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Bench to bedside: Translating pre-clinical research into clinical trials for childhood brain tumors

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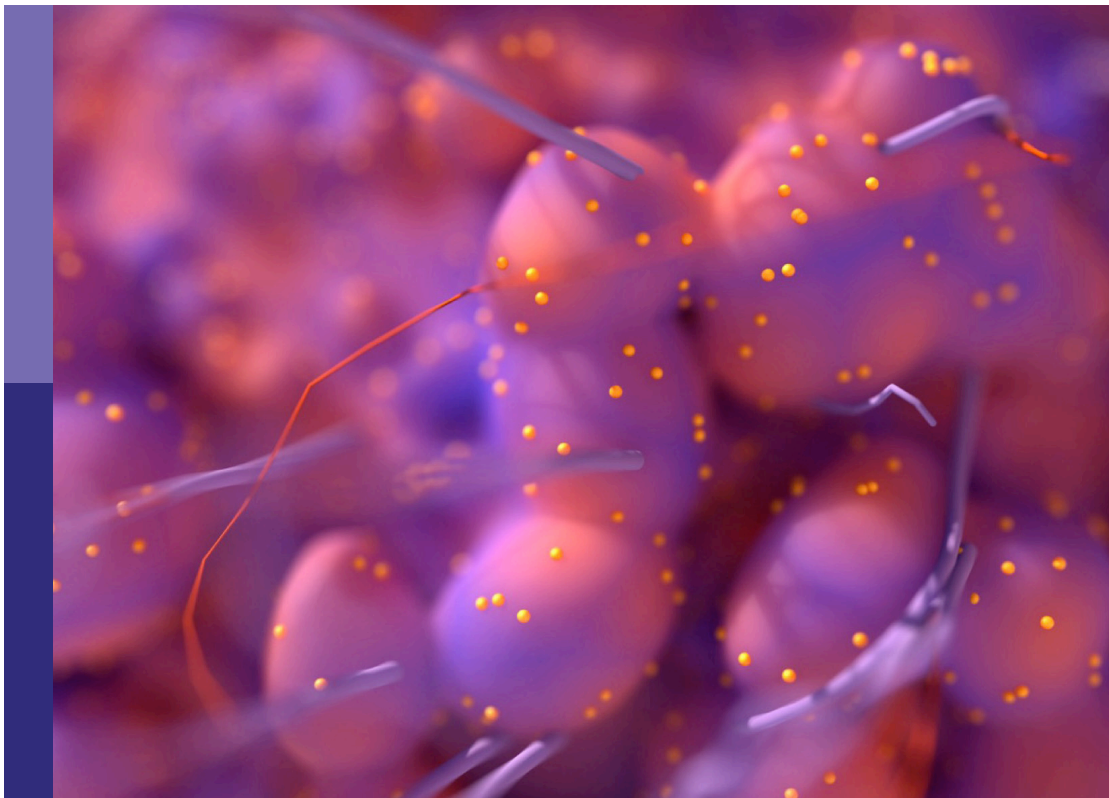

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Bench to bedside: Translating pre-clinical research into clinical trials for childhood brain tumors

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Editorial: Bench to bedside: translating pre-clinical research into clinical trials for childhood brain tumors

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Editorial on the Research Topic
Bench to bedside: translating pre-clinical research into clinical trials for childhood brain tumors

Tumors arising in the brain are the most common solid cancers in children (1). They are the major cause of childhood cancer deaths and worryingly, malignant brain tumor incidence rates have increased 0.5% to 0.7% annually among children and adolescents from 2008 to 2017 (1). Despite improvement in cure rates towards the end of the 20th century, survival statistics have remained unchanged over the past two decades and remain at a level well below that of other childhood cancers, such as leukemia (2), and this remains a major unmet clinical need. Also, survivors have a high risk of significant permanent adverse side effects that require a lifetime of clinical management, significantly impacting health systems and quality of life for patients (3).

The lack of advancements in childhood brain cancer treatment had previously been due to deficiencies in knowledge about the underlying biological causes. However, pediatric neuro-oncology has undergone an exciting and dramatic transformation during the past 20 years, driven by advances in genomic technology, international collaboration, and the generosity of families willing to share tissue samples for research. Next-generation sequencing and other molecular profiling techniques, have revealed that the underlying biology of many childhood brain cancer entities are varied and complex (4–7). Armed with this new knowledge, there is a tremendous opportunity to personalize brain cancer treatment by developing novel therapeutic approaches that are tailored to each molecular subtype of these devastating brain cancers to improve the cure rate while minimizing toxicities.

Most childhood brain tumors are still treated using three main therapeutic modalities: surgery, radiotherapy, and chemotherapy (8). However, there now exists a huge number of new cancer drugs that more precisely target the molecular abnormalities in cancer cells that

drive tumor growth or immunotherapies. We now have a major task ahead in identifying the best new drug or therapy for each type of brain cancer and ensuring that clinical trials are applying new treatments in patients with the most likely chance of benefit. Added to this is challenge now is that we have far more new drugs to assess through clinical trials than the number of patients available. We believe the answer to this conundrum is to increase the rigor of preclinical testing to identify only the most effective drugs and prioritize only those drugs with the best chance of success for clinical translation. It is important to note that to date, very few new drugs are yet to demonstrate improved clinical outcomes when translated to early phase trials. There are two major reasons for this. Firstly, there has been a failure to fully evaluate these new drugs in model systems that accurately reflect the disease, or specific molecular subtypes of central nervous system (CNS) cancers, prior to clinical trial. Secondly, not adequately assessing drugs for their ability to penetrate through the brain's natural protective barrier, the Blood-Brain Barrier (BBB). This means that the drugs fail to reach their target, the tumor.

This Research Topic aims to address these challenges and raises important considerations in preclinical childhood brain cancer research. Importantly, within this Research Topic, Walker provides important perspective, highlighting that a focus on survival as the primary outcome for clinical trials can often fail to recognize the acquisition of brain injury that occurs and persists for the remaining life of brain cancer survivors. From a societal perspective, the economic benefit of reducing cancer therapy-induced late effects is significant, yet preclinical studies often fail to assess or report on long term side effects. While seeking new clinical trials to improve survival, the long term impacts of therapy and quality of life for survivors must also remain a priority. There is an opportunity for the field to better assess the developmental impacts of experimental therapies using preclinical models, and such studies – potentially investigating neurostructural impacts of treatment or behavioral changes – should be valued and encouraged. An example of the balance between brain tumor therapy and quality of life is described by Walker et al. in the setting of optic pathway and hypothalamic glioma (OPHG). Guided by careful analysis of retrospective clinical data, they pose that multi-disciplinary factors should be taken into account when selecting patients for observation versus treatment. Especially given that up to half of OPHG cases are associated with the inherited cancer predisposition syndrome neurofibromatosis type 1 (NF1), treatment decisions should include oncologists, ophthalmologists, endocrinologists, neurodevelopmentalists and geneticists, among other specialties. Such a "total care" approach is broadly adopted in many world class pediatric oncology centers and is the gold standard internationally.

There are many more promising cancer therapies emerging than can be tested clinically for rare cancers like pediatric CNS tumors. As such preclinical modelling is an essential step that can focus the field on agents active in the brain and prevent the evaluation of ineffective or sub-optimal agents in children. For preclinical models to inform clinical response more accurately, it is important that the methodologies applied in the research setting better represent those of the clinic. Preclinical radiotherapy has been hampered in the past by the inability of small animal radiotherapy devices to precisely target tissues of interest, while sparing normal healthy tissue. Moreover, the linear quadratic model (9), which attempts to simplify preclinical experimentation by replacing fractionated dosing with a mathematically estimated, bioequivalent single dose of radiotherapy, is relied on heavily for ease of application. However, such differences in dosing schedule, especially when assessing combinations of small molecules with radiotherapy can potentially lead to misleading results. More recent preclinical radiotherapy equipment, such as SARRP (Xstrahl) and SmART+ (Precision X-ray Irradiation) platforms, enable collimated and more accurate delivery of radiotherapy to small animals, which reduces off-target exposure and facilitates fractionated dosing. Such systems are now considered the gold standard for preclinical radiotherapy research. In this Research Topic, Knox et al. report a fractionated radiotherapy protocol they designed and evaluated in a preclinical model of diffuse midline glioma (DMG). Studies such as these are essential to improve how we evaluate radiosensitizing strategies for childhood brain cancers.

In vivo preclinical testing is highly valued as it is better able to mimic drug distribution, metabolism, and excretion compared to *in vitro* testing. Different routes of administration and schedules can be evaluated, with pharmacokinetic studies and pharmacodynamic measures of drug action within tumor cells essential in the interpretation of any effect of a novel compound against CNS tumor cells in an orthotopic setting (or lack thereof). While the use of small animals remains fundamental in cancer research, there is an obligation to consider alternative methods that may reduce the number of animals used in experimentation, to support their welfare and enable researchers to use animal models only when strictly necessary. As mentioned above, the BBB limits the brain penetration of many anti-cancer drugs. However, it is well established that the integrity of the BBB can be compromised in tumors and is often more accurately referred to as the blood-brain-tumor-barrier (BBTB). Several recent studies, summarized in this Research Topic by Morris et al., report that the BBTB has different properties across pediatric brain cancer types. They also discuss innovative and complementary model systems that can be used as an alternative to small mammal research, such as zebrafish or intricate 3-dimentional co-culture models of vasculature and tumor cells. Given experimentation in mice is laborious, costly, and requires specialist research and veterinary skills, the application of diverse model systems in preclinical CNS cancer research may facilitate a more rapid and refined selection of which agents should progress to small mammal experimentation. Such an approach is consistent with the 3 Rs (Replacement, Reduction, and Refinement), and ensures the rational and respectful use of laboratory animals.

Translational research is often described as bench to bedside, however a more comprehensive perspective is to think of cancer research as a cycle from bedside to bench and back around. Here, Lazow et al. describe their investigation of a radionuclide therapy, ^{177}Lu-DOTATATE (Lutathera®), which binds somatostatin receptors and has demonstrated clinical efficacy in adult neuroendocrine tumors, in a pediatric CNS cohort. They show

that the targets of Lutathera, somatostatin type-2A receptors (SST2A), are highly expressed in certain high-risk pediatric CNS tumors and meningiomas. This agent is currently being investigated in a phase I/II trial (NCT05278208), and there is now a preclinical opportunity to begin assessing Lutathera in combination with current standard of care therapies with the goal of providing additional information to guide future clinical trial design. Their observation that SST2A has highest expression in non-SHH medulloblastomas, enables this preclinical assessment to be undertaken in the most appropriate models.

As an example of the value of preclinically assessing novel therapies in combination with standard of care therapies, Sengupta et al. describe the assessment of a telomerase inhibitor in the context of high-risk, *MYC*-amplified Group 3 medulloblastoma. In this study, they show that even with very promising *in vitro* and *in vivo* evidence that telomerase inhibition can slow medulloblastoma growth; when the drug imetelstat was combined with radiotherapy, it appeared to antagonize the anti-tumor effects of radiotherapy, although some tumor growth delay was observed (Sengupta et al.). With early phase clinical trials data suggesting this drug is not well tolerated in children (10), this study highlights the value of using preclinical models to undertake proof of concept studies that confirm certain proteins are good therapeutic targets, even if the right clinical agent/formulation is not yet available.

Numerous high quality preclinical studies have provided compelling and convincing evidence of the efficacy of several new drug combinations in a range of pediatric cancer types (examples include (11–13) among others), and these combinations are now being assessed in early phase clinical trials (such as SJ-ELIOT/NCT04023669, SJ-DAWN/NCT03434262, PNOC022/NCT05009992). However, it is too soon to determine if the preclinical effort and data are useful in predicting clinical efficacy. Indeed, the jury is still out regarding the amount and type of preclinical data needed to accurately inform new clinical trials that are more likely to succeed compared to their predecessors. Tackling pediatric brain cancer requires international effort and collaboration. This is especially critical for conducting clinical trials for very rare brain cancer entities. To facilitate and inform clinical trial decision making, Jones et al. brought together multiple international clinical and preclinical consortia to provide a set of specific assessment criteria with respect to *in vitro* and *in vivo* evidence designed to aid prioritization of ideas. These well-considered guidelines provide a benchmark for preclinical pediatric brain cancer studies and encourage rigorous validation of results in multiple different institutions – especially important given non-reproducibility of preclinical data is recognized as a major concern in cancer research (14). While demonstration of treatment efficacy in multiple preclinical models (such as multiple patient-derived orthotopic xenografts (PDOX), murine allograft models, and/or genetically engineered mouse models) is possible for certain high-risk childhood brain cancer entities, this is a significant limitation for other brain cancers where very few models exist, and/or those that do exist fail to represent the spread of diverse molecular subtypes. This is well described by Whitehouse et al., who report on the challenges of developing PDOX models for ependymoma. Pooling data from three different institutions, they describe the poor engraftment rate of human ependymoma in the brain of immune-deficient mice. Moreover, through molecular analyses, their data suggests that certain molecular features might facilitate PDOX model establishment. Clearly, refinements in preclinical modelling are required, with several groups now suggesting that improved success may be achieved in age-appropriate animals that better replicate the developmental stage of the brain in which these tumors arise (Jones et al.; Whitehouse et al.; 15).

Overall, there is significant reason to be optimistic with more clinical options available for children newly diagnosed with CNS cancers or with relapsed disease. Several clinical trials have recently been established based on rationally selected molecularly-targeted therapies and preclinical evidence of efficacy. An exemplary example is PNOC022, an adaptive platform trial for children and young adults with DMGs including diffuse intrinsic pontine gliomas (DIPGs). This has been facilitated in large part through the generous donation of surgical or autopsy tumor tissue for research, establishment of a large number of preclinical DMG/DIPG models, combined with the international collegiality, collaboration and sharing of these models. The molecular analyses of large numbers of high-grade gliomas (HGGs) have identified key mutations which drive gliomagenesis, with certain molecular features now officially recognized in the latest 2021 WHO classification of CNS tumors (16). Of note, is that histone H3.1/H3.3, ATRX and IDH1/2 mutations are frequent in HGG, but currently the diversity of mutations is not well represented in preclinical HGG models, and additional work is required to expand models that harbor H3.3 G34R/V variants. Voon and Wong provide a comprehensive overview of the mechanisms of action for these mutations in HGG, improving our understanding of how these molecular alterations might be targetable therapeutically.

Importantly, epidemiological and molecular data for pediatric brain cancers has mostly relied on North American or European populations, and while these data hint at racial differences among brain tumors, limited global information is available. Yang et al. provide critical data in this Research Topic regarding the experience of the national health center for children in China highlighting some notable differences compared to other reports. With ongoing international generation and sharing of preclinical models, laboratory techniques and molecular data, alongside careful monitoring of ongoing clinical trial findings, and robust scientific discussion, achieving international consensus on the optimal clinical trials to benefit children affected by brain cancer will be achievable.

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RE: Conceptualization, Writing – original draft, Writing – review & editing. BW: Writing – review & editing. NG: Conceptualization, Writing – review & editing.

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Conflict of interest

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Childhood brain tumors: It is the child's brain that really matters

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Context of research in childhood brain tumors

The context for research into brain tumors of childhood over the past three decades has focused upon developing an understanding of the biological mechanisms of tumor formation (1). This has been pursued in the belief that it will be the key that will unlock the tumors' vulnerability to therapeutic approaches. The "driver for change" has been improving overall survival. In childhood this has gratifyingly been associated, in high income countries (HIC), with a rise in survival rates from 40-70% (2–4). Within this statistic there are significant variations between European countries. Clinical trials have shown remarkable advances, such as intra-cranial germ cell tumors (5) and medulloblastoma (6), which have improved with combined standard approaches of well delivered chemotherapy, radiotherapy and rational approaches to surgery. Radiotherapy research and trials in the past decades have focussed upon optimising radiation doses to the tumour and surrounding brain to minimise the cognitive consequences (7).

Bio-characterization of these tumors offers hope of further stratification of outcomes with biologically targeted therapies (8). There have been surprises, such as chemo-sensitivity of low grade glioma, offering control of this early onset, self-limiting tissue growth disorder of astrocytes (9). The targeted effect of mTOR inhibitors have controlled progression of sub-ependymal giant cell astrocytoma (SEGA) complicating tuberous sclerosis (10). Bio-characterization of these benign tumors has identified single pathway mutations suitable for drug targeting (11). There have been disappointments with limited or no progress in drugs contributing to cure of ependymoma (12), diffuse intrinsic pontine glioma (DIPG) (13), atypical teratoid rhabdoid tumor (ATRT) (14, 15) and high grade glioma (HGG) (16, 17). Each of these tumor types have been bio-characterised with the intention of identifying targetable mutations to contribute to improved responses; a

strategy yet to provide improvements in cure rates. These are tumors with high levels of primary drug and radiation resistance. The complexity of their diverse bio-characterization profiles, which commonly change after successive treatments, are compounded by superimposed anatomically-determined diversity of mutational patterns. This seems to undermine the rationale for bio-target driven therapies. Contemporary bioscience thinking has responded in particular to the challenges of this primary resistant group by highlighting seven research strategies to look for new therapies (1) which include:

1. Redesigning the research pipeline
2. Leveraging neuroscience research
3. Enhancing understanding of the tumor microenvironment including the blood brain barrier
4. Developing predictive models for research
5. Developing drugs for complex targets in a shifting tissue landscape
6. Developing precision medicine
7. Reducing treatments for sensitive tumor types

This comprehensive proposal is staggering in its scope and has no identifiable timetable or funding stream. The children and their families, the funders and their governments are given no guarantees on delivery or success. Is this outline a safe basis for planning a successful assault on children's brain tumors or is it simply a backdrop for neuro-oncology research practitioners to justify anything they might suggest, in the hope that something will emerge by chance alone?

Biology and therapy of benign versus malignant brain tumors

Biological research has clearly demonstrated that brain tumors in childhood are products of embryologically-sensitive mutations linked to age and precise neuro-anatomical locations (18–21). It is notable that over the past 4 decades there have only been 5 drugs licensed for brain tumors in adults and children, of which 4 are still in production: CCNU (22), temozolomide (23), carmustine wafers (Gliadel) (24)and everolimus (10). The first 3 are licensed for HGG, each has been selected or developed with their capacity to penetrate or bypass the blood brain barrier. Of these, only temozolomide has been licensed for children. Everolimus was licensed for SEGAs that present in Tuberous Sclerosis during late childhood and early adulthood (9). There are trials in progress to evaluate MAP Kinase inhibtors (MEKi) in low grade glioma and NF1-associated neurofibroma (25–29). It is possible therefore that MEKi will join the list of licensed drugs for children for this tumor sub-group. There are trials studying WNT medulloblastoma subtype that may offer enhanced drug penetration across the blood brain barrier and therefore greater sensitivity to standard chemotherapy (30).

