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ABSTRACT BOOK

ORAL PRESENTATIONS IN PLENARY SESSIONS

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PS1.1

Spontaneous regression and differentiation in neuroblastoma lacking telomerase

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Plenary session I: Genetic defects and dependencies in neuroblastoma, May 15, 2023, 9:15 AM - 10:15 AM

Background

Clinical courses of neuroblastoma vary greatly, ranging from fatal progression to spontaneous regression or maturation into benign ganglioneuroma. High-risk neuroblastoma is associated with activation of telomere maintenance mechanisms, mostly conferred by telomerase, however, their mechanistic contribution to neuroblastoma pathogenesis have remained unclear.

Aims

We aimed to examine the mechanistic relevance of telomerase in determining neuroblastoma phenotypes using <I>Tert</I>-deficient and -proficient mouse models driven by <I>Th-MYCN</I> and <I>Th-ALK^{F1174L}</I> transgenes.

Methods

We crossbred <I>Th-MYCN;Th-ALK^{F1174L}</I> mice with constitutively <I>mTert</I>-deficient mice (<I>mTert^{-/-}</I>), and monitored tumor development and growth by magnetic resonance imaging. Murine tumors were characterized by histological assessment, immunohistochemistry, RNA-sequencing, and low-coverage whole-genome sequencing. Alternative lengthening of telomeres (ALT) was determined by detection of ALT-associated PML nuclear bodies. Phenotypic and molecular characteristics of murine tumors were compared to those of human neuroblastic tumors, including ganglioneuroblastoma and ganglioneuroma.

Results

We found that neuroblastoma occurred at the same frequency and age in <I>Tert</I>-proficient and -deficient mice. In the latter, however, tumor growth ceased over time, which was followed by spontaneous regression. While early <I>Tert</I>-deficient tumors resembled <I>Tert</I>-proficient tumors both histologically and in their gene expression patterns, we noted that <I>Tert</I>-deficient tumors developed into mature ganglioneuroblastoma later, which was accompanied by massive transcriptional reprogramming. We observed induction of senescence, down-regulation of cell cycle associated genes (e.g.,

<I>Mki67</I>, <I>Ccnb1</I>, <I>Cdk1</I>), and up-regulation of Schwann cell, neuronal and chromaffin differentiation markers (e.g., <I>S100b</I>, <I>Sox10</I>, <I>Plp1</I>, <I>Ntrk1</I>, <I>Chgb</I>) in late <I>Tert</I>-deficient tumors. Comparison of gene expression profiles from mouse tumors with those of human neuroblastoma revealed high concordance of the various clinical and histological subtypes across species. Furthermore, the dynamic changes of both histology and transcriptional profiles observed in murine <I>mTert</I>-deficient tumors were exactly recapitulated in six human neuroblastomas with poorly-differentiated histology that spontaneously matured to ganglioneuroblastoma or ganglioneuroma, which was accompanied by partial regression in four of the cases.

Conclusion
Our data demonstrate that silencing of telomerase causes both spontaneous regression and maturation into ganglioneuroblastoma in mouse and human neuroblastoma. Furthermore, our data show that telomerase is essential for continuous progression of <I>MYCN</I>-driven tumors, thereby providing a starting point for targeted therapeutic interventions in these malignancies.

PS1.2

TFIIIC and MYCN link the three-dimensional chromatin structure of promoters to transcription termination of stalled RNA polymerase II

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Plenary session I: Genetic defects and dependencies in neuroblastoma, May 15, 2023, 9:15 AM - 10:15 AM

Background: In eukaryotic nuclei major biochemical reactions take place in spatially separated subnuclear compartments. Transcription by RNA polymerase II (RNAPII) occurs at several thousand sites per nucleus which form foci to concentrate the factors and enzymes needed for transcription.
MYC oncoproteins drive the development of most human tumors and recurrent amplifications of MYCN is correlated with a poor prognosis in neuroblastoma. MYC proteins bind to virtually all active promoters transcribed by RNAPII. They interact with many general transcription factors and thereby facilitating several steps of the RNAPII transcription cycle. We previously showed that MYC proteins also form complexes with TFIIIC, which was first identified as a general transcription factor for RNA polymerase III. Beyond its role in RNAPIII transcription TFIIIC is also described to be involved in chromatin architecture.

Aims: MYCN/TFIIIC complexes are prominent during G1 and G2 phase but are partly replaced by MYCN/Aurora-A complexes during S phase. We know that MYCN/TFIIIC have several joint chromatin binding sites but whether they interact with the three-dimensional chromatin architecture is unknown. We aim to decipher the involvement in chromatin architecture and the function of this complex switch.
Methods: Upon manipulation of MYCN AND TFIIIC5 expression we used ChIP as well as HiChIP techniques to catalogue the binding and three-dimensional chromatin interactions of MYC and TFIIIC.

Results: We found that MYCN localizes to three-dimensional hubs formed by active promoters and enhancers. In these hubs, MYCN interacts with TFIIIC. MYCN recruits TFIIIC to promoters when transcription elongation is inhibited, and the complex of both proteins induces premature transcription termination. Termination correlates closely with the TFIIIC-dependent removal of MYCN from promoter hubs. This limits DNA damage by removing RNAPII that stalls proximal to double-strand breaks. Binding of TFIIIC to MYCN is limited by competition with Aurora-A and this protects genes involved in mRNA processing from termination

Conclusion: Our data define a MYCN/TFIIIC-dependent pathway that links the dynamics of transcription factories to promoter-proximal transcription termination. It shows that MYCN contributes to the unusual proliferative capacity of neuroblastoma cells via removing stalled RNAPII from promoter hubs and via increasing the capacity for RNA processing.

PS1.3

Inactivating mutations of MKK7 in a subgroup of high-risk neuroblastoma

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Plenary session 6: Genetic drivers of resistance and relapse, May 18, 2023, 9:00 AM - 10:00 AM

Background/Introduction

The mechanisms underlying clinical courses in high-risk neuroblastoma are still incompletely understood. Activation of telomere maintenance mechanisms has been found as hallmark of high-risk disease, and is associated with <I>MYCN</I> amplification or <I>TERT</I> rearrangements in telomerase-positive tumors, and inactivating <I>ATRX</I> mutations in ALT-positive cases. While such alterations occur in roughly 80% of high-risk neuroblastoma, it is unclear which genomic events may drive malignant transformation and relapse in the remaining tumors.

Aims

Here, we aimed to uncover genetic alterations in high-risk neuroblastomas lacking commonly known mutations to identify novel oncogenic drivers in this subtype of neuroblastoma.

Methods

Whole-genome, whole-exome and RNA sequencing data were examined in 140 neuroblastoma cases. Loss of chromosomal target regions was confirmed by fluorescence in situ hybridization. Loss of protein expression was validated by immunohistochemistry. Knock-out cell lines were generated by CRISPR/CAS9 gene editing. Cell viability and apoptosis of knock-out cells was examined by flow cytometry, TUNEL assay and western blot analysis.

Results

We identified compound heterozygous somatic mutations of the <I>MKK7</I> gene, encoding for the Map Kinase Kinase 7 (MKK7) in six cases of high-risk neuroblastoma that lacked known alterations of <I>MYCN</I>, <I>TERT</I>, and <I>ATRX</I>. MKK7 is a kinase that activates JNK through phosphorylation, which in turn can induce various transcription factors that affect cell differentiation, proliferation, survival as well as apoptosis. In all mutated cases, one <I>MKK7</I> allele was deleted, while the other one was affected by single nucleotide variations (nonsense, n=3; missense, n=2; splice site, n=1), suggesting biallelic inactivation of <I>MKK7</I> in these tumors. RNA-expression analysis revealed enrichment of mesenchymal markers in <I>MKK7</I> mutated tumors when compared to corresponding high-risk cases, indicating adrenergic to mesenchymal transition (AMT). When challenged with chemotherapy or UV radiation, <I>MKK7</I> knock-out neuroblastoma cells showed enhanced survival compared to parental cells. These results were reproduced with the JNK inhibitor SP600125, thereby confirming the relevance of MKK7 for JNK-induced cell death in neuroblastoma.

