent studies have shifted focus on trying to find evidence of the initiation from early stages of NC development when it has not yet committed to the sympathoadrenal lineage. Similarly, our knowledge on normal NC development is increasing, which provides understanding to what goes awry during malignant transformation. Even though the NC is derived from the ectodermal germ layer post-gastrulation, it is pluripotent and gives rise to very different cell types including the peripheral nervous system, pigment, bone, cartilage and chromaffin cells. This contradicts the developmental dogma according to which the ability of the embryo to contribute to pluripotent progeny is lost after gastrulation.

Aims: We aimed to understand the very early steps of NC development right after gastrulation when the ectoderm is patterned into the domains of future central nervous system, epidermis and the NC. We specifically focused on the highly contested question of how the pluripotency is formed – by maintenance from pre-gastrula stage or by re-gaining the potential later during neural crest development.

Methods: We monitored transcriptional changes in the embryonic ectoderm from gastrulation to neurulation using high resolution single-cell-Multiplex-Spatial-Transcriptomics (scMST) complemented with scRNA-sequencing.

Results: Unexpectedly, we find maintenance of undecided Nanog/Oct4-PouV/Klf4-positive pluripotent pan-ectodermal stem-cells spanning the entire ectoderm late in the neurulation process with ectodermal patterning completed only at the end of neurulation when pluripotency becomes restricted to NC, challenging our understanding of gastrulation. Furthermore, broad ectodermal pluripotency is found at all axial levels, including the posterior parts where neuroblastoma forms, unrelated to the NC lineage the cells later commit to, suggesting a general role in stemness enhancement and proposing a mechanism by which the NC acquires its ability to form derivatives beyond “ectodermal-capacity” in chick and mouse embryos.

Conclusion: We proposes a model for how NC gains its exceptionally high stem cell potential. As tumors often utilize regulatory circuitries adopted from their stem cell phase, these results will provide insight on how pluripotency regulation may be altered during neuroblastoma formation.

O12.4

Modelling neuroblastoma using germline ALK-R1275Q mutant patient-derived induced pluripotent stem cells

Liu M[1], Zubillaga V[1], Marin Navarro A[1], Kogner P[2], Falk A[3,4], Wilhelm M[1]
[1]Department of Microbiology, Tumor and Cell biology, Karolinska Institutet, Stockholm, Sweden, [2]Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden, [3]Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden, [4]Department of Experimental Medical Science, Lund University, Lund, Sweden

Parallel session 12: Stem cell differentiation in vitro at age, May 17, 2023, 10:00 AM - 10:50 AM

Background
Amplification of Anaplastic Lymphoma Kinase (ALK) or activating mutations in the tyrosine kinase domain of the ALK gene is a common somatic alteration in neuroblastoma (NB) and has been correlated with poor prognosis in intermediate and high-risk patients. Although hereditary NB is rare, germline gain-of-function mutations have been found in ALK, mimicking common sporadic ALK mutations in NB.

Aim
Our aim is to study the role of ALK mutations in NB initiation.

Method
The induced pluripotent stem (iPS) cell technology allows somatic cells to be reprogrammed into pluripotent stem cells with the ability to self-renew and differentiate to almost all cell types. To study the contribution of ALK mutations in NB development, we reprogrammed non-cancerous fibroblast from NB patients carrying a germline ALK-R1275Q mutation and healthy individuals to iPS cells. The origin of NB is thought to be neural crest cells (NCC) and its derivative sympathoadrenal (SA) cells. Importantly, we established a robust NCC and SA differentiation protocol deriving trunk NCC and SA cells from iPS cells to analyze the impact of ALK-R1275Q mutation during SA differentiation.

Results and Conclusion
No differences in reprogramming capacity, expression of pluripotency markers, or ability to differentiate to migratory trunk NCC were observed, suggesting that ALK-R1275Q mutation does not interfere with early human embryonic development. Analysis of the transcriptomic landscape during the differentiation process from NCC to SA cells shows that trunk NCC-relevant markers, like SOX10, TFAP2A, NGFR (p75), and HOXC9, are mainly expressed in the NCC-stage and downregulated in SA cells. On SA-lineage commitment, we observe upregulation of SA-lineage markers PHOX2B, ISL1, and CHGA, suggesting differentiation to both sympathoblasts and chromaffin cells. Interestingly, we observe increased expression of ALK after SA-lineage commitment which rapidly decreases during SA differentiation in cells derived from healthy individuals but remains highly expressed in cells derived from ALK-R1275Q NB patients. Pathway analysis identifies significant downregulation of neural differentiation and p53 signaling pathway with concomitant upregulation of DNA replication and protein translation pathways in patient cells compared to control cells, suggesting a decrease or delay in differentiation and a lingering of ALK-R1275Q cells in a proliferative state.

O13.1

Combining venetoclax with lorlatinib leads to complete responses in multiple neuroblastoma models with high BCL-2 expression and ALK mutation.

Vernooij L[1], Alles L[1], van Arkel J[1], Koopmans B[1], Essing A[1], Gürgen D[2], Keller K[1], Schubert N[1], Langenberg K[1], Schild L[1], van der Kraak P[3], Castigliego D[3], Mao J[4], Kamili A[4,5], Bladergroen R[1], Lelieveld S[1], Kuiper R[1], Koster J[6], van Hooff S[1], van den Boogaard M[1], Eising S[1], van Noesel M[1], Molenaar J[1,7], Dolman E[1]

[1]Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands, [2]EPO, Berlin, Germany, [3]University Medical Center Utrecht, Utrecht, the Netherlands, [4]Children's Cancer Institute, Lowy Cancer Centre, UNSW Sydney, Kensington, NSW, Australia, [5]School of Clinical Medicine, UNSW Medicine & Health, UNSW Sydney, Kensington, NSW, Australia, [6]Department of Oncogenomics, Amsterdam UMC, location AMC, Amsterdam, the Netherlands, [7]Department of Pharmaceutical sciences, Utrecht University, Utrecht, the Netherlands

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Background

Overexpression of the anti-apoptotic protein BCL-2 is frequently observed in neuroblastoma and can be targeted by venetoclax. Unfortunately, venetoclax monotherapy causes therapy resistance and is therefore unlikely to eradicate the whole tumor. Personalized medicine for subgroups of neuroblastoma patients could help in overcoming this problem, by targeting multiple tumor-specific aberrations simultaneously. A subgroup of particular interest is neuroblastoma with high BCL-2 expression and an ALK mutation. The relatively high incidence in which these abnormalities occur together, combined with the poor survival of high-risk neuroblastoma patients indicate that there is a large subset of patients that could benefit from a combination therapy targeting both BCL-2 and ALK.

Aims

We investigated targeted combination strategies for neuroblastoma tumors with BCL-2 overexpression and genomic aberrations of ALK.

Methods

To this end, we performed high-throughput drug screening, testing the BCL-2 inhibitor venetoclax in combination with a large library of targeted compounds. Based on the screening results, we selected compounds showing high effectivity or synergy together with venetoclax. The most effective compounds were subsequently tested in vivo and the best performing combination was selected for extensive in vivo analysis and biomarker validation.

Results

Combinations of venetoclax with lorlatinib (ALK inhibitor), talazoparib (PARP inhibitor), fimepinostat (PI3K/HDAC inhibitor) and prexasertib (CHK1 inhibitor) were selected based on their beneficial effects in the drug screen. In vivo comparison in KCNR xenografts identified venetoclax with lorlatinib as the most promising combination therapy for our subgroup of interest. Protein validation studies revealed that both compounds worked on-target and that the combination promoted apoptosis. Subsequently, the combination of venetoclax and lorlatinib was studied in two additional patient-derived xenograft (PDX) models with high BCL-2 expression and an F1174L or R1275Q ALK mutation. Both models showed promising effects, with mice reaching complete remission during treatment.

Conclusion

Our study demonstrates that the combination of venetoclax with lorlatinib is a promising therapeutic approach for a subgroup of neuroblastoma patients with high BCL-2 expression and an additional ALK mutation, as evidenced by the observed synergistic effects and complete responses. The extend of these results select the combination of venetoclax with lorlatinib for further clinical development.

O13.2

Combining Notch blockade with High-dose radiation therapy (HDRT) and immunotherapy (IO) results in a synergistic growth inhibition of NB tumors.

Banerjee D[1], Wang Q[2], Connolly E[3], Connolly E[2], Yamashiro D[1]
[1]Columbia University Irving Medical Center, Pediatrics, New York, United States of America , [2]Columbia University Irving Medical Center, Radiation Oncology, New York, United States of America , [3]Georgia Institute of Technology, The School of Biological Sciences, Atlanta, United States of America

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Background: New therapies are needed for patients suffering from high-risk neuroblastoma. High-dose radiation therapy (HDRT) combining with immunotherapy (IO) are currently being studied for several adult and pediatric cancers. The Notch inhibitor AL101 has been granted fast track designation by the FDA for the treatment of patients with recurrent/metastatic adenoid cystic carcinoma.
Aim: We study the effect of combining Notch inhibition, by AL101, with HDRT and IO on tumor microenvironment (TME) and tumor response.
Methods: 10^6 mouse neuroblastoma 9464D cells were implanted subcutaneously into the flank of a C57BL/6 mouse. Tumors (200 mm3) were randomly enrolled (day 0) and treated with AL101 (6.5mg/kg) once per day for 10 days. Mice were treated with HDRT (12Gy) on Day 3, and with anti-PD1 antibody (200 g) on days 0, 3, 6. A cohort of mice was sacrificed at day 10, and the rest sacrificed when tumor reached 1cm3. Tumors were sorted to isolate CD45+ immune cells that were subjected to 10x Genomics single-cell library construction for next generation sequencing and data analysis (scRNA-seq).
Results: Anti-PD1 or AL101 treatment alone had no effect on 9464D tumor growth. Adding AL101 with HDRT increased median survival of mice bearing 9464D tumors (RT vs RT+AL101; 37d vs 50d, P=0.0018). Strikingly, combining Notch inhibition by AL101 with anti-PD1 therapy and HDRT, results in a synergistic and durable tumor growth inhibition that significantly prolongs mice survival (AL101+RT+anti-PD1 vs HDRT+anti-PD1; 87 days vs 46 days, P=0.0003). ScRNA-seq analysis shows 12Gy increases pro-angiogenic, tumor promoting M2-type TAMs including angio TAM, MRC1+ TAM. Combining AL101 with HDRT minimizes the angio TAM, and MRC1+ TAM populations and increases immunostimulatory, anti-tumor M1-type MHC and NOS2+ TAMs. Immunohistochemistry shows AL101 reduces the HDRT-induced M2-type F/480+ MRC1+ TAM population in 9464D tumors. Flow cytometry further shows that HDRT doses of 12Gy increases Treg population, by > 3-fold, at day 10. AL101, decreases the 12Gy induced Treg.
Conclusion: Combining Notch blockade with HDRT and anti-PD1 therapy results in a synergistic inhibition of murine neuroblastoma tumors. Mechanistically, Notch blockade minimizes HDRT-induced intratumoral immunosuppressive M2-type TAM and Treg populations