What is emerging from this experience is that benign brain tumors are brain development disorders which respond to systemically administered drugs, whilst malignant brain tumors require strategies to penetrate or bypass the blood brain barrier for existing drugs to be effective. If bio-targeted drugs are to be used, a wide variety of targeted drugs will need to be tested in combinations to cover diversity of mutations and their evolution over time. Furthermore, they will need to be specifically delivered across the blood brain barrier if they are to be effective. A whole range of drug delivery techniques are emerging for further study, including intra-CSF delivery, intra-cavity/interstitial delivery, ultrasound BBB disruption, electric field therapy, immunotherapy and transmucosal delivery (31–33). They will require careful selection for study in childhood brain tumors as the biology of children's tumor types and the state of the brain's environment differ markedly from the adult experience and so progress will be determined by specialist paediatric centers adopting techniques for study, ideally as part of an international collaborative strategy (34).

Selecting outcome measures as "drivers for change"

The historical reliance on overall survival as the "driver for change" in the strategy has failed to recognize the incremental acquisition of brain injury by all children with brain tumor for as long as they live and therefore its major health impact for all children from diagnosis (Figure 1). A strategy that omits the consequences of brain injury is therefore deficient and needs review (35). The authors of the seven challenges have not identified brain injury as a target for their research priorities. Brain injury starts with symptom onset prior to diagnosis, is a recognized consequence of brain surgery, radiotherapy and drug therapy and can be exacerbated in its impact in the absence of effective rehabilitative support during childhood and adolescence (36–38). Brain injury is the experience that colors the children's lives for as long as they live and is therefore the most important clinical target for research intervention as it applies to all children not just those who are curable.

Strategies to minimize acquired brain injury

Accelerating diagnosis (36, 37), predicting surgical risks (39, 40) and preventing them, modifying radiation doses and techniques (41), designing trials and outcomes measures to measure neurological and disability outcomes (42) targeting drug therapy precisely (31) and promoting rehabilitative

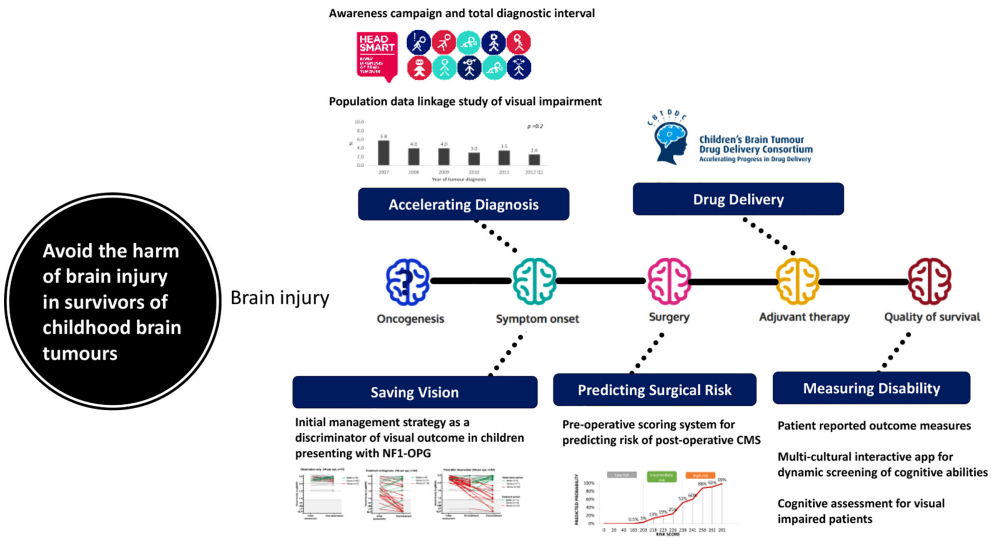

FIGURE 1
A model identifying sources of cumulative brain injury with examples of data and interventions during the management of childhood brain tumours.

effectiveness (35) can all be considered as legitimate interventions to reduce the risk and degree of acquired brain injury, as well as other toxicities (Figure 1). They can be advanced as strategies immediately as they are about using real clinical data to drive change. If these strategies are to be tested, whilst they are being introduced and studied for their impact across health systems. There are promising developments in the design of trials in optic pathway glioma (26, 27) and evaluating surgical strategies in medulloblastoma (43) (M. Mynarek, personal communication). A key "driver for change" will be the selection of primary outcome measures for neurological and quality of life outcomes during childhood, adolescence and early adulthood that reflect this cumulative brain injury (42, 44).

A global health challenge

The World Health Organisation (WHO) Child Cancer Initiative has recognized brain tumor specifically as a global priority (45). The Lancet Commission identifies the economic potential of tripling returns of investment in childhood cancer, particular in low and middle income countries (46). The material cost of acquired brain injury has been quantified by legal processes to range from £2m-26m per child. In the absence of a legal award this is the type of cost needed to support a child after treatment for brain tumor from family, health, social and community services budgets (37). The time is right therefore, to build upon the previously identified research challenges by focusing upon strategies to measure and minimize acquired brain injury in parallel with these initiatives as a sincere effort to minimize the suffering in the immediate future for the children with brain tumor and their families.

Whether the seven challenges will ever be overcome to deliver the new targeted therapies hoped for by the bio-science community remains to be seen. The children need us to deliver change soon to help them and their families have more hope for the future in the next decade. Preventing the acquisition of cumulative brain injury seems a good target for now.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

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Immunohistochemical assessment and clinical, histopathologic, and molecular correlates of membranous somatostatin type-2A receptor expression in high-risk pediatric central nervous system tumors

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Introduction: ^{177}Lu-DOTATATE, a radionuclide therapy that binds somatostatin type-2A receptors (SST2A), has demonstrated efficacy in neuroendocrine tumors and evidence of central nervous system (CNS) penetration, supporting potential expansion within pediatric neuro-oncology. Understanding the prevalence of SST2A expression across pediatric CNS tumors is essential to identify patients who may benefit from somatostatin receptor-targeted therapy and to further elucidate the oncogenic role of SST2A.

Methods: SST2A immunohistochemistry (IHC) was performed on tumor specimens and interpreted by an experienced pathologist (blinded), utilizing semi-quantitative scoring of membranous expression within viable tumor. Immunoreactive cell percentage was visually scored as 0 (none), 1 (<10%), 2 (10-50%), 3 (51-80%), or 4 (>80%). Staining intensity was scored as 0 (none), 1 (weak), 2 (moderate), or 3 (strong). Combined scores for each specimen were calculated by multiplying percent immunoreactivity and staining intensity values (Range: 0-12).

Results: A total of 120 tumor samples from 114 patients were analyzed. Significant differences in SST2A IHC scores were observed across histopathologic diagnoses, with consistently high scores in medulloblastoma (mean ± SD: 7.5 ± 3.6 [n=38]) and meningioma (5.7 ± 3.4 [n=15]), compared to

minimal or absent expression in ATRT (0.3 ± 0.6 [n=3]), ETMR (1.0 ± 0 [n=3]), ependymoma (grades I-III; 0.2 ± 0.7 [n=27]), and high-grade glioma (grades III-IV; 0.4 ± 0.7 [n=23]). Pineoblastoma (3.8 ± 1.5 [n=4]) and other embryonal tumors (2.0 ± 4.0 [n=7]) exhibited intermediate, variable expression. Among medulloblastomas, SST2A IHC scores were higher in non-SHH (8.5 ± 3.1) than SHH (5.0 ± 3.3) molecular subgroups (p=0.033). In a subset of paired primary and recurrent specimens from four patients, SST2A IHC scores remained largely unchanged.

Discussion: High membranous SST2A expression was demonstrated in medulloblastoma, meningioma, and some rarer embryonal tumors with potential diagnostic, biologic, and therapeutic implications. Somatostatin receptor-targeted therapy such as ^{177}Lu-DOTATATE deserves further investigation in these highly SST2A-expressing pediatric CNS tumors.

KEYWORDS

somatostatin receptor, SST2A, immunohistochemistry, pediatric CNS tumors, embryonal tumors, medulloblastoma, somatostatin receptor-targeted therapy, DOTATATE

Introduction

High-grade central nervous system (CNS) tumors remain a leading cause of cancer-related death in children and adolescents (1). While cure can sometimes be achieved with conventional chemotherapy, surgery, and/or radiation, the prognosis for patients with recurrent or progressive disease despite these treatments is dismal (2–6). There is therefore a critical need to develop new, effective therapies for pediatric patients with refractory CNS tumors. Somatostatin receptors regulate cell growth through complex downstream modulation of both proliferation (i.e., mitogen-activated protein kinase, protein tyrosine phosphatase) and apoptosis signaling pathways, and thus represent a potential therapeutic target (7–9). Lutetium (^{177}Lu)-DOTATATE, a radionuclide therapy which binds type-2A somatostatin receptors (SST2A) and delivers local radiation *via* beta particle emission, has gained FDA approval for the treatment of adult patients with gastroenteropancreatic neuroendocrine tumors (10, 11), a disease characterized by consistent SST2A expression (8). There is emerging evidence that certain pediatric CNS tumors express SST2A, with corresponding uptake on somatostatin-receptor radiolabeled nuclear imaging (12–39). SST2A expression has been described in medulloblastoma (13, 26–31), other embryonal tumors (17, 28, 31), meningiomas (25, 32, 33), high-grade gliomas (17, 27, 34–38), and ependymomas (17, 39), though with variable frequencies and lower levels in the latter two histologic diagnoses. Case reports/series have demonstrated treatment response (disease stabilization or regression) to somatostatin receptor-targeted therapy in children and young adults with relapsed medulloblastoma, high-grade glioma, meningioma, and brain metastases of neuroendocrine tumors (19, 22–25, 40–46), suggesting sufficient CNS penetration to achieve therapeutic benefit.

Understanding the prevalence, heterogeneity, and key correlates of SST2A expression across pediatric high-grade CNS tumors is essential to determine which patients are most likely to respond and to further elucidate the oncogenic role of somatostatin receptor pathways within these aggressive diseases. Although aforementioned reports of SST2A expression in pediatric CNS tumors support investigation of somatostatin receptor-targeted therapy (13, 17, 27–38), findings were limited by small sample sizes, varied measures of receptor levels (including SST2A mRNA, an imperfect surrogate for functional protein expression) (47, 48), and inconsistent definitions of SST2A positivity by immunohistochemistry (IHC). Several prior studies evaluating SST2A expression by IHC in CNS tumors used polyclonal anti-SST2A antibodies, which may yield less specific results due to cross-reactivity with other antigens (49, 50). Moreover, associations between SST2A expression and tumor stage, histologic grade, presence of prognostically-significant genetic alterations, response to ^{177}Lu-DOTATATE, and/or survival have been established in neuroendocrine tumors, neuroblastoma, and adult anaplastic oligodendrogliomas (36, 37, 48, 51–55), but corresponding data are lacking in pediatric CNS tumors.

Identifying patients with high-risk CNS tumors who may benefit from somatostatin receptor-targeted therapy demands

rigorous assessment of membranous (i.e., targetable) SST2A protein expression *via* a validated, functionally-relevant SST2A IHC scoring system. Within a large, representative cohort of pediatric high-grade and/or difficult-to-treat CNS tumors, we applied SST2A IHC scoring methodology adapted from neuroendocrine tumors, which demonstrated correlation with somatostatin autoradiography quantification *in vitro* as well as uptake on somatostatin receptor nuclear imaging and response to somatostatin analog therapy *in vivo* (12, 49, 51, 56–58). Additionally, SST2A IHC was performed with a newer monoclonal anti-SST2A antibody (UMB-1), which offers improved sensitivity and specificity compared to earlier polyclonal antibodies (48–50). Utilizing these tools and scoring approach, we evaluated the prevalence of membranous SST2A expression and potential clinical, histopathologic, and molecular correlates across high-risk pediatric CNS tumors.

Materials and methods

Clinical cohort

This retrospective study was performed at Cincinnati Children's Hospital Medical Center (CCHMC) and included pediatric, adolescent, and young adult patients enrolled in the CCHMC institutional review board-approved tumor tissue repository. The patient cohort was selected based on availability of adequate tumor specimens from diagnosis and/or recurrence, with a confirmed histologic diagnosis of CNS embryonal tumor, high-grade glial neoplasm, ependymoma (any grade), and meningioma (any grade) by pathology review. This histopathologic distribution was chosen to ensure inclusion of diagnoses with previously reported evidence suggesting SST2A expression as well as tumors with high histologic grade and/or limited therapeutic options at recurrence. The following clinical data were abstracted from patients' electronic health records and subsequently de-identified: age, sex, presence of metastases, molecular profiling results if applicable, treatment details, event-free survival (defined as time from diagnosis to disease progression, recurrence, secondary malignancy, death, or censoring), and overall survival (defined as time from diagnosis to death or censoring). Patients without an event at last known follow-up were considered censored. All tumor samples and clinical data were collected after informed consent was provided by patients or legal guardians.

Tissue and SST2A IHC preparation

Tumor samples had been preserved as formalin fixed paraffin embedded (FFPE) tissue. To ensure adequate tumor content and viability, hematoxylin and eosin (H&E) slides were first reviewed from the same FFPE block that SST2A IHC was to be performed. Four micron-thick sections were subjected to SST2A IHC preparation following the College of American Pathologists (CAP)/Clinical Laboratory Improvement Amendments (CLIA)-validated clinical assay utilized at CCHMC. Heat-Induced Epitope Retrieval was performed with Ethylenediaminetetraacetic acid and samples were stained, *via* an automated Ventana Ultra IHC stainer at a dilution of 1:200 using the monoclonal anti-SST2A antibody UMB-1 (a rabbit monoclonal antibody targeting the C-terminus of the SST2A protein [Abcam catalog# 134152]), as introduced above (49, 50). Samples were then processed using a secondary antibody and 3,3'-Diaminobenzidine chromogens (Roche: ultraView Universal DAB Detection Kit, catalog# 760-500) for signal visualization.

SST2A IHC scoring and interpretation

SST2A IHC was interpreted by an experienced pathologist (SS) together with a pediatric neuro-oncologist (MAL) in all cases, both blinded to clinical data. A semi-quantitative scoring system was utilized, which incorporated the following SST2A IHC staining profile characteristics: presence and completeness of membranous (versus cytoplasmic) staining, percent of immunoreactive tumor cells, and staining intensity (12, 49, 51, 56–58). Specifically, immunoreactive tumor cell percentage was visually scored as 0 (none), 1 (<10%), 2 (10-50%), 3 (51-80%), or 4 (>80%). Staining intensity was scored as 0 (none), 1 (weak), 2 (moderate), or 3 (strong). Combined scores for each specimen were calculated by multiplying percent immunoreactivity and staining intensity values (possible range: 0-12; Figure 1), as has been implemented in SST2A immunoreactivity assessments of neuroendocrine tumors (51), pituitary adenomas (58), and adult high-grade gliomas (37). Scores of 0-1 were considered negative; scores ≥2 were considered positive. Only membranous staining within viable tumor was considered for scoring purposes. However, non-membranous staining and/or staining in non-tumor cells were recorded for descriptive purposes (and the latter often provided internal negative or positive [e.g., endothelial] controls). Unevaluable areas of hemorrhagic or ischemic tumor were excluded from scoring. Staining distribution, heterogeneity within individual tumor specimens, morphologic patterns, and other relevant histopathologic features were also assessed. Additionally, to evaluate inter-rater reliability of this SST2A IHC scoring system within this pediatric CNS tumor cohort, a second pathologist (CF, blinded to the first pathologist's scores) reviewed digitally uploaded SST2A IHC slides for a subset of 50 tumors, applying the same scoring rules. This sample was intentionally selected to include the range of histopathologic diagnoses from the entire cohort as well as a variety of specimens with high, intermediate, and minimal to absent membranous SST2A expression, as interpreted by the first pathologist. Cases with discordant impressions were subsequently re-examined by both reviewers together, with further collective inspection and discussion to reach consensus final score.

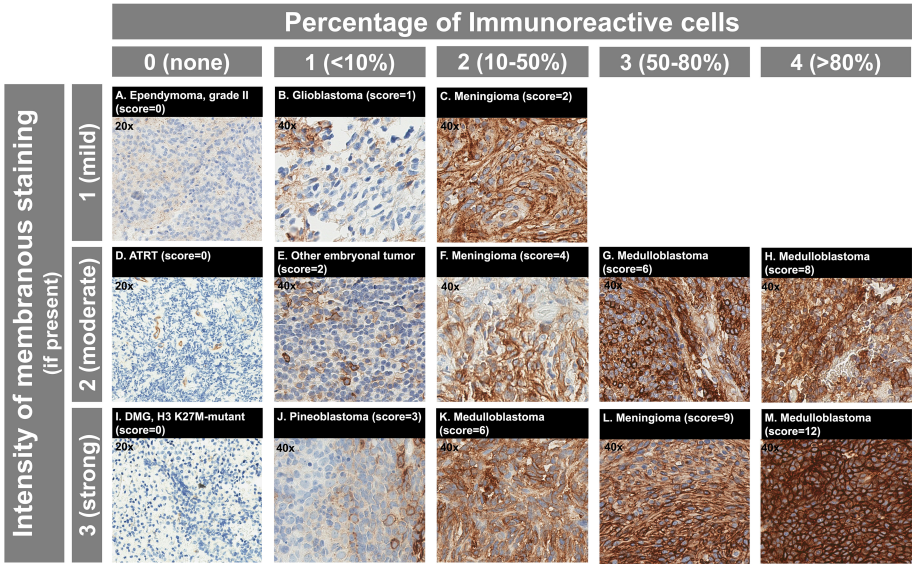

FIGURE 1
SST2A IHC scoring system with example cases from the analyzed pediatric CNS tumor cohort. **(A–M)** Immunoreactive cell percentage is illustrated horizontally, with scores ranging from 0 (none) to 4 (>80%) shown. Staining intensity is illustrated vertically, with scores ranging from 1 (mild) to 3 (strong) shown for tumors with membranous expression present. Total SST2A IHC score (calculated from multiplying immunoreactive cell percentage and staining intensity scores) is noted in parentheses for each example, with the respective histopathologic diagnosis specified. Note that all three cases in the first column **(A**, **D**, **I)** had entirely absent membranous SST2A expression within viable tumor and received total SST2A IHC scores of 0; images for these cases are shown at 20x magnification. Endothelial staining serves as a positive internal control **(D)**. For all other cases, images are shown at 40x magnification.