Conclusion
Our data indicate that inactivation of <I>MKK7</I> may contribute to malignant transformation in a fraction of neuroblastomas, and may promote relapse and progression by mediating AMT, which is known to be associated with therapy resistance.

PS1.4

BARD1 germline variants induce haploinsufficiency and DNA repair defects in neuroblastoma

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Plenary session I: Genetic defects and dependencies in neuroblastoma, May 15, 2023, 9:15 AM - 10:15 AM

Background: High-risk neuroblastoma is a complex genetic disease. Through genome-wide association studies (GWAS) and next-generation sequencing, we have identified common single-nucleotide polymorphisms (SNPs) and rare, pathogenic (P) or likely pathogenic (LP) germline loss-of-function (LOF) variants in BARD1 enriched in neuroblastoma patients. The functional implications of these findings remain poorly understood.

Aims: To define the functional relevance of BARD1 germline variation in children with neuroblastoma.

Methods: We correlated BARD1 genotype with expression in normal tissues and neuroblastomas, along with the burden of DNA damage in tumors. To validate the functional consequences of rare germline P-LP BARD1 variants, we used CRISPR/Cas9 to generate isogenic neuroblastoma (IMR-5) and control (RPE1) cellular models harboring heterozygous BARD1 LOF variants (R112*, R150*, E287fs, and Q564*) and quantified genomic instability in these cells via whole-genome sequencing and with multiple functional assays measuring the efficiency of DNA repair.

Results: Both common and rare BARD1 germline variants were associated with significantly lower levels of BARD1 mRNA and an increased burden of DNA damage in neuroblastoma. The most significant BARD1 common risk allele (T) at SNP rs17487792 from our GWAS was associated with an increased number of DNA double-strand-breaks (DSBs) in two independent cohorts of primary neuroblastomas (n=134 and 383 tumors). Using neuroblastoma cellular models engineered to harbor heterozygous BARD1 LOF variants we further functionally validated this association with inefficient DNA repair. We found that neuroblastoma cells with heterozygous BARD1 LOF variants displayed enhanced genomic instability, including harboring an increased number of large-scale copy number alterations, structural variants, and DNA DSBs. Functionally, BARD1 LOF variant isogenic cells exhibited reduced efficiency in repairing Cas9-induced DNA damage, ineffective RAD51 focus formation at DNA DSBs, and enhanced sensitivity to cisplatin and poly-ADP ribose polymerase inhibition both in vitro and in vivo.

Conclusion: At least 1 in 10 children with cancer carry a pathogenic variant in a cancer predisposition gene, thus it is critically important to understand their functional relevance. Here, we demonstrate that germline BARD1 variants enriched in neuroblastoma patients disrupt DNA repair fidelity. This is a fundamental molecular mechanism contributing to neuroblastoma initiation that may have important therapeutic implications.

PS2.1

Tracing the dynamics of neuroblastoma transcriptomic states across the metastatic progression with high depth single cell approaches

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Plenary session II: Plasticity in neuroblastoma and normal development, May 15, 2023, 2:00 PM - 3:00 PM

Background
Metastatic neuroblastoma (NB), representing half of cases, are detected as of the diagnosis which strongly limits our understanding of the disease etiology. Such a broad and immediate extension opens the question of unique dissemination properties related to NB origin. Single cell RNA sequencing and lineage tracing largely contributed to characterize the continuum of cell transcriptomic states transiting in the neural crest (NC)-derived sympatho-adrenal (SA) lineage and highlighted transcriptional proximity of NB with immature neuroblasts. However, the precise origins of NC-derived tumoral neuroblasts remain debated. Hence, whether NB metastatic properties are reminiscent of their origin or are features acquired during disease progression is unknown. Moreover, whether NB states are shaped by signals provided by the successive microenvironments they encounter during metastasis remains obscure.

Aims
We hypothesized that NB metastatic routes and features are intrinsically linked to their NC-derived embryonic origin. We aimed at tracing both physically and transcriptomically NB adaptations to the embryonic tissues encountered during disease progression.

Methods
We previously setup an avian embryo model in which human NB samples, grafted back within the NC, form SA primary tumors that disseminate in a few days and form metastatic foci in the bones and bone marrow. We conducted longitudinal high-depth single cell RNA sequencing of NB collected at key steps of disease progression -in primary tumor sites, routes of dissemination, and bone marrow-.

Results
Over time, three plastic phenotypical states emerged which revealed that, in their embryonic original context, NB cells replay a specific branch of the SA-derived neuroblast progenitor program. We confirmed in series of patient samples that the identified states account for most of NB intratumor heterogeneity. Using a genomic-based strategy, we mapped the dissemination paths taken by NB cells and identified central adaptations of all three phenotypical states to the successive microenvironments. Finally, we extracted a set of genes dynamically regulated during metastatic progression whose clinical relevance was supported by single cell data obtained from pairs of primary tumor / bone marrow patient samples.

Conclusion
Our approach provides an unprecedented view of transcriptional events underlying NB metastatic process and paves the way for novel therapeutic entry points.

PS2.2

Spatial and single-cell transcriptomics characterize tumor heterogeneity and early stage tumors in mouse neuroblastoma models

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Plenary session II: Plasticity in neuroblastoma and normal development, May 15, 2023, 2:00 PM - 3:00 PM

Background/Introduction: MYCN and ALK oncogenes are major drivers of neuroblastoma oncogenesis. Our previous work documented prolonged neurogenesis in sympathetic ganglia in Knock-in AlkF1178L mice and demonstrated a strong oncogenic cooperation between MYCN overexpression and AlkF1178L mutation. The obtained tumors were median and perivascular, likely emerging from the coeliac ganglia. The adrenal gland of these animals did not present any macroscopically visible abnormalities.

Aims: The present study aims at deciphering how MYCN overexpression and Alk activation impacts the homeostasis of the sympathetic nervous system in TH-MYCN/AlkF1178L mice.

Methods: We combined histology analysis, immunochemistry, single-cell (Chromium, 10X) and spatial (Visium, 10X) transcriptomics to characterize the adrenal gland, sympathetic ganglia and tumors at various ages in double heterozygous TH-MYCN/AlkF1178L mice.

Results: Single-cell transcriptomic documents many neuroblasts and few chromaffin cells in the adrenal gland of TH-MYCN/AlkF1178L mouse at P30, whereas at P4 chromaffin cells were more abundant than neuroblasts. The observed histology at P30 is consistent with an in situ high-risk, stroma-poor and poorly differentiated neuroblastoma. At P4, both cycling chromaffin and cycling neuroblasts were identified, yet no trajectories between both cell types could be highlighted.
Spatial transcriptomic data uncovers spatial heterogeneity in a TH-MYCN/AlkF1178L mouse at P20 in a section comprising a tumor area likely arising from the coeliac ganglia and two regions corresponding to normal sympathetic ganglia, from which high cell density areas appear to emerge. A UMAP analysis indicates that the expression profile of the high cell density regions is in between the profile of the normal ganglia area and the one of the large tumor. Our present hypothesis is that these high cell density regions correspond to early tumor stages. In-depth analysis of the obtained transcriptomic data, including deconvolution of spots using single-cell data will allow to further characterize these cells and decipher their specific properties.

Summary/Conclusions: Our data suggest that the MYCN and Alk oncogenes are able to drive neuroblastoma oncogenesis both from sympathetic ganglia and adrenal gland, yet with a different time frame. Spatial transcriptomic analysis allows the characterization of the expression profiles of normal sympathetic ganglia as well as early and late tumor stages.

PS2.3

Expansion of an early SOX2 positive cell population in germline ALK mutant iPSCs during in vitro differentiation towards sympathoadrenal progenitors.