O13.3

The CDK7/CDK9 and CKIα co-inhibitor exerts efficacious anticancer effects against TERT gene-rearranged neuroblastoma

Chen J[1], Xi Z[2], Wong M[1], Kamili A[1], Fletcher J[1], Combaret V[3], Boeva V[4], Janoueix-Lerosey I[5], Norris M[1], Haber M[1], Pickett H[6], Reddel R[6], Marshall G[1], Liu T[1]

[1]Children's Cancer Institute Australia, Randwick, Sydney, Australia, [2]School of Pharmacy, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [3]Laboratoire de Recherche Translationnelle, Centre Léon-Bérard, Lyon, France, [4]Department of Computer Science, Institute for Machine Learning, ETH Zürich, Zurich 8092, Switzerland, [5]Institut Curie, Paris Sciences et Lettres Research University, INSERM, Paris, France, [6]Children's Medical Research Institute, Faculty of Medicine and Health, The University of Sydney, Sydney, Australia

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Background: TERT gene rearrangement with transcriptional super-enhancers results in TERT over-expression and neuroblastoma. There is currently no targeted therapy for clinical trials in TERT-rearranged neuroblastoma patients. The transcriptional kinases CDK7 and CDK9 control transcriptional initiation and elongation of super-enhancer-associated oncogenes respectively, and the tumor suppressor protein p53 is degraded by CKIα. The novel anticancer agent A51 co-inhibits CDK7, CDK9 and CKIα, show promising anticancer effects against leukemia, and is now in clinical trials in leukemia patients.

Aims: To define the anticancer efficacy of the CDK7/CDK9 and CKIα co-inhibitor A51 against TERT-rearranged neuroblastoma and to identify the mechanism of action.

Material and Methods: The effects of A51 on TERT gene transcriptional suppression and p53 protein stabilization and up-regulation were examined by RT-PCR, immunoblot, chromatin-immunoprecipitation assays of TERT gene transcription and pulse-chase assays of p53 protein half-life. The anticancer effects of A51 were investigated in TERT-rearranged neuroblastoma cell lines and in mice xenografted with TERT-rearranged neuroblastoma cells or patient-derived xenograft (PDX) tumor cells. The effects of CDK7, CDK9 and CKIα on TERT gene expression, p53 protein degradation, TERT-rearranged neuroblastoma cell proliferation and survival in vitro and tumor progression in mice were then examined.

Results: Treatment with A51 reduced CDK7 and CDK9 binding at the TERT gene promoter and 3'-untranslated region respectively, blocked p53 protein degradation, activated p53 protein expression and induced TERT-rearranged neuroblastoma cell apoptosis which was rescued by TERT over-expression and p53 knockdown. In mice xenografted with TERT-rearranged neuroblastoma cell lines or PDX tumor cells, A51 treatment significantly induced tumor cell apoptosis, suppressed tumor progression, and improved mouse survival. In addition, CDK7 or CDK9 knockdown down-regulated TERT gene expression, CKIα knockdown activated p53 protein expression, and CDK7/CDK9/CKIα co-knockdown resulted in more considerable apoptosis than CDK7, CDK9 or CKIα single knockdown. In mice xenografted with TERT-rearranged neuroblastoma cells, CDK7/CDK9/CKIα co-knockdown more significantly inhibited tumor progression than CDK7, CDK9 or CKIα single knockdown.

Conclusions: CDK7 and CDK9 co-operatively up-regulate TERT gene transcription, and CKIα induces p53 protein degradation and absent expression. The CDK7, CDK9 and CKIα co-inhibitor A51 is an efficacious anticancer agent for targeted therapy against TERT gene-rearranged neuroblastoma.

O13.4

Dual-selective pharmacotherapy achieves profound and lasting tumor growth suppression in experimental models of MYCN-amplified neuroblastoma

Alferiev I[1], Guerrero D[1], Bratinov G[1], Nguyen F[1], Guan P[1], Kolla V[1], Fishbein I[1], Brodeur G[1], Chorny M[1]
[1]Divisions of Oncology and Cardiology, Children's Hospital of Philadelphia and the University of Pennsylvania, Philadelphia, United States

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Background. Neuroblastoma (NB) pharmacotherapies relying on a single biological mechanism for their tumor selectivity and mode of action often fail to durably suppress high-risk disease without causing severe toxicity. In this study, we showed that a macromolecule-linked prodrug designed to integrate two selectivity mechanisms by targeting the norepinephrine transporter (NET) and by acting exclusively on cycling cells can achieve lasting tumor growth inhibition without significant adverse effects in models of MYCN-amplified, newly diagnosed or recurrent NB.
Aims. Our goal was to evaluate in clinically relevant models of pre-therapy and relapsed MYCN-driven high-risk NB a new prodrug-based pharmacotherapeutic strategy combining tissue affinity with a replication-dependent mode of action as a way of enhancing therapeutic efficacy and tumor selectivity.
Methods. For efficacy studies, we used orthotopic and pseudo-metastatic xenograft models of aggressive disease established using MYCN-amplified NB cells. Drug uptake was determined in orthotopic chemo-refractory NB tumors established using p53-mutant SK-N-BE(2)C cells derived at relapse after intensive chemo-radiotherapy. The results were analyzed by Kruskal-Wallis one-way ANOVA and the log-rank test.
Results. NET-targeted delivery of polymer-linked prodrug of SN-38, a topoisomerase I inhibitor selectively and potently killing proliferating cells, resulted in accumulation and stable presence of the bioactive drug in established orthotopic NB tumors at levels two orders of magnitude greater than its IC90 threshold for lastingly suppressing the growth chemoresistant SK-N-BE(2)C NB cells. The prodrug administered twice a week over 4 weeks completely eliminated MYCN-amplified tumors with wild-type p53. It also caused rapid regression and durably suppressed regrowth of MYCN-amplified orthotopic xenografts and pseudo-metastatic tumor deposits with an acquired loss-of-function p53 mutation, extending survival in both cohorts beyond 14 weeks. This is in contrast to the clinically used precursor of SN-38, irinotecan, having no significant effect on the disease progression in either model.
Conclusion. The results of our studies show remarkable effectiveness of tumor-guided delivery using a NET-targeted prodrug designed to integrate two selectivity mechanisms against pre-therapy and relapsed, multidrug-resistant forms of high-risk, MYCN-amplified NB. Dual-selective pharmacotherapy with tumor-targeted prodrugs has the potential to provide a new, clinically viable strategy for treating aggressive NB that shows no durable response to conventional therapeutics.

O14.1

Circulating adrenergic neuroblastoma mRNAs predict outcomes in children with relapsed and refractory neuroblastoma; a BEACON-Neuroblastoma biomarker study

Burchill S[1], Weston R[2], Viprey V[1], Berry A[1], Tchirkov A[3], Corrias M[4], Lammens T[5], Gray J[6], Owens C[7], Laidler J[2], Gambart M[8], Caňete A[9], van Eijkelenburg N[10], Nysom K[11], Laureys G[12], Castellano A[13], Gerber N[14], Ladenstein R[15], Valteau-Couanet D[16], Wheatley K[2], Moreno L[17]

[1]Leeds Institute of Medical Research, University of Leeds, Leeds, United Kingdom, [2]Cancer Research UK Clinical Trials Unit, University of Birmingham, Birmingham, United Kingdom, [3]Cytogénétique Médicale CHU Estaing, Clermont-Ferrand, France, [4]IRCCS Istituto Giannina Gaslini, Genova, Italy, [5]Department of Pediatric Hematoloy-Oncology and Stem Cell Transplantation, Ghent University Hospital, Ghent, Belgium, [6]Cancer Immunology Centre, University of Southampton, Southampton, United Kingdom, [7]Our Lady's Children's Hospital, Crumlin, Ireland, [8]Département d'Onco-hématologie Pédiatrique, Hôpital des Enfants, Toulouse, France, [9]University Hospital La Fe, Valencia, Spain, [10]Princess Máxima Centre for Paediatric Oncology, Utrecht, The Netherlands, [11]Department of Paediatrics and Adolescent Medicine Rigshospitalet, Copenhagen, Denmark, [12]Department of Pediatrics, Ghent University Hospital, Ghent, Belgium, [13] Division of Hematology, Ospedale Pediatrico Bambino Gesù, Rome, Italy, [14]Department of Oncology, University Children's Hospital, Zurich, Switzerland, [15]Children's Cancer Research Institute (CCRI), Vienna, Austria, [16]Département de Cancérologie de l'enfant et de l'adolescent, Gustave Roussy, Paris, France , [17]Division of Pediatric Hematology & Oncology, Val d'Hebron Barcelona Hospital Campus, Barcelona, Spain

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Background

Children with relapsed and refractory neuroblastoma (RR-NBL) have poor outcomes, with overall survival (OS) less than 20%. Early identification of children at greatest risk of relapse could mean timelier modifications of their treatment and improved outcomes. We report on the predictive value of adrenergic neuroblastoma mRNAs in blood from children treated in the BEACON-Neuroblastoma trial (NCT02308527).

Aims

To prospectively evaluate the predictive power of the adrenergic neuroblastoma mRNAs paired-like homeobox 2B (PHOX2B) and tyrosine hydroxylase (TH) in blood from children with RR-NBL.

Methods

Blood samples collected at trial entry (n=87), after cycle 2 (n=53) and cycle 6 (n=33) of treatment were analysed by reverse transcriptase polymerase chain reaction for PHOX2B and TH mRNAs as previously described (doi:10.1200/JCO.2013.53.3604). The predictive power of PHOX2B and TH mRNAs, alone and in combination, was evaluated using Kaplan-Meier survival curves and Cox proportional hazards regression.