Statistical analysis

Continuous and categorical variables are described by mean (± standard deviation [SD]) or median (range) and frequency (percent), respectively. T-tests and one-way ANOVA or Wilcoxon rank-sum and Kruskal Wallis tests were used for comparisons of mean SST2A IHC score based on specific clinical, histopathologic, and molecular features. Pearson's correlation was used to evaluate associations between patient age and SST2A IHC within the medulloblastoma cohort. To assess potential associations between SST2A expression and outcome (event-free survival and overall survival, as defined above), univariate and multivariable Cox proportional hazards regression analyses were performed, with SST2A IHC score and histologic diagnosis as covariates; hazard ratios (HR) and corresponding 95% confidence intervals (CI) were reported. Survival outcomes were summarized using the Kaplan-Meier method and log-rank analyses were used to compare survival between patients divided into three SST2A IHC score categories (0-1 [negative], 2-5, 6-12). The weighted kappa statistic and Spearman's correlation were used to evaluate measures of inter-reviewer reliability between pathologists, All p-values were two-sided and those less than 0.05 were considered statistically significant. Statistical analyses were completed in SAS software, version 9.4 (SAS Institute, Cary, NC) or base R statistical software (R Foundation for Statistical Computing, Vienna, Austria) with the "survival" and "survminer" packages.

Results

Cohort characteristics

A total of 120 tumor samples from 114 patients were included in the analysis. Demographic and clinical characteristics are summarized for the entire cohort and by histopathologic diagnosis in Table 1. Median age at diagnosis was 7.1 years (range: 0.1-29.3 years), and 39% of the patients were female. Metastatic disease was identified in 13% of patients at initial diagnosis, and 25% experienced subsequent recurrence or progression. Eighty-nine percent were alive at last follow-up (median 7.7 years from diagnosis).

Assessment of membranous SST2A expression across and within histopathologic diagnoses

Comparison across histologic subgroups:

The distribution of membranous SST2A expression by histopathology is illustrated in Figure 2 and summarized in Table 2, with significant differences in SST2A IHC scores between histopathologic diagnosis groups ($p<0.001$). Higher total SST2A IHC scores were observed among embryonal tumors (mean ± SD: 5.8 ± 4.2), albeit with variation described

TABLE 1 Cohort Characteristics.

	Number (%) of patients	Age at diagnosis (median [range] in years)	Gender distribution (n [%] female)	Metastatic disease at diagnosis (n [%])	Subsequent recurrence or progression (n [%])	Number (%) Alive at Last Follow-up	Time to last follow-up (median [range] in years)
Entire cohort	114	7.1 (0.1 – 29.3)	44 (39%)	15 (13%)	28 (25%)	102 (89%)	7.7 (0.2 – 30.3)
By Histopathologic diagnosis:							
Meningioma	13 (11%)	14.3 (6.0 – 29.3)	7 (54%)	0 (0%)	4 (31%)	13 (100%)	11.2 (0.8 – 25.5)
Medulloblastoma	38 (33%)	6.5 (0.9 – 17.6)	6 (16%)	8 (21%)	3 (8%)	37 (97%)*	9.0 (0.2 – 26.7)
Pineoblastoma	4 (4%)	6.8 (0.8 – 21.9)	3 (75%)	2 (50%)	0 (0%)	4 (100%)	2.3 (1.5 – 15.3)
ATRT	3 (3%)	1.3 (0.8 – 4.9)	2 (67%)	0 (0%)	0 (0%)	2 (67%)#	11.3 (1.3 – 13.8)
ETMR	3 (3%)	2.6 (2.2 – 3.7)	2 (67%)	1 (33%)	1 (33%)	2 (67%)	0.3 (0.3 – 6.9)
Other embryonal tumors[a]	7 (6%)	2.1 (1.2 – 15.7)	2 (29%)	3 (43%)	2 (29%)	5 (71)	10.4 (1.8 – 22.1)
Ependymoma	25 (22%)	7.0 (1.0 – 18.1)	13 (52%)	0 (0%)	5 (20%)	25 (100%)	8.3 (0.7 – 30.3)
High-grade glioma	21 (18%)	10.3 (0.1 – 27.8)	9 (43%)	1 (5%)	13 (62%)	14 (67%)	4.4 (0.3 – 22.8)

Demographic and clinical features of patients included in the analysis, summarized overall and within histopathologic diagnosis subgroups.
[a]The subgroup designated "other embryonal tumors" includes six patients with tumors formerly classified as CNS primitive neuro-ectodermal tumor (PNET) and one patient more recently diagnosed with embryonal tumor, not otherwise specified (NOS).
*,# Cause of death was disease recurrence or progression for deceased patients in the cohort except one patient with medulloblastoma (sequalae of treatment-related co-morbidities) and one patient with ATRT (complications of brainstem necrosis).

below, and meningioma (5.7 ± 3.4), compared to ependymomas (0.7 ± 0.1) and high-grade gliomas (0.4 ± 0.7), both with minimal or absent expression (Figure 2).

Embryonal tumors

Medulloblastoma

The 38 medulloblastoma samples collectively comprised the tumors with the highest SST2A expression in our cohort (Figure 2). Positive SST2A IHC scores (≥2) were reported in 35 (92%) medulloblastoma tumors (Figures 1G, H, K, M), with all 35 demonstrating moderate-strong staining intensity and most (22 [63%]) exhibiting >50% tumor cell immunoreactivity.

There were significant differences in membranous SST2A expression between medulloblastoma histopathologic subgroups, with the highest SST2A IHC scores observed in large cell/anaplastic tumors (11 ± 1.7 [n=3]), followed by classic histology (7.9 ± 3.3 [n=17]], and lowest, yet often still positive, SST2A IHC scores in nodular/desmoplastic tumors (4.4 ± 3.1 [n=7]; p=0.012 for comparison across 3 groups; Figure 3A).

When comparing the 24 medulloblastoma specimens with available methylation results enabling molecular classification (Figure 3B), there was a trend toward differences in mean SST2A IHC score across the four molecular subgroups (group 3: 10.5 ± 1.9: [n=4] > WNT: 8.7 ± 2.3 [n=3] > group 4: 7.7 ± 3.5 [n=12] > Sonic Hedgehog (SHH): 5.0 ± 3.3 [n=5]; p=0.096), and significantly higher scores in non-SHH versus SHH tumors (8.5 ± 3.1 vs. 5.0 ± 3.3; p=0.033). Correspondingly, three of the four tumors with the lowest SST2A IHC scores (1–2) within the medulloblastoma cohort were classified as nodular/desmoplastic histologically, with two confirmed as SHH-activated by methylation testing (not performed in third).

An inverse correlation was observed between SST2A IHC score and patient age at diagnosis when analyzed across all 38 medulloblastoma cases (R=-0.32, p=0.048). There were no significant differences in membranous SST2A expression by sex (female: 9.5 ± 2.6 [n=6], male: 7.0 ± 3.6 [n=32]; p=0.13), presence of metastatic disease at diagnosis (metastatic: 6.9 ± 3.0 [n=8], localized: 7.6 ± 3.7 [n=30]; p=0.58), or likelihood of relapse (among patients followed ≥2 years from diagnosis: relapse: 9.0 ± 1.0 [n=3]; no relapse: 7.2 ± 3.8 [n=29]; p=0.45). One recurrent, post-treatment specimen was analyzed, with a SST2A IHC score of 10 (corresponding diagnostic sample was not available).

Morphological patterns of SST2A expression were identified across medulloblastoma tumors and specifically within the nodular/desmoplastic subset, which enabled comparison of IHC positivity along the intratumoral spectrum of cell differentiation. In several cases, including two infant SHH tumors with higher SST2A IHC scores, SST2A expression inversely correlated with tumor cell maturation, with more immature cells demonstrating strong, complete, circumferential SST2A IHC positivity, compared with absent membranous expression in the more mature cells comprising the tumor nodules (Figure 4A). Conversely, in two other nodular/desmoplastic histology cases, including one adolescent SHH tumor with a low SST2A IHC score, the more primitive cells lacked SST2A positivity, whereas expression was observed within the nodules' differentiating cells (Figure 4B).

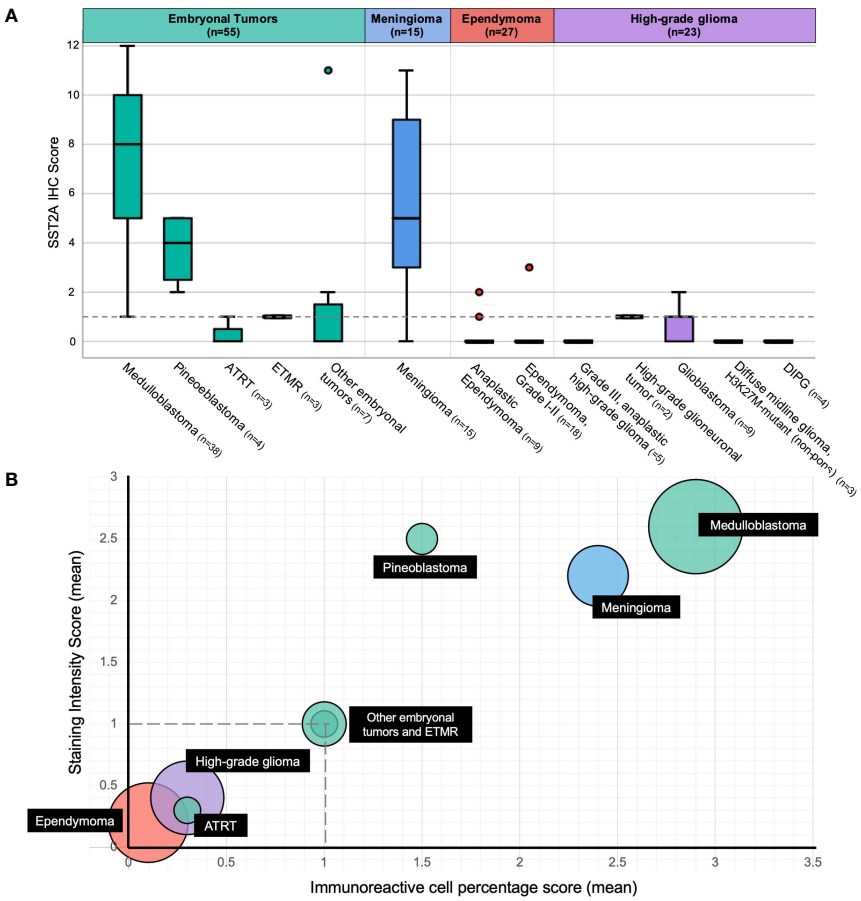

FIGURE 2
Membranous SST2A expression by histopathology. **(A)** Boxplot of total SST2A IHC score (y-axis) for pediatric CNS tumors of different histopathologic diagnoses (x-axis). The dashed gray line (corresponding to a total SST2A IHC score of 1) distinguishes between negative (0-1) and positive (≥2) SST2A IHC scores. **(B)** Bubble plot of mean SST2A IHC subscores (staining intensity score on y-axis, immunoreactivity score on x-axis) for different histopathologic diagnoses (indicated in black boxes), with circle size proportional to the number of patients analyzed within each respective histopathology group. The dashed horizontal and vertical gray lines (corresponding to staining intensity and immunoreactivity scores of 1, respectively) illustrate the subscore thresholds below which total SST2A IHC scores would be considered negative (0-1).

Pineoblastoma

All four (100%) pineoblastoma tumors had positive SST2A IHC scores, with at least moderate staining intensity in each specimen (Figure 2). The two tumors with higher membranous SST2A expression [both with scores of 5 (Figure 5A)] had diffuse leptomeningeal metastases at diagnosis; in one of these specimens, SST2A IHC positivity appeared to correlate with focal papillary morphology. The remaining two tumors (with scores of 2 and 3, both with localized pineal disease) exhibited small (<10%), focal areas of moderate to strong SST2A expression (Figure 1J). All four patients are alive without evidence of recurrence.

ATRT and ETMR

Negative SST2A IHC scores (0–1) were observed in all six (100%) cases of atypical teratoid/rhabdoid tumor (ATRT) and embryonal tumor with multilayered rosettes (ETMR), irrespective of sex, age, metastatic disease, or likelihood of relapse (Figure 2). The three ATRT tumors exhibited largely absent membranous SST2A expression, with scores of 0 in two samples (Figure 1D) and at most 1 in the remaining specimen. Minimal SST2A expression was similarly demonstrated in the three ETMR tumors, which all received scores of 1 for very small (<10%) areas of light and/or incomplete membranous positivity, with otherwise absent staining throughout (Figure 5B). Morphological rosette structures lacked membranous SST2A IHC positivity.

Other embryonal tumors

Among seven additional embryonal tumors not classified in the above histologic categories (i.e., most formerly diagnosed as CNS primitive neuro-ectodermal tumor [PNET]), there was heterogeneous membranous SST2A expression, with positive IHC scores in two specimens (29%) (Figure 2). Notably, one

TABLE 2 Summary of SST2A IHC immunoreactive cell percentage, staining intensity, and combined scores based on histopathologic diagnosis.

	Immunoreactive cell percentage score (Range: 0-4)	Staining intensity score (Range: 0-3)	Combined SST2A IHC score (Range: 0-12)
Entire cohort (n=120)	1.4 (± 1.5)	1.4 (± 1.2)	3.5 (± 4.1)
Meningioma (n=15)	2.4 (± 1.2)	2.2 (± 0.9)	5.7 (± 3.4)
Embryonal tumors (n=55)	2.3 (± 1.4)	2.1 (± 1.0)	5.8 (± 4.2)
• Medulloblastoma (n=38)	2.9 (± 1.2)	2.6 (± 0.6)	7.5 (± 3.5)
• Pineoblastoma (n=4)	1.5 (± 0.6)	2.5 (± 0.4)	3.8 (± 1.5)
• ATRT (n=3)	0.3 (± 0.6)	0.3 (± 0.6)	0.3 (± 0.6)
• ETMR (n=3)	1.0 (± 0.0)	1.0 (± 0.0)	1.0 (± 0.0)
• Other embryonal tumors (n=7)[a]	0.8 (± 1.3)	0.9 (± 1.2)	2.0 (± 4.0)
Ependymoma (n=27)	0.1 (± 0.3)	0.2 (± 0.7)	0.2 (± 0.7)
• Ependymoma, grade I-II (n=18)	0.1 (± 0.2)	0.2 (± 0.7)	0.3 (± 0.8)
• Anaplastic ependymoma (n=9)	0.2 (± 0.4)	0.3 (± 0.7)	0.1 (± 0.3)
High-grade glioma (n=23)	0.3 (± 0.5)	0.4 (± 0.7)	0.4 (± 0.7)
• Grade III, anaplastic glioma (n=5)*	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)
• High-grade glioneuronal tumor (n=2)	1.0 (± 0.0)	1.0 (± 0.0)	1.0 (± 0.0)
• Glioblastoma (n=9)	0.7 (± 0.5)	0.9 (± 0.8)	0.9 (± 0.8)
• Diffuse midline glioma, H3K27M-mutant (non-pontine) (n=3)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)
• DIPG (n=4)[#]	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)

Scores are shown as mean (± SD) for the entire cohort and within respective histopathologic diagnosis subgroups.
[a]The subgroup designated "other embryonal tumors" includes six patients with tumors formerly classified as CNS primitive neuro-ectodermal tumor (PNET) and one patient more recently diagnosed with embryonal tumor, not otherwise specified (NOS).
* Including tumors classified as anaplastic astrocytoma (n=3), anaplastic pleomorphic xanthoastrocytoma (n=1), and anaplastic oligodendroglioma (n=1).
[#] Including tumors diagnosed as DIPG on the basis of classic radiographic and clinical features, histologically consistent with diffuse midline glioma, H3K27M-mutant (n=3) and anaplastic astrocytoma (n=1) [all with tissue available from biopsy].

tumor, characterized histologically as malignant embryonal tumor, not otherwise specified (NOS), with especially primitive morphology, received one of the highest IHC scores of the entire cohort, with nearly 100% membranous immunoreactivity of strong intensity (Figure 5C); this patient presented in adolescence with extensive intracranial and spinal metastases and remains recurrence-free approximately one year post-completion of craniospinal irradiation and adjuvant chemotherapy. The other embryonal tumor with SST2A IHC positivity (score of 2, primary supratentorial location) exhibited focal areas of moderate staining intensity (Figure 1E). Minimal to absent membranous SST2A expression was observed in the remaining five embryonal tumor specimens). Neither genetic sequencing nor methylation testing was available on these tumors, limiting further molecular characterization.

Meningiomas

Membranous SST2A expression was consistently identified in meningiomas (Figure 2), with positive SST2A IHC scores in 14 of 15 (93%) tumors analyzed (Figures 1C, F, L). Scores were variable, ranging from 2 to 11, with no significant correlation with histologic grade (grade I: 5.2 ± 3.7 [n=6]; grade II: 5.6 ± 3.4 [n=8], p=0.81 [the one grade III meningioma received a score of 9]). There was no association between SST2A IHC score and patient age at diagnosis, gender, likelihood of progressive disease, or prior treatment exposure (p>0.05 for all). Intratumoral heterogeneity of membranous SST2A expression was frequently observed, with focal areas of positivity in several cases. In at least one tumor (grade II), SST2A staining correlated with morphology, present on most meningioma cells with classic appearance and absent in most spindle-shaped, sarcomatous cells, but this was not universally seen.