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Plenary session II: Plasticity in neuroblastoma and normal development, May 15, 2023, 2:00 PM - 3:00 PM

Background: Studies defining normal and disrupted human neural crest cell development have been challenging. Accurate human model systems are lacking and insight into the early disruptive events causing neural crest related disease such as neuroblastoma (NB) is limited. We therefore developed an in vitro differentiation model to recapitulate the normal in vivo developmental process of the sympathoadrenal lineage which gives rise to NB.

Methods/materials: In vitro differentiations of wild type (WT) and ALKR1275Q-mutant human pluripotent stem cells (hPSCs) into sympathoadrenal progenitors (SAPs) were analyzed using bulk and scRNA-seq; data were compared with publicly available human in vivo scRNAseq data to map the resulting cell populations and ALKR1275Q-mutant data were integrated with the WT data in a comparative UMAP analysis to analyze the effect of the mutation on the developmental tracks. We performed pseudotime analysis to pinpoint transition states between cell subpopulations.

Results: For both WT and ALKR1275Q-mutant differentiation tracks, we confirmed the development of SAPs based on emerging cells and upregulation of transcription factors such as ASCL1, PHOX2B, and STMN2. Comparison of our in vitro results with public in vivo human embryo data, confirmed the presence of known in vivo SAP developmental subpopulations thus indicating that our model recapitulates the essential steps observed for human in vivo cell and subpopulation-specific state transitions. We exploited the power of our model to perform a comparative UMAP analysis of the ALKR1275Q-mutant and WT scRNA-seq data sets and revealed the presence of an abnormal ALKR1275Q-mutant-specific subpopulation, characterized by the absence of SAP developmental markers and persistent expression of SOX2, a pluripotency marker implicated in cancer. Finally, we performed preliminary syngeneic transplantation studies, showing that induced MYCN expression in developing SAPs, generated employing our system, leads to tumor formation upon injection into SCID mice.

Conclusion: We developed an in vitro differentiation system to study dynamically regulated transcriptomes during sympathoblast development by conducting serial scRNA-seq. Next, we successfully applied this model to study early developmental impact of the germline ALKR1275Q-mutation showing an early

expanding effect on SOX2-expressing cells and demonstrate that in vivo generated SAPs can generate tumors through forced MYCN overexpression.

PS2.4

Identification, characterization and therapeutic targeting of chemotherapy resistant high-risk neuroblastoma persister cells

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Plenary session II: Plasticity in neuroblastoma and normal development, May 15, 2023, 2:00 PM - 3:00 PM

BACKGROUND. The mechanisms of high-risk neuroblastoma chemotherapy resistance are largely unknown and the cells responsible for relapse have not been identified.

AIMS. To discover, characterize, and target the cells responsible for relapse in high-risk neuroblastoma.

METHODS. We used single nucleus RNA and bulk whole genome sequencing to identify and characterize the malignant cells that survive chemotherapy (persister cells) from a cohort of 20 matched diagnostic and definitive surgical resection high-risk neuroblastoma samples. Confirmatory functional studies using flow cytometry, qPCR, immunoblotting, CRISPR-CAS9 knock out, and small molecule inhibition were performed in eight representative cell lines derived from neuroblastomas at diagnosis and treated with standard-of-care chemotherapy.

RESULTS. Five subtypes of persister cells were computationally identified through pathway-based clustering. Subtypes with high MYC(N) activity were determined in two patients with early progressive disease. Sixteen patients had low MYC(N) activity persisters even in the presence of MYCN amplification. These persisters showed NF⍰B and lineage specific (neuronal) pathway activation, an observation that was recapitulated in a diagnostic PDX with MYCN amplification treated with chemotherapy. M2 macrophages showed significant TGFβ expression, a known activator of NF⍰B signaling.

We validated our findings in vitro, confirming a decrease in both MYCN or MYC protein level as well as activation of NF⍰B signaling following chemotherapy, which was enhanced by co-culture with M2 macrophages. Pharmacologic inhibition of the NF⍰B pathway with the small molecule ML120B, a specific I⍰B kinase inhibitor, or genetic depletion of RelA using CRISPR-CAS9, both resulted in increased killing of persister cells when combined with chemotherapy. As BCL-XL is a downstream effector of NF⍰B signaling and was significantly upregulated in persister cells, we combined the selective BCL-XL small molecule inhibitor A-1331852 with topotecan and observed an 8.6-fold increase in cancer cell killing ($p<0.0001$).

CONCLUSIONS. Cellular persistence is mediated by tumor cell-intrinsic and -extrinsic activation of NF�B signaling and BCL-XL expression. We are currently developing a BCL-XL-targeting PROTAC and expanding our efforts to define the immunopeptidome of persister cells.

PS4.1

Phase 2 trial of the Wee1 inhibitor Adavosertib and Irinotecan in Neuroblastoma

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Plenary Session 4: Precision therapy, May 16, 2023, 2:30 PM - 3:30 PM

Background: Inhibition of the DNA damage response Wee1 kinase by adavosertib (AZD1775) potentiates replicative stress from genomic instability or chemotherapy. Alternative lengthening of telomeres (ALT) is associated with replication stress and is enriched in tumors from older patients with relapsed refractory neuroblastoma.
Aims: 1. To report the anti-tumor activity of ADVL1312 therapy combining irinotecan and adavosertib in neuroblastoma; 2. To descriptively assess ALT as a potential correlative biomarker of response.
Methods: Patients with measurable or MIBG-evaluable relapsed/refractory neuroblastoma were treated with irinotecan (90 mg/m2/dose) and adavosertib (85 mg/m2/dose) orally for 5-days every 21-days. The combination was considered effective if there were at least three objective responses in 20 enrolled patients, confirmed by central radiology review. Tumor tissue was analyzed for ALT by multiplex spatial in situ fluorescent co-localization of ultrabright telomeric foci, Phox2b and ATRX.
Results: Twenty eligible patients with neuroblastoma had a median age of nine years (6 – 19), were heavily pretreated and 95% had a history of prior irinotecan. There were three objective responses (9, 11 and 18 cycles) meeting the protocol defined efficacy endpoint (estimated response rate of 16.7%). Three additional patients had prolonged stable disease of 8, 11 and 13 cycles. The combination was well tolerated and there were no dose limiting toxicities at cycle 1 or beyond in any parts of ADVL1312 at the RP2D. The objective response rate was 25% for patients whose tumors were ALT positive (2 of 8), 0% if ALT negative (0 of 4) and 12.5% (1 of 8) if ALT data was not available.
Conclusion: Adavosertib and irinotecan was tolerable and demonstrates early signal of activity in relapsed neuroblastoma. Tumor tissue was not available from all enrolled patients limiting correlative biology results. Additional clinical studies are needed to determine whether adavosertib, novel WEE1 inhibitors or agents that target replicative stress will have activity in neuroblastoma and other ATRX mutant, ALT activated tumors.

PS4.2

Mutated fibroblast growth factor receptor 1 is a strong oncogenic driver and a therapeutic target in neuroblastoma

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Plenary Session 4: Precision therapy, May 16, 2023, 2:30 PM - 3:30 PM

Background
Fibroblast growth factor receptor 1 (<I>FGFR1</I>) is recurrently mutated in neuroblastoma, albeit at low frequencies. Mutations preferentially occur at hot-spot position N546 within the tyrosine kinase domain of the protein, which is also affected in other cancer types, suggesting that they are oncogenic drivers. The impact of such alterations on neuroblastoma pathogenesis, however, has not been determined yet.

Aims
We aimed to determine the oncogenic potential of <I>FGFR1^{N546K}</I> and its potential value as therapeutic target.

Methods
We screened sequencing data of 331 neuroblastoma patients for <I>FGFR1</I> mutations. Ba/F3 cells stably expressing <I>FGFR1^{N546K}</I> were generated to evaluate the transforming capacity. We also developed an <I>R26-LSL-FGFR1-N546K^{fl/wt};Th-IRES-Cre^{tg/wt}</I> transgenic mouse model, in which an <I>FGFR1^{N546K}</I> transgene is integrated into the <I>Rosa26</I> safe harbor locus and expressed after Cre-mediated recombination in tyrosine hydroxylase expressing cells. <I>R26-LSL-FGFR1-N546K^{fl/wt};Th-IRES-Cre^{tg/wt}</I> mice were crossbred with <I>Th-MYCN</I> mice to evaluate the effect of <I>FGFR1^{N546K}</I> transgene alone and in combination with <I>MYCN</I> on tumor development and progression.