Results

TH and PHOX2B mRNAs were detected in 48/87 (55%) and 52/87 (60%) of blood samples respectively at trial entry. Expression of either mRNA was associated with progression (TH, hazard ratio (HR) 1.45, 95% CI 1.25-1.69; PHOX2B, HR 1.46, 95% CI 1.25-1.70) and reduced OS (TH or PHOX2B, HR 1.47, 95% CI 1.22-1.77). Detection of TH and PHOX2B mRNAs was more strongly associated with progression (HR 2.68, 95% CI 1.65-4.35) and OS (HR 2.84, 95%CI 1.71-4.72) than either mRNA alone. TH or PHOX2B mRNAs were detected in 17/53 (32%) of samples post-cycle 2. Detection of TH (HR 1.45) or PHOX2B (HR 1.87) alone or in combination (HR 5.47) was associated with progression. Post cycle 6, the detection of TH (11/33; 33%) or

PHOX2B (7/33; 21%) was associated with progression (TH alone, HR 2.76; PHOX2B alone HR 3.80; TH and PHOX2B, HR 6.45) and reduced OS (TH alone, HR 2.61; PHOX2B alone HR 3.19; TH and PHOX2B HR 8.80).

Conclusion
Detection of TH, PHOX2B or TH and PHOX2B mRNAs in blood at trial entry or during treatment, identified children with RR-NBL at greatest risk of progression or death. In the RR-NBL setting, this simple blood test could be used to fast-track children expressing these mRNAs into early phase clinical trials.

O14.2

Significantly improved PFS in relapsed/refractory neuroblastoma adding molecular targeted drugs to Irinotecan/Temozolomide: Results of a randomized phase II trial (RIST)

Corbacioglu S[1], Lode H[2], Jakob M[1], Suttorp M[4], Escherich G[5], Gruhn B[6], Ellinger S[1], Simon T[7], Lang P[8], Rohde M[15], Zeman F[35], Debatin K[9], Steinbach D[9], Beilken A[36], Ladenstein R[10], Spachtholz R[1], Heiss P[32], Stroszczynski C[32], Hellwig D[33], Troeger A[1], Koller M[35], Menhart K[33], Riehmenschneider M[32], Zoubaa S[32], Turowski P[1], Kietz S[1], Babor F[11], Fröhlich B[12], Bielack S[13], Bernig T[16], Greil J[17], Niktoreh N[14], Cario G[18], Faber J[19], Lauten M[31], Frühwald M[20], Jorch N[21], Paulussen M[22], Schneider D[23], Metzler M[24], Leipold A[25], Nathrath M[26], Imschweiler T[27], Christiansen H[28], Schmid I[29], Crazzolara R[30], Eggert A[3], Foell J[1]

[1]University Medical Center Regensburg, Germany; Department of Pediatric Hematology, Oncologa and Stem Cell Transplantation, Regensburg, Germany, [2]University Medical Center Greifswald, Germany; Departmen of Pediatrics, Greifswald, Germany, [3]Charité, University Medical Center Berlin, Germany; Department of Pediatric Hematology and Oncology, Berlin, Germany, [4]University Medical Center Dresden, Germany; Department of Pediatric Hematology and Oncology, Dresden, Germany, [5]University Medical Center Eppendorf Hamburg, Germany; Department of Pediatric Hematology, Hamburg, Germany, [6]Jena University Hospital; Department of Pediatrics, Jena, Germany, [7]University Medical Center Cologne, Germany; Department of Pediatric Hematology and Oncology, Cologne, Germany, [8]University Medical Center Tuebingen, Germany; Department of Pediatric Hematology and Oncology, Tuebingen, Germany, [9]University Medical Center Ulm, Germany; Department of Pediatric Hematology and Oncology, Ulm, Germany, [10]St. Anna Children's Cancer Research Institute, University Medical Center Vienna, Austria, Vienna, Austria, [11]University Medical Center Düsseldorf, Germany; Department of Pediatric Hematology, Oncology and Clinical Immunology, Düsseldorf, Germany, [12]University Medical Center Muenster, Germany; Department of Pediatric Hematology and Oncology, Muenster, Germany, [13]Olgaspital Stuttgart, Germany; Department of Pediatric Hematology and Oncology, Stuttgart, Germany, [14]University Medical Center Essen, Germany; Department of Pediatric Hematology and Oncology, Essen, Germany, [15]University Medical Center Giessen, Germany; Department of Pediatric Hematology and Oncology, Giessen, Germany, [16]University Medical Center Halle, Germany; Department of Pediatric Hematology and Oncology, Halle, Germany, [17]University Medical Center Heidelberg, Germany; Department of Pediatric Hematology, Oncology and Immunology, Heidelberg, Germany, [18]University Medical Center Kiel, Germany; Department of Pediatric Hematology and Oncology, Kiel, Germany, [19]University Medical Center Mainz, Germany; Department of Pediatric Hematology and Oncology, Mainz, Germany, [20]University Medical Center Augsburg, Germany; Department of Pediatrics , Augsburg, Germany, [21]University Medical Center Bielefeld, Germany; Department of Pediatrics , Bielefeld, Germany, [22]University Medical Center Witten/Herecke, Germany; Vestische Children's Hospital , Datteln, Germany, [23]University Medical Center Dortmund, Germany; Department of Pediatrics , Dortmund, Germany, [24]University Medical Center Erlangen, Germany; Department of Pediatric Hematology and Oncology, Erlangen, Germany, [25]Medical Center Karlsruhe, Germany; Department of Pediatrics, Karlsruhe, Germany, [26]Medical Center Kassel, Germany; Department of Pediatrics, Kassel, Germany, [27]Helios Hospital Krefeld, Germany; Department of Pediatrics, Krefeld, Germany, [28]University Medical Center Leipzig, Germany; Department of Pediatric Hematology and Oncology, Leipzig, Germany, [29]Dr. von Haunersches Kinderspital, University Medical Center Munich; Department of Pediatric Hematology and Oncology , Munich, Germany, [30]University Medical Center Innsbruck, Austria; Department of Pediatric Hematology and Oncology, Innsbruck, Austria, [31]University Medical Center Lübeck, Germany; Department of Pediatric Hematology and Oncology, Lübeck, Germany, [32]University Medical Center Regensburg, Germany; Department for Neuropathology, Regensburg, Germany, [33]University Medical Center Regensburg, Germany; Department for Radiology, Regensburg, Germany, [34]University Medical Center Regensburg, Germany; Department of Nuclear Medicine, Regensburg, Germany, [35]University Medical Center Regensburg, Germany; Center for Clinical Trials, Regensburg, Germany, [36]University Medical Center Hannover Germany; Department of Pediatric Hematology and Oncology, Hannover, Germany

Parallel session 14: Clinical trials for relapse neuroblastoma, May 17, 2023, 11:20 AM - 12:25 PM

Background: The outcome of children with relapsed or refractory neuroblastoma (r/rNB) remains dismal. Irinotecan and temozolomide (I/T) are the current backbone for novel treatment options. RIST represents a

metronomic molecular-targeted treatment strategy, combining I/T with the multikinase inhibitor dasatinib (S) and the mTORC1-inhibitor rapamycin (R).
Methods: RIST-rNB-2011, a randomised Phase II trial (NCT01467986), assessed RIST (experimental arm) in comparison with an I/T regimen (control arm; CA). Patients with r/rNB (stage IV and all MYCN amplified stages; MYCN+) were eligible. A block-wise randomization (block length of 6) stratified by MYCN status was performed. The treatment in the experimental arm consisted of two treatment phases, each with four repetitive cycles of 1 and 2 four-day-courses of R/S, respectively, and 5 day-courses of I/T. In the control arm, R/S was substituted by rest days between I/T courses. The primary endpoint was progression-free survival (PFS), using a multivariable cox-regression. Primary analysis was based on the intention-to-treat principle (ITT). Toxicity was assessed in all participants who received at least one dose of protocol therapy.
Results: One hundred twenty-four subjects were randomized between August 2012 - September 2020. Median age was 5.37 years (range 1.1 – 24.6 years). Disease characteristics were relapse in 80% (<18 months after diagnosis: 40%) and refractory disease in 20%, MYCN+ was present in 39%. The median overall follow-up was 72 months. Median PFS in the ITT population was 11 months in RIST vs 5 in CA (HR: 0.62 (95%-CI: 0.42, 0.92), p=0.019), and 16 months vs 4 months in the PP population (HR: 0.53 (95%-CI: 0.31, 0.90), p=0.018). Subgroup analyses (ITT) regarding MYCN showed the main effect in the MYCN+ subgroup (HR: 0.45 (95%-CI: 0.24, 0.84), p=0.012) compared to the MYCN- subgroup (HR: 0.84 (95%-CI: 0.51, 1.38), p=0.492). Even in the ultra-high-risk population (MYCN+/relapse <18 months) PFS remained significantly different (p=0.048). No differences in outcome were observed in the MYCN- population.
Conclusion: RIST demonstrated significant and sustained anti-tumour activity, selectively in the highest-risk group of MYCN+ patients with r/rNB. Further evaluation of MYCN+ associated biomarkers may help to identify patients most likely to respond to this metronomic molecular targeted treatment strategy.

O14.3

Phase I Study of 131I-MIBG with Dinutuximab +/- Vorinostat for Patients with Relapsed or Refractory Neuroblastoma (NANT 2017-01)

Cash T[1], Marachelian A[2], DuBois S[3], Chi Y[2], Groshen S[4], Nargizyan A[2], Shamirian A[2], Crowley M[2], Macy M[5], Pinto N[6], Desai A[7], Granger M[8], Vo K[9], Sondel P[10], Asgharzadeh S[2], Weiss B[11], Mosse Y[12], Matthay K[9], Park J[6], Goldsmith K[1]

[1]Department of Pediatrics, Aflac Cancer & Blood Disorders Center, Children's Healthcare of Atlanta, Emory University School of Medicine, Atlanta,, United States, [2]Department of Pediatrics, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles,, United States, [3]Department of Pediatrics, Dana-Farber/Boston Children's Cancer and Blood Disorders Center and Harvard Medical School, Boston,, United States, [4]Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles,, United States, [5]Department of Pediatrics, Children's Hospital Colorado, University of Colorado Anschutz Medical Campus, Aurora,, United States, [6]Department of Pediatrics, Seattle Children's Hospital, University of Washington School of Medicine, Seattle,, United States, [7]Department of Pediatrics, University of Chicago Medical Center, Chicago,, United States, [8]Cook Children's Medical Center, Fort Worth,, United States, [9]Department of Pediatrics, UCSF Benioff Children's Hospital, University of California, San Francisco School of Medicine, San Francisco,, United States, [10]Department of Pediatrics, University of Wisconsin-Madison School of Medicine and Public Health, Madison,, United States, [11]Department of Pediatrics, Cincinnati Children's Hospital Medical Center, University of Cincinnati School of Medicine, Cincinnati,, United States, [12]Department of Pediatrics, Children's Hospital of Philadelphia, Perelman School of Medicine at the University of Pennsylvania, Philadelphia,, United States

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Background: The NANT conducted a phase 1 study of 131I-metaiodobenzylguanidine (MIBG) with dinutuximab for patients with relapsed/refractory (R/R) high-risk neuroblastoma (HRNB; Part A), and established 18 mCi/kg of MIBG with 17.5 mg/m2/dose of dinutuximab as the recommended phase 2 dose (RP2D). A randomized phase 2 trial demonstrated that the HDAC inhibitor vorinostat, when combined with MIBG, nearly doubled objective response rates compared to MIBG monotherapy. Vorinostat preclinically upregulates neuroblastoma cell surface GD2 and enhances anti-GD2 responses. We therefore conducted Part B to determine the RP2D, safety, and preliminary best overall response (BOR; CR + PR) of vorinostat in combination with MIBG and dinutuximab in R/R HRNB.