Ependymomas

Minimal to absent membranous SST2A expression was demonstrated in the 27 ependymoma samples evaluated (Figure 2), with negative SST2A scores in 25 (93%) tumors (Figure 1A). The remaining two ependymomas (grade II) had scores of 2 and 3, respectively, with very small (<10%), focal areas of membranous (Figure 5D). Largely absent SST2A expression was consistently observed across ependymomas of different histologic grades (grades I-II: 0.2 ± 0.7 [n=18]; grade III/anaplastic: 0.3 ± 0.7 [n=9]; p=0.57) and primary tumor locations (posterior fossa: 0.2 ± 0.5 [n=19]; supratentorial: 0.5 ± 1.2 [n=6]; p=0.32). Six tumors had molecular profiling (posterior fossa group A [n=3], posterior fossa group B [n=2],

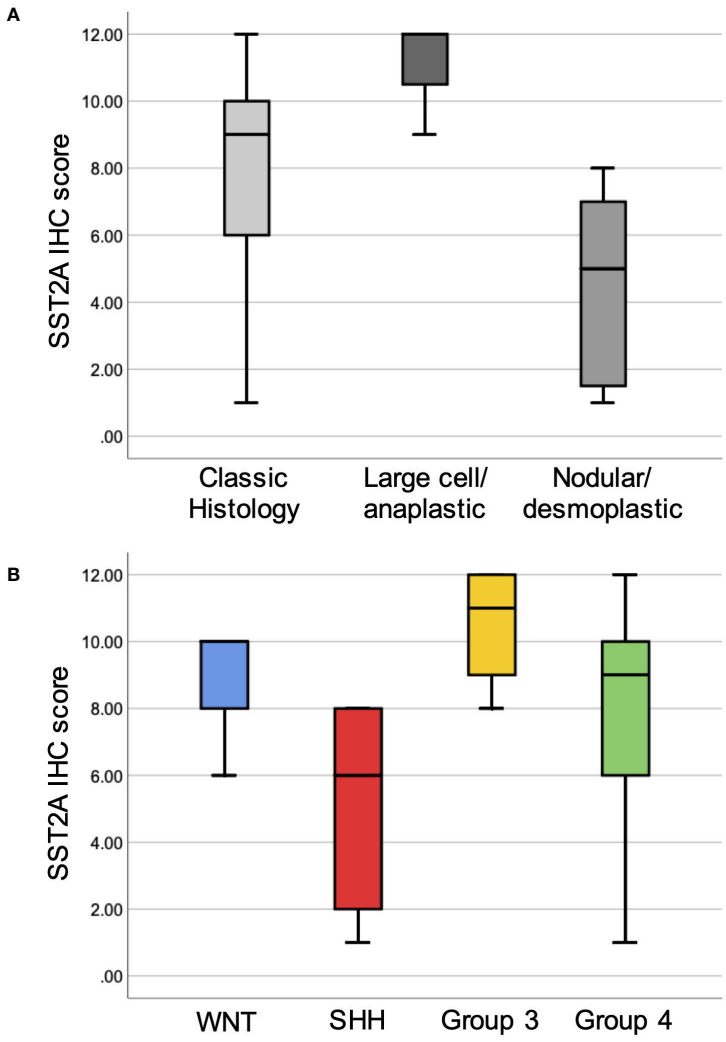

FIGURE 3
Membranous SST2A expression across histopathologic and molecular subgroups of medulloblastoma. Boxplots illustrate total SST2A IHC score by histologic **(A)** and molecular **(B)** classification.

supratentorial *ZFTA-RELA* fusion-positive [n=1]), all with SST2A IHC scores ≤2.

High-grade gliomas

Membranous SST2A expression was minimal to absent in the 23 pediatric high-grade gliomas analyzed (Figures 1B, I, Figure 2), which included the following specific histologic diagnoses: (a) anaplastic astrocytoma, anaplastic pleomorphic xanthoastrocytoma, and anaplastic oligodendroglioma (n=5), (b) high-grade glioneuronal tumor (n=2), (c) glioblastoma (n=9), (e) non-pontine diffuse midline glioma, H3 K27M-mutant (n=3), and (f) DIPG (n=4; diffuse midline glioma, H3 K27M-mutant [n=3] and anaplastic astrocytoma [n=1]). Twenty-one (91%) tumors had negative SST2A IHC scores; the remaining two high-grade glioma samples received scores of 2 (both glioblastoma, one of which was *IDH1*-mutant), with interpretation limited somewhat by specimen quality and diffuse non-tumoral, non-membranous background staining (see below) in both cases. Negative SST2A scores were consistently observed across the aforementioned five high-grade glioma diagnosis subgroups, without differences by specific histology.

Paired diagnostic and recurrent tumor samples

Four patients had paired diagnostic and recurrent tumor specimens analyzed, enabling preliminary assessment of

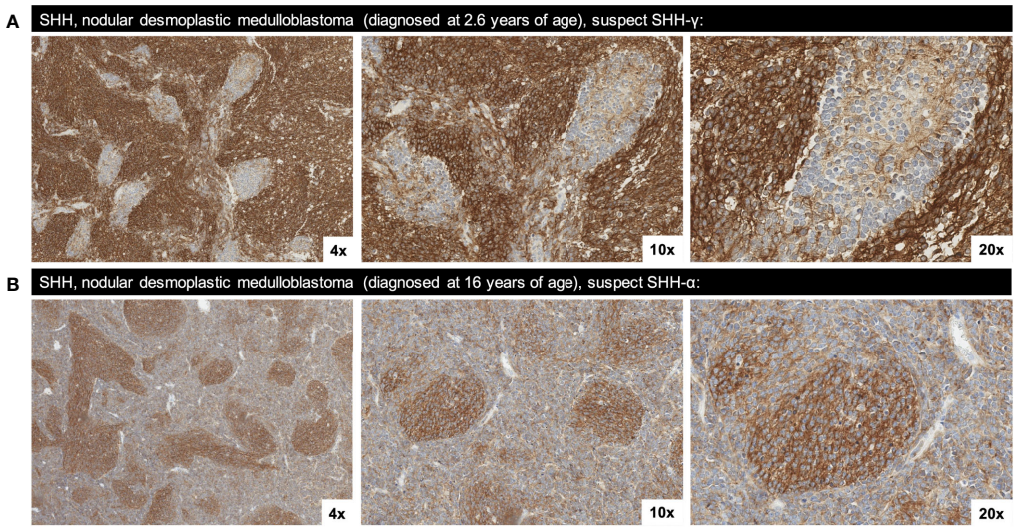

FIGURE 4
Two morphological patterns of SST2A staining observed in SHH, nodular/desmoplastic medulloblastoma. **(A)** SST2A IHC of SHH medulloblastoma (in a patient diagnosed at 2.6 years of age) demonstrating an inverse correlation between membranous SST2A expression and tumor cell maturation; the more immature cells exhibit strong, complete, circumferential SST2A IHC positivity, whereas the more mature cells comprising the nodules lack membranous expression (yet have cytoplasmic granularity). **(B)** SST2A IHC of SHH medulloblastoma (in a patient diagnosed at 16 years of age), with more primitive cells lacking membranous SST2A expression while the nodules' differentiating cells exhibit SST2A positivity (though not consistently membranous). Images in both cases are shown at 4x, 10x, and 20x magnification.

temporal heterogeneity in SST2A expression profiles. SST2A IHC scores were largely conserved over time within this small subset. One patient with a multiply progressive atypical meningioma had three tumor specimens evaluated, all with similar membranous SST2A expression: 10-20% tumor cell immunoreactivity and intratumoral heterogeneous staining intensity (light to strong) was demonstrated in each of the diagnostic and two recurrent specimens (latter following radiation and reirradiation, respectively) (Figure 6). The remaining paired specimens all exhibited conserved absence of SST2A expression. Specifically, two patients with recurrent ependymomas (grade II and grade III/anaplastic) had specimens submitted from diagnosis and post-radiation local relapses, all with SST2A IHC scores of zero. One patient with an H3 K27M-mutant diffuse midline glioma had tumor specimens evaluated from diagnosis (pre-treatment biopsy of spinal lesion) and metastatic progression (biopsy of extraneural osseous metastasis), both entirely lacking membranous SST2A expression.

Association between membranous SST2A expression and survival:

Preliminary analyses evaluating association between SST2A IHC score and outcomes (event-free survival and overall survival) were performed, recognizing interpretation is limited by the relatively low rates of recurrence/progression and death in the cohort as well as by heterogeneity in histologic diagnoses. When assessed in univariate analyses across the entire cohort (all histopathologic diagnoses), SST2A IHC score (analyzed as a continuous variable) was associated with improved event-free survival (HR=0.85 [95% CI: 0.76-0.96]; p=0.009) and overall survival (HR=0.64 [0.42-0.97]; p=0.034). However, in multivariable analyses adjusting for histopathologic diagnosis (i.e., classification as embryonal tumor, meningioma, ependymoma, or high-grade glioma), SST2A IHC score was no longer predictive of event-free survival (HR=0.87 [0.74-1.01], p=0.07), but remained associated with overall survival (HR=0.33 [0.12-0.95]; p=0.039).

Inter-rater reliability

Among the subset of 50 tumors reviewed (via digital slide upload) in blinded fashion by a second pathologist, there was moderate agreement in ordinal score measurements, with a weighted kappa of 0.54 (p<0.0001), and strong positive correlation in score absolute values (Spearman's Rho=0.81, p<0.0001). Subsequent retrospective review of discordant cases by the two pathologists revealed that most discrepancies occurred in cases with poor, compromised tumor tissue quality and/or diffusely positive background, non-membranous staining which potentially limited visualization of the tumor cell surface.

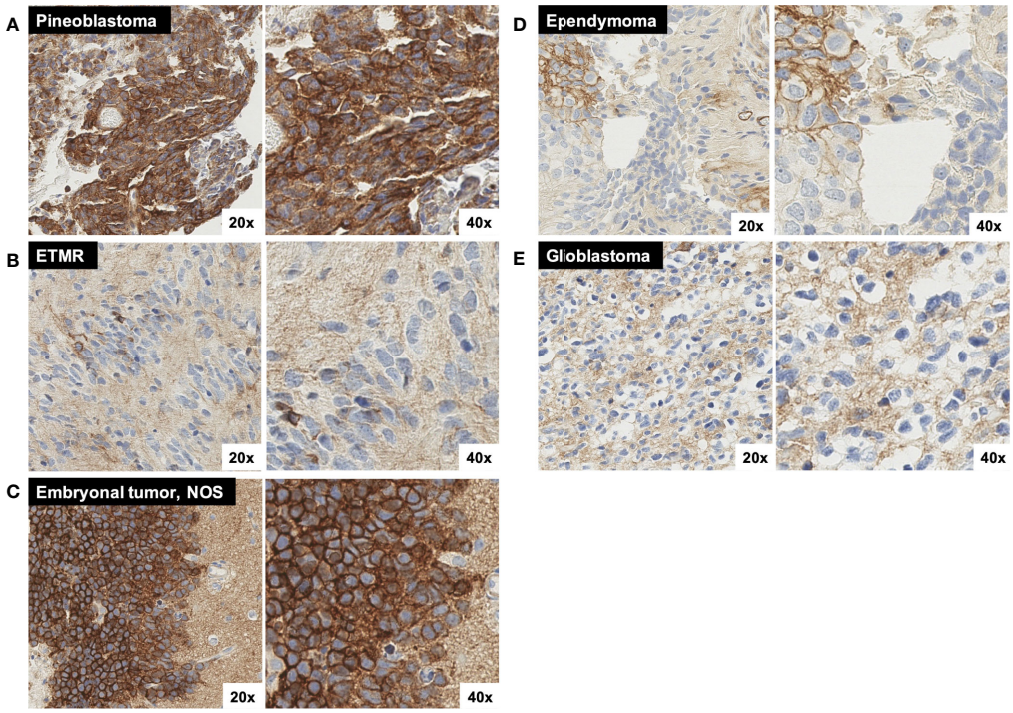

FIGURE 5
Additional representative SST2A IHC images. **(A)** Pineoblastoma sample with moderate to strong SST2A staining in 10-50% of tumor cells. **(B)** ETMR sample with very small (<<10%) areas of light and/or incomplete membranous positivity, with otherwise absent tumoral staining throughout. Background granular and/or cytoplasmic staining is demonstrated. **(C)** Embryonal tumor, NOS with strong SST2A staining intensity in nearly 100% of tumor cells. Tumor cells (left) are shown in proximity to background cortical tissue (right), which demonstrates non-specific (non-membranous) staining. **(D)** Ependymoma (grade II) sample with very small (<10%), focal areas of membranous positivity (on upper left), but otherwise absent tumoral staining. Background granular and/or cytoplasmic staining is demonstrated. **(E)** Glioblastoma sample with minimal to absent tumoral membranous staining, yet diffuse background staining of non-neoplastic tissue. All images are shown at 20x and 40x magnification.

Discussion

Potential incorporation of somatostatin receptor-targeted therapy in the treatment of children, adolescents, and young adults with refractory CNS tumors requires an understanding of the prevalence and key correlates of SST2A expression across these aggressive diseases. To our knowledge, this study represents one of the first detailed immunohistochemical assessments of membranous SST2A expression within a representative cohort of pediatric high-risk CNS tumors utilizing previously validated, functionally-relevant IHC scoring methodology. Whereas SST2A was largely absent from the tumoral cell surface of pediatric high-grade gliomas and ependymomas, high membranous SST2A expression was demonstrated in medulloblastoma, meningioma, and some rarer embryonal tumors with important diagnostic, biologic, and therapeutic implications.

Medulloblastomas in our cohort consistently expressed tumoral cell surface SST2A, in accord with previous reports describing SST2A expression within this histopathologic diagnosis when evaluated through a combination of different assays, including IHC, mRNA levels, somatostatin receptor autoradiography, somatostatin receptor scintigraphy, and/or SST2A-radiolabeled nuclear imaging (e.g., DOTATATE PET) (13–16, 20, 21, 26–31). Our findings confirm and expand upon this earlier work by illustrating a high prevalence of membranous (i.e., functional and targetable) SST2A protein expression among medulloblastoma cases assessed by strict immunohistochemical measures. More than 90% of the 38 analyzed medulloblastoma specimens had positive SST2A IHC scores, all with moderate-strong staining intensity and most exhibiting >50% tumor cell immunoreactivity.

Although nearly all medulloblastoma tumors expressed membranous SST2A to some extent, differences in expression were detected across histopathologic and molecular subgroups. The highest SST2A IHC scores were observed in large cell/anaplastic tumors (albeit the smallest sample size), followed by classic histology, and lowest in the nodular/desmoplastic variant. Correspondingly and in agreement with the findings of Remke et al. (29), non-SHH medulloblastoma tumors had significantly higher membranous SST2A expression than SHH tumors, with a trend toward greater SST2A IHC scores in cases further classified by methylation testing as group 3. Additionally,

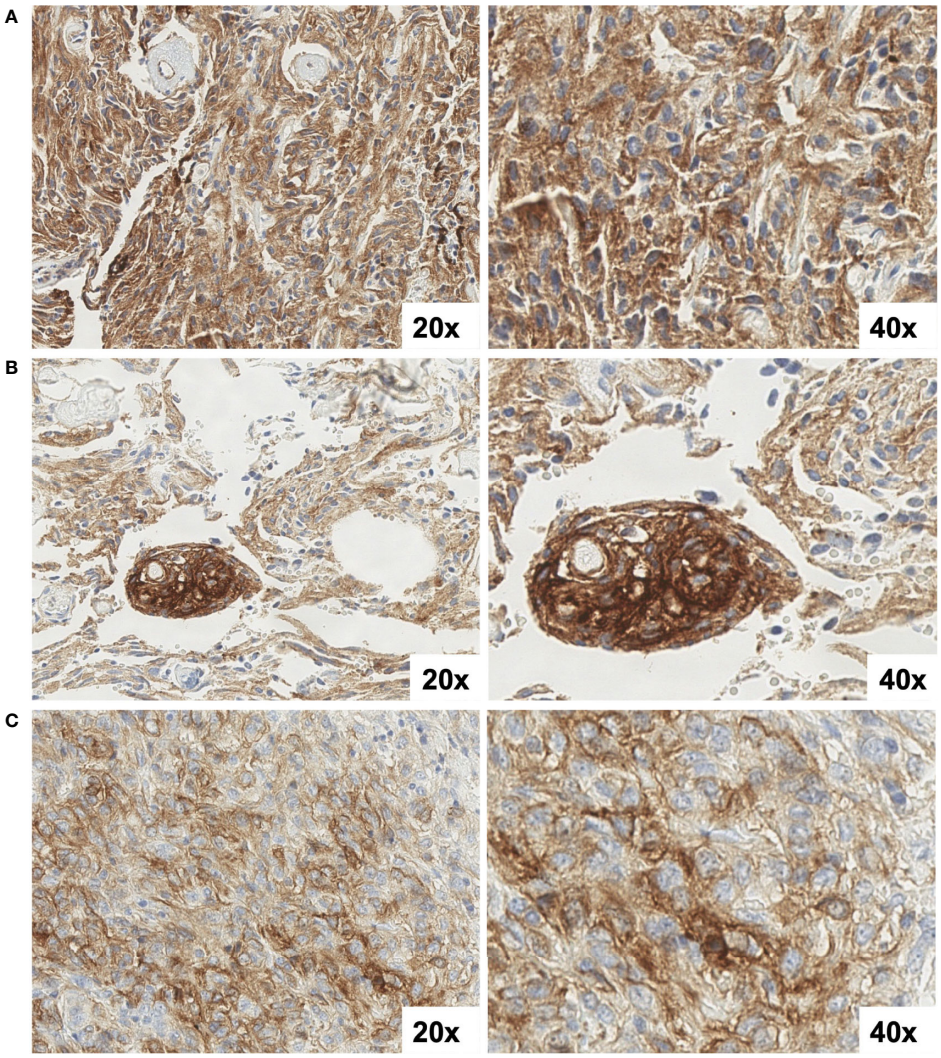

FIGURE 6
SST2A IHC images of a multiply recurrent atypical meningioma from three time-points: **(A)** initial diagnosis, prior to treatment, **(B)** post-radiation, five years after diagnosis, and **(C)** following re-irradiation two years later **(C)**. All specimens demonstrated 10-20% tumor cell immunoreactivity and intratumoral heterogeneous staining intensity, ranging from light to strong. Non-specific background staining is also observed. All images are shown at 20x and 40x magnification.

SST2A IHC positivity correlated with morphology in some medulloblastoma specimens, most notable when assessed across the intratumoral spectrum of differentiation within nodular/desmoplastic histology. In several nodular/desmoplastic tumors (including two infant SHH cases with higher SST2A IHC scores), more immature cells highly expressed membranous SST2A, whereas more differentiated cells comprising the nodules lacked membranous SST2A positivity (yet exhibited cytoplasmic granularity). These findings provide support for a proposed association between SST2A overexpression and genomically-defined dedifferentiated, proneural, and/or primitive neuronal precursor lineage from studies in adult CNS tumors (37). However, an almost inverse SST2A IHC staining pattern was occasionally noted (including in one adolescent SHH tumor which received a low IHC score) with absent SST2A expression in more primitive cells, yet positivity (however still usually cytoplasmic, not membranous) in the nodule's maturing cells. This variation of SST2A expression both within SHH (known to comprise many subtypes [e.g., SHH-γ, SHH-α]) and across medulloblastoma subgroups likely reflects the biological intra- and inter-tumoral heterogeneity of this disease (59, 60), for which the emerging oncogenic role of somatostatin receptor pathways warrants continued research.