Results
We detected <I>FGFR1^{N546}</I> mutations in five high-risk tumors. All five patients had rapid disease progression and fatal outcome after onset of the <I>FGFR1</I> mutation. Growth of Ba/F3 cells became independent of IL-3 after transduction with an <I>FGFR1^{N546K}</I> transgene, indicating oncogene addicted proliferation. Downstream targets of the FGFR signaling pathway were phosphorylated in <I>FGFR1^{N546K}</I>-transformed Ba/F3 cells, and treatment with FGFR inhibitors effectively impaired both proliferation and pathway activation. In mice, we found that co-

expression of the <I>FGFR1^{N546K}</I> and <I>MYCN</I> transgenes caused early development of neuroblastomas within the first days of life with 100% penetrance, leading to death at the age of 18-22 days. In contrast, mice with <I>Th-MYCN^{tg/wt};Th-ALK^{F1174/wt}</I>-driven neuroblastomas lived more than twice as long, indicating that <I>FGFR1^{N546K}</I> has a stronger oncogenic potential than <I>ALK^{F1174L}</I>. Treatment of murine <I>FGFR1^{N546K}</I>-driven neuroblastoma with FGFR inhibitors had anti-tumor activity and led to prolonged survival <I>in vivo</I>.

Conclusion
Our data demonstrate that <I>FGFR1^{N546K}</I> mutations are strong oncogenic drivers in neuroblastoma. The early onset of neuroblastoma development and rapid lethality in <I>R26-LSL-FGFR1-N546K^{fl/wt};Th-IRES-Cre^{tg/wt};Th-MYCN^{tg/wt}</I> mice correspond to the aggressive clinical course of <I>FGFR1</I>-mutated neuroblastoma in patients. Our data provide a rationale for implementing FGFR-directed targeted therapies in <I>FGFR1</I>-mutated neuroblastoma patients.

PS4.3

Combined targeting of metabolic dependencies of proline translation in neuroblastoma

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Plenary Session 4: Precision therapy, May 16, 2023, 2:30 PM - 3:30 PM

Background: Cancer growth is supported by nutrient uptake and the local metabolic network. Increasing evidence links genetic alterations with nutrient dependencies, enabling metabolism targeted treatments. Hyperactivation of the transcription factors MYC or MYCN are prime examples, driving metabolic programmes including glutaminolysis and protein biosynthesis while portending poor clinical outcome. Whereas gene expression regulation by MYC has been studied in detail, the in vivo functional metabolic phenotypes and dependencies in MYC driven cancers remain incompletely understood.
Methods: Primary human neuroblastoma tumors, cell line xenografts and spontaneous TH-MYCN tumors were studied using high resolution LC-MS to identify MYCN characteristic metabolite signatures. Data was correlated to publicly available gene-expression datasets from primary tumors. Functional validation of metabolic phenotypes was performed using the TH-MYCN model for in vivo 13C-stable isotope tracing and metabolic intervention studies. Quantitative whole body metabolic networks were computed. Ribo-seq, RNA-seq, proteomics, and immunoaffinity were performed for mechanistical validation.
Results: Untargeted metabolomics revealed a defined metabolic signature including high proline content across primary MYCN-amplified tumors and models. Transcriptomics supported de-novo proline biosynthesis as the primary source and upregulation of polyamine metabolism in MYCN-amplified tumours. In contrast, functional evaluation of metabolic networks by in vivo stable isotope tracing identified external proline uptake as the primary tumor source. Proline, arginine, and polyamines are interconvertible. To apply therapeutic stress on this dependency we treated TH-MYCN+/+ mice with difluoromethylornithine (DFMO) and/or dietary depletion of the non-essential proline and arginine. Despite significantly reducing tumor growth, diet alone was unable to prolong mouse survival. Combining DFMO and dietary depletion markedly enhanced survival ($p<0.0001$) with ~30% living tumor-free beyond therapy end at day 200. Dual-treated tumors showed reduced polyamine-activation of translation factor eIF5A, and ribosome profiling revealed specific stalling enriched at proline and polyproline motifs that require functional eIF5A for effective translation. Integration of matched RNA-Seq and proteomics identified both, global protein biosynthetic deficiencies and specific enrichment in the proline-centric translatome.
Conclusions: This study pioneers a rational designed targeting of synergistic metabolic dependencies of translation in neuroblastoma. In vivo assessment of protein biosynthetic needs opens the door for a functional precision oncology approach targeting metabolic vulnerabilities of translation.

PS4.4

Low GD2 expression as a mechanism of resistance to chemoimmunotherapy with dinutuximab

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Plenary Session 4: Precision therapy, May 16, 2023, 2:30 PM - 3:30 PM

Introduction: Chemoimmunotherapy with anti-GD2 antibodies is used for treating progressive disease (PD) neuroblastoma and is being evaluated with induction chemotherapy. While most neuroblastomas express GD2, low or negative GD2-expressing neuroblastomas occur and may not benefit from anti-GD2 therapy. We sought to determine the relationship between GD2 expression and response to chemoimmunotherapy in patient-derived xenografts (PDXs) and to determine the frequency of low GD2 expression in neuroblastoma patients.
Methods: We quantified dinutuximab binding to neuroblastoma cells in marrow by multi-color flow cytometry, gating out CD45+ cells and gating on cells positive for the non-GD2 neuroblastoma antibody HSAN (binds to 100% of neuroblastomas) to define % positive and median fluorescence intensity (MFI). We assessed 104 bone marrow, 9 tumor, and 15 blood samples (88 pretherapy, 40 at PD) from neuroblastoma patients on Children's Oncology Group protocol ANBL00B1. Event-free survival (EFS) was assessed for subcutaneous PDXs in nu/nu mice treated with 15 mg/kg temozolomide (TMZ) + irinotecan 7.5 mg/kg (IRN) (days 1-5 and 21-25) +/- 15 mg/kg dinutuximab (days 2, 4, 22, 24).
Results: Based on % positive and intensity of dinutuximab binding we defined 4 groups of patient samples: high, intermediate, low, and very low dinutuximab binding. GD2 expression was very low in 13% pretherapy and 15% PD patient samples, and low in 29% pretherapy and 40% PD samples. We assessed activity of TMZ/IRN +/- dinutuximab in neuroblastoma PDXs (3 PD, 1 established pretherapy). In COG-N-480x (pre-therapy PDX, high GD2 expression) dinutuximab increased 300-day EFS to 100% vs 20% with TMZ/IRN (p=0.04). In COG-N-452x (intermediate GD2 expression) dinutuximab increased 300-day EFS to 50% vs 0% for TMZ/IRN (p=0.01). Dinutuximab did not enhance TMZ/IRN activity in COG-N-519x (very low GD2) or Felix PDX (low GD2).
Conclusions: Low GD2 expression occurs in neuroblastoma. Neuroblastoma PDXs in nu/nu mice provide a preclinical model to assess antibody activity when combined with chemotherapy. In > 10% of patient marrow samples analyzed, dinutuximab binding was comparable to levels seen in PDX's that did not benefit from dinutuximab. Quantifying GD2 expression by flow cytometry is a potential biomarker of activity for dinutuximab in patients treated with anti-GD2 based chemoimmunotherapy.