Methods: R/R HRNB patients 1-29 years of age with MIBG uptake in ≥1 site were eligible. Prior anti-GD2 mAb not administered with MIBG and one prior MIBG therapy were allowed. Prior HDAC inhibitor with MIBG was excluded. Vorinostat was administered orally days 0-13 at 180 mg/m2/dose in combination with the Part A RP2D of MIBG (day 1), dinutuximab (days 8-11 and 29-32), and GM-CSF (days 8-17 and 29-38). Autologous stem cells were infused on day 15. Up to two courses were allowed.

Results: Fourteen patients were enrolled in Part B, including 12 dose-limiting toxicity (DLT)-evaluable patients who were treated at 180 mg/m2/dose. Median age was 10 (range: 3-24) years. All patients had previously received chemoimmunotherapy with a median of 7 (range: 1-22) courses. One patient (7%) received prior MIBG. There were no course 1 DLTs. Among the 10 (71%) patients who received a second course (2 with data pending), 2 DLTs occurred: grade 3 alanine aminotransferase increased and grade 5 pneumonitis. Common grade 3/4 treatment-related toxicities were hematologic toxicities attributable to MIBG and expected non-hematologic toxicities attributable to vorinostat, dinutuximab or GM-CSF. Among the 11 response evaluable patients, the institutional BOR rate was 45% (1 CR, 4 PR). In addition, there were 3 minor responses for a CR/PR/MR rate of 73%.

Conclusion: The RP2D of vorinostat in combination with MIBG and dinutuximab/GM-CSF is 180 mg/m2/dose. This regimen was generally well-tolerated, though one patient had fatal pneumonitis of unclear etiology. Encouraging preliminary responses were observed.

O14.4

Norepinephrine transporter and vesicular monoamine transporter-2 tumor expression as a predictor of response to 131I-MIBG in patients with relapsed/refractory neuroblastoma

Batra V[1], Gikandi A[2], Pawel B[3], Martinez D[1], Granger M[4], Marachelian A[5], Park J[6], Maris J[1], Vo K[7], Matthay K[7], DuBois S[8]

[1]Children's Hospital of Philadelphia and Perelman School of Medicine of the University of Pennsylvania, Philadelphia,, United States, [2]Harvard Medical School, Boston,, United States, [3]Department of Pathology, Children's Hospital Los Angeles and USC Keck School of Medicine, Los Angeles,, United States, [4]Department of Pediatrics, Cook Children's Hospital, Fort Worth,, United States, [5]Children's Hospital Los Angeles and USC Keck School of Medicine, Los Angeles,, United States, [6]Department of Pediatrics, Seattle Children's Hospital and University of Washington School of Medicine, Seattle,, United States, [7]Department of Pediatrics, UCSF Benioff Children's Hospital and UCSF School of Medicine, San Francisco,, United States, [8]Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Harvard Medical School, Boston,, United States

Parallel session 14: Clinical trials for relapse neuroblastoma, May 17, 2023, 11:20 AM - 12:25 PM

Background

Prior studies suggest that norepinephrine transporter (NET) and vesicular monoamine transporter 2 (VMAT2) mediate metaiodobenzylguanidine (MIBG) uptake and retention in neuroblastoma tumors. We hypothesize that expression levels of these transporters may predict response to ^{131}I-MIBG therapy.

Aims

The primary aim of the current study was to evaluate the association between NET and VMAT2 protein expression with response to therapeutic ^{131}I-MIBG in patients with relapsed or refractory neuroblastoma. Secondary aims included evaluating the association between tumor NET and VMAT2 protein expression with clinical and biological features of neuroblastoma as well as with whole-body radiation exposure.

Methods

Immunohistochemistry (IHC) was used to evaluate NET and VMAT2 protein expression levels on archival tumor samples (obtained at diagnosis or relapse) from patients with relapsed or refractory high-risk neuroblastoma treated with ^{131}I-MIBG. IHC results were used to determine a composite protein expression H-score by multiplying a semi-quantitative intensity value (0-3+) by the percentage of tumor cells expressing the protein. Wilcoxon rank sum and Fisher exact tests were used to compare NET and VMAT2 protein expression with response to ^{131}I-MIBG as well as clinicopathological features.

Results

Tumor samples and clinical data were available for 106 patients, of whom 28.3% had >partial response (PR). NET H-score was not significantly associated with response (>PR), though the percentage of tumor cells expressing NET was lower among responders (median 80% for >PR vs. 90% for <PR; p=0.0014). VMAT2 H-score was not significantly associated with >PR vs. <PR, though patients with >PR had lower VMAT2 staining intensity (p=0.005). VMAT2 H-score was significantly lower in patients with complete response (median 40 vs. 210 for patients with < complete response; p=0.0049). VMAT2 H-scores were significantly higher in ganglioneuroblastoma (vs. neuroblastoma; p=0.037), differentiated/poorly differentiated tumors (vs. undifferentiated; p=0.0047), and tumors lacking MYCN amplification (vs. MYCN amplified; p=0.0011).

Conclusions
Markers of lower NET and VMAT2 protein expression are associated with higher likelihood of response to ^{131}I-MIBG therapy in patients with relapsed/refractory neuroblastoma. Increased VMAT2 protein expression is associated with a more differentiated disease phenotype.

O14.5

Lorlatinib with or without chemotherapy in ALK-driven refractory/relapsed neuroblastoma: phase I trial results and insights into mechanisms of resistance through tracking of serial circulating tumor DNA

Goldsmith K[1,2], Berko E[8,27], Park J[3,4], Witek G[8], Matkar S[8], Petrova Z[29,30], Kayser K[5], Malvar J[6], Chi Y[6,7], Groshen S[7], Villablanca J[6,7], Krytska K[8], Lai L[9], Acharya P[7,10], Goodarzian F[7,10], Pawel B[7,11], Shimada H[12], Ghazarian S[6], States L[28], Marshall L[13,14], Chesler L[13,14], Granger M[15], Desai A[16], Mody R[17], Morgenstern D[18,19], Shusterman S[20], Macy M[21,22], Pinto N[3,4], Schleiermacher G[23,24], Vo K[25], Thurm H[26], Chen J[26], Liyanage M[26], Peltz G[26], Matthay K[25], Maris J[8,28], Radhakrishnan R[31], Lemmon M[29,30], Marachelian A[6,7], Mosse Y[8,28,29]

[1]Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Atlanta, Georgia, USA, , , [2]Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia, USA 3 Seattle Children's Hospital, Seattle, Washington, USA, , , [3]Seattle Children's Hospital, Seattle, Washington, USA, , , [4]Department of Pediatrics, University of Washington School of Medicine, Seattle, Washington, USA, , , [5]Department of Psychiatry and Behavioral Sciences, Vanderbilt University Medical Center, Nashville, Tennessee, USA, , , [6]Cancer and Blood Disease Institute, Children's Hospital Los Angeles, Los Angeles, California, USA, , , [7]Keck School of Medicine, University of Southern California, Los Angeles, California, USA, , , [8]Division of Oncology and Center for Childhood Cancer Research; Children's Hospital of Philadelphia; Philadelphia, Pennsylvania, USA, , , [9]Department of Radiology, University of Iowa Hospital and Clinics, Iowa City, Iowa, USA, , , [10]Department of Radiology, Children's Hospital Los Angeles, Los Angeles, California, USA, , , [11]Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles, Los Angeles, California, USA, , , [12]Department of Pathology and Pediatrics, Stanford University School of Medicine, Stanford, California, USA, , , [13]The Royal Marsden Hospital, London, UK, , , [14]The Institute of Cancer Research, London, UK, , , [15]Cook Children's Medical Center, Fort Worth, Texas, USA, , , [16]Department of Pediatrics, Section of Hematology/Oncology/Stem Cell Transplantation, University of Chicago, Chicago, Illinois, USA, , , [17]Department of Pediatrics, University of Michigan, Ann Arbor, Michigan, USA, , , [18]Division of Haematology and Oncology, Hospital for Sick Children, Toronto, Ontario, CAN, , , [19]Department of Paediatrics, University of Toronto, Toronto, Ontario, CAN , , , [20]Dana Farber Cancer Institute, Boston Children's Cancer and Blood Disorders Center, Harvard Medical School, Boston, Massachusetts, USA, , , [21]University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA, , , [22]Center for Cancer and Blood Disorders, Children's Hospital Colorado, Aurora, Colorado, USA, , , [23]RTOP (Recherche Translationelle en Oncologie Pédiatrique), INSERM U830, Research Center, PSL Research University, Institut Curie, Paris, FRA, , , [24]SIREDO Oncology Center (Care, Innovation and Research for Children, Adolescents and Young Adults with Cancer), Institut Curie ; Paris, FRA, , , [25]Department of Pediatrics, UCSF Benioff Children's Hospital, University of California, San Francisco School of Medicine, San Francisco, California, USA, , , [26]Global Product Development, Clinical Pharmacology, Pfizer Oncology, Pfizer Inc, New York, New York, USA, , , [27]Division of Pediatric Hematology and Oncology, Schneider Children's Medical Center, Petach Tikva, Israel; Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel, , , [28]Perelman School of Medicine at the University of Pennsylvania; Philadelphia, PA, USA, , , [29]Department of Pharmacology, Yale University School of Medicine, New Haven, CT, USA, , , [30]Yale Cancer Biology Institute, Yale University, West Haven, CT, USA, , , [31]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA, USA, ,

Parallel session 14: Clinical trials for relapse neuroblastoma, May 17, 2023, 11:20 AM - 12:25 PM

Background: Neuroblastomas harbor ALK aberrations clinically resistant to crizotinib yet sensitive preclinically to the third generation ALK inhibitor, lorlatinib.

