ition to cycling populations and those associated with lineage-specificity, we identified 'aggressive' subpopulations defined by significant upregulation of immediate early response genes such as FOS/FOSL1/JUN, those associated with promotion of invasion-migration such as SERPINs and MMPs. These subpopulations could be mapped to isolated single-cell-derived subclones with highly proliferative or motile phenotypes, defined by comprehensive profiling of expressed and secreted proteins. Differential cis-regulation driving cell identity-tumorigenesis was found in one example to occur via a trans-histone mechanism mediated by an H4-lysine-methyltransferase, KMT5B. Application of functionally-defined interventional strategies aimed at disrupting the interactions between these subpopulations based upon evolutionary biology principles may offer a novel approach to treat these otherwise incurable tumours in children and young adults.

DIPG-42. DIFFUSE MIDLINE GLIOMAS, H3K27-ALTERED AS AN INTERDISCIPLINARY CHALLENGE

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INTRODUCTION: Diffuse midline gliomas represent a particular challenge in treatment. DIPG behave like highly malignant glioma with extremely poor prognosis due to location and inoperability. Molecular genetic studies, complementary to classical histopathology, have found an entity for midline gliomas, newly described ten years ago and entered the WHO classification in 2016. This group of childhood tumor with DMG's, H3K27-altered will be demonstrated in the following with 14 case reports from our clinic. METHODS: Clinical data of four patients with tectum/thalamic gliomas, six with diffuse intrinsic brainstem glioma, two with cerebellar, one with suprasellar, one with spinal glioma were retrospectively studied. MRI data, volume increase, contrast behavior was also analyzed. Tumor tissue was obtained by various surgical procedures and diagnostic workup included histopathology as well as genetics and epigenetics. RESULTS: 14 pediatric patients were treated from 2012 to 2021, median age 7,5 years. Leading symptoms were hydrocephalus, movement disorders, cranial nerve disorders. Four patients (29%) were partially resected, two (14%) received extended biopsy, seven (50%) were (stereo tactically) biopsied, one diagnosed by liquid biopsy (7%). Histological results revealed the presence of GBM in four cases (29 %). Subsequent methylome analyses confirmed that the tumors belonged to the group of diffuse midline gliomas, H3K27-altered. The other ten tumors (71%) were primarily assigned to this H3K27 group. CONCLUSION: The pediatric tumors of the brainstem, the further midline structures, including intraspinal manifestation show different MRI findings, histology, and clinical course. Complementary molecular genetic diagnosis is essential and a meaningful addition to the histological assignment. It is considered proven that the exclusivity of H3K27 – altered tumors of children and adolescents differs from that of IDH mutated gliomas and glioblastomas by their localization of hemispheric processes. Possible therapeutic approaches using targeted therapy require understanding of these oncological mechanisms.

DIPG-43. GLUCOSYLCERAMIDE SYNTHASE INHIBITORS INDUCE CERAMIDE ACCUMULATION AND SENSITISE H3K27 MUTANT DIFFUSE MIDLINE GLIOMA TO IRRADIATION