PS5.1

Bispecific SNIPER Antibody Improves Tumor Targeting and Reduces Toxicity while Maintaining Efficacy for Neuroblastoma

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Plenary session 5: Immune therapy, May 17, 2023, 9:00 AM - 10:00 AM

Background:
The current “standard of care” for neuroblastoma involves an immunotherapy regimen that includes a mAb, such as dinutuximab, that recognizes disialoganglioside (GD2) which is expressed at high levels on neuroblastoma tumors. While GD2 is expressed minimally on normal tissues, it is expressed on nerve cells. Thus, dinutuximab treatment causes substantial, dose-limiting, neuropathic pain.
Aims and Methods:
To increase tumor specificity, we developed a bispecific SNIPER-Ab that binds well to cells only when it simultaneously binds to two tumor antigens (GD2 and B7H3). B7H3 is overexpressed on multiple tumor types, with minimal expression on most normal cells, and is absent on nerve cells.
Results:
We tested the specificity of this GD2/B7H3-SNIPER-Ab by flow cytometry and found that it specifically targets B7H3+/GD2+ tumor cells, but it does not bind to GD2+/B7H3- cells (which simulate nerves). Separately, we tested in vivo tumor specificity using positron emission tomography with mice bearing 4 tumors, each with variant expression of GD2/B7H3. We found that 89Zr-radiolabeled SNIPER-Ab specifically targeted B7H3+/GD2+ tumors, but it was not taken up into GD2+/B7H3-, GD2-/B7H3+ or GD2-/B7H3- tumors. We tested antibody dependent cellular-cytotoxicity (ADCC) on tumors co-expressing GD2 and B7H3; we found that SNIPER-Ab was as effective at ADCC as dinutuximab, and superior at ADCC than anti-B7H3 mAb. An afucosylated version of SNIPER showed significantly enhanced ADCC compared to dinutuximab. In vivo efficacy studies of SNIPER were tested against mice bearing either melanoma or neuroblastoma tumors that co-express GD2+/B7H3+. SNIPER was similarly effective as dinutuximab when given at the same dose. Finally, using mouse burrowing assays to assess neuropathic pain, we have found that SNIPER-Ab does not cause pain compared to controls, but dinutuximab does.
Conclusion:
Our preclinical research shows that this B7H3/GD2-SNIPER-Ab has similar efficacy but reduced toxicity compared to dinutuximab. Because SNIPER should not bind to nerves, it may be possible to administer increased doses of SNIPER beyond the tolerable dose of dinutuximab, which could further improve efficacy. By retaining anti-tumor activity and eliminating off-target binding to nervous tissue, our goal is to improve the anti-tumor efficacy and quality of life for patients receiving anti-GD2 treatment.

PS5.2

Integrative analysis of neuroblastoma by single-cell RNA sequencing identifies the NECTIN2-TIGIT axis as a target for immunotherapy

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Plenary session 5: Immune therapy, May 17, 2023, 9:00 AM - 10:00 AM

Background: Children with high-risk neuroblastoma have poor survival rates and urgently need more effective treatments with less side effects. Immunotherapies may fill this need, yet show limited clinical efficacy.

Aims: We aimed to provide a comprehensive overview of neuroblastoma's immune environment to identify strategies for improving immunotherapy efficacy.

Methods: 25 neuroblastomas from 20 patients (17 high-risk, 6 MYCN-amplified), were collected pre-treatment (n=10) or after induction chemotherapy (n=15). Samples were enzymatically digested, FACS sorted and single-cell RNA-sequenced. Killing assays were performed with patient-derived neuroblastoma organoids and healthy donor PBMCs. Checkpoint inhibition was tested in vivo in three syngeneic models (Neuro2a, N1E-115, N18) and one chemotherapy-resistant syngeneic model (Th-ALKF1174L/MYCN 129/SvJ).

Results: Neuroblastomas were infiltrated by dendritic cells, monocytes, and macrophages with an M2-like differentiation, associated with immunosuppressive and pro-tumorigenic features. Lymphoid cells in neuroblastoma consisted of NK, B, and various T cells including highly suppressive Tregs. Among two CD4+ non-Treg clusters, one likely contained tumor-reactive cells and was significantly enriched for genes associated with T cell dysfunction (TIGIT, CTLA4). CD8+ T had significantly upregulated LAG3 and PDCD1, also associated with T cell dysfunction. Overall, T cells showed signs of dysfunction/exhaustion particularly post-chemotherapy, with enhanced expression of immune checkpoint receptors. NK cells had impaired cytotoxicity (GZMB, PRF1, GNLY), particularly in pre-treatment tumors, which correlated with TGF-β1 signaling and a disbalance between inhibitory receptors TIGIT and CD96 and activating CD226. To identify functional targets for reinvigorating T/NK cell function, we constructed an unsupervised interaction network. This predicted an abundance of immunoregulatory interactions in the tumor microenvironment affecting T/NK cell function, including CLEC2D-KLRB1, PD1-PDL1 and NECTIN2-TIGIT. Since also in T cells the TIGIT/CD226 balance proved disturbed, we tested combined TIGIT/PD-L1 blockade in vitro, which significantly increased killing of neuroblastoma organoids. Moreover, TIGIT/PD-L1 blockade in vivo in three syngeneic models induced complete remissions in a subset of animals and significantly improved survival. Lastly, addition of TIGIT blockade to the standard backbone treatment for relapse/refractory neuroblastoma significantly improved survival in a chemotherapy-resistant model mimicking relapse/refractory tumors.

Conclusion: We provided a comprehensive atlas of neuroblastoma's immune environment and identified TIGIT as a promising target for (combination) immunotherapy.

PS5.3

Multi-antigen targeting of neuroblastoma with CAR T-cells secreting bispecific innate immune cell engagers

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Plenary session 5: Immune therapy, May 17, 2023, 9:00 AM - 10:00 AM

Background: The long-term efficacy of chimeric antigen receptor (CAR) T-cells targeting the neuroblastoma oncoprotein GPC2 can be limited by both GPC2 downregulation and an immunosuppressive tumor microenvironment (TME).

Aims: We designed a bicistronic construct enabling T-cell expression of a CAR targeting GPC2 and secretion of a bispecific innate immune cell engager (BiCE) targeting a second tumor antigen, GD2, and CD16a to both circumvent CAR-induced GPC2 downregulation and recruit/activate natural killer (NK) cells and macrophages to restructure the TME.

Methods: Lentiviral vectors encoded a GPC2.CAR or a GPC2.CAR-GD2.BiCE. In vitro, we assessed GPC2.CAR-GD2.BiCE T-cell cytotoxicity, BiCE secretion kinetics and binding, NK-cell cytotoxicity and polyfunctionality, and macrophage phagocytosis. In vivo, we quantified GD2 BiCE biodistribution, NK-cell intratumor accumulation, and CAR.BiCE efficacy in neuroblastoma patient-derived xenografts (PDXs).

Results: GPC2.CAR-GD2.BiCE T-cells had comparable CAR expression and induced similar cytotoxicity compared to GPC2.CARs alone when co-incubated with GPC2-high neuroblastoma cells (n=4; 85.6±13.4% killing at 5:1 ratio) but not against GPC2-low cells (n=3). Secretion of GD2.BiCEs was only detected in the supernatants of GPC2.CAR-GD2.BiCE T cells and increased 12-35-fold when T-cells were activated by GPC2-high cell lines (n=6). GD2.BiCEs bound to both neuroblastoma cells and human NK cells and facilitated NK cell killing of GD2-high neuroblastoma cells (n=3; 81.1±21.1% killing at 10:1 ratio). Using single-cell proteomics, we found that GD2 BiCEs induced a comparable number of polyfunctional NK cells to dinutuximab (9% vs 13%). Further, GD2.BiCEs also induced macrophage phagocytosis of GFP-labeled NB-EbC1 cells. In mice, GPC2.CAR-GD2.BiCE T-cells delivered GD2 BiCEs specifically to the tumor bed, but not normal murine tissues, and increased the intratumor retention of CD16a-high NK92 cells. Finally, we demonstrate that while both GPC2.CAR and GPC2.CAR-GD2.BiCE T-cells induce comparable GPC2-high/GD2-high COG-N-421x PDX tumor regressions, in antigen heterogeneous PDXs (COG-N-561x; GPC2-moderate/GD2-high and COG-N-603x; GPC2-low/GD2-high), GPC2.CAR-GD2.BiCE T-cells had superior antitumor efficacy compared to GPC2.CAR T-cells alone. No clinically apparent toxicities were observed in either GPC2.CAR or GPC2.CAR-GD2.BiCE T-cell treated mice.