Aims: We conducted a first-in-child study evaluating lorlatinib with and without chemotherapy in children and adults with relapsed/refractory ALK-driven neuroblastoma.

Methods: The trial is ongoing, and we report here on three cohorts that have met pre-specified primary endpoints: lorlatinib as a single agent in children (12mo to <18yrs), lorlatinib as a single agent in adults (>18yrs), and lorlatinib in combination with topotecan/cyclophosphamide in children (<18yrs). Primary

endpoints were safety, pharmacokinetics, and recommended phase 2 dose (RP2D). Secondary endpoints were response rate and 123I-metaiodobenzylguanidine (MIBG) response. Lorlatinib was evaluated at 60-115mg/m2/dose in children and 100-150mg in adults. To track evolutionary dynamics and heterogeneity of tumors, and to detect early emergence of lorlatinib resistance lorlatinib, we collected serial circulating tumor DNA (ctDNA) samples from patients enrolled on this trial.

Results: Common adverse events (AEs) were hypertriglyceridemia (90%), hypercholesterolemia (79%), and weight gain (87%). Neurobehavioral AEs occurred mainly in adults and resolved with dose hold/reduction. The RP2D of lorlatinib with and without chemotherapy in children was 115mg/m2. The single agent adult RP2D was 150mg. The single agent response rate (complete/partial/minor) for <18yrs was 30%, for >18yrs was 67%, for chemotherapy combination in <18yrs was 63%, and 13/27 (48%) responders achieved MIBG complete responses. We discovered off-target resistance mutations in 11 patients (27%), predominantly in the RAS-MAPK pathway. We also identified novel secondary compound ALK mutations in 6 (15%) patients, all acquired prior to disease progression. Functional cellular and biochemical assays plus computational studies elucidated lorlatinib resistance mechanisms.

Summary/Conclusion: These data have supported lorlatinib's rapid translation into active phase 3 trials for patients with newly diagnosed high-risk ALK-driven neuroblastoma. ClinicalTrials.gov registration: NCT03107988. Our results also establish serial the clinical utility of ctDNA sampling to track response and progression and to discover novel resistance mechanisms that can be leveraged to develop therapeutic strategies to overcome lorlatinib resistance.

O16.1

Loss of p16INK4a in neuroblastoma induces shift to an immature state with mesenchymal characteristics and increases sensitivity to EGFR inhibitors

van den Boogaard M[1], Schubert N[1], van Hooff S[1], Schild L[1], Ober K[1], van den Handel K[1], Essing A[1], Koopmans B[1], Jansky S[2], Stainczyk S[2], Toprak U[2], Westermann F[2], Eising S[1], Molenaar J[1]
[1]Princess Máxima Center for Pediatric Oncology, Utrecht, Nederland, [2]Hopp Children's Cancer Center Heidelberg (KiTZ) and Division of Neuroblastoma Genomics, German Cancer Research Center (DKFZ), Heidelberg, Germany

Parallel sessions 16: Genetic drivers of resistance and relapse, May 18, 2023, 11:15 AM - 12:05 PM

Background

Homozygous inactivation of the CDKN2A locus is one of the most common genomic aberrations in human cancer and the most frequent potential actionable event in relapse pediatric cancers including neuroblastoma. The locus codes for two unrelated and distinctly regulated proteins: p14ARF and p16INK4a, which inhibit MDM2 and CDK4/6, respectively. However, CDKN2A inactivation does not function as a biomarker for sensitivity to MDM2 inhibitors or CDK4/6 inhibitors indicating the need to identify potential new therapeutic interventions specific for CDKN2A inactivated tumors.

Aims and methods

To examine the consequences of the loss of the two distinct gene transcripts in neuroblastoma and to identify potential therapeutic interventions, we used the CRISPR-Cas9 system to knockout p14, p16 and p14+p16 in neuroblastoma cells and used RNA profiling and compound screening to functionally analyze the isogenic systems. Subsequently we validated the compound hits in vitro and in vivo

Results

RNA sequencing of the transcriptome revealed a striking shift towards an immature Schwann cell precursor-like phenotype with mesenchymal characteristics, specifically in the p16 and p14+p16 knockouts. The shift in phenotype could be confirmed in a large patient cohort with primary and relapse neuroblastoma including tumors with CDKN2A inactivating events. High-throughput drug screening of p16 and p14+p16 knockout clones identified increased sensitivity to multiple EGFR inhibitors. On protein level, we were able to confirm that EGFR pathway activation is higher in p14+p16 knockout cells and that treatment with the EGFR inhibitor afatinib resulted in higher levels of apoptosis. Afatinib also reduced tumour growth in xenografts transplanted with p14+p16 knockout SY5Y cells.

Conclusion

Overall, our study suggests that CDKN2A deletion in neuroblastoma relates to a phenotypic shift towards a more progenitor like state with mesenchymal characteristics. These findings suggest a relation between tumor evolution events and phenotype switching which has also been suggested for the RAS-MAPK pathway. Isogenic systems with CDKN2A inactivation show increased sensitivity to EGFR inhibitors which should be further explored to prioritize for clinical trials.

O16.2

Clonal Decomposition and DNA Replication States Defined by Scaled Single-Cell Genome Sequencing in Neuroblastoma

Bhalshankar J[1,2], Bellini A[1,2], Jimenez I[1,2], Saberi-Ansari E[1,2], Iddir Y[1,2], Saint-Charles A[1,2], Bohec M[3], Lameiras S[3], Baulande S[3], Surdez D[4], Zaidi S[4], Janoueix-Lerosey I[5], Thirant C[5], Pierron G[6], Guerin C[7], Montero Carcaboso A[8], Eugenia Marques da Costa M[9], Geoerger B[9], Zinovyev A[10], Delattre O[1,6], Schleiermacher G[1,2]

[1]INSERM U830, Laboratoire de Génétique et Biologie des Cancers, Research Center, PSL Research University, Institut Curie, Paris, Ile de France, France, [2]SiRIC RTOP « Recherche Translationelle en Oncologie Pédiatrique », Translational Research Department, Research Center, PSL Research University, Paris, Ile de France, France, [3]Institut Curie Genomics of Excellence (ICGex) Platform, Paris, Ile de France, France, [4]INSERM U830, Équipe Labellisé LNCC, PSL Université, SIREDO Oncology Centre, Institut Curie, Paris, Ile de France, France, [5]Institut Curie, PSL Research University, Inserm U830, Equipe Labellisée Ligue contre le Cancer, F-75005, Paris, Ile de France, France, [6]Unité de Génétique Somatique, Institut Curie, Paris, Ile de France, France, [7]Institut Curie, Flow Cytometry Department, Paris, Ile de France, France, [8]Preclinical Therapeutics and Drug Delivery Research Program, Department of Oncology, Hospital Sant Joan de Déu, Barcelona, Spain, [9]Gustave Roussy Cancer Campus, Department of Paediatric and Adolescent Oncology, INSERM U1015, Université Paris-Saclay, Villejuif, France, [10]INSERM U900, Cancer et génome: Bioinformatique, biostatistiques et épidémiologie des systèmes complexes, Research Center, PSL Research University, Institut Curie, Paris, Ile de France, France

Parallel sessions 16: Genetic drivers of resistance and relapse, May 18, 2023, 11:15 AM - 12:05 PM

Background: How cell-to-cell allele specific copy number alterations underpin genetic Intratumor heterogeneity (ITH), drive genomic and phenotypic variation, and consequently the evolution of neuroblastoma (NB), remains understudied.

Aims: Here we investigate ITH, timing of specific genomic aberrations, single-cell replication timing and the co-evolution of the genome and transcriptome in NB tumors at single-cell resolution. Further, we study subclonal dynamics and clone specific response or resistance under targeted therapeutic pressure.

Methods: In addition to germline/tumor bulk whole exome sequencing (WES), ultra-low depth (0.25x) single-cell whole-genome DNA sequencing (scDNAseq) was performed using 10x genomics Chromium single-cell CNV kit and 9435 tumor cells were characterized from 14 patient-derived xenografts (PDX) NB-models and 4 tumor biopsies from NB-patients, either at diagnosis (n=7), progression (n=3) or relapse (n=8). Single-cell RNA sequencing (scRNAseq) data was obtained from the same PDX and patient tumor samples as published recently (Thirant Cécile et al, bioRxiv, 23/03/2021). 6/14 PDX models were subjected to different treatment combinations (targeted treatment with/without chemotherapy) and bulk WES was performed at two time-points, pre- and post-treatment.

Results: Polyclonal (n=12) and monoclonal (n=6) genomes were determined by allele and haplotype specific copy number (CN) alteration using both scDNAseq and scRNAseq data analysis. 2 to 6 clones were observed per polyclonal NB tumor. Whole genome duplication events (n=7) were observed in both polyclonal and monoclonal genomes. Known driver CN (segmental loss in chr1p and chr11q and gain at chr17q, or MYCN/ALK amplification) or somatic mutations (ALK/ATRX/TP53/NF1) were early clonal events. scDNAseq analysis showed parallel copy number evolution of two distinct clones, Clone A/B, in one PDX model. Data integration of clonal mutational profiles with pre and post targeted therapy (lorlatinib) reveals clone specific treatment response. Clone-A was partially responding with extinction of a sub-set of somatic alterations, whereas no change was observed in Clone-B. The replication timing (RT) profile of these two clones, Clone-A (late-RT) and Clone-B (early-RT) were mutually exclusive. Study on relationship between DNA RT and gene transcription is on-going.

Conclusion: Together, these results determine the evolutionary trajectories of NB tumors, linked to distinct replication timing and highlight opportunities for targetable early clonal alteration detection.