There is a critical need to develop novel therapies for children and young adults with relapsed medulloblastoma, who currently

have limited treatment options, no standard salvage regimen, and an especially poor prognosis, with overall survival <15% (3, 5, 6). Promising results of somatostatin receptor-targeted therapy (both somatostatin analogues like octreotide as well as SST2A peptide receptor radionuclide treatment) have been observed in small series of recurrent medulloblastoma cases, with sustained radiographic and clinical responses in tumors refractory to conventional radiation and chemotherapy (40–42). Moreover, positive correlations between extent of membranous SST2A expression, evaluated using similar immunoreactive IHC scoring as implemented here, and response to somatostatin receptor-targeted therapy were detected in gastrointestinal neuroendocrine tumors and pituitary adenomas (51, 58, 61, 62). Improved response rates, progression-free survival, and overall survival following such treatment were demonstrated in tumors with SST2A IHC scores of at least 5-6 (51, 61), which corresponds to >75% of the medulloblastoma cases in our cohort, further supporting investigation of somatostatin receptor-targeted therapy such as ^{177}Lu-DOTATATE in these patients. Development of an early phase clinical trial of ^{177}Lu-DOTATATE in children and young adults with refractory SST2A-expressing high-grade CNS tumors, including medulloblastoma, is currently underway, using the immunohistochemical evaluation of membranous SST2A expression described here for eligibility screening (NCT05278208). If somatostatin receptor-targeted therapy proves effective in medulloblastoma and other SST2A-expressing CNS tumors, this could eventually be incorporated into upfront treatment backbones for these aggressive diseases, potentially presenting a modality to deliver targeted, localized radiation in younger patients with high-risk tumors. Importantly, prevalent membranous SST2A expression was observed in aforementioned medulloblastoma tumors with known poor prognostic molecular, histopathologic, and clinical features (group 3, large cell/anaplastic histology, metastatic disease) (63, 64), representing a possible therapeutic target in the upfront setting for these more challenging subgroups.

Heterogeneous membranous SST2A expression was identified across other pediatric embryonal tumors. Earlier reports described mixed results regarding SST2A expression by IHC or mRNA in small series of supratentorial CNS-PNETs, with SST2A positivity noted in some studies (17, 28, 31), but absent expression in others (29). Our findings expand upon this previous work, demonstrating varied membranous SST2A expression in non-medulloblastoma embryonal tumors —present in pineoblastoma, absent in ATRT and ETMR, wide-ranging in remaining cases. Despite shared histopathologic features, the observed heterogeneity in SST2A expression among these rarer pediatric embryonal tumors likely parallels their divergent molecular landscapes and distinct DNA methylomes (65–68). Detailed genetic sequencing or methylation array were not available on these tumors, precluding molecular characterization. Further exploration in a larger cohort with comprehensive genomic profiling is necessary, but these results suggest a potential role for somatostatin receptor-targeted therapy in certain embryonal tumors, including pineoblastoma.

Membranous SST2A expression was prevalent in pediatric meningiomas in our cohort, with positive IHC scores in nearly all cases. These findings corroborate previous reports of SST2A overexpression in most meningiomas with corresponding uptake on somatostatin-receptor radiolabeled nuclear imaging (25, 32, 33). Whereas this earlier work largely focused on meningiomas in adult patients, we assessed SST2A expression in meningiomas diagnosed during childhood, adolescence, or young adulthood (median age at diagnosis: 14 years [range: 6-29]), with similar IHC positivity as older counterparts. Although most pediatric meningiomas expressed SST2A to some extent, both intra- and inter-tumoral heterogeneity in expression was observed. Potential correlations between immunohistochemical SST2A expression and histologic grade, microvessel density, and/or morphologic features have been suggested in adult meningiomas (25, 69, 70), yet not consistently shown. We did not identify significant associations between meningioma SST2A IHC scores and histopathologic or clinical characteristics, albeit possibly limited by the small sample size and thus deserving continued investigation. Nonetheless, pediatric and young adult patients with recurrent meningiomas face poor outcomes with limited effective treatments, especially in cases where surgery and conventional radiation are not feasible or confer excessive toxicity (25, 71, 72). Somatostatin receptor-targeted therapy represents a promising consideration for refractory and/or unresectable pediatric meningiomas, given frequently detected membranous SST2A expression in these tumors as well as emerging reports of response or prolonged disease stabilization in treated adult patients (22–25).

Minimal to absent membranous SST2A expression was consistently demonstrated in all pediatric ependymomas and high-grade gliomas in our cohort, irrespective of histology, tumor location, or patient clinical features. Although prior reports describe the presence of SST2A within some pediatric ependymomas and high-grade gliomas (17, 27, 34, 39), positive findings were largely limited to cytoplasmic IHC staining and/or mRNA expression, which exhibited poor correlation with membranous immunolabeling and functional protein levels, likely due to post-translational modification (39, 47, 48). Our results confirm the general paucity of targetable, tumoral cell-surface SST2A in pediatric ependymomas and high-grade gliomas seen in earlier studies (17, 27, 29, 38, 39), evaluated here through utilization of stringent IHC measures, a more specific, monoclonal anti-SST2A antibody, and a larger sample size. Whereas membranous SST2A expression was lacking, many pediatric high-grade glioma specimens in our cohort [especially those classified histologically as glioblastoma or diffuse midline glioma, H3 K27M-mutant (Figure 5E)] as well as some ependymomas (Figure 5D) exhibited non-specific, background staining—suspected to represent normal glial

processes in close proximity to malignant cells in these highly infiltrative tumors and/or endothelial and inflammatory cells in the setting of prominent vascular proliferation or necrosis, in accord with Cervera et al. (27).

Additionally, within a small group of patients with paired diagnostic and recurrent tumor specimens analyzed, SST2A expression profiles were conserved over time, including after treatment. Although interpretation is limited by the sample size and absent expression in most paired specimens (ependymomas and high-grade gliomas), these results support lack of temporal heterogeneity in tumoral SST2A, yet continued research in a larger cohort of recurrent tumors will be necessary.

The favorable prognostic impact of SST2A overexpression in gastrointestinal neuroendocrine tumors, neuroblastoma, and adult anaplastic oligodendrogliomas has been demonstrated (36, 37, 51–55), but little outcome data has been reported thus far within pediatric neuro-oncology. Remke et al. showed improved overall survival in medulloblastoma cases with high (>50% immunoreactivity) SST2A levels (significant correlation in their institutional cohort, trend in their larger validation cohort) (29). We observed a correlation between higher SST2A IHC scores with increased event-free survival and overall survival in univariate analyses when evaluated across the entire cohort, though in multivariable analyses adjusting for histolopathology, only the association with overall survival remained significant. Caution must be applied when drawing conclusions due to the relatively low number of patients with recurrent/progressive disease or death as well as significant heterogeneity of assesed histologic diagnoses and corresponding outcomes. However, these preliminary findings suggest potential prognostic significance of membranous SST2A expression within some high-risk pediatric CNS tumors that demands further investigation and corroboration in larger-scale studies.

Finally, in a subset of tumors with SST2A IHC interpreted by two pathologists (blinded to one another), moderate inter-reviewer reliability, with strong correlation in score absolute values, was demonstrated. Discordant impressions of membranous SST2A positivity were limited to cases of poor, compromised tumor tissue quality and/or diffusely positive background, non-membranous staining, indicating potential for technical and biological factors to impede interpretation; adjudication by consensus between two reviewers is likely necessary in these rare, but challenging cases. This further highlights the importance of IHC-based scoring being performed by a neuropathologist highly familiar with SST2A staining patterns, especially when results may have therapeutic implications. This is the case for the aforementioned clinical trial investigating ^{177}Lu-DOTATATE in high-risk pediatric CNS tumors (NCT05278208), which mandates central pathology review as part of eligibility screening. Additionally, SST2A IHC scoring excludes inevitable non-specific background staining commonly encountered in IHC methodology in general (73), as well as cytoplasmic positivity seen in diseased or healthy brain tissue, likely corresponding to endothelium, inflammatory cells, and/or glial processes, as described herein and in other studies (27, 74, 75). This non-membranous (and thus non-targetable) staining should not confer increased toxicity risk with ^{177}Lu-DOTATATE, but given the potential to confound IHC impressions, efforts should be taken to select an anti-SST2A antibody with superior binding affinity and minimal cross-reactivity with other antigens. The commercially available rat monoclonal anti-SST2A, UMB-1, was used in the present study because it has demonstrated more distinct membranous staining and less diffuse background staining compared to alternative agents (49, 50, 76).

This study expands our understanding of the prevalence, correlates, and therapeutic implications of membranous SST2A expression across high-risk pediatric CNS tumors. Medulloblastoma (especially non-SHH subgroups), meningioma, and some rarer embryonal tumors highly expressed SST2A, suggesting a potential role for somatostatin receptor-targeted therapy such as ^{177}Lu-DOTATATE in these aggressive diseases. Pediatric ependymomas, high-grade gliomas, ATRT, and ETMR consistently lacked membranous SST2A expression. SST2A variation within and across these histopathologic diagnoses provides valuable insight into their underlying biological and molecular heterogeneity. Taken together, these findings support utilization of membranous SST2A as a diagnostic tool, therapeutic target, and potential biomarker in some high-risk pediatric CNS tumors, which will be essential to explore in future research.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Cincinnati Children's Hospital Medical Center IRB. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

All authors contributed to study design. ML and SS analyzed all SST2A IHC cases. CF analyzed a subset of cases. ML abstracted clinical data and performed analyses, with statistical guidance from JS. All authors participated in interpreting results, writing the manuscript, and revising the manuscript critically for

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chromatin mutations in pediatric high grade gliomas

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Pediatric high grade gliomas (HGG) are lethal tumors which are currently untreatable. A number of recent studies have provided much needed insights into the mutations and mechanisms which drive oncogenesis in pediatric HGGs. It is now clear that mutations in chromatin proteins, particularly H3.3 and its associated chaperone complex (ATRX), are a hallmark feature of pediatric HGGs. We review the current literature on the normal roles of the ATRX/H3.3 complex and how these functions are disrupted by oncogenic mutations. We discuss the current clinical trials and pre-clinical models that target chromatin and DNA, and how these agents fit into the ATRX/H3.3 mutation model. As chromatin mutations are a relatively new discovery in pediatric HGGs, developing clear mechanistic insights are a key step to improving therapies for these tumors.

KEYWORDS

histone H3.3, H3.3 K27M, DMG = diffuse midline glioma, H3.3 G34R/V, ATRX, pediatric gliomas, KDM4, alternative lengthening of telomeres (ALT)

Introduction

Gliomas are the most common form of primary brain tumors and are currently lethal in both children and adults. Over the past decade, a number of large-scale genome sequencing studies have identified key mutations which drive oncogenesis in these tumors (1–4). From these studies, it has become increasingly clear that adult and pediatric gliomas are distinct biological entities with specific mutational profiles. These differences are now officially recognized in the latest 2021 WHO classification of CNS tumors (5). One of the clearest features which distinguishes pediatric from adult gliomas, are the high rates of mutations in chromatin-related proteins in pediatric tumors (6). Specifically, mutations in histone genes have been officially designated as diagnostic subgroups of pediatric-type diffuse high-grade gliomas: diffuse midline glioma, H3K27-altered; diffuse hemispheric glioma, H3 G34-mutant (5). The overwhelming majority of these histone point mutations occur on the histone variant H3.3 (83% of K27M mutations, 100% of G34R/V mutations) (4), with rare mutations in canonical histone H3.1.

In addition, H3.3 mutations in pediatric gliomas frequently occur in conjunction with inactivating mutations in ATRX (20% H3.3 K27M, >90% of H3.3 G34R) (4). ATRX is a SNF2 helicase/ATPase (7) that partners with DAXX (8, 9) to form an H3.3 chaperone complex (10, 11). Taken together, this suggests that H3.3 and its ATRX chaperone complex are core contributors to oncogenesis in pediatric gliomas. Furthermore, inactivating mutations in ATRX are also found in conjunction with point mutations in isocitrate dehydrogenase (IDH) in adult-type diffuse gliomas (>86%) (12, 13), recently designated as "astrocytoma, IDH-mutant" (5). Mutations in IDH1/2 (mIDH) generate an oncometabolite which inhibits a range of chromatin modifiers and, similar to H3.3 mutations, severely disrupt chromatin profiles. IDH mutations are most common in adolescents (14) and younger adults (<55 years) (15), possibly indicating a graded continuum between these chromatin mutations and age-of-onset. This review will focus on the current understanding of these glioma-associated chromatin mutations which affect younger age groups.

Histone H3.3

Histones are the protein component of nucleosomes, which form the basic repeated structural unit of chromosomes. Each nucleosome consists of ~146 bp of DNA wrapped around a histone octamer comprised of two units each of histone H2A, H2B, H3, and H4 (Figure 1A). The majority of nucleosomes are comprised of "canonical" histones such as histone H3.1/2 which are encoded by 13 genes in the human genome (16). Histone H3.1/2 are synthesized only during S-phase of the cell cycle and are rapidly assembled behind the DNA replication fork (16). Unlike canonical replication-dependent H3.1/2, histone variant H3.3 is expressed throughout the cell-cycle in a replication-independent manner (17) and is thought to replace histones which are displaced outside of S-phase (18).

Histone H3.3 is encoded by two genes in the genome (*H3F3A* and *H3F3B*) and differs from canonical H3.1/2 at 5/4 amino acid residues respectively at positions 31, 87, 89, 90, with an additional difference at 96 between H3.3 and H3.1. The so-called "AAIG" motif at positions 87, 89, and 90 determine the interactions with chaperone assembly complexes (11) (Figure 1B). The combination of replication-independent synthesis and specific chaperones means that H3.3 has a genome localization pattern and function, which is unique and distinct from canonical H3.1/2. As H3.1/2 are linked to DNA replication, the canonical histones are uniformly distributed across the genome in the wake of replicating DNA polymerase. In contrast, H3.3 is most often associated with the promoters of active genes where it replaces histones which have been displaced by the passage of RNA polymerase (18–20). In addition, H3.3 is also associated with unusual chromatin environments such as telomeres (10, 20, 21), ERV repeats (22, 23), and imprinted genes in mouse embryonic stem (ES) cells (22); the V_H locus which undergoes V(D)J recombination (24); primordial germ cells (25), and complete remodeling of the paternal genome post-fertilization (26, 27).

As a result, H3.3 has been associated with diverse functions including fertility, embryogenesis, maintenance of stem cell states, and execution of differentiation programs (28). It is not entire clear why H3.3 is uniquely important for maintaining or altering chromatin states but it is evident that despite the high

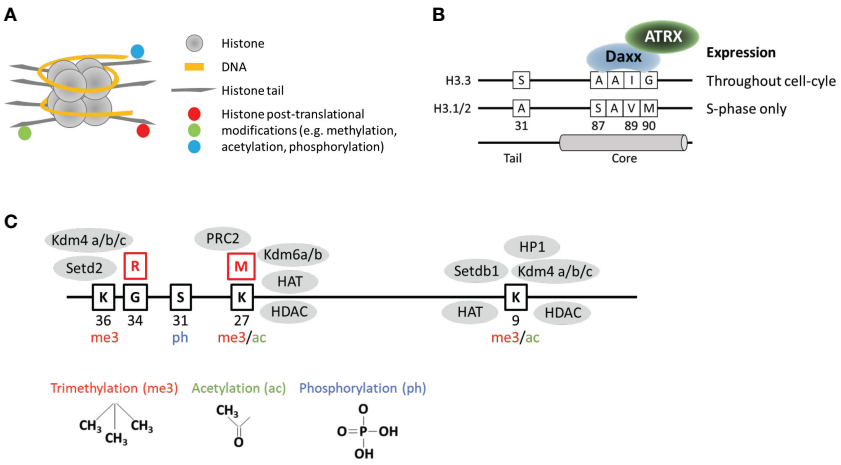

FIGURE 1
Key structural and functional features of histone H3.3. **(A)** DNA wrapped around nucleosome comprised of histones arranged into an octamer configuration with protruding tails. **(B)** Key features which distinguish histone variant H3.3 from canonical H3.1/2. **(C)** Selected amino acid residues on the H3.3 tail which are regulated by post-translational modifications. Red boxes show the position of frequently mutated residues. Grey ovals represent examples of epigenetic readers, writers, and erasers known to interact with mutated and surrounding residues.

degree of sequence similarity, H3.3 is functionally distinct from canonical H3.1/2. The unexpected discovery of oncogenic H3.3 point mutations further emphasized this difference and shifted our collective understanding of chromatin biology and associated mutations in cancer. The first studies in this area discovered heterozygous substitution mutations at position 27 (lysine) and 34 (glycine) exclusively on *H3F3A* of H3.3 (2, 3), as well as a minority on K27M (lysine to methionine) mutation on HIST1H3B (H3.1). Interestingly, the H3.3 K27M mutations appear to be distinct from H3.1 K27M counterparts as they are associated with different secondary mutations (29), age-of-onset (29), and chromatin and gene expression profiles (30–32). It is therefore highly likely that the H3.3 mutations in pediatric gliomas are functionally significant in a manner that is related to the normal endogenous functions of H3.3.

H3.3 K27M mutations

Of these histone mutations, the H3.3 K27M mutations are the most common and therefore also the most well-studied. Early studies reported H3.3 K27M mutation rates of >90% in tumors which were then classified as Diffuse Intrinsic Pontine Gliomas (DIPG). The WHO CNS tumors classification schema has since been updated and H3.3 K27M is now considered a defining feature of a class of pediatric high-grade gliomas dubbed "diffuse midline glioma, H3 K27-altered" (5). H3.3 K27M mutations most often arise in midline structures including the thalamus, pons and brainstem, with a median age of diagnosis of around 9-10 years (4). The nature and location of these tumors severely limits treatment options and the 2-year survival rate is <10%, with a median survival time of 11 months (4). The H3.1 K27M mutations are specific to the pons and occur in a younger age group (median 5 years) and are associated with a median survival time of 15 months (4).

Early studies suggested that the H3.3 K27M mutation was acting primarily through inhibition of the Polycomb Repressive Complex 2 (PRC2) (33, 34), a methyltransferase which mediates trimethylation of lysine 27 (35). The H3K27me3 modification is primarily associated with the promoters of silenced genes and is important for regulation of gene expression, particularly through differentiation (35, 36). The H3.3 K27M mutation consistently triggers the global loss of H3K27me3 and *in vitro* studies indicated that the K27M mutation was capable of binding and inhibiting PRC2 (33, 34). However, direct interactions between H3.3 K27M and PRC2 have proven difficult to detect *in vivo* (37, 38) and these proteins have distinct localization profiles (31). PRC2 primarily localizes to promoters of silenced genes (36) while histone H3.3 is primarily associated with regions of high nucleosome turnover, notably the promoters of active genes (20).