Conclusions: A CAR.BiCE approach enables dual GPC2/GD2 targeting of neuroblastomas by inducing both CAR T-cell cytotoxicity and BiCE-mediated activation and recruitment of CD16a-expressing anti-tumor innate immune cells.

PS5.4

A novel B7H3 targeting chimeric antigen receptor regulated by an IMiD drug sensitive degron tag, optimised for neuroblastoma immunotherapy

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Plenary session 5: Immune therapy, May 17, 2023, 9:00 AM - 10:00 AM

Background
A recently completed clinical study of second-generation chimeric antigen receptor targeting GD2 in relapsed/refractory neuroblastoma showed clinical activity but lack of sustained engraftment or remission. A common determinant of CAR-T failure in solid cancers is T cell exhaustion associated with sustained signaling in the solid tumour environment. Reversible downregulation of CAR expression during manufacture or following infusion holds promise as an approach to allow CAR-T recovery. B7H3 is an oncogene and immune checkpoint ligand over expressed in neuroblastoma and many other childhood solid cancers

Methods
A novel anti-B7H3 antibody (TE9) optimal for CAR-T function was selected from phage ScFv library and compared with current clinical B7H3 binders in identical formats (376.96 and MGA271 binders). CAR functional and biochemical properties were determined by avidity (Lumicks), organoid co-culture repeat stimulation assays, and in vivo tumour growth assays. An optimised novel degron tag sensitive to IMiD drug downregulation (designated iTAG2) was obtained by mutational screening of known zinc finger binding degron motifs.

Results
TE9 was found to have optimal sustained activity in second generation format with CD28 and CD3-zeta endodomains. TE9 and 387.96 have similar avidities, which are much higher than MGA271. Whilst all CARs are active against high antigen density neuroblastoma organoids and in vivo models, MGA271 selectively fails to activate and eliminate in models with low antigen density. TE9-28Z showed superiority in vivo over the clinical trial huk666-28Z CAR evaluated in phase I trial. The novel iTAG2 degron was added to TE9-28Z CAR and showed reversible degradation at nanomolar concentrations of IMiD agents. Use of iberdomide during manufacture switched off CAR expression preventing tonic CAR signaling and improving effector function.

Conclusion
We have developed and optimised a novel B7H3 CAR for neuroblastoma and other solid tumours that can be reversibly switched on and off by the immunodulatory IMiD drugs. This design may be optimal for clinical combination trials in which pulses of IMiD drug simultaneously control tumour growth and modulate tumour microenvironment, whilst allowing CAR-T cell rest and recovery.

PS6.1

Investigating neuroblastoma evolution by matched diagnosis, relapse and cfDNA sequencing as part of the Stratified Medicine for Paediatrics study (SMPaeds)

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Plenary session 6: Genetic drivers of resistance and relapse, May 18, 2023, 9:00 AM - 10:00 AM

Background. Despite intensive front-line therapy, over 40% of patients with neuroblastoma either relapse or are treatment resistant. Studies comparing pre-treatment and relapsed tumours are required to elucidate the molecular basis of neuroblastoma evolution. Given the inherent difficulties of acquiring multiple tissue biopsies, circulating free DNA (cfDNA) offers a less invasive alternative for molecular profiling and also has the potential to assess tumour heterogeneity missed by individual tissue biopsies.
Aims. To investigate evolution of neuroblastoma from diagnosis to relapse and to assess the utility of cfDNA profiling at relapse.

Methods. The SMPaeds study conducts molecular profiling for relapsed paediatric cancers, including neuroblastoma to drive enrolment onto clinical trials and generate research data. Targeted capture panel and low coverage WGS (lcWGS) sequencing was performed on tumour tissue and cfDNA collected at relapse. Archival diagnostic tissue samples were also sequenced where available.

Results. Of the first 500 patients enrolled on the SMPaeds study, paired diagnostic and relapse tissue samples were available for 252 patients. We showed that these tumours undergo significant genomic changes from diagnosis to relapse, accumulating SNVs, indels and complex genome-wide copy number changes. We observed positive selection and gain of mutations in TP53, ATRX and NF1 at relapse across the whole cohort. Interestingly, the 47 neuroblastoma patients with paired samples showed a different pattern: ATRX mutations were stable between diagnosis and relapse with no enrichment seen at relapse. On the contrary, missense ALK mutations were only acquired at relapse in patients with neuroblastoma. Most mutations acquired at relapse in neuroblastoma were in ALK and TP53/RAS/MAPK pathway genes. cfDNA at relapse was analysed in 60 neuroblastomas. Variants found in tissue were successfully detected in cfDNA for most patients; at least one tissue SNV was detected in cfDNA for 83.9% cases. cfDNA unique variants were detected in 54% of neuroblastomas, including pathogenic ALK mutations.

Conclusions. Paediatric tumours evolve significantly from diagnosis to relapse; however, neuroblastoma shows unique features at relapse. Molecular profiling of cfDNA yields comparable results to tissue biopsy. The high number of cfDNA-unique variants detected in neuroblastoma highlights the potential for cfDNA sequencing to compliment clinical diagnostic tissue profiling.

PS6.2

The transcriptional co-repressor Runx1t1 is essential for N-Myc-driven neuroblastoma tumorigenesis

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Plenary session I: Genetic defects and dependencies in neuroblastoma, May 15, 2023, 9:15 AM - 10:15 AM

Background: Using a large-scale in vivo mutagenesis screen in Th-MYCN transgenic mice, we recently identified a single germline point mutation in the transcriptional corepressor Runx1t1, leading to a loss-of-function (LOF) and abolition of N-Myc-driven neuroblastoma tumorigenesis (Murray et al, ANR 2020). This LOF effect was independently confirmed using Runx1t1 knockout in the Th-MYCN mouse model, where Runx1t1 haploinsufficiency alone prevents neuroblastoma in Th-MYCN mice.

Aims: To investigate the mechanism by which RUNX1T1 contributes to N-Myc-driven neuroblastoma tumorigenesis.

Methods: RNAseq and GSEA were performed following shRNA-inducible knockdown of RUNX1T1. Mass spectrometry and co-immunoprecipitation (Co-IP) were used to define the RUNX1T1 protein interactome and ChIP-seq to map the genomic binding sites.

Results: Using human neuroblastoma cell lines and primary-patient samples, we found that N-Myc drives increased RUNX1T1 protein translation rather than RUNX1T1 transcription. Silencing of RUNX1T1 using shRNA in Kelly and BE(2)-C cells resulted in decreased colony formation in vitro, and inhibited tumour growth ($P<0.0001$) in vivo, highlighting a role for this gene in progression of human neuroblastoma. Mass-spectrometry and Co-IP following transfection of BE(2)-C cells with wild-type RUNX1T1 showed interaction of this co-repressor with the transcription factor HAND2 as well as the key CoREST factors LSD1/KDM1A, CoREST3/RCOR3 and HDAC1-3. However, introduction of RUNX1T1 LOF point mutation, led to reduced interaction with HAND2, LSD1 and HDAC3, and complete loss of CoREST3 interaction. ChIP-seq showed RUNX1T1 binding occurring almost exclusively within intergenic regions of the genome, with 24% of all RUNX1T1 peaks co-localizing with HAND2, LSD1 and CoREST3 peaks. Gene ontology processes strongly associated with this common set of peaks included neuronal development and differentiation. ChIP-seq with histone markers (H3K4me1, H3K4me3, H3K27me3, H3K27ac) showed that downregulation of RUNX1T1 led to increases in regions associated with active enhancers and primed enhancers, but no change in poised enhancer regions. RUNX1T1 downregulation also reversed an embryonal stem cell-like genotype characteristic of aggressive MYCN-amplified neuroblastoma.