O16.3

Direct targeting of RAS via small-molecule inhibition in RAS-driven and lorlatinib-resistant ALK-driven high-risk neuroblastomas

Gerelus M[1,2], Balyasny S[1], Casey C[1], Groff D[1], Matkar S[1], Berko E[1], Daniels A[1], Polkosnik G[1], Krytska K[1], Maris J[1,2], Mosse Y[1,2]

[1]Children's Hospital of Philadelphia, Philadelphia, United States, [2]Pharmacology Graduate Group, University of Pennsylvania, Philadelphia, United States

Parallel sessions 16: Genetic drivers of resistance and relapse, May 18, 2023, 11:15 AM - 12:05 PM

Background: Mutations in the RAS/MAPK pathway in NB are rare at diagnosis but detectable using ultra-deep sequencing (Berko et al. abstract submitted). Pathway activation is enriched in a subset of patients upon relapse after upfront cytotoxic therapy and ALK inhibition therapy. Importantly, this pathway is controlled by numerous negative and positive feedback loops which limits the activity of single-agent MEK inhibitors in NB and other contexts, but we anticipate targeting RAS directly may prevent reactivation. We hypothesize that direct targeting of RAS in lorlatinib-resistant and RAS-driven NB tumors will reveal optimal combination strategies to improve and sustain therapeutic efficacy.
Aims: We aimed to identify superior therapeutic combinations targeting the RAS/MAPK pathway in lorlatinib-resistant RAS/MAPK-mutant, and RAS-driven NB xenografts using novel small molecules.
Methods: We tested recently developed small molecule inhibitors (to be disclosed at meeting) in vivo targeting SHP2 (SHP2i) and RAS (RASi). Lorlatinib-resistant and RAS/MAPK-driven models were engrafted into CB-17 SCID mice to determine efficacy and pharmacodynamics of single agent RASi, SHP2i, and lorlatinib, and combinations of each.
Results: Using our deep sequencing assay, we identified a newly acquired HRASQ61K mutation (variant allele frequency of 30%) in the lorlatinib-resistant NB1643-LRX (ALKF1174L, MYCN-amp., HRASQ61K). Dual RASi/lorlatinib elicited significant growth delay in NB1643-LRX, remaining under 0.5cm³ during 4 weeks of treatment. Vehicle, RASi, and lorlatinib arms reached 2cm³ in 2.17±0.23, 3.57±0.69, and 2.57±0.29 weeks, respectively. Dual RASi/SHP2i elicited antitumor activity in SK-N-AS (NRASQ61K, TP53H168R), regressing to 0.02cm³ during 3 weeks of treatment, while the RASi arm remained cytostatic. Vehicle and SHP2i arms reached 2cm³ in 2.57±0.99 and 2.94±0.50 weeks, respectively. Dual RASi/SHP2i elicited growth delay in NB-EBc1 (KRASG12D), reaching 0.75cm³ during 4 weeks of treatment. Vehicle, RASi, and SHP2i arms reached 2cm³ in 1.57±0.43, 3.20±1.10, and 2.08±0.19 weeks, respectively. Biochemical experiments revealed robust and sustained downregulation of phospho-ERK1/2 in NB1643-LRX only upon dual RASi/lorlatinib, and SK-N-AS and NB-EBc1 only upon dual RASi/SHP2i combinations.
Conclusions: Targeting the RAS-MAPK pathway in NB remains clinically challenging. While in vivo combination therapies revealed promising early activity, these studies shed light on the narrow therapeutic window of RAS/MAPK pathway inhibition, requiring optimization of dosing and schedule.

O16.4

A genetic study of paired diagnosis and relapse neuroblastoma samples identifies a novel relapse-specific mutational signature

Allinson L[1], Gabriel A[1], Raven J[1], Goodman A[2], Chaidrata S[1], Beckett E[1], Whiteman S[1], Stevenson L[1], Harding F[2], Fong F[1], Mundwadkar A[1], Potts A[2], Underhill A[1], James E[1], Basta N[3], Williamson D[1], McAnulty C[2], McNally R[3], Tweddle D[1,4]

[1]Translational & Clinical Research Institute, Centre for Cancer, Newcastle University, Newcastle upon Tyne, United Kingdom, [2]Newcastle Genetics Lab, Newcastle upon Tyne Hospitals NHS Trust, Newcastle upon Tyne, United Kingdom, [3]Population Health Sciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom, [4]Great North Children's Hospital, Newcastle upon Tyne, United Kingdom

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Background: Neuroblastoma is a highly heterogenous disease, therefore patients are stratified into high-, intermediate- and low-risk groups based on clinical and genetic factors. More than 50% of high-risk patients relapse, after which survival is <10%. Most paired neuroblastoma relapse studies focus on high-risk patients alone, here we present a risk-group agnostic cohort.

Aims: To study paired diagnosis and relapse neuroblastoma cases to elucidate the genetic basis of relapse.

Methods: From a cohort of 39 relapsed neuroblastoma patients (17 high-, 10 intermediate- and 12 low-risk), chromosomal copy number aberrations (CNAs) were assessed in 33 patients using single-nucleotide polymorphism (SNP) arrays or array comparative genomic hybridisation and visualised using Nexus Copy Number software. Whole exome sequencing (WES) was performed on 34 cases with two or more paired DNA samples. Mutational signatures were generated using SigProfiler and compared to COSMIC signatures for patients with constitutional DNA (n=18). Clonal clustering of single nucleotide variants was performed using Sequenza and QuantumClone (n=5).

Results: CNA analysis identified nine MYCN amplified cases, four with ALK co-amplification and one with MDM2 and CDK4 co-amplification, all present at diagnosis and relapse. Typical neuroblastoma SCAs were present in all but one case at relapse. Intragenic ATRX deletions were observed in three patients, one relapse-specific. WES mutational analysis revealed nine ALK mutated cases (four relapse-specific) and two cases with TP53 mutations (diagnosis and relapse). Germline mutation analysis identified six potentially pathogenic germline mutations in five cases (ALK, MAX, ATM x2, BRCA1 and FANCA). Mutational signature analysis of WES identified COSMIC clock-like signatures SBS1 (8/18) and SBS5 (4/18) as well as SBS3 (defective homologous recombination) (14/18) at diagnosis/pre-treatment. Analysis of post-chemotherapy and relapse samples revealed additional signatures, SBS35 (platinum treatment) (6/30) and a novel relapse specific signature, SBS96B (17/30). Although not linked to known chemotherapy signatures, all but one case with the SBS96B signature had undergone chemotherapy including platinum-based chemotherapy. Clonality analysis in five patients identified between three to five subclones with mixed branching.

Conclusion: We have identified a novel relapse-specific mutational signature. SBS3 could be used to predict sensitivity to DNA damage repair inhibitors. Further investigation into mutational signatures is on-going.

O17.1

Performing [18F]mFBG Long Axial Field Of View (LAFOV) PET-CT without sedation or general anaesthesia for imaging of children with neuroblastoma.

Borgwardt L[1], Brok J[2], Andersen K[1], Madsen J[1], Gillings N[1], Fosbøl M[1], Denholt C[1], Enevoldsen L[1], Oturai P[1], Johannesen H[1], Czyzewska D[1], Højgaard L[1], Andersen F[1], Fischer B[1]

[1]Copenhagen University Hospital, Rigshospitalet, Department of Clinical Physiology and Nuclear Medicine, Copenhagen, Danmark, [2]Copenhagen University Hospital, Rigshospitalet, Department of Paediatrics, Copenhagen, Denmark

Parallel session 17: Novel diagnostics: imaging, May 18, 2023, 3:20 PM - 4:10 PM

Background/Aims: For diagnosing and monitoring disease in clinical routine meta-[123I]iodobenzylguanidine ([123I]mIBG)-scintigraphy with single-photon emission computed tomography-CT (SPECT/CT) is used. Application of the new PET-tracer meta-[18F]fluorobenzylguanidine ([18F]mFBG) allows for fast and high-resolution imaging of tumours expressing the norepinephrine transporter, and the new LAFOV-PET/CT-scan can be performed without sedation or GA and with a much higher resolution. This prospective study will be investigating the value of [18F]mFBG LAFOV-PET/CT compared to [123I]mIBG-scintigraphy with SPECT/CT for imaging in neuroblastoma.

Methods: We have started a prospective, single-center study, and are recruiting children with neuroblastoma, referred for [123I]mIBG-scintigraphy with SPECT/CT. Within 1 week of [123I]mIBG-scintigraphy, [18F]mFBG LAFOV-PET/CT (Siemens Biograph Vision Quadra PET/CT) is performed at 1 h p.i. of [18F]mFBG (1.5-3 MBq/kg) without sedation or GA, hence the younger children are scanned during naptime for 2 min. (this in contrast to the 24 h p.i. of [123I]mIBG and 2.5 h acquisition time with GA often needed). In the LAFOV-PET/CT-scanner the sensitivity is increased more than 20 times compared to mCT PET/CT, on top of the increase from SPECT- to PET-methodology. Tumour localisations and extension on paired scans will be compared.

Results: In the initially scanned children (n=16), intraspinal involvement, retroperitoneal lymph node involvement and bone marrow involvement are the most frequently diagnosed lesions on the [18F]mFBG LAFOV-PET/CT, that are not seen on the [123I]mIBG-scintigraphy. And if the [123I]mIBG-scintigraphy do show suspicion of these involvements, it is diagnosed with a much higher diagnostic certainty on [18F]mFBG LAFOV-PET/CT. The PET acquisition time is of 10 min., with only 2 min. of reconstruction required, being the minimum required scanning time in order to provide a clinically useful image. By scanning 10 min. the period without motion artefacts can be reconstructed. As a result of the short acquisition time, the PET-scans can be performed without sedation or GA.

Conclusion: Initial results shows that more neuroblastoma localisations are diagnosed on [18F]mFBG LAFOV-PET/CT compared to [123I]mIBG-scintigraphy with SPECT/CT and that GA seems not to be needed due to the short acquisition time of the LAFOV-PET/CT. [18F]mFBG LAFOV-PET/CT shows promise for future staging and response assessment in neuroblastoma in children.