In addition, it has become increasing clear that a broad range of chromatin modifications are disrupted in the presence of this mutation. As well as reductions in H3K27me3, the K27M mutation is also associated with reduced DNA methylation (hypomethylation) across the genome (39), increased H3K27ac (38, 40, 41), reduced H3K36me2 (42, 43), and increased H3K9ac and H3K4me3 (44). As there is no consensus on proteins which interact directly with the K27M mutation, the primary chromatin alterations associated with this mutation are currently unknown. Given the conflicting studies and the diverse range of chromatin alterations, it is possible that there are multiple interacting partners which are disrupted by the K27M histone tail mutation. The H3K27 residue is important for gene regulation and is a target for both post-translational methylation and acetylation, which regulate silencing and activation respectively (45) (Figure 1C). The substitution mutation could potentially affect the activity of K27 methyl- and acetyl- transferases as well as the demethylases and deacetylases. It is also possible that chromatin readers, writers and erasers which target neighboring residues could be disrupted by the K27 substitution (Figure 1C). Furthermore, as the H3.3 K27M mutation appears to be distinct from the H3.1 mutations, there may be histone-specific interactors which have thus far been overlooked.

H3.3 G34R mutations

A second frequent histone mutation in pediatric gliomas is a substitution of the glycine residue at position 34, most often to arginine (G34R, 94%) and less frequently to valine (G34V, 6%) (4). The G34R/V mutations occur exclusively on histone H3.3 (3, 6, 46) and frequently overlap with inactivating mutations in ATRX and TP53 (90%) (4). The H3.3 G34R/V mutations are most often found in high grade gliomas localized to the cerebral hemispheres with a median age of diagnosis of 15 years and a median survival time of 17-18 months (4, 47). Additional G34 substitutions have also been reported in giant cell tumor of bone (G34W/L/R/V/M) (48) and osteosarcomas (G34W/R) (49).

Unlike the K27 residue, G34 is not a direct target for post-translational modifications but is located close to the K36 residue which is trimethylated (K36me3) (Figure 1C). H3K36me3 predominantly localizes to the bodies of active genes and is associated with elongating RNA polymerase II (50). This modification is thought to suppress cryptic initiation of transcription (51) by suppressing histone turnover within gene bodies (52). The substitution of a small glycine to a bulky arginine residue has been suggested to inhibit the activity of the H3K36 methyltransferase (SETD2) (53) and the K9/K36 demethylase (KDM4) (54) (Figure 1C). Inhibition of SETD2 reportedly occurs *in cis* and would therefore only affect the K36 residue on the mutated histone (53). In contrast, the chromatin alterations (increased H3K9me3 and H3K36me3) associated with inhibition of KDM4 were observed across the genome which is consistent with a dominant negative effect expected

from these mutations, although the specific mechanism remains unclear (54). In addition to H3K36me3 and H3K9me3, the H3.3 G34R mutations have also been associated with altered patterns of H3K27me3 (53), and DNA methylation (55). Furthermore, the G34R substitution also interferes with reader proteins such as ZMYND11 (56) and ZMNYD8 (57) which bind this region of the histone tail, and has been associated with altered splicing (58).

As for the K27M substitutions, there is no single clear unifying model to account for the primary defects and downstream effects of the H3.3 G34 substitution mutation. Indeed, the current observations suggest that G34 mutations could affect multiple chromatin pathways simultaneously, which is likely to also be the case for the K27M mutation. Given that the G34 substitutions occur exclusively on histone H3.3, it is likely that the oncogenic mechanism is specific to this variant histone. In support of this, the H3.3 G34R mutation often overlaps with inactivating mutations in ATRX which is part of an H3.3 chaperone complex.

ATRX mutations

ATRX is a chromatin remodeler which forms a complex with DAXX, an H3.3-specific chaperone, to deposit H3.3 and maintain H3K9me3 heterochromatin silencing at repetitive DNA. This complex is frequently mutated across a range of cancers, and mutations are strongly associated with activation of a telomere maintenance pathway known as Alternative Lengthening of Telomeres (ALT) (59). A recent study found around 17% of all pediatric high grade gliomas (pHGG) have inactivating mutations in ATRX (4). Of the ATRX-mutated HGGs, 33% overlap with H3.3 G34R/V and 50% overlap with H3.3 K27M mutations (4). There is no overlap between ATRX and H3.1 K27M mutations, which are instead associated with mutations in ACVR1 (4, 29, 60). These findings strongly suggest that histone H3.3 plays an important oncogenic role in pHGG.

Unlike the histone mutations, ATRX-mutated pHGGs show no particular regional or temporal specificity. Indeed, ATRX mutations extend into young adulthood and occur at high frequency in adult low grade gliomas (13, 61), as well as other cancers such as pancreatic neuroendocrine tumors (62), pediatric osteosarcomas (63), sarcomas (64–66), pheochromocytomas and paragangliomas (67, 68). These mutations are strongly associated with the ALT telomere maintenance pathway (59), most likely due to disruption of H3.3 incorporation at telomeres. Puzzlingly, patients with ATR-X syndrome who inherit germline mutations in ATRX, do not appear to have an increased risk of cancer (69). This suggests that ATRX mutations are necessary but not sufficient to activate ALT, and additional mutations are likely required.

Given that ATRX mutations frequently co-occur with H3.3 mutations in pediatric gliomas, H3.3 mutations are good candidates for potential partners in ALT activation. Consistent with this, H3.3 G34R has been reported to consistently activate ALT when combined with inactivating mutations in ATRX, TP53 and telomerase (TERT) in mouse ES cells (70). This was attributed to inhibition of the H3 K9/K36 demethylase, KDM4B, by the H3.3 G34R mutation. It appears that the loss of telomeric H3.3 (ATRX KO) combined with increased H3K9me3 through inhibition of KDM4B, results in a chromatin environment that supports the formation of ALT-associated PML bodies (APBs) which are essential for telomere maintenance (70). PML bodies are naturally occurring phase-separated nuclear condensates (71) which become abnormally large and localise to telomeres in ALT-positive cancers (72). One of the main drivers of phase-separation is heterochromatin protein 1 (HP1α) (73), a protein which binds to the H3K9me3 modification (74). It seems that inhibition of the KDM4B demethylase results in increased H3K9me3 to facilitate phase-separation and APB formation.

IDH1/2 mutations

Interestingly, point mutations in a citric-acid cycle enzyme, isocitrate dehydrogenase (IDH1/2), are also known to inhibit this family of lysine demethylases (75). IDH mutations are relatively rare (~6%) in pediatric HGGs and tend to occur in the forebrain of older patients with a median age of 17 years (4). However, IDH mutations occur at very high frequency (~80%) in adult low-grade gliomas (aLGG, WHO grade II and III) (13) and tend to be associated with younger cohorts (76). The majority (52%) of IDH-mutated aLGGs overlap with ATRX/TP53 inactivating mutations while the remainder are predominantly oligodendrogliomas which co-occur with 1p/19q co-deletion mutations (13). The high frequency overlap between IDH and ATRX mutations is reminiscent of the H3.3/ATRX mutations in pediatric high-grade gliomas, and hints at similarities between the IDH and H3.3 mutations.

The IDH1/2 enzymes catalyze the oxidative decarboxylation of isocitrate to 2-oxoglutarate/α-ketoglutarate (2-OG/α-KG), which is a key reaction in the citric acid cycle. In addition, α-KG serves as a cosubstrate for αKG-dependent dioxygenases, which include the TET family of 5-methylcytosine hydroxylases and histone lysine demethylases such as the KDM4 family of enzymes (75). Oncogenic mutations in IDH most often occur as heterozygous, dominant negative point mutations at the active site of IDH1 (R132) or IDH2 (R172) (77). These mutations convert αKG to R(-)-2-hydroxyglutarate (2HG), an oncometabolite that inhibits αKG-dependent dioxygenases, including the KDM4 family that is affected by H3.3 G34R mutations (75, 78).

Inhibition of histone lysine demethylases could therefore be a common factor which unites histone H3.3 and IDH mutations. Consistent with this, both mutations frequently co-occur with ATRX inactivation in gliomas, and both H3.3 G34R and IDH1

R132H have been shown to promote ALT when combined with inactivation of ATRX/TP53 and telomerase (70). In addition, much like the histone mutations, IDH1/2 mutated gliomas also exhibit broad disruptions in chromatin modifications including DNA methylation (79) and histone methylation (H3K9me2, H3K27me2, H3K79me2) (75), which ultimately results in a failure in differentiation (78). At present, the common theme across the H3.3/IDH mutations appears to be inhibition of demethylases, coupled to defects in H3.3 either directly or through inactivation of ATRX, leading to widespread chromatin alterations that block differentiation. Although the specific pathways which are most affected by H3.3/IDH mutations are currently under investigation, it is clear that chromatin disruption is a common feature across these gliomas. This would potentially render these cancers vulnerable to DNA damaging agents and epigenetic drugs regardless of specific targets, and a number of these agents are currently being trialled.

Clinical trials

The current management of pediatric gliomas typically includes a combination of surgical resection and radiotherapy. However, due to the location and infiltrative nature of high grade gliomas, complete resection is often not possible and treatment is usually palliative. Chemotherapy has proven ineffective for pediatric high-grade gliomas thus far. In addition to the universal issues of efficacy, selectivity and acceptable adverse side-effects, effective drugs must also be capable of crossing the blood brain barrier. A range of potential candidates are currently being trialed but clear leads or principles have yet to emerge.

Developing a clearer understanding of mutations and molecular mechanisms should provide some guidance on the best strategies to trial, with the ultimate goal of developing targeted and specific therapies. In accordance with this, there are a number of ongoing trials which attempt to target the K27M peptide specifically through a neoantigen peptide (NCT04749641) or a peptide vaccine in combination with the PD-1 inhibitor, nivolumab (NCT02960230) (80). In addition, the IDH mutations are also an attractive target and trials are somewhat more mature as the IDH mutations are more common, occurring at high frequency in adult low grade gliomas as well as acute myeloid leukemia (AML). Inhibition of mutant IDH2 with enasidenib (81) or IDH1 with ivosidenib (82) is effective at treating IDH-mutated AML providing clinical evidence that inhibition of the mutant enzyme is beneficial. Phase I trials in low grade gliomas found that ivosidenib was well tolerated and reduced tumor volume (83). Trials with vorasidenib, a mutant IDH1/2 inhibitor with improved blood brain penetration, was similarly well tolerated and showed preliminary antitumor activity (84). While IDH inhibitors have not yet been trialed specifically in IDH mutated pediatric high grade gliomas, positive outcomes in adult gliomas would likely be translated to the pediatric cohort.

In addition, there are trials underway for inhibiting EZH2 (PRC2) with tazemetostat (NCT03155620), and the polycomb protein BMI1 with PTC596 (NCT03605550) (85). Although it is not entirely clear that K27M acts through PRC2, it is clear that both the histone and IDH mutations cause widespread disruptions across the genome regardless of the specific mechanisms. Therefore, it is possible that chromatin and genome targeting agents would further exacerbate this phenotype and trigger cell death. Indeed, the two strategies which have some proven efficacy in gliomas both rely on DNA damage. Radiation is a potent DNA damaging agent and temozolomide which is used to treat adult gliomas, is an alkylating agent that damages DNA by methylating purine (guanine, adenine) bases. Although efficacy is obviously limited and treatment only extends lifespan by months, trials are currently underway to test re-radiation and combinations with other drugs including chromatin and DNA damaging agents [reviewed in (80)].

A number of chromatin, epigenetic, and DNA damaging drugs are routinely used in chemotherapy regimes across different cancers but none have proven effective as single agents in pediatric high-grade gliomas. As a result, most current trials involve testing these drugs in combinations with other agents. The most frequently used class of chromatin and epigenetic drugs are the histone deacetylase inhibitors (HDACi) which include panobinostat (NCT02717455, NCT04341311) and a nanoparticle formulation, MTX110 (NCT03566199, NCT04264143), fimepinostat (NCT02909777, NCT03893487), and vorinostat (NCT02420613, NCT01189266). Drugs that target the genome include agents such as nucleoside analogues (gemcitabine, NCT02992015), topoisomerase inhibitors (etoposide NCT04049669; irinotecan NCT01837862; and topotecan NCT03709680), and alkylating agents (temozolomide NCT03709680, NCT04049669, NCT03243461; lomustine NCT04049669; carboplatin NCT01837862). In addition, inhibition of DNA repair pathways using poly ADP-ribose polymerase (PARP) inhibitors have been hypothesized to complement IDH inhibition and DNA alkylation by blocking the break-excision repair pathway and a number of these are now in trials (BGB-290 NCT03749187; olaparib NCT03233204; veliparib NCT03581292). It will be interesting to see if any of these trials yield positive results.

Pre-clinical models

It should be noted that the majority of the compounds which are currently in clinical trials are already used in the treatment of other cancers. However, there is potential for developing entirely novel compounds with improved specificity, and this process

would be greatly expedited by pre-clinical models which accurately reflect pediatric HGGs. Pre-clinical models can be roughly divided into three groups each with their own advantages and shortcomings: patient-derived cell lines, patient-derived xenograft (PDX) animal models, and animal models with clinically-relevant endogenous mutations.

While patient-derived models should theoretically mirror individual tumors (86), it is inevitable that *in vitro* or *ex vivo* cell culture and transplantation will induce and/or select for alterations in the tumor cells relative to *in situ* counterparts. Amongst the primary concerns are tumor heterogeneity and clonal selection. It is impossible to capture the complex endogenous environment of tumors and every manipulation from cell culture to transplantation applies artificial selective pressure which alters the morphology and clonality of the patient-derived cells (87, 88). While PDX models of pediatric gliomas have been established (89, 90), these models have only undergone limited molecular characterization and it is not currently clear how well these systems reflect endogenous tumors.

One alternative to patient-derived models is the creation of engineered mouse models with clinically relevant mutations which develop equivalent tumors. Given the high frequency of H3.3 mutations in pediatric HGGs, it is very clear that these mutations are oncogenic drivers yet it seems that they are not sufficient to drive tumorigenesis in mouse models (33, 91). Constitutive expression of H3.3 K27M is embryonically lethal (92) and expression must be limited to neural lineages. Expression of H3.3 K27M in isolation does not result in tumor formation (91, 92) but adding a TP53 mutation induces HGG formation at low frequencies (91, 92). The frequency of HGG formation can be boosted with the addition of PDGFRA (91, 92) but these mutations rarely co-occur in patient tumors. The difficulties in establishing model systems has been attributed to restricted developmental stages and cell lineages which are vulnerable to H3.3 mutations (93). Further research into mutations and mechanisms may improve these mouse models in the future however it still remains to be seen if these models can accurately reflect the human tumors.

No model system can completely capture the complexity of patient tumors and all models will suffer from unavoidable pitfalls. It is therefore vitally important that multiple model systems are developed in parallel so that potential therapies can be tested across a range of systems. As with all experimental strategies, orthogonal approaches are the gold-standard for maximizing the chances of identifying efficacious agents while minimizing potential for harm.

Concluding remarks

Given that pediatric high grade gliomas have proven resistant to all interventions, any degree of improvement would be welcome at this stage. Based on the high rates of mutations in chromatin protein and the adverse effects of these mutations on the genome, it is almost certain that genome targeting agents would prove beneficial as part of combinatorial strategies. However, given the non-specific effects of these drugs and the sensitive nature of neural tissues, off-target effects are likely to pose an issue. As is true for most therapies, targeted delivery and boosting specificity will play an important role in improving overall outcomes and developing accurate pre-clinical models will greatly expedite this process. In addition, further studies into the exact molecular mechanisms behind these mutations could help to uncover pathways that can be targeted with greater specificity and efficacy.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Limitations of radiosensitization by direct telomerase inhibition to treat high-risk medulloblastoma

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Medulloblastoma (MB) is the most common malignant pediatric brain tumor. Previous studies have elucidated the genomic landscape of MB leading to the recognition of four core molecular subgroups (WNT, SHH, group 3 and group 4) with distinct clinical outcomes. Group 3 has the worst prognosis of all MB. Radiotherapy (RT) remains a major component in the treatment of poor prognosis MB but is rarely curative alone and is associated with acute and long-term toxicities. A hallmark of cancer cells is their unlimited proliferative potential which correlates closely with telomere length. The vast majority of malignant tumors activate telomerase to maintain telomere length, whereas this activity is barely detectable in most normal human somatic tissues, making telomerase inhibition a rational therapeutic target in the setting of cancer recurrence and therapy resistance. We and others have previously shown that short telomeres confer sensitivity to ionizing radiation (IR) suggesting that telomerase inhibition mediated telomere shortening will improve the efficacy of RT while minimizing its side effects. Here, we investigated the efficacy of the combination of IR with IMT, a potent telomerase inhibitor, in an *in vivo* model of group 3 MB. Our results indicate that although IMT inhibited MB telomerase activity resulting in telomere shortening and delayed tumor growth, the combination with IR did not prevent tumor recurrence and did not improve survival compared to the treatment with IR alone. Together, these findings suggest that the radiosensitization by direct telomerase inhibition is not an effective approach to treat high-risk pediatric brain tumors.

KEYWORDS

telomerase inhibition, ionizing radiation, radiosensitization, high-risk medulloblastoma, therapy

Introduction

Telomeres are the physical ends of eukaryotic linear chromosomes and, in mammals, are composed of several kilobases of tandem TTAGGG repeats that are bound by the shelterin protein complex (1). With each cell cycle, telomeres shorten until they reach a critical length that triggers cellular senescence or apoptosis. This is counteracted by the activity of the telomerase enzyme. Human telomerase consists of at least two essential components, a protein catalytic subunit (hTERT) and an RNA template (hTERC) that contribute to the synthesis of telomeric repeats, thereby renovating telomeres. Telomerase activation, a feature of the vast majority of cancers, is essential for maintaining an immortal phenotype by conferring unlimited replicative potential, whereas in most normal somatic cells, this activity is not detectable, supporting the rationale of targeting telomerase and telomeres to treat cancer (2–4). We and others have shown that telomere shortening enhances sensitivity to ionizing radiation (IR) by altering the kinetics of the DNA damage response (5, 6). We previously conducted a molecular biology and phase II study of Imetelstat (IMT), a potent inhibitor of telomerase (7, 8) to estimate inhibition of tumor telomerase activity and efficacy in children with recurrent central nervous system (CNS) malignancies (9). The regimen proved intolerable and ineffective due, at least in part, to toxicities that prevented more frequent dosing of IMT to allow sustained inhibition of telomerase and tumor burden reduction.