Conclusion: RUNX1T1 is essential for tumorigenesis and forms part of a transcriptional LSD1-CoREST3-HDAC repressive complex recruited by HAND2 to regulate chromatin accessibility, controlling neuron-specific pathway genes and maintaining an undifferentiated state in MYCN-amplified neuroblastoma cells.

PS6.3

Frequency and clinical significance of subclonal and clonal driver mutations in high-risk neuroblastomas at diagnosis: A Children's Oncology Group Study.

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Plenary session 6: Genetic drivers of resistance and relapse, May 18, 2023, 9:00 AM - 10:00 AM

Background: Relapsed neuroblastomas are enriched for targetable mutations in the ALK and RAS-MAPK pathways, but the frequency and clinical significance of clonal and subclonal aberrations in newly diagnosed high-risk neuroblastoma (HR-NBL) patients remained undefined.

Aims: To define the frequency, spectrum, and clinical significance of clonal (VAF≥20%) and subclonal pathogenic alterations in neuroblastoma-associated genes in tumors from patients enrolled on ANBL0532, a randomized Phase 3 trial for newly diagnosed HR-NBL patients that studied tandem vs. single autologous stem cell transplantation.

Methods: We developed a neuroblastoma-specific deep sequencing panel to assay selected domains of the ALK, NRAS, KRAS, HRAS, BRAF, PTPN11, TP53 and ATRX genes. We validated limit of detection to a VAF of 0.1%, and sequenced DNA from pre-therapy tumors. Event-free survival (EFS) was compared using the log-rank test.

Results: Of 242 patients: 87.6% (n=212) had INSS Stage 4 disease, 47.9% (n=102) were randomized to tandem transplant, and 63.2% (n=153) received immunotherapy. Tumors were MYCN amplified in 50.6%.

We detected ALK activating mutations in 21.9% of tumors (n=53, 30 clonal, 10 with VAF>5%-<20%, and 13 with VAF<5%), with 10 tumors harboring ≥2 activating ALK mutations concurrently; 3.3% (n=8) had ALK genomic amplification. Five-year EFS for patients with any ALK-aberrant tumor (n=60) was inferior relative to patients with ALK wild-type (WT; n=182) tumors (34.9% vs 50.6%; p=0.0193). Patients with tumors harboring activating ALK mutations with VAF of >5% (n=40) had a 5-year EFS of 33.2% compared to 50.7% for ALK WT patients (n=195; p=0.0586). 5-year EFS for patients with ALK amplified tumors was 25.0% (p=0.0858).

We identified pathogenic mutations in RAS pathway genes in 7.9% of tumors (n=19; clonal=4, subclonal= 15). Five-year EFS for patients with tumors harboring a clonal RAS pathway mutation was inferior to that of patients with tumors harboring subclonal mutations or no aberrations (0 vs 53.3% vs 46.8%, p=0.0457).

Summary/conclusion:
Ultra-deep sequencing of diagnostic HR-NBLs demonstrates that aberrations in ALK and RAS-MAPK pathway genes are more prevalent at diagnosis than previously recognized and confer inferior prognosis. Further analyses of the impact of RAS pathway clonality, TP53 and ATRX aberrations on survival are ongoing and will be reported.

PS6.4

Genomic landscape of a national neuroblastoma relapse cohort and its implications

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Plenary session 6: Genetic drivers of resistance and relapse, May 18, 2023, 9:00 AM - 10:00 AM

BACKGROUND
Neuroblastoma relapse treatment is one of the biggest challenges in pediatric oncology. While survival rates in low-risk tumors are high, high-risk tumors frequently relapse and prognosis of these relapsed tumors remains dismal. Which molecular mechanisms drive relapsed neuroblastoma and how treatment can be improved is still subject of intensive investigation. Detailed analysis of relapsed tumors is required to understand this extremely aggressive disease and help setting up new and fruitful clinical studies.

AIMS
Our goal was to give an unbiased description of a relatively large representative relapse cohort. This description should lead to a deeper understanding of this very specific patient collective. Results of this analysis should find their way into the planning of prospective clinical studies in neuroblastoma relapse cohorts and may support clinicians in their decision making.

METHODS
We describe here a disease specific view on the German neuroblastoma cohort of the INFORM registry. We investigated patients (n=124) for mutational status, telomere maintenance mechanisms (TERT rearrangement, MYCN, ‘long-telomere’) and combined those results with clinical data. Tumor samples were analysed by low-coverage WGS, WES, RNA sequencing and 850k DNA methylation. Paired primary and relapse comparisons were performed for n=20 patients.

RESULTS
Different therapy protocols for relapse patients were evaluated such as RIST or targeted therapy. We found a significant proportion of our patients to harbour mutations in targetable cell signalling pathways. For example, 28 of 124 (22,5%) patients showed ALK pathway activation, either by gain-of-function (GOF) mutation or gene amplification, 38 patients (30,6%) harboured potentially activating mutations in the RAS/MAPK pathway with NF1 being the most frequently affected gene. ALK and RAS/MAPK were mutually exclusive, except for 1 initially ALK-mutated tumour that relapsed with a de novo NRAS mutation. P53

pathway alterations, especially CDKN2A deletions, were also among the mutations regularly found in this cohort.

CONCLUSION
We molecularly described, to our knowledge, the largest neuroblastoma relapse cohort, so far, and found that targetable cell signalling pathways are frequently mutated in patients with relapse. This implies that molecularly targeted therapy might be a feasible therapy option in neuroblastoma and that further studies into this are required.

PS7.1

Liquid biopsies for monitoring tumor evolution and pharmacodynamic assessment of vorinostat effects during neuroblastoma therapy.

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Plenary session 7: Novel diagnostics: liquid biopsies, May 18, 2023, 1:50 PM - 2:50 PM

Background: Neuroblastoma (NB) is the most frequent tumor in infants and prognosis for patients with high-risk disease remains poor. NANT-2011-01 was a phase II trial testing the effects of a chromatin remodeling agent, vorinostat, added to 131I-MIBG for relapsed NB. In our study, we hypothesize that changes in chromatin accessibility and gene expression can be detected in cell-free DNA (cfDNA) and circulating tumor cells (CTCs) and may predict the impact of adding vorinostat to MIBG therapy.
Methods: We performed WGS on cfDNA samples isolated from patients in the NANT study. We used the novel Griffin algorithm to measure transcription factor activity in cfDNA, based on DNA fragmentation patterns and detection of nucleosome positioning. To identify differences in chromatin accessibility sites and gene expression changes in response to vorinostat therapy, we performed ATAC-Seq and RNA-seq bulk profiling on NB cell lines treated with vorinostat for five days, which reflect the schedule of blood samples collected from patients enrolled in the NANT-2011-01 trial. We will validate in vivo the vorinostat transcriptomic signature in CTCs from metastastic sites after injection of NB cells via tail vein in immunocompromised mice.
Results: Fourty-four percent (37/84) of all blood samples at baseline yielded detectable tumor DNA in the cfDNA and levels correlated with disease burden. In these pre-treatment cfDNA samples, we demonstrated an enrichment of chromatin accessibility at NB-specific transcription factor binding sites including PHOX2B, compared to cfDNA profiled from healthy donors. In vorinostat-treated cells, we found that changes in chromatin accessibility occurred at the transcriptional start sites of genes previously associated with vorinostat treatment. Multi-omics data integration revealed a correlation between the differentially expressed genes and the differential chromatin accessibility sites. We also demonstrated that we could successfully isolate CTCs from mouse blood and bone marrow and measure gene expression through scRNA-Seq, validating our mouse model.
Conclusions: We showed that nucleosome profiling of cfDNA enables prediction of gene expression and transcription factor activity. We also identified chromatin signatures that characterize NB transcriptional programs in NB cells. This approach will be applied to liquid biopsies from NB patients enrolled on an upcoming phase II NANT-2021-02 trial.