O17.2

18F-MFBG PET imaging in patients with neuroblastoma: lesion targeting and comparison with MIBG

Modak S[1], Mauguen A[2], Basu E[1], Price A[3], Behr G[3], Min R[3], Lyashchenko S[3], Schwartz J[4], Pandit-Taskar N[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]Memorial Sloan Kettering Cancer Center, Department of Radiology, New York, United States, [4]Memorial Sloan Kettering Cancer Center, Department of Medical Physics, New York, United States

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Background/Introduction: ^{123}I-MIBG gamma camera imaging (MIBG) is routinely used for the imaging of neuroblastoma. Limitations include need for two-day imaging schedule and lack of quantitative uptake measurements. Phase I study showed that ^{18}F-MFBG (MFBG), a positron-emitting analog of MIBG, is safe and feasible (PMID:28705916). In the follow-up phase II trial (NCT02348749) we evaluated MFBG PET imaging, comparing neuroblastoma lesion between the two modalities.
Methods: Patients with known neuroblastoma underwent whole- body PET CT or PET-MR imaging one hour post-injection with 8mCi/1.7m² of MFBG. All patients had concurrent MIBG scan with planar and SPECT-CT imaging without intervening therapy. MFBG and MIBG images were independently analyzed for lesion targeting; lesion number, site and uptake was noted for each patient and compared. Discordant lesions were further investigated with correlative imaging and/or clinical follow up. MFBG score was generated, similar to the modified Curie score for MIBG, for each patient.
Results: 25 patients (median age 8 y) underwent MFBG scans at a median of 2 (range 0-24) days after MIBG scans; in one patient MFBG scan was performed 1 day before MIBG scan. No MFBG-related adverse events were noted. 24 patients had both MIBG and MFBG positive scans while 1 patient had both MIBG and MFBG negative scans. A total of 367 lesions were visualized on MFBG as against 217 on MIBG. There were no MIBG-positive that were negative on MFBG. A median number of 11 (range 0-57) lesions per patient were detected on MFBG scan. Uptake was noted in osteomedullary only, (n=13), soft tissue only (n=1) and both soft tissue and osseous lesions (n=10). MFBG detected a median of 6 (range 1-24) additional lesions in 19 patients. In 16/19 patients where follow up was available, the discordance was related to true positive disease, including at least 116 sites. Respective median curie scores were 3 (range:0-22) and 9 (range:0-24) with MIBG and MFBG respectively.
Conclusions: MFBG scan detected disease in all patients with MIBG positive disease. MFBG detected similar or more lesions compared to MIBG in patients with neuroblastoma, with overall increased scores. Further analysis to evaluate impact on patient management is underway.

O17.3

Prognostic value of tumor heterogeneity in neuroblastoma using pre-treatment MIBG SPECT/CT: is there a place for radiomics?

Luporsi M[1,2], Brisse H[2,3], Nioche C[2], Jehanno N[1,2], Fokem-Fosso H[2], Buvat I[2], Schleiermacher G[4,5], Orlhac F[2]
[1]Department of nuclear medicine, Institut Curie, PSL Research University, Paris, France, [2]Institut Curie, Inserm , LITO-U1288, Orsay, France, [3]Department of radiology, Institut Curie, PSL Research University, Paris, France, [4]SiREDO Integrated Pediatric, adolescents and young adults Oncology Center, Paris, France, [5]Laboratory of translational research in pediatric oncology INSERM-U830, Institut Curie, Paris, France

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BACKGROUND
Genetic heterogeneity and cellular plasticity of neuroblastoma (NB) are promising biomarkers for future therapeutic developments. MIBG scan is one of the diagnostic pillars in patients' management but its interpretation is currently limited to visual analysis, mainly qualitative, using only part of the information. Recent studies have suggested that NB's metabolic activity characterized using radiomic features reflecting the volume, the shape or signal distribution within tumors could be promising prognostic tools. Yet, radiomic studies focusing on MIBG scintigraphy are sparse and have only involved small series so far.

AIMS
To assess the prognostic significance of primary tumor heterogeneity in NB based on pre-treatment 123I-MIBG SPECT/CT radiomic features.

METHODS
We retrospectively included 92 patients with a newly diagnosed NB treated in our institution from 2012 to 2020 who underwent their pre-therapeutic 123I-MIBG scan with a SPECT/CT (Discovery GE 670). Genomic, histological, biological and CT data as well as survival (> 2 years of follow-up) were collected from medical records. Radiomic features of the primary lesion were extracted using the LIFEx software (v7.0).

RESULTS
92 patients were studied: 32 female, mean 27±32 months at diagnosis (range [1-178], < 18 months: 52%). 52/92 patients presented with metastatic disease at diagnosis (42 stage M, 10 Ms). 36/92 patients had high-risk NB.
All the well-established prognostic features (age at diagnosis, INRGSS stage, metastatic site, LDH, MKI, INPC and genomic profiles, notably the MYCN amplification status), were predictive of survival, as well as the MSI score and the baseline SIOPEN score ($p \leq 0.05$). Interestingly, MIBG-radiomic features reflecting metabolic volume (MV), asphericity (AS) or aggressiveness (Normalized distance from Hotspot to Centroid-NHOC) of the primary tumor were also significantly predictive of survival ($p \leq 0.05$). The combination of the SIOPEN score, MV, AS and NHOC led to the identification of 3 prognostic profiles ($p < 0.0001$), regardless of the MYCN status.

CONCLUSION
In this largest series to date, radiomic features extracted from MIBG SPECT/CT in NB patients are associated with survival and might be used for as new biomarkers for prognostic stratification. Studies involving advanced imaging features and external validation cohorts are underway to further explore the potential of radiomics in NB.

O17.4

Multispectral short-wave infrared (SWIR) fluorescence imaging as a new versatile tool to enhance intra-operative delineation of tumour margins.

Privitera L[1,2], Waterhouse D[2], Da Pieve C[3], Barisa M[1], Ogunbiyi O[4], Hutchinson C[4], Ogunlade O[5], Sebire N[4], Cross K[6], Beard P[5], Kramer-Marek G[3], Anderson J[1], Giuliani S[1,2,6]

[1]Cancer Section, Developmental Biology and Cancer Programme, UCL Great Ormond Street Institute of Child Health, LONDON, United Kingdom, [2]Wellcome / EPSRC Centre for Interventional and Surgical Sciences (WEISS), LONDON, United Kingdom, [3]Preclinical Molecular Imaging, Division of Radiotherapy and Imaging, The Institute of Cancer Research, LONDON, United Kingdom, [4]Department of Histopathology, Great Ormond Street Hospital for Children NHS Trust, LONDON, United Kingdom, [5]University College London, Department of Medical Physics and Biomedical Engineering, LONDON, United Kingdom, [6]Department of Specialist Neonatal and Paediatric Surgery, NHS Foundation Trust, Great Ormond Street Hospital for Children, LONDON, United Kingdom

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Background: Targeted fluorescence-guided surgery (FGS) has the potential to revolutionize Neuroblastoma (NB) surgery by providing real-time visualisation of the tumour. Short-wave infrared (SWIR, >1000nm) fluorescence imaging has shown advantages over conventional near-infrared I (NIR-I, 700-950nm) fluorescence due to reduced tissue scattering and negligible autofluorescence.

Aim: Using two NIR-I anti-GD2 probes with long tails emitting in the SWIR range, our preclinical study aims to demonstrate that SWIR fluorescence imaging enables superior depth penetration and better visualisation of tumour margins compared to NIR-I.

Methods: A clinical-grade anti-GD2 antibody was conjugated with two NIR-I dyes (anti-GD2-IR800 and anti-GD2-IR12), and a multispectral NIR-I/SWIR imaging device was constructed to allow objective comparison between the two fluorescence windows. System sensitivity and depth penetration were assessed with the use of a tissue-mimicking phantom model consisting of cells pellets stained with the anti-GD2 conjugates, obscured beneath tissue scattering medium. In-vivo, NSG mice bearing subcutaneous NB tumours were intravenously injected with either anti-GD2-IR800 (n=4), anti-GD2-IR12 (n=4) or saline-solution (n=2). One mouse from each group was imaged using the multispectral NIR-I/SWIR device at four different time points. Tumour mean fluorescence intensity (MFI) and tumour-to-background ratio (TBR) were evaluated.

Results: In-vitro, the novel NIR-I/SWIR fluorescence device showed a minimum detectable number of 2.5×10^5 cells. While the peak signal height of the fluorescence decreased rapidly with the depth of the tissue scattering medium, the full-width half maximum of the signal was smaller for longer wavelengths, demonstrating the sub-surface potential of SWIR fluorescence. In-vivo, anti-GD2-IR800 showed higher MFI compared to anti-GD2-IR12 (MFIIR800/MFIIR12=3.0±0.7, p=0.013, Friedman test for dye effect). The tumours remained detectable above background tissue at all time points for both conjugates (2.3<TBR<4.1), with neither time nor conjugates being a significant source of variance. SWIR fluorescence showed significantly higher TBR than NIR-I fluorescence (TBR_{1300}=4.6±1.0 vs TBR_{900}=2.6±0.4, p=0.00003), allowing for a higher contrast delineation of the tumour margins.

Conclusion: Our study demonstrates, for the first time, the potential for GD2-targeted SWIR FGS to improve NB surgery. Crucially, the use of a clinically approved antibody, combined with the availability and translatability of NIR-I dyes, supports a straightforward translation of SWIR imaging techniques into clinical practice.

O18.1

Mastering the game of hide and seek: epigenetic modulation as a strategy to increase T-cell immunogenicity of high-risk neuroblastoma

Cornel A[1,2], Dunnebach E[1,2], Hofman D[1], van Dinter J[1], Das S[3,4], Sengupta S[3,4], van den Ham F[1], Wienke J[1], van den Beemt D[1,2], Essing A[1], Engels S[1], Lo Presti V[1,2], Szanto C[1], George R[3,4], Molenaar J[1], van Dierselhuis M[1], van Heesch S[1], Nierkens S[1,2]

[1]Prinses Máxima Center, Utrecht, The Netherlands, [2]UMC Utrecht, Center for Translational Immunology, Utrecht, The Netherlands, [3]Dana-Farber Cancer Institute, Department of Pediatric Oncology, Boston, USA, [4]Harvard Medical School, Department of Pediatrics, Boston, USA

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Background

Immunotherapy in high-risk neuroblastoma (HR-NBL) does not live up to its full potential due to extensive immunomodulation of HR-NBL and its microenvironment. We hypothesize that (immuno)therapy efficacy can be enhanced by effectively engaging T-cells, thereby stimulating anti-tumor cytotoxicity and creating immunological memory to prevent future relapse. Even though the absence of MHC-I surface expression on NBL cells initially limits cytotoxic T-cell engagement, we previously showed that MHC-I can be upregulated by cytokine-driven immune modulation. Next, we questioned whether modification using FDA-approved drugs can increase the immunogenicity of HR-NBL.

Aim

To identify pharmacological strategies to enhance T-cell engagement and therewith immunogenicity of HR-NBL.

Method/Materials

Drug repurposing libraries were screened to identify compounds enhancing MHC-I surface expression in NBL cells using high-throughput flow cytometry. Hits were confirmed in a panel of NBL patient-derived organoids. Compound-treated NBL cell lines and organoids were subsequently co-cultured with PRAME reactive tumor-specific T-cells and healthy-donor NK-cells to determine the effect on T- and NK-cell cytotoxicity. Additional immunomodulatory effects of histone deacetylase inhibitors (HDACi) in organoids were identified using RNA- and ribosome sequencing (RNA-/riboseq), and flow cytometry.