Medulloblastoma (MB) is the most common pediatric brain tumor in the posterior cranial fossa, accounting for approximately 25% of all brain tumors in children (10). Previous studies have elucidated the genomic landscape of MB leading to the recognition of four core molecular subgroups (WNT, SHH, group 3 and group 4) with distinct clinical outcomes (10, 11). Group 3 MB overall displays the worst prognosis. Radiotherapy is a standard treatment in older children with group 3 MB but is rarely curative alone and is associated with acute and long-term toxicities (12–14). We have previously shown that over 90% of MB patients express *hTERT* and demonstrated telomerase activity (15). High expression levels were associated with worse progression free survival and overall survival. Group 3 patients had the highest *hTERT* expression. To test the efficacy of radiation therapy while minimizing its side effects, we investigated the efficacy of the combination of IMT, as a radiosensitizer, in *in vitro* and *in vivo* models of group 3 MB telomerase-positive stem-like cells derived from high-risk group 3 pediatric MB (10) (harboring *MYC* amplification). Our results indicate that although IMT inhibited tumor telomerase activity resulting in telomere shortening and delayed tumor progression, the combination with IR did not prevent tumor recurrence and did not improve survival compared to the treatment with IR alone. These findings indicate that the direct telomerase inhibition combined with IR has limited efficacy and new approaches utilizing this quasi-universal cancer target are required to treat high-risk pediatric brain tumors. This is the first report evaluating the combination of telomerase inhibition combined with IR in high-risk group 3 medulloblastoma.

Materials and methods

Primary tumor cell culture

High-risk group-3 medulloblastoma primary patient-derived neurospheres MB004 (*TP53* mutated, *c-MYC* amplified) (16, 17) were cultured in neurosphere stem cell media as previously described (18). Briefly, cells were cultured in tumor stem media in serum-free condition consisting of DMEM/F12, Neurobasal-A, B27 (Gibco); human-basic EGF, FGF (Shenandoah biotech); and Leukemia Inhibitory Factor (Millipore). For sphere formation assay, MB004 neurospheres were dissociated by TrypLE express (Gibco), and single cells were seeded in 96-well plate in serial dilution up to single cell per well. Sphere re-growth or self-renewal was monitored by microscopy. Cells were cultured in the presence of 10% FBS to test adherence and differentiation ability.

Drug treatment

Imetelstat (GRN163L; Geron Corp.) was dissolved in 1X PBS to prepare a 282 μM stock solution for *in vitro* use, and 1 mg/mL stock for *in vivo* studies. Mismatch (MM) control oligonucleotide was prepared the same way as Imetelstat. After reconstitution, drug was aliquoted and stored in -20°C. *In vitro*, short-term treatments were conducted for 72 hours, and long-term studies were conducted for up to 6 weeks then cells were reseeded with fresh media (with or without Imetelstat). *In vivo*, Imetelstat (15 mg/kg) was intraperitoneally (i.p.) administered.

Telomerase activity assay

Telomerase activity was assayed using the TRAPeze Telomerase Detection Kit (Millipore). Cell extracts were prepared according to the manufacturer's protocol. A total of 50 to 100 ng of total protein was used to assess the telomerase activity.

Telomere restriction fragment analysis

Telomere lengths were determined by Southern blot using the TeloTAGGG Telomere Length Assay Kit (Roche Diagnostics). Genomic DNA was extracted from Imetelstat treated or untreated MB004 cells or xenograft tissue using the Gentra Puregene kit (Qiagen). 1 μg genomic DNA was digested, separated by gel electrophoresis, and transferred to a charged nylon membrane. Hybridization and detection were carried out following the manufacturer's instructions. Mean telomere length was determined by comparing the mean size or the maximum intensity of the smear relative to the molecular weight marker provided in the kit, using TeloTool version 1.3 (19). Genomic DNA with known telomere length supplied in the kit was used as positive control.

Patient-derived mouse xenograft and treatments

Six-seven weeks-old athymic Ncr-*nu/nu* female mice (J:NU (*Foxn1*^{nu}/*Foxn1*^{nu}, The Jackson Laboratory) were subcutaneously injected in single flank with 10,000 MB004 cells as previously described (18). Ten days postimplantation, Imetelstat dosing was initiated intraperitoneally at 10 mg/kg, and 15 mg/kg doses twice a week along with a vehicle control (PBS). Tumors were measured every other day by slide calipers taking two longest tumor-diameters (length and width) perpendicular to each other and volumes were calculated by using the formula: $(\pi/6) \times d^3$, where d = mean diameter. For irradiation (IR) experiments, IR doses were started when the tumors reached the volume of 500 mm^3 and were given in fractions of 2 Gy per day for five days (Monday – Friday) a week. Localized mouse irradiation device with shielding apparatus was used to deliver focal irradiation to the tumors as described elsewhere (20). All animal procedures were conducted according to our IACUC protocol (#IACUC2015-0066, CCHMC).

Immunofluorescence and immunohistochemistry

Immunostaining was performed as described previously (18, 21). For immunofluorescence, primary antibodies used were against Ki67 (Abcam), GFAP (DAKO), γH2A.X (Cell Signaling), Nestin (Millipore), and/or TRF2 (NOVUS), at 1:500 (rabbit) or 1:200 (mouse) dilutions as applicable. Corresponding secondary antibodies (Alexa-Fluor 488- or 594-conjugated donkey anti-rabbit, or anti-mouse (1:500) (Jackson ImmunoResearch) were used for 1 hour and washed with TBS (x3) before mounting with DAPI (Vector Laboratories H1200). Images were captured on Nikon Eclipse Ti confocal microscope. Formalin-fixed paraffin-embedded (FFPE) tissue sections of MB004 patient-derived xenograft were used for histopathological staining (H&E and Synaptophysin IHC). Tissues were mounted with Permount (Fisher Scientific) and imaged by Nikon eclipse 80i microscope.

Statistical analysis

Student's *t-test* or multiple-way ANOVA were applied as required, and Kaplan Meier Survival Analysis was performed using the GraphPad Prism (version 7.02). Each *in vitro* experiment was repeated at least twice. Error bars represent standard deviation from different animals considered as biological replicates. Differences were considered significant at $^*P < 0.05$. Log-rank test was applied to compare the differences in event-free survival between treated or control groups *in vivo*. Response, recurrence, or treatment failure rates, and multiple comparison for the Log-rank test were performed by the Center for Biostatistics, The Ohio State University, and as described elsewhere (22, 23).

Results

Characterization of patient-derived high-risk group 3 medulloblastoma cells

MB004 cells, derived from high-risk group 3 medulloblastoma patient were cultured in serum-free tumor stem cell media in a neurosphere culture system as described previously (18). This system allowed the selection of primary cancer stem-like cells, also known as the tumor initiating cells (TICs), representing a small sub-population of tumor, responsible for tumor growth and recurrence. We first tested the presence of cancer stem cell properties such as self-renewal, proliferation, neuronal origin, differentiation ability, and tumorigenicity. The patient-derived neurospheres, when dissociated into single cells, were able to form secondary neuropsheres demonstrating self-renewal property (Figure 1A). Further characterization of the cells detected the expression of markers of proliferation, neuronal precursor, and differentiation such as Ki67, nestin, and GFAP respectively (Figure 1B). Tumorigenic property of the primary cells was verified by the establishment of patient-derived xenograft (PDX), that retained high cellularity and evidence of neuronal origin evidenced by H&E, and synaptophysin staining respectively (Figure 1B).

Treatment of group 3 medulloblastoma patient-derived xenograft with radiation reduced tumor growth, improved survival but did not prevent recurrence and subsequent animal death

To assess IR response, subcutaneous PDX tumors were irradiated with clinically relevant focal radiation given at 2 Gy/day, 5 days/week, Monday to Friday for 5, 10, 15 and 20 days to complete the cumulative doses of 10, 20, 30 Gy, and 40 Gy respectively (Figure 1C). Although initially responding to IR, most tumors recurred. Response to radiation and overall survival (up to 30 Gy) was directly proportional to the cumulative IR doses (Figures 1D, E). The 10 Gy group showed the least response with the majority of the mice showing no tumor regression. All mice in 20 Gy group showed either some stabilization or regression in tumor growth for 2 weeks after the last fraction of 2 Gy. However, all tumors regrew, and no complete regression was observed. In both 30 Gy, and 40 Gy groups, at least 1 out of 3 mice (33%) showed complete regression with no instance of recurrence. The remaining mice (~67%) in both groups showed tumor volume stabilization or sustained regression for 2 weeks after the last dose. Of note, tumor regrowth was either observed in the field of irradiation at primary injection site or in distant locations (OOF, out of field, Figure 1D). Taken together, group 3 MB004 patient-derived neurosphere cells grown in stem-cell media represented an appropriate medulloblastoma model retaining cancer stem-cell like properties and tumorigenicity *in vitro* and *in vivo* and radiation alone did not prevent tumor recurrence and animal death.

The hallmark of telomere dysfunction is the formation of DNA damage foci localized at telomeres called TIFs (telomere dysfunction-induced foci). TIFs are focal accumulations of DNA damage response factors such as ATMS1981-P, γH2AX, and 53BP1 at dysfunctional telomeres (24). We visualized TIFs by FISH combined staining using γH2AX colocalization with a telomere-specific PNA probe. As predicted, from MB group 3, MB004 cells displayed high levels of telomerase activity which was inhibited by IMT in a dose-dependent manner (0.1 to 2.0 μM) (Figure 2A). Long-term treatments (2, 4 and 6 weeks) of MB004 cells with IMT led to sustained telomerase inhibition, telomere shortening, and subsequent telomere damage

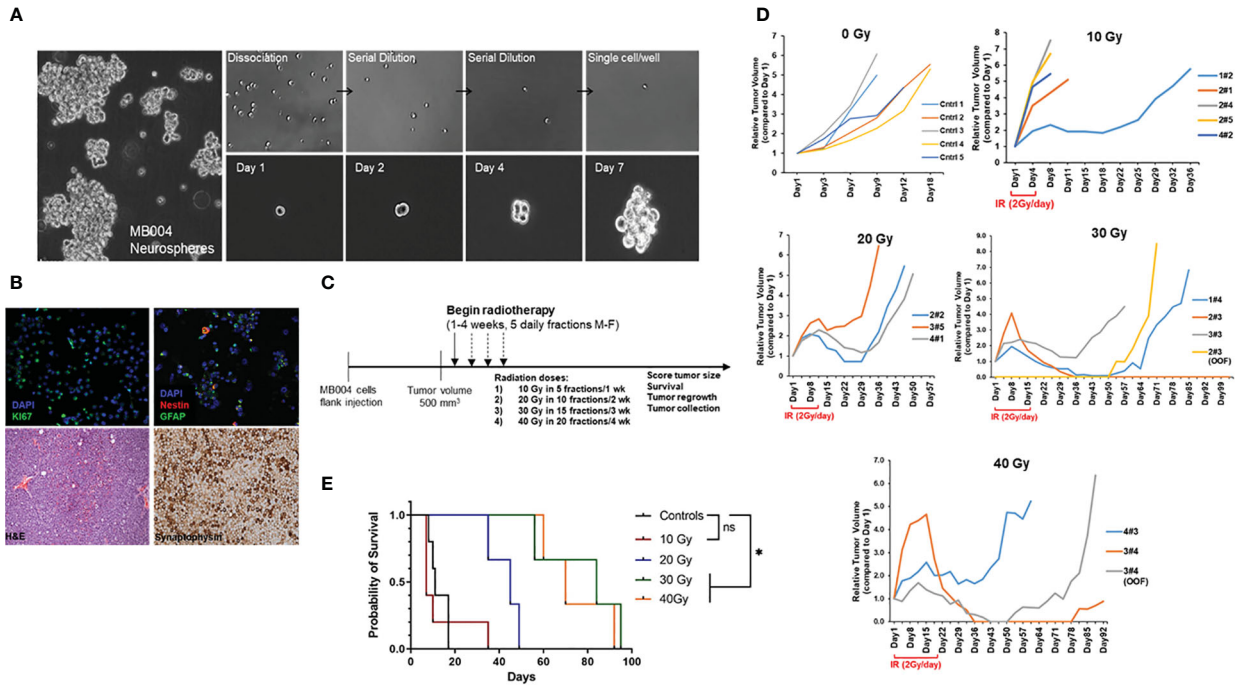

FIGURE 1
Characterization of patient-derived MB004 cells. **(A)**, images of neurospheres (left); serial dilution of dissociated single cells (right upper panel) and secondary sphere formation from a single cell cultured from day 1 to day 7 (right lower panel). **(B)**, upper panel, representative IF images of MB004 cells showing Ki67 in green (left), nestin and GFAP in red and green respectively (right). DAPI (blue) indicates nucleus staining. Lower panel, H&E (left) and synaptophysin staining (right) of MB004 patient-derived xenograft. **(C)**, scheme representing the experiment design to assess the effect of different IR doses in a flank xenograft model of MB004 cells. (M-F indicates Monday to Friday) **(D)**, tumor growth plots showing relative tumor volume (RTV) of MB004 PDX tumors untreated (0 Gy, n=5) or treated with 10 Gy (n=5), 20 Gy (n=3), 30 Gy (n=3), and 40 Gy (n=3) irradiation (IR). Duration of IR in each dosed group is indicated (2 Gy/day x 5 days per week). Each line indicates tumor growth per mouse. OOF indicates out of field of irradiation. **(E)**, survival plots of treated mice. * $p< 0.0332$; ns, not significant.

evidenced by TIFs formation (Figures 2B–D). Furthermore, treatment with IMT did not affect the ability of MB004 cells to form neurospheres and did not induce their differentiation as shown in Figure 2E when cells were cultured in 10% serum, suggesting that IMT inhibited the canonical function of telomerase and did not affect the stemness of MB004 cells. Together, these results indicate that IMT treatment inhibits telomerase activity in MB004 stem-like cells leading to telomere shortening and telomere dysfunction-induced foci (TIFs) without affecting MB004 stemness, and prolonged treatments sustain this inhibition resulting in telomere shortening.

Imetelstat delayed MB004 tumor progression, induced intratumoral telomerase inhibition and telomere shortening in patient-derived mouse xenograft model

Next, we tested the ability of IMT to inhibit telomerase activity *in vivo* in MB004 PDX and induce tumor growth inhibition. Athymic nude mice were subcutaneously injected with 10,000 MB004 cells, and IMT dosing was initiated intraperitoneally at 10 mg/kg and 15 mg/kg doses twice a week along with a vehicle control (PBS) group ten days postimplantation. Compared to the control group, IMT treatment delayed tumor growth (Figure 3A) and inhibited in tumor telomerase activity (Figure 3B) leading to a decrease in telomere length (Figure 3C).

Imetelstat treatment combined with radiation delayed tumor recurrence but did not significantly improve survival outcome compared to radiation alone

The aim of our initial experiment with radiation (Figure 1) was to determine the IR doses to be used in combination with IMT. The minimal dose that induced minor response (25% tumor volume reduction in average) and partial response (50% volume reduction in average) were used with IMT. In the combination study, prior to IR, IMT was intraperitoneally administered, 15 mg/kg twice per week for two weeks. Tumors were then irradiated with the doses that induced minor and partial response, 20 and 30 Gy respectively (Figure 1). The objective of the combination treatment was to test the ability of IMT to sensitize tumors to IR by enhancing the minor and the partial response to IR and improving survival in comparison with IR alone. Thus, lowering the IR doses to achieve a complete or better response. We distributed athymic nude female mice into 6 groups (n=10 each): (a) untreated (vehicle), (b) IMT (15mg/kg), (c) 20 Gy IR, (d) 30 Gy IR, (e) IMT (15mg/kg) + 20 Gy IR, and (f) IMT (15mg/kg) + 30 Gy IR. Following injection of the MB004 cells subcutaneously, (IMT) treatment (15mg/kg, twice a week) was started intraperitoneally at day 10 upon tumor initiation evidence. When the tumors reached 500 mm^3, the indicated radiation cumulative doses (2 Gy/day, 5 days a week) were started focally. IMT administration was continued until the last IR dose in both the

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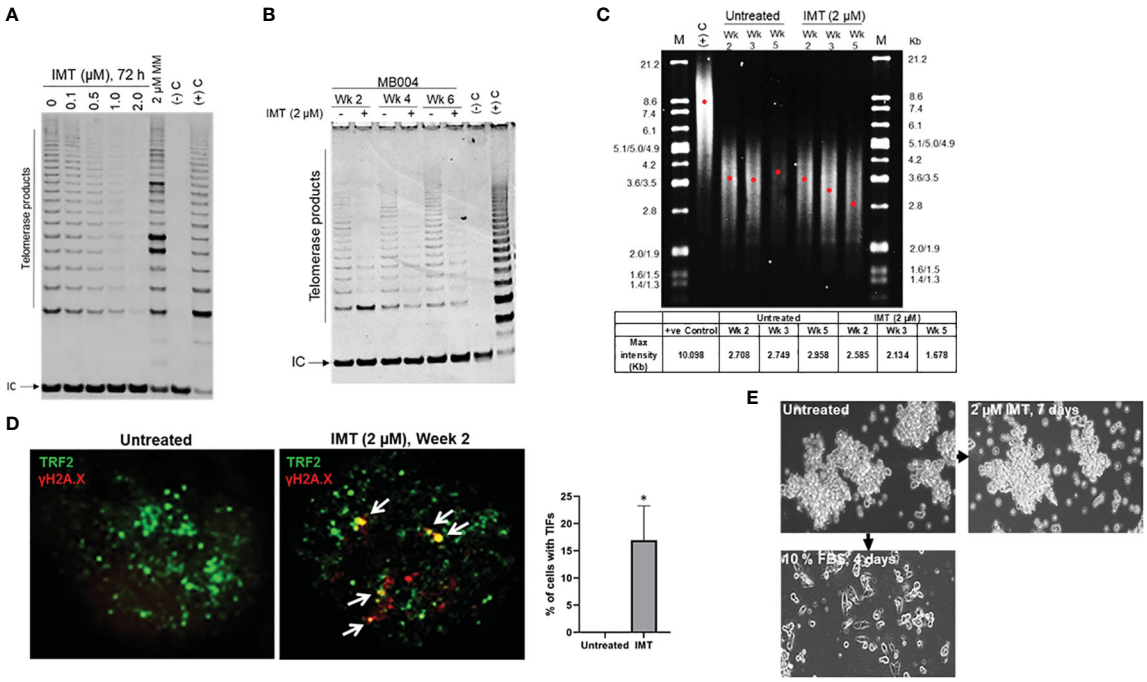

FIGURE 2
Prolonged telomerase inhibition leads to sustained telomere shortening. **(A)**, telomerase activity in MB004 cells, untreated and treated with Imetelstat (IMT) from 0.1 to 2 μM for 3 days, or with 2 μM mismatch (MM, IMT negative control) for 3 days. (-)C and (+)C, TRAP assay negative and positive controls respectively. IC, indicates internal PCR control. Telomerase products are indicated. **(B)**, TRAP assay evaluating telomerase activity in MB004 cells untreated or treated with 2 μM Imetelstat (IMT) for 2, 4, and 6 weeks. Wk, week, (-)C and (+)C, TRAP assay negative and positive controls respectively. IC, indicates internal PCR control. Telomerase products are indicated. **(C)**, telomere restriction fragment analysis of MB004 cells untreated or treated with 2 μM Imetelstat for 2, 4, and 5 weeks. along with positive (+ve) control. M, molecular weight marker with their associated sizes denoted in Kilobases (Kb). Each red dot denotes the maximum intensity of each smear in the respective lane indicative of the mean telomere length. Table (bottom) shows the maximum intensity values of the respective wells as indicated. wk, week.**(D)**, representative IF images of MB004 cells untreated and treated with 2 μM Imetelstat (IMT) for 2 weeks showing TRF2 (green, telomere marker), γH2AX (red, DNA damage marker). White Arrows indicate the colocalization of TRF2 and γH2AX, indicative of telomere dysfunction-induced foci (TIFs, yellow). The percentage of cells with TIFs were quantified. Error bars represent the SD from two different fields (10-15 cells/field). **(E)**, images of MB004 neurospheres untreated or treated with 2 μM Imetelstat (IMT) for 7 days or cultured in 10% FBS for 4 days as indicated. *p< 0.0332.