PS7.2

Quantitative and sensitive neuroblastoma minimal residual disease detection using extrachromosomal DNA (ecDNA) MYCN breakpoints

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Plenary session 7: Novel diagnostics: liquid biopsies, May 18, 2023, 1:50 PM - 2:50 PM

Background: More than half of the patients with high-risk neuroblastoma relapse, implying the persistence of therapy-resistant cells as minimal residual disease (MRD). Extrachromosomal circular oncogene amplification is of prognostic relevance in many tumors including neuroblastomas. MYCN amplification is a determinant of high risk and its gene copies are commonly found on highly rearranged extrachromosomal DNA (ecDNA), harboring patient-specific breakpoints.
Aims: PCR-based assays to detect MRD levels are already incorporated in clinical routine for patients with leukemia. We hypothesized that individual ecDNA MRD assays for patients with neuroblastoma are of high prognostic relevance and could aid treatment adaptations.
Methods: Here, we employed a neuroblastoma targeted sequencing panel providing high-resolution of the ecDNA MYCN sequence from FFPE and snap-frozen tumor. For ecDNA structural reconstruction, Illumina short-read Circle-seq and long-read Nanopore sequencing was used. EcDNA MYCN breakpoint sequences served as template for patient-specific RQ-PCR and droplet digital PCR, enabling longitudinal assessment of MRD in bone marrow and liquid biopsy samples from patients with high-risk neuroblastoma.
Results: For workflow establishment, 21 ecDNA MYCN breakpoints identified by targeted sequencing in 13 MYCN-amplified cell lines were orthogonally validated using PCR following Sanger sequencing. To estimate the MRD sensitivity limits, 9 breakpoint PCR amplicons were detected by RQ-PCR and ddPCR in single cells among up to 10^6 reference cells. In primary tumor samples of 22 patients, we found 47 ecDNA MYCN breakpoints. MRD detection for one target per patient revealed a sensitivity of a single tumor cell in 10^3 to 10^6 normal cells. Importantly, ecDNA MYCN breakpoints identified in primary neuroblastomas remained stable in relapsed neuroblastomas. Our strategy for personalized MRD monitoring enabled us to track tumor cells in bone marrow aspirates at diagnosis and during therapy. As proof-of-concept, we detected ecDNA MYCN breakpoints within liquid biopsies from 5 patients, showing target specificity and distinct positive droplets.
Conclusion: We show that highly sensitive PCR-based detection of patient-specific ecDNA MYCN breakpoints enables MRD quantification of neuroblastomas. Evaluating MRD levels in tissue or liquid biopsies could

expose dynamic changes associated with response to therapy and might allow re-assessment of risk and adjustment of therapy intensity in a personalized approach.

PS7.3

Application of liquid biopsy approaches in neuroblastoma: Whole-exome sequencing and targeted approaches for diagnostic, prognostic and minimal residual disease detection

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Plenary session 7: Novel diagnostics: liquid biopsies, May 18, 2023, 1:50 PM - 2:50 PM

Background and aims: Liquid biopsies allow detailed analysis of the genomic tumor profile in body fluids, by investigating cell-free tumor DNA (ctDNA), circulating tumor cells and tumor-derived mRNA. We previously showed that sensitive minimal residual disease (MRD) monitoring by combining real-time quantitative PCR (qPCR) mRNA detection and droplet digital PCR (ddPCR) cfDNA detection, is associated with outcome in neuroblastoma. We here investigated the potential application of Whole-Exome Sequencing (WES) to detect CNV and SNVs in liquid biopsies in comparison with qPCR and ddPCR.
Methods: Peripheral blood (PB) and Bone Marrow (BM) samples of 20 high-risk neuroblastoma patients at diagnosis and two at end-of-induction were studied. cfDNA from PB (n=17) and BM (n=12) was analyzed using both WES and methylated RASSF1A (RASFFA-M) ddPCR. PB- and BM-samples were also studied by qPCR using an adrenergic (ADRN) NB-mRNA panel (PHOX2B, TH, DDC, CHRNA3 and GAP43 and DBH for BM and PB), and a mesenchymal (MES) NB-mRNA panel (FMO3, PRRX1 and POSTN).
Results: cfDNA sequencing results mimic the matched tumor DNA for almost all recurrent aberrations in neuroblastoma (e.g. MYCN and ALK amplification, 1p loss, 11q loss and 17q gain). However, in 4/17 samples additional copy number aberrations, e.g. MDM4 and CCND1 were detected in the plasma. Moreover, cfDNA sequencing data of paired PB and BM plasma (n=12) were concordant, although MYCN amplification was higher in the BM-plasma than the matched PB-plasma. The ADR mRNA-panel was detected in 24/29 paired samples. 2/29 samples were positive for the MES mRNA-panel. Both MES-positive samples had a high calculated infiltration according to the ADRN qPCR-panel. Lastly, in the tested samples, the level of RASSF1A-M detected by ddPCR mimicked the aggressiveness of the tumor as analyzed by WES.
Conclusions: This proof-of-principle study demonstrates the feasibility to study liquid biopsy approaches at diagnosis and for MRD testing, in paired samples, by WES, ddPCR and mRNA qPCR. To establish the optimal liquid biopsy panel, this is an important outcome in the ongoing SIOPEN HR-NBL2 study. This will also help to determine the exact clonal and (sub)clonal compositions of the neuroblastoma tumor and to develop both tumor and patient-specific evolutionary trajectories.

PS7.4

Integrating correlative biology into a phase III clinical trial for high-risk neuroblastoma to study mechanisms of tumor evolution.

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Plenary session 7: Novel diagnostics: liquid biopsies, May 18, 2023, 1:50 PM - 2:50 PM

Background:
Outcomes for high-risk neuroblastoma (HR-NB) have improved but causes of treatment-refractory disease progression are poorly understood. ANBL1531 is a Children's Oncology Group (COG) phase 3 study that will enroll approximately 700 patients with HR-NB. Longitudinal tissue samples are being collected on this study to identify prognostic biomarkers and study patterns of tumor heterogeneity and evolution that contribute to treatment resistance. We describe sequencing approaches and initial progress in this ongoing work.
Methods:
Tumor and germline samples are submitted to the Mardis lab for whole-exome sequencing (WES) and the Reynolds lab to define telomere maintenance and establish PDXs. Serial plasma samples are submitted to the Crompton lab for cell-free DNA sequencing by ultra-low passage whole genome sequencing (ULP-WGS) and WES to estimate circulating tumor DNA (ctDNA) content and identify mutations. Aliquots of cell-free DNA are sent to the Applebaum lab for detection of genome-wide 5-hmC DNA modifications associated with prognostic subtypes of neuroblastoma and to the Pugh lab to quantify immune cell repertoire. Thus far, the Crompton lab has received 1,850 serial plasma samples and distributed aliquots to the Applebaum lab. We report the initial findings of ctDNA sequencing from 380 patients.
Results:
ULP-WGS showed that 274/277 patients (98.9%) with a pre-treatment sample had detectable ctDNA ranging between 0-97% of total cell-free DNA. ctDNA remained detectable in 86%, 36%, 26%, 3.7%, 3.9% and 0% of cases at pre-induction cycle 2, 3, 4, end-induction, start of post-consolidation, and end post-consolidation respectively. Samples analyzed at disease progression were positive in 58% of cases, with ctDNA levels 0-89% of total cell-free DNA. Deep sequencing of baseline ctDNA samples demonstrated mutational heterogeneity compared to tumor biopsies, including subclonal variants in ALK (3 of 28 cases) and TP53 (2 of 28 cases). Deep sequencing of samples obtained during treatment and at progression is ongoing.
Conclusions:
This interim report demonstrates robust patient sample collection on a COG phase 3 trial, enabling an unprecedented number of interactive laboratory correlative studies that will maximize the potential for novel discoveries. Initial sequencing demonstrates changes in serial ctDNA levels and evidence of mutational heterogeneity otherwise undetected in tumor biopsies.