Results

Drug library screening revealed that various HDACi induce MHC-I upregulation in NBL cells. The HDACi Entinostat was further evaluated, as it is currently in trials in pediatric setting. Entinostat induced MHC-I expression in a panel of NBL cell lines and patient-derived organoids and enhanced in vitro T-cell activation and cytotoxicity.

Interestingly, RNAseq revealed that Entinostat induced an immune activation signature not limited to MHC-I presentation, which coincided with a switch towards a mesenchymal NBL phenotype. Among others, we observed enhanced expression of NK-cell engaging receptors, resulting in increased in vitro NK-mediated cytotoxicity, induction of previously unannotated neoantigens (validated on a translatome level with riboseq), and activation of the immunoproteasome.

Conclusion

This study shows that epigenetic modulation by Entinostat results in a tumor cell lineage switch that is accompanied by increased immunogenicity of HR-NBL. Additionally, we identified Entinostat-induced expression of novel neoantigens unique to NBL. These results substantiate the combination of (immuno)therapy with HDACi to enhance adaptive immune engagement and therewith immunological memory to improve outcome for children with HR-NBL.

O18.2

Copper chelation disrupts the immunosuppressive neuroblastoma microenvironment to enhance anti-GD2 immunotherapy.

Rouaen J[1,2], Luciani F[3], Salerno A[1,2], Murray J[1,2], Shai-Hee T[1,2], Tedla N[3], Faridi P[4], Siebert N[5], Lode H[5], Haber M[1,2], Trahair T[1,2,6], Vittorio O[1,2]

[1]Children's Cancer Institute, Lowy Cancer Research Centre, University of New South Wales, Sydney, Australia, [2]School of Clinical Medicine, Faculty of Medicine, University of New South Wales, Sydney, Australia, [3]School of Medical Sciences, University of New South Wales, Sydney, Australia, [4], School of Clinical Sciences at Monash Health, Monash University, Melbourne, Australia, [5]Department of Pediatric Hematology and Oncology, Greifswald University Medicine, Greifswald, Germany, [6]Kids Cancer Centre, Sydney Children's Hospital, Randwick, Australia

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Background: Anti-GD2 immunotherapy has improved the survival of high-risk neuroblastoma patients, however efficacy is strongly hampered by the immunosuppressive tumor microenvironment. Neuroblastomas often exhibit low mutational burden, scarce major histocompatibility complex (MHC) class I expression, increased immune checkpoint markers and soluble mediators to reduce immune cell infiltration and activity, all contributing to dampening of the anti-tumor immune response.
Aims: Given the emerging link between copper and immune evasion (PMID: 32816860), we evaluated the impact of copper chelation on neuroblastoma using the agents tetraethylenepentamine (TEPA) and the clinically approved analogue triethylenetetramine (Trientine-Cuprior™). We first sought to characterize the effect on the tumor microenvironment before assessing therapeutic efficacy of TEPA and Trientine in combination with anti-GD2 immunotherapy using two preclinical models of neuroblastoma.
Methods: Using the immunocompetent Th-MYCN transgenic model, animals were treated daily with saline or TEPA (400mg/kg) for seven days before tumor resection. To assess cellular and molecular heterogeneity, tumors and immune cells were processed and analyzed using single-cell RNA sequencing, Nanostring digital spatial profiling and mass spectrometry-based immunopeptidomics. This was further complemented by Opal multiplex immunohistochemistry and cytokine profiling. Therapeutic efficacy of combination therapy of TEPA and Trientine with anti-GD2 monoclonal antibody therapy was evaluated using the Th-MYCN and subcutaneous syngeneic NXS2 murine models of neuroblastoma.
Results: Transcriptomic analyses revealed that copper chelation reversed the metabolic and immunosuppressive activities of tumor cells and enhanced immune cells activation, favoring the anti-tumor immune response. This data notably suggested treatment-induced restoration of tumor cell immunogenicity through the upregulation of MHC Class I and stimulation of neoantigen production, facilitating tumor clearance. These findings were supported by immunohistochemistry and cytokine profiling which demonstrated enhanced cytotoxic T-cell, natural-killer cell and neutrophil infiltration and activation. Combination of copper chelation with anti-GD2 immunotherapy led to increased long-term survival, with ~40% of mice exhibiting complete remission in both in vivo models.
Conclusion: Collectively, this study highlights the important role of copper in modulating the neuroblastoma tumor microenvironment and anti-tumor immune response. Findings provide the first evidence for repurposing the clinically approved copper chelation agent Trientine-Cuprior™ as an immunomodulatory agent to potentiate anti-GD2 immunotherapy.

O18.3

A humanized anaplastic lymphoma kinase (ALK)-directed antibody-drug conjugate demonstrates potent cytotoxicity in neuroblastoma (NB) and other ALK-expressing cancers

Osborne B[1], Acholla T[1], Casey C[1], Krytska K[1], Guerra A[1,2], Matkar S[1], Kennedy A[1], Witek G[1,2], Chen C[3], Li W[3], Balyasny S[1], Polkosnik G[1], Dimitrov D[3], Jelev D[3], Mosse Y[1,2]

[1]Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, United States, [2]University of Pennsylvania, Perelman School of Medicine, Philadelphia, United States, [3]University of Pittsburgh, Department of Medicine, Pittsburgh, United States

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Background: Full length ALK is expressed on the cell surface of most NB cells, as well as the majority of alveolar rhabdomyosarcoma (aRMS) and a subset of colorectal cancers (CRC), but not on normal tissue, supporting development of immune-based ALK-targeting approaches.
Aim: Evaluate the efficacy of a humanized ALK-directed antibody-drug conjugate, KTN0239-PBD, across a comprehensive panel of NB, CRC and aRMS patient-derived xenograft (PDX) models and define biomarkers of tumor response.
Methods: A highly specific, humanized ALK antibody KTN0239 was developed with site-specific conjugation of an amine-azido linked conjugated to pyrrolobenzodiazepine (PBD) dimers using click chemistry. We tested KTN0239-PBD in vitro in three, well-characterized ALK-expressing NB patient-derived cell lines with serial dilutions of ADC (100fM to 10nM). Flow cytometry determined ALK cell-surface density in ten PDXs and we prioritized seven models (4 NB, 2 CRC, and 1 aRMS) with low, medium, and high ALK expression for in vivo efficacy studies. Tumor-bearing CB17 SCID mice received once weekly, intraperitoneal injections for three weeks of KTN0239-PBD at 1mg/kg, 0.5mg/kg, or 0.1mg/kg, or one of six control injections. Mice were measured bi-weekly for tumor volume and weight.
Results: We demonstrate on-target binding of KTN0239 to cell surface ALK with dose- and ALK expression-dependent cytotoxicity of NB cells lines. In vivo studies demonstrate complete tumor regression after three doses with maintained regression lasting 56-98 days in NB models (NB-1 [ALK-amplified, MYCN-amplified, high ALK expression], COG-N-424x [ALK-WT, MYCN- amplified, low ALK expression], and NB-SD [ALK-F1174 mutant, MYCN-amplified, medium ALK expression]), maintained regression lasting 56-91 days in CRC models (SW-48 and HCT116), and maintained regression lasting 28 days in one aRMS model (RH41). The low ALK-expressing, ALK-WT, MYCN non-amplified NB model, SK-N-AS, shows modest tumor reduction with regrowth on day 58 supporting ALK-specificity of KTN0239-PBD. Dose de-escalation studies show further specificity and activity at clinically relevant doses. Tumors continue to be measured bi-weekly through study endpoint.
Conclusions: Findings underscore the importance of novel immunotherapeutic strategies that exploit tumor specific ALK expression. In parallel, we are engineering ALK chimeric antigen receptors and optimizing delivery in NB and other ALK-expressing cancers to maximize clinical benefit from antibody-based immunotherapy.

O18.4

Targeting neuroblastoma using Aurora-A inhibition and CAR T-cells

Müller M[1,2], Barisa M[3], Anderson J[3], Eilers M[1], Büchel G[1,2]
[1]Theodor Boveri Institute, Department of Biochemistry and Molecular Biology, Biocenter, University of Würzburg, Würzburg, Germany, [2]Mildred Scheel Early Career Center, University Hospital Würzburg, Würzburg, Germany, [3]Department of Developmental Biology and Cancer, UCL Great Ormond St Institute of Child Health, London, United Kingdom

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Background: Amplification of MYCN is a driver and associated with high-risk cases of neuroblastoma. MYCN can be stabilized by complex formation with the Aurora-A kinase during S-phase. Previous work showed that treatment with the Aurora-A inhibitor MLN8237 is effective at high doses. In combination with an ATR inhibitor, MLN8237 significantly improved the survival of mice harboring a neuroblastic tumor. The therapy engaged the immune system for tumor eradication. This involvement of the immune system opens new immunotherapeutic treatment options since neuroblastoma belong to the class of immunological unresponsive tumors.

Aims: We aim to characterize treatment with Aurora-A inhibitors in combination with immunotherapy in vitro and in vivo. For this we use chimeric antigen receptor (CAR) expressing T-cells to specifically target an overexpressed antigen.

Methods: We infected T-cells with retroviruses for expression of the CAR. In co-cultivation assays we investigated cytotoxicity and killing ability of the engineered T-cells.

Results: We characterized the novel Aurora-A inhibitor AK01 (LY 3295668). Treatment of neuroblastoma cells with AK01 induced apoptosis and cell cycle arrest, with a higher amount of cells arresting in S-phase compared to MLN8237. These phenotypes of AK01 treatment can be rescued by introduction of a point mutation at the published inhibitor binding site in the murine Aurora-A domain, which further indicates high on-target activity. We found that T-cells are as well affected by Aurora-A inhibitor treatment resulting in compromised proliferation and expression of activation marker with higher inhibitor concentrations, but are substantially less sensitive compared to tumor cells. Furthermore, we engineered CAR T-cells to recognize the human B7-H3 antigen. In co-cultivation assays we observed that the engineered CAR T-cells were able to eradicate hB7-H3-overexpressing tumor cells. When combining the CAR T-cells with Aurora-A inhibitor treatment a five times lower effector to target ratio resulted in a complete elimination of tumor cells.

Conclusion: We propose that treatment with Aurora-A inhibitor dissociates the packed tumor mass which as a consequence creates accessibility for immune cells. Combined with tumor antigen specific CAR T-cells we predict a complete eradication of the tumor resulting in event free survival of the treated mice in vivo.