IMT and combination groups. The mice were observed for tumor growth, regression, recurrence, and survival for up to ~20 weeks after the end of treatments (Figure 4A). Overall, there was no striking difference observed between the IR only and combination groups, as the majority (90%) of the tumors in all treatment groups recurred after an initial regression (Figure 4B). The regression was slightly prolonged in IMT+20 Gy and IMT+30 Gy groups compared to the corresponding cumulative doses alone. This regression was more

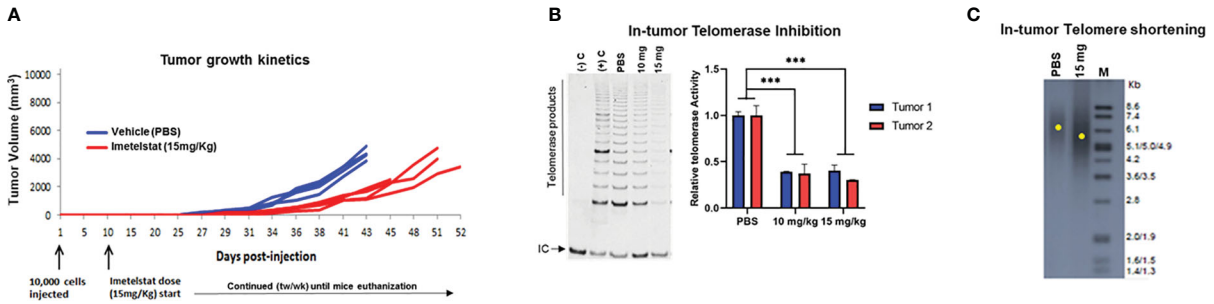

FIGURE 3
Evaluation of Imetelstat treatment in MB004 patient-derived xenograft (PDX). **(A)**, tumor growth kinetics of mice treated with PBS (vehicle) or 15mg/Kg Imetelstat. Each line denotes tumor growth per mouse. Mice were subcutaneously injected with 10,000 neurosphere cells. Imetelstat or PBS were intraperitoneally injected twice per week 10 days post-implantation for the indicated period of time. Each line denotes tumor growth per mouse. tw/wk, twice per week. **(B)**, left, representative TRAP gel image showing in-tumor telomerase activity levels in control (PBS, vehicle) and in 10 or 15 mg/Kg of Imetelstat treated MB004 PDX tumors collected at the end of the study. (-)C and (+) C are the negative and the positive controls of the TRAP assay respectively. IC, Internal PCR control; telomerase products are indicated. Right, corresponding plot showing quantitation of relative telomerase activity in two collected tumors. Error bars represent the standard deviation from two independent TRAP assays **(C)**, representative telomere restriction fragment analysis of MB004 PDX tumors treated with PBS (vehicle) or 15mg/Kg Imetelstat. M, molecular weight marker. Each yellow dot indicates the maximum intensity of each smear in the respective lane indicative of the mean telomere length. *** p< 0.0002.

pronounced in IMT+30 Gy group (Figure 4B). In IMT+30 Gy group 30% of the tumors (3 out of 10) showed a delayed recurrence compared to the 30 Gy tumors (Figure 4B). The IMT treated tumors were evaluated to confirm the in-tumor telomerase inhibition (Figure S1). Treatment with IMT and 30 Gy accentuated telomerase inhibition compared to IMT alone. Interestingly, IR treatment inhibited telomerase activity as previously shown (25, 26). When the mean tumor volumes were compared, IMT+30 Gy group showed a better regression curve and a significant delay in the recurrence (Figure 4C). However, both groups eventually reached exclusion criteria (due to tumor burden). Importantly, the survival plot did not reveal any significant improvement of survival benefit in the IMT+IR groups compared to the IR only (Figure 4D) (20 Gy vs 20 Gy + IMT, $p = 0.5685$; 30 Gy vs 30 Gy + IMT, $p = 0.5500$). Together, these findings do not support the hypothesis that the direct telomerase inhibition will sensitize cancer cells to IR hence making radiation more effective at lower doses, thus minimizing the devastating effects of the radiation therapy.

Discussion

Medulloblastoma (MB) accounts for approximately 25% of all brain tumors in children. Group 3 MB is refractory to multimodal therapy and displays the worst prognosis. Radiotherapy is a standard treatment in older patients with group 3 MB but is rarely curative and is associated with acute and long-term toxicities. Craniospinal RT, at a young age leads to devastating neurocognitive decline. Achieving a cure for children with poor-prognosis MB while minimizing radiotherapy-associated sequelae remains a major goal of pediatric neuro-oncology. Given the role played by telomerase reactivation in oncogenesis, telomeres and telomerase are relevant therapeutic targets in children with high-risk brain tumors. With the aim to improve radiation efficacy and minimizing the associated toxicities, we sought to sensitize MB tumors to radiation by using a direct telomerase inhibitor. We found here that IMT, used as a radiosensitizer, had a limited effect on tumor growth, recurrence, and survival. Similar results were observed using a murine orthotopic model of human glioblastoma (27). IMT, is the only telomerase inhibitor tested in adults and children (9, 28–30). We have conducted the first phase I and II clinical trials with IMT in children (9, 28). Our phase II clinical trial of IMT proved intolerable and ineffective in children with recurrent or refractory CNS malignancies due, at least in part, to toxicities that prevented more frequent dosing of IMT to allow sustained inhibition of telomerase (9). Specifically, that there were two deaths due to intracranial hemorrhage associated with thrombocytopenia. Importantly, IMT treatment led to in-tumor inhibition of telomerase activity, indicating that IMT crosses the blood–brain barrier. Targeting telomerase directly, would result in a significant lag period from the initiation of treatment until telomeres shortened sufficiently to reduce tumor burden, while stopping therapy with IMT would lead to rapid telomere regrowth. Therefore, for these reasons and based on our present findings, we do not recommend direct telomerase inhibition as a radiosensitization approach to treat high-risk pediatric brain tumors. We have tested a new approach of telomere targeting strategy consisting of the incorporation of 6-thio-2'-deoxyguanosine (6-thio-dG), a telomerase substrate precursor nucleoside analogue, into telomeres by telomerase (31). Because this

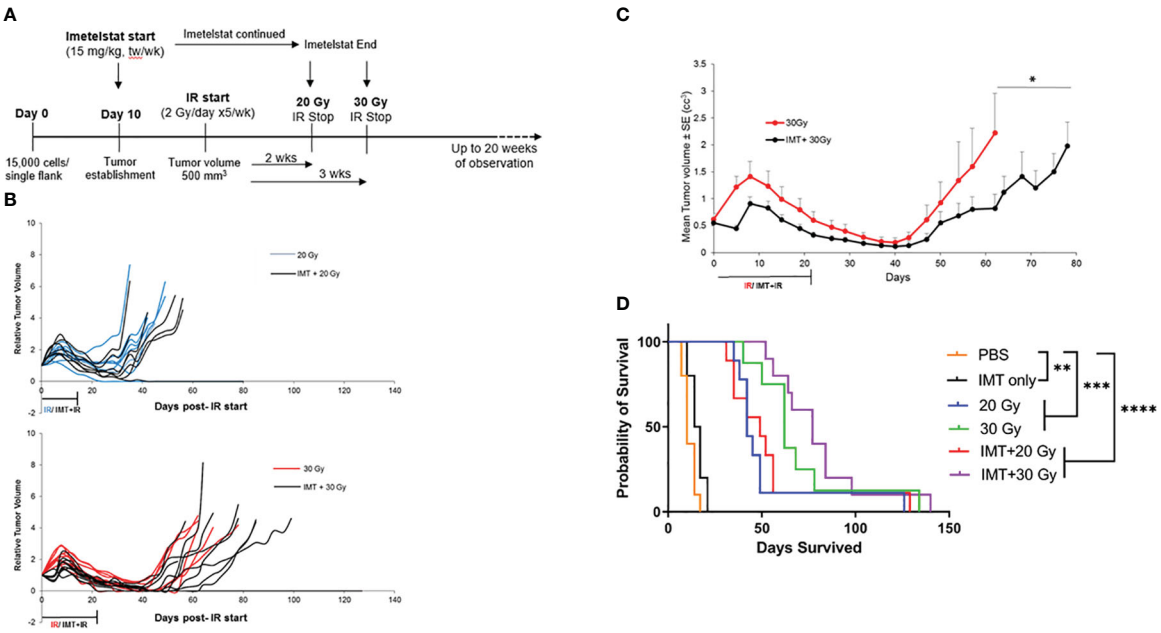

FIGURE 4
Limited effect of Imetelstat treatment combined with radiation in MB004 patient-derived xenografts. **(A)**, schematic diagram summarizing the workflow of combination treatment with Imetelstat (15mg/Kg) and IR (20Gy and 30Gy). tw/wk, twice per week. **(B)**, Relative tumor volumes (RTV) of MB004 PDX tumors treated with 20 Gy IR (blue) versus Imetelstat (IMT) + 20Gy IR (black) (upper plot); and 30Gy (red) versus IMT + 30Gy IR (black) (lower plot). Each line indicates tumor growth per mouse. **(C)**, Average tumor growth kinetics of 30 Gy IR only (red) and IR (30 Gy) + IMT (black) treated mice. The duration of IR or IMT + IR treatments are indicated. *P*-value is indicated, * $P<0.05$. Error bars represent the standard error mean between the tumor sizes from all the mice in their respective treatment groups, collected at each timepoint. **(D)**, corresponding survival plot of mice treated with vehicle (PBS), IMT, IR (20Gy or 30Gy), and IMT + IR (20Gy or 30Gy). * $p< 0.0332$; ** $p< 0.0021$; *** $p< 0.0002$, **** $p< 0.0001$.

effect appears to be telomere length independent, the prediction using this novel approach is that treatment with 6-thio-dG will require a shorter time period to achieve a rapid effect on tumor growth and progression than direct telomerase inhibition-based therapy (32).We recently tested the *in vitro* and *in vivo* effect of 6-thio-dG in telomerase-positive stem-like cells derived from poor-prognosis pediatric brain tumors (18). Treatment with 6-thio-dG induced persistent telomere dysfunction and cell death within days in all telomerase-positive cell lines tested. Furthermore, 6-thio-dG crossed the blood–brain barrier and could specifically targeted tumor cells in an orthotopic mouse model of diffuse intrinsic pontine glioma, another deadly tumor in children. Our findings documented that 6-thio-dG is a promising novel approach to treat therapy-resistant pediatric brain tumors and provided a rationale for 6-thio-dG testing as a single agent or in combination with radiotherapy.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by IACUC2015-0066, Cincinnati Children's Hospital Medical Center.

Author contributions

Conception and design: RD. Development of methodology: SS, RD. Acquisition of data: SS, SK, MS, KB. Analysis and interpretation of data: SS, SK, KB, XM, RD. Writing, review, and/or revision of the manuscript: SS, SK, RD. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): SS, SK, MS, XM, RD. Study supervision: RD. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1104670/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Effect of IR and IMT, and IR on in-tumor telomerase activity. Quantification of telomerase products were normalized with the internal control (IC) using Image Studio Lite (LI-COR Biosciences) and represented as bar graphs in arbitrary units (AU). Error bars represent the SD between different collected tumor samples (n=3-7 for each treatment arm). Multiple comparisons were conducted using One-way ANOVA and corrected using the Tukey method. * $p< 0.0332$; ** $p< 0.0021$; *** $p< 0.0002$, **** $p< 0.0001$.

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Addressing blood-brain-tumor-barrier heterogeneity in pediatric brain tumors with innovative preclinical models

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Brain tumors represent the leading cause of disease-related mortality and morbidity in children, with effective treatments urgently required. One factor limiting the effectiveness of systemic therapy is the blood-brain-barrier (BBB), which limits the brain penetration of many anticancer drugs. BBB integrity is often compromised in tumors, referred to as the blood-brain-tumor-barrier (BBTB), and the impact of a compromised BBTB on the therapeutic sensitivity of brain tumors has been clearly shown for a few selected agents. However, the heterogeneity of barrier alteration observed within a single tumor and across distinct pediatric tumor types represents an additional challenge. Herein, we discuss what is known regarding the heterogeneity of tumor-associated vasculature in pediatric brain tumors. We discuss innovative and complementary preclinical model systems that will facilitate real-time functional analyses of BBTB for all pediatric brain tumor types. We believe a broader use of these preclinical models will enable us to develop a greater understanding of the processes underlying tumor-associated vasculature formation and ultimately more efficacious treatment options.

KEYWORDS

medulloblastoma, pediatric brain tumor, blood brain barrier, neurovascular unit, zebrafish, endothelial cells, microfluidics

Introduction

The BBB is established through physical and functional interactions of different cell types, referred to as the neurovascular unit (NVU) (1–3), including non-fenestrated endothelial cells (ECs), pericytes, astrocytes and microglia (2–4). In addition to these cellular components, the BBB is further supported by a specialized extracellular matrix (ECM) (5). Here we focus on the structure and function of these BBB components, highlighting complexities within tumor vasculature of pediatric brain cancer and advances in innovative vasculature modelling. For detail into physiological structure and function of NVU components we refer to Table 1. In the context of brain tumors, the BBB is commonly referred to as the blood-brain tumor barrier (BBTB) (3) and is generally thought to be more permeable and 'leaky' (3, 35) than the BBB under

TABLE 1 Structure and function of neurovascular unit components.

	Structure	Function
Endothelial Cells	• Continuous monolayer of ECs that are tightly connected *via* transmembrane tight junction proteins (3, 6, 7) • ↑ Mfsda2 expression, ↓ transcytosis (8–10) • Lack desmosomes and fenestrae (6, 10)	• Facilitates bi-directional transport of substances between brain parenchyma and blood (9, 10) • Transport mediated *via* (6, 10–12): ○ Paracellular diffusion ○ Carrier and receptor mediated transcytosis (9, 11) • Secrete PDGF-B to recruit pericyte anchorage (10, 13)
Astrocytes	• Endfeet processes encapsulate all CNS capillaries and arterioles (3, 10) • Fine processes extend to synapses, nerve cell bodies, and nodes of Ranvier (7, 14, 15) • Astrocyte to astrocyte connection and communication *via* gap junctions (5, 14) • Two types (14–16): ○ protoplasmic astrocytes: uniform distribution within grey matter • Complex cells that envelope synapses and microvasculature ○ Fibrous astrocytes: distributed along white matter tracts • Contact nodes of Ranvier and Oligodendroglia	• Facilitate bi-directional signalling between ECs and neurons controlling blood flow and neural activity (3, 14, 17) • Regulator of ion and water homeostasis (3, 10, 14, 15) • Phagocytic functions: clearing synaptic debris and protein aggregates (15) • Promotes and maintains BBB integrity • Regulator of immune cell entry in the brain (17–19): ○ physiological (restrict) ○ pathological (promote)
Pericytes	• Envelop capillaries, highly abundant in the CNS (7, 10) • Connect with ECs *via* tight, gap and adherens junctions at peg-socket contacts (20, 21) • Physiologically static, can remodel upon loss of neighbouring pericytes (7)	• Promote and maintain angiogenesis *via* crosstalk with ECs (10, 21, 22) • Regulates expression of tight and adherens junction proteins in ECs, thereby controlling BBB permeability (22) • Regulate capillary blood flow *via* neuronal coupling (23, 24) • Redirect and modulate polarisation of astrocyte endfeet on capillary wall (13, 21)
Microglia	• Resident immune cells of the CNS (25, 26) • Symmetrical extension and retraction of processes allows for surveying of the environment during physiological conditions (27, 28) • Little turnover during physiological conditions, remain quiescent (18, 26) • Activation of resting microglia gives rise to M1 (pro-inflammatory) and M2 (anti-inflammatory) microglial (18)	• Survey and respond to pathophysiological stressors within the brain microenvironment to maintain homeostasis (18, 25, 28) • Mediate tissue repair, activation of inflammation, and neuronal degradation/repair (18, 28) • Rapidly respond to threats in pathological conditions, indicative of morphological changes and chemokine release (28, 29) • Secrete cytokines to upregulate EC adhesion molecules and activate an immune response (25)
Basement Membrane	• Highly organised protein sheet comprised of extracellular matrix proteins (5, 30–32) • Two types of BM in the brain: endothelial BM and parenchymal BM (5, 31, 33) • Highly dynamic structure (30, 31) • Two main families of ECM receptors that aid in cell-cell and cell-matrix connections: dystroglycans and integrins (5, 33)	• Provides structural support, cell anchoring and signalling transduction between cells (5, 30, 33) • Physical barrier restricting paracellular transport of cells and larger molecules and proteins (including infiltrating leukocytes) (5) • Mediates tissue shape and cell polarity (34)

normal physiological conditions. Leakiness is generally considered to be a consequence of cancer cells disrupting the function or distribution of cells that make up the NVU (36, 37). This relationship is displayed in Figure 1. Clinically, BBB dysfunction in brain tumors is assessed by contrast enhanced magnetic resonance imaging (CE-MRI) using a gadolinium-based contrast agent (38). Studies over the past decade have highlighted that not all pediatric brain tumors alter BBTB similarly. Instead, BBTB function is heterogenous between tumor types as well as within individual tumors (39–42). Understanding BBTB heterogeneity will allow the development of more targeted and effective treatments of distinct tumors. Here we discuss observations made in the most common and also most lethal malignant pediatric brain tumors, Medulloblastoma (MB) and Diffuse Intrinsic Pontine Glioma (DIPG).