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BACKGROUND AND AIMS: Glycosphingolipids (GSL) are amphipathic lipids particularly abundant in the brain where their amount and expression patterns change drastically during the embryonic to postnatal stages and during tumorigenesis. The biosynthesis of GSL begins with the formation of glucosylceramide from ceramide, a step catalysed by the glucosylceramide synthase (UGCG). UGCG can be inhibited by eliglustat, which is used for treating children with Gaucher's disease. We have previously shown that the GSL composition is deregulated in H3K27M mutant diffuse midline glioma (H3K27M mut) and that eliglustat inhibits cell proliferation. Here we analysed the mechanism of action of eliglustat in H3K27M mut and its effect on irradiation. METHODS: The concentration of different components of the sphingolipid metabolism (ceramide, ceramide-1-Phosphate (CIP), sphingomyelin, Sphingosine and Sphingosine-1-Phosphate (S1P)) was assessed by mass spectrometry in the H3K27M mut cell line SF8628, before and after treatment with eliglustat. The combination of eliglustat with ionizing radiation was analysed by clonogenic assay. RESULTS: The treatment of H3K27M mut cells with eliglustat resulted in a significant increase in the concentration of ceramide, Sphingosine, C1P, but not S1P. The increase was concentration and time dependent and was not observed after longer incubation. Eliglustat treatment reduced the colony formation ability after irradiation. CONCLUSIONS: Ceramide is a known mediator of apoptosis involved in the molecular mechanisms underlying cellular response to irradiation. Increased endogenous ceramide levels, induced by blocking the synthesis of GSL, may sensitize H3K27M mut cells to irradiation. However, ceramide can be converted in C1P, a potent inhibitor of apoptosis and inducer of cell survival. Thus, the time and concentration dependent shift to ceramide and C1P requires further investigation in order to achieve an appropriate balance between the levels of these two metabolites and identify the optimal therapeutic window for combination with irradiation and potentially chemotherapy

DIPG-44. H3K27-ALTERED DIFFUSE MIDLINE GLIOMAS WITH SECONDARY DRIVER MOLECULAR ALTERATIONS

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INTRODUCTION: Large-scale sequencing led to the identification of driver molecular alterations such as FGFR1 and BRAF in occasional diffuse midline gliomas (DMGs) H3K27-altered, but their significance is not completely explored. We evaluated these associations in our institutional cohorts. MATERIALS AND METHODS: We searched our sequencing data base (2013-2020) for H3K27M-mutant gliomas and analyzed the co-occurring genetic alterations. The demographics, clinical information, and pathology were reviewed. Copy number profiles were evaluated using BioDiscovery's Nexus Copy Number software package. Oncoplots and Kaplan-Meier survival curves were generated with the maftools R package. RESULTS: We identified 77 patients (age range 2-68, median 26). The diagnosis was DMG (n=55), anaplastic astrocytoma/glioblastoma (n=19), low-grade glioma (n=1), low-grade glioneuronal tumor (n=1), and ganglioglioma (n=1). Recurrent alterations were seen in TP53 (n=42), ATRX (n=17), NF1 (n=15), PDGFRA (n = 4). Five cases had BRAF V600E (1 ganglioglioma; 4 DMG); twelve had FGFR1 mutations (9 DMG; 3 anaplastic astrocytoma/glioblastoma). The most common location in the BRAF group was the brainstem and in the FGFR1 was the thalamus. Survival ranged from 0 to 97 months, median 12.9 months (28.8 months for FGFR1 and 22.8 for the BRAF V600E). This was not significantly different from OS reported in the literature for DMG.The BRAF V600E ganglioglioma patient is alive 37 months after diagnosis. CONCLUSION: There was no significant difference in outcomes for patients with secondary molecular drivers when compared with conventional H3K27M DMG. The outcome of the BRAF V600E tumors seemed to correlate with the histology. These findings and the possibility of targeted therapy argue for comprehensive sequencing of H3K27-altered gliomas.

DIPG-45. RADIATION INDUCES A ROBUST INTERFERON RESPONSE IN DIFFUSE MIDLINE GLIOMA (DMG), IMPROVING THE POTENTIAL FOR COMBINATION IMMUNOTHERAPY

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Diffuse Midline Glioma (DMG), H3K27M altered, confers a dismal survival of 9-15 months and has a non-inflammatory tumor immune microenvironment (TIME). Radiation therapy (RT) is the mainstay treatment for DMG and has been shown in other cancers to recruit an immune component. However, the effect of RT on the DMG TIME has not been explored. In a syngeneic murine model of pontine DMG (PDGFB+, H3.3K27M, p53–/–), mice were treated with single fraction 15Gy RT or sham control, four mice per group. We performed single cell sequencing after CD45 isolation to evaluate the TIME 4 days post RT and compare to untreated tumor (sham control). Unsupervised clustering of 14,848 CD45+ cells revealed 16 immune cell subsets, most abundantly microglia at 75% of cells, with four subtypes representing a spectrum of homeostatic to activated. Microglia from RT are more concentrated in the activated subtypes with an upregulation of interferon response (i.e. Isg15, Ifit3) compared to untreated tumor with an increase in several interferon pathways using REACTOME. Consistent with

RT response, RT treated tumors have increase in cell cycle regulatory genes such as Cdkn1a, across all clusters. In non-resident myeloid cells, compared to untreated tumor, RT is associated with a robust upregulation of interferon response genes in both macrophages (Isg15 Fold Change (FC) 2.30; Ifit1 FC 1.64; Ifit3 FC 2.02; Cxcl10 FC 2.29) and dendritic cells (Isg15 FC 2.67; Ifit1 FC 1.72; Ifit3 FC 2.06; Cxcl10 FC 1.50). We also find differential expression of immune checkpoints in RT-treated versus untreated tumor with decreased expression of Lag3, Tim3 (Havcr2), and Csf1R and increased expression of Cd47, Sirpa and Gitr (Tnfrsf18) post RT. In summary, RT stimulates a pro-inflammatory TIME response and alters immune checkpoints in DMG, highlighting the potential for combining RT and immunotherapy in these tumors.

DIPG-46. RADIATION INDUCED SENESCENCE IN DIFFUSE INTRINSIC PONTINE GLIOMA CELLS REVEALS SELECTIVE VULNERABILITY TO BCL-XL INHIBITION

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Diffuse intrinsic pontine glioma remains a devastating condition with a dismal five year survival rate less than 5%. New approaches for treating this aggressive disease are critical to driving progress. Conventional radiotherapy remains the cornerstone of treatment, with no chemotherapeutic agent found to improve survival. However, radiotherapy is often delivered as a palliative treatment, and disease often recurs 3-6 months after. Radiation causes DNA damage and oxidative stress yielding a senescent state of replicative arrest in susceptible cells. However, increasing evidence demonstrates malignant cells can escape senescence leading to tumour recurrence. Targeted ablation of non-replicating senescent tumour cells following radiation could negate tumour recurrence. It remains unknown whether DIPG undergoes senescence following radiation, and furthermore, whether senolytics can be utilised to target senescent DIPG cells. We employed radiation to induce a senescent state in primary human DIPG cell lines. Senescence was confirmed using SA-β-gal staining, lack of EdU incorporation and qRT-PCR to characterise the SASP in three primary human DIPG cell lines. RNA-sequencing on DIPG cells following radiation revealed senescence and SASP signatures. Likewise, expression of senescence markers has been detected in human tumours. Viable cells that survive radiation were then utilised to screen candidate senolytic drugs, only Bcl-XL inhibitors demonstrated reproducible senolytic activity in radiation treated DIPG cells. In addition, Bcl-XL degradation using PROTACs (proteolysis targeting chimeras) resulted in a significant increase in senolysis of susceptible tumour cells. Conversely, Bcl-2 inhibitors failed to show any consistent senolytic activity. We are currently performing preclinical studies in the mouse to test the efficiency of senolytics against DIPG. These results demonstrate future possibilities of targeting radiation induced senescence in DIPG, using novel senolytic therapies and highlight Bcl-XL dependency as a potential vulnerability of surviving DIPG cells following exposure to radiation.

DIPG-47. TSO500CTDNA SEQUENCING REVEALS ONCOGENIC MUTATIONS AND COPY NUMBER VARIATIONS IN THE LIQUID BIOME OF CHILDREN WITH DIFFUSE MIDLINE GLIOMA

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BACKGROUND: Molecular profiling of childhood CNS tumors is critical for diagnosis and clinical management, yet tissue access is restricted due to sensitive neuroanatomical locations. Moreover, CNS tumors including diffuse midline glioma (DMG) exhibit mutational heterogeneity and clonal evolution, which cannot be captured by upfront diagnostic biopsy alone. To address the lack of tumor visibility, and tprovide opportunity for longitudinal sampling, we validated and optimized a commercially available deep sequencing platform for analysis of circulating tumor DNA (TSO500ctDNA™). METHODS: In a proof-of-concept study, we defined the sensitivity, specificity, and clinical relevance of our novel ctDNA platform via analysis of paired tissue, CSF, and blood from children with DMG (n=10). Paired samples were assessed for concordance and sequencing results were compared to digital droplet PCR (ddPCR) detection of prognostic H3K27M mutation. RESULTS: DMG associated mutations in genes including *H3-3A, H3C2, TP53*, and *ACVR1* were detected in ctDNA, including in CSF samples with low (<5ng) starting DNA input. Of 9 H3K27M mutations identified in tumor, 8 were present in CSF and 3 in plasma/serum, for a positive percent agreement with tumor results of 89% and 33%, respectively. Among CSF samples, H3.3K27M was detected in 6/6 cases, and H3.1K27M in 2/3 cases, with variant allele frequencies comparable to ddPCR results. CNVs including *PDGFRA, KIT*, and *MDM4* gains were detected in CSF and paired tumor. Low frequency events including ACVR1, PIK3CA activating mutations and *KRAS* amplification were detected in CSF but absent from paired tumor, indicating tissue heterogeneity. Strategies to optimize ctDNA detection, including optimization of ctDNA isolation and adjustment of library QC metrics, were identified. **CONCLUSION**: Targeted ctDNA deep sequencing is feasible, can inform on clinically relevant tumor mutation and CNV profiling, and provides an opportunity for longitudinal monitoring of tumor genomic evolution in the liquid biome of children with CNS tumors.

DIPG-48. MRI VOLUMETRIC AND MACHINE LEARNING BASED ANALYSES PREDICT SURVIVAL OUTCOME IN PEDIATRIC DIFFUSE MIDLINE GLIOMA

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INTRODUCTION: Diffuse midline glioma (DMG) is a fatal childhood CNS tumor. Magnetic resonance imaging (MRI) is the gold standard for DMG diagnosis and monitoring of response to therapy. Leveraging novel MRI analytical approaches, including volumetric and machine learning based analyses, may aid in the prediction of patient overall survival (OS) and help to identify high-risk cases. METHODS: T1- and T2-weighted MR images were retrospectively collected from children and young adults diagnosed with DMG (n=43). MRI features, including manually determined 3D tumor volume (T2), T1 contrast-enhancing tumor volume, T1 relative to T2 volume (T1/T2), tumor relative to whole brain volume, tumor average intensity, and tumor heterogeneity (i.e., intensity skewness and kurtosis), were evaluated at upfront diagnosis. MRI features were analyzed to identify significant predictors of OS outcome, which was defined as OS shorter, or longer, than one year from diagnosis. A support vector machine was used to predict OS outcomes using combinations of these features. RESULTS: The presence of T1 contrast-enhancing tumor at diagnosis (p=0.01), and a high T1/T2 ratio (>25%, p=0.009), predicted significantly shorter median OS. Moreover, feature selection identified T2 mean intensity (p<0.001), T2 image intensity skew (p=0.006), T1/T2 ratio (p=0.02), and T1 volume relative to whole brain (p=0.03) as significant predictors of OS outcome (short versus long). Combining T2 mean intensity, T2 image skew, T1 segment kurtosis and patient gender resulted in OS outcome prediction accuracy of 83.3% (sensitivity=85%, specificity=81.8%, n=42 cases).CONCLUSION: We have identified MRI volume and imaging features that significantly predict OS outcome in children diagnosed with DMG. Our findings provide a framework for incorporating MRI volumetric and machine learning analyses into the clinical setting, allowing for the customization of treatment based on tumor risk characteristics.

DIPG-49. INTERNATIONAL PRECLINICAL DRUG DISCOVERY AND BIOMARKER PROGRAM INFORMING AN ADOPTIVE COMBINATORIAL TRIAL FOR DMG

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