EPEN-24. BIOLOGICAL MARKERS OF EPENDYMOMA IN CHILDREN AND ADOLESCENTS (BIOMECA): SYSTEMATIC COMPARISON OF METHODS FOR THE PRECISE EVALUATION OF BIOMARKERS FOR EPENDYMOMA DIAGNOSIS AND PROGNOSTICATION

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The identification and validation of prognostic and diagnostic biomarkers is a key element of The SIOP Ependymoma II trial, realised through the Biomarkers of Ependymoma in Children and Adolescents study (BIOMECA). BIOMECA aims to identify and validate biomarkers for prediction of outcome whilst enhancing stratification for the next generation of ependymoma trials. We outline our findings from the first 147 consecutive BIOMECA cases (posterior fossa, PF=111; supratentorial, ST=32; spinal, SP=4). We compared various methods for biomarker assessment, across six European laboratories to determine key analysis methods. Methods included: methylation-based classification (EPIC 850K DNA methylation array) (n=141); immunohistochemistry (IHC) for nuclear p65-RELA (n=32), H3K27me3 (n=115), and Tenascin-C (TNC) (n=147); copy number (CN) analysis by FISH, MLPA (1q, *CDKN2A*) (n=147), and MIP (molecular inversion probe) and DNA methylation array (1q, *CDKN2A*, 6q, 11q, 13q, 22q) (n=141); analysis of *ZFTA*- and YAP1-fusions by RT-PCR, sequencing, Nanostring assays and break-apart FISH (n=32). Using DNA methylation-based classification, 91% (n=101/111) of PF cases classified as PF ependymoma group A (PFA) and 69% (n=22/32) of ST cases as ST ependymoma, ZFTA fusion-positive (ZFTA). Most PFAs demonstrated inter-centre agreement for loss of H3K27me3, and were TNC positive, representing surrogate markers for PFA identification. Combinations of p65-RELA IHC, FISH analysis, and RNA-based methods were suitable to identify *ZFTA*- and *YAP1*- fused ST ependymomas. Predictive CN alterations were identified by high-resolution, quantitative MIP technology.The integration of histopathology assessment and molecular typing is now critical as the updated 2021 WHO CNS5 classification of ependymomas lists seven molecularly distinct entities. This study highlights the importance of evaluating different methods in a prospective trial cohort. Here, advanced molecular techniques represent powerful tools for the classification of ependymoma entities (DNA methylation array) and for the detection of CN alterations (MIP) and specific fusions, enabling the correct classification and identification of prognostic markers.

EPEN-25. A NOVEL SPONTANEOUS MODEL OF ZFTA-RELA FUSION EPENDYMOMA

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Ependymomas driven by the ZFTA-RELA fusion account for >70% of all supratentorial ependymomas. These tumours are now recognised in the WHO classification of CNS tumours and have been associated with a poor prognosis. Seven ZFTA-RELA fusion variants have been described: around two thirds of cases are fusion 1. No spontaneous genetically modified mouse models (GEMMS) have been described and current models require invasive intracranial injection (of transduced cells or RCAS-TVA system). Here we describe the first spontaneous GEMM of ZFTA-RELA fusion-driven ependymoma. Nestin-Flx-STOP-Flx-ZFTA-RELA (Fusion 1) or E1alpha-Flx-STOP-Flx-ZFTA-RELA open reading frames were targeted together with luciferase to the Rosa-26 locus. Breeding these mice with Nestin-CreERT2 or Blbp-Cre lines that drive recombination in neural progenitor cells resulted in forebrain tumours that could be tracked with bioluminescence imaging from P20. Tumours displayed NF-kB and L1CAM expression and ZFTA-RELA protein was detected using a novel in-house antibody. Tumours display expression of a known ZFTA-RELA fusion ependymoma transcriptomic signature. ZFTA-RELA tumours can be grown as neurospheres and passaged as allografts in nude mice. We provide the first spontaneous GEMM of this important group of ependymomas. We are now characterising these tumours histologically and transcriptomically relative to the human disease and using these to understand the lineage origins of ependymoma and plan use of conventional and novel treatments.

EPEN-26. CHEMOKINE RECEPTOR BLOCKADE REVERSES CCL2 MEDIATED IMMUNOSUPPRESSION AND RESTORES CAR-T CELL FUNCTION IN POSTERIOR FOSSA EPENDYMOMA

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Trastuzumab-based HER2 CAR-T constructs have demonstrated preclinical efficacy in medulloblastoma and are being evaluated for use in children and young adults with recurrent or refractory CNS tumors. Preliminary results demonstrate immune activation but no objective tumor response in three patients, including two patients with posterior fossa (PF)-EPN. A key finding in the serum and CSF of all three patients was very high levels of the inflammatory chemokine CCL2 following treatment with CAR-T cells. Preclinical studies suggest that high levels of CCL2 may impede T cell mediated anti-tumor activity in CNS tumors. The role of CCL2 to enhance or diminish CAR-T cell efficacy for CNS tumors is unknown. We evaluated a second generation trastuzumab-based HER2 CAR construct with a 4-1BB co-stimulatory domain in two ultra-high-risk patient-derived xenograft (PDX) models that faithfully recapitulate PFA-EPN. In contrast to preclinical studies in other cancers, treatment with trastuzumab-based HER2 CAR-T cell alone causes only partial regression of tumors and robust infiltration of immunosuppressive monocytes in PFA-EPN PDX mouse models. We studied constitutive NF-kB activation because it is a hallmark of PFA-EPN that drives dysregulation of inflammatory genes and forms an immunosuppressive tumor microenvironment. Upon tumor recognition, CAR-T cells produce high amounts of the cytokine tumor necrosis factor-alpha, which is an extracellular stimulus that propagates NF-kB activation in PFA-EPN. We show that HER2 CAR-T cell treatment causes increased nuclear translocation of the RELA NF-kB subunit, which induces CCL2 gene transcription and chemokine release. This results in CCL2-CCR2 ligand/receptor mediated influx of inflammatory monocytes and regulatory T cells, impairing CAR-T cell effector function. Inhibition of CCR2 restores anti-tumor CAR-T cytotoxicity against bulky orthotopic tumors by decreasing the infiltration of inflammatory monocytes and regulatory T cells. Combinatorial strategies addressing tumor mediated immunosuppression should be evaluated in upcoming CAR-T cell trials for patients with high-risk CNS tumors.

EPEN-27. EPIGENETIC DISSECTION OF SPINAL EPENDYMOMAS (SP-EPN) SEPARATES TUMORS WITH AND WITHOUT *NF2* MUTATION

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Ependymomas encompass multiple, clinically relevant tumor types based on localization, genetic alterations, and epigenetic and transcriptomic profiles. Tumors belonging to the methylation class of spinal ependymoma (SP-EPN) represent the most common intramedullary neoplasms in children and adults. However, molecular data of SP-EPN are scarce, and clear treatment recommendations are lacking. The only known recurrent genetic events in SP-EPN are loss of chromosome 22q and *NF2* mutations. Yet, it remains unclear whether SP-EPN with germline or sporadic *NF2* mutations or with *NF2* wild type status differ clinically or molecularly. To provide a comprehensive molecular profile of SP-EPN, we integrated epigenetic, genomic, transcriptomic, and histological analyses of up to 237 cases. Clustering of methylation data revealed two distinct molecular SP-EPN subtypes. The distribution of *NF2* mutated cases differed significantly across these subtypes ($p < 0.0001$): The vast majority of tumors harboring either a previously known *NF2* germline mutation or a sporadic mutation were assigned to subtypes A, whereas subtype B tumors mainly contained *NF2* wild type sequences. In addition, subtype A tumors showed a lower frequency of MGMT promoter methylation ($p = 0.018$) and contained almost all pediatric patients of the cohort. Whole-exome sequencing (30 cases) identified numerous mutations in *NF2* wild type and mutated tumors. Mutated genes in *NF2* wild type tumors were enriched for genes associated with cell cycle and cytoskeleton. RNA sequencing revealed two distinct transcriptional groups with upregulation of proliferative genes in one group and upregulation of cilial genes in the other group. The molecular subtypes displayed subtle, but significant differences in the appearance of histopathological characteristics, such as surfaces, inflammation, and hyalinized vessels. Investigation of clinical parameters is ongoing and will complete the picture of SP-EPN heterogeneity as an important basis for future clinical decision-making.

EPEN-28. ONCOGENIC DEPENDENCY OF PEDIATRIC EPENDYMOMAS ON EXTRACELLULAR VESICLE PATHWAYS

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INTRODUCTION: The majority of pediatric ependymoma (EPN) comprise either supratentorial EPN characterized by ZFTA-fusions (ST-EPN-ZFTA) or posterior fossa group A EPN (PF-EPN-A), for both of which only limited therapeutic options are available. Because pediatric EPNs have a relatively low mutational burden, identification and characterization of tumor-associated pathways and molecular processes are of critical importance to reveal potential therapeutic targets. Data from previous transcriptional studies and a cross-species in vivo screen implied aberrant vesicular pathways in ST-EPN-ZFTA, prompting further investigation of their putative role in EPN pathogenesis. METHODS: We investigated EPN group-specific differences in extracellular vesicle (EV) biogenesis pathways in human EPN transcriptome and proteome datasets. In addition, we characterized isolated EPN EVs by mass spectrometry. EPN-specific EV cargo was further investigated by immunofluorescence staining and western blotting. This enhanced understanding of EPN vesicular signaling allowed for a pre-selection of inhibitors targeting specific EV biogenesis pathways. *In vitro* proliferation and invasion assays as well as *in vivo* treatment studies were performed on EPN model systems. RESULTS: Integration of multi-omic data from both EPN tissues and EPN-EV-associated proteome led to the identification of ST-EPN-ZFTA-specific EV populations. We could spatially map specific EV markers to the perivascular niche that primarily harbors undifferentiated ST-EPN-ZFTA cell populations. Targeting EV biogenesis pathways by inhibiting factors of the lipid metabolism reduced the abundance of released EVs resulting in altered growth behavior and decreased invasion of tumor cells *in vitro*. I*n vivo* validation of EV release inhibitors in an orthotopic ST-EPN-ZFTA PDX model significantly reduced tumor growth and increased survival. OUTLOOK: In summary, we have leveraged ST-EPN-ZFTA-specific EV pathways as a potential therapeutic vulnerability. Further mechanistic investigations on EPN EV biogenesis, release, or uptake are expected to improve our understanding of the cross-talk between tumor cells and cells of the microenvironment and may lead to potential new therapeutic avenues.

EPEN-29. SPATIAL TRANSCRIPTOMIC ANALYSIS OF EPENDYMOMA IMPLICATES UNRESOLVED WOUND HEALING AS A DRIVER OF TUMOR PROGRESSION

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Ependymoma is a childhood brain tumor that remains incurable in approximately 50 percent of cases, most commonly in posterior fossa subgroup A (PFA). Uncovering how heterogeneous cell types within the tumor microenvironment (TME) interact is crucial to a complete understanding of PFA disease progression. The underlying cellular components of the PFA TME have been revealed by single-cell RNAseq (scRNAseq), identifying divergent epithelial differentiation and epithelial-mesenchymal transition (EMT) lineages. Here, we utilize spatial transcriptomics (Visium) of 14 PFA samples, integrated with scRNAseq, to chart neoplastic and immune cell architecture, with a higher resolution of cellular heterogeneity than scRNAseq alone. At a gross level, all PFA were primarily comprised of neoplastic epithelial and mesenchymal transcriptomic spatial zones, each containing a diversity of hierarchical cellular stages. In all samples we revealed spatially and transcriptomically-distinct mesenchymal zone-associated subclusters, including a quiescent undifferentiated progenitor-like subpopulation and clusters with characteristics of early and late stage EMT. Two early stage EMT clusters were distinguished by signatures of either myeloid cell interaction or hypoxia, and both were demonstrated to be EMT-initiating processes in in-vitro PFA experimental models. Myeloid cell interaction is the predominant initiating stage of EMT in PFA, occurring in zones that are spatially distinct from hypoxia induced EMT. Other mesenchymal clusters represent later EMT stages characterized by wound repair and tissue remodeling. Increased proliferation was a general characteristic of epithelial zone clusters, which included a second undifferentiated progenitor-like population that showed a particularly high mitotic rate and was associated with histologically hypercellular areas. Given the biological parallels with normal wound healing, we propose that mesenchymal and epithelial zones interact to create a cycle of persistent tissue damage response and mitogenic re-epithelialization signals. Unresolved wound repair is therefore a potential driver of PFA progression, a new concept that could provide novel targets for effective therapeutic intervention.

EPEN-30. 5FU WITH RADIATION FOLLOWED BY MAINTENANCE OF 5FU AND ATRA SIGNIFICANTLY IMPROVES SURVIVAL OF 1Q+/6Q- PFA EPENDYMOMA XENOGRAFT MODELS

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In a screen of over 100 FDA approved drugs on PFA 1q+ EPN cells, 5-fluorouracil (5FU) and All-Trans-Retinoic Acid (ATRA) were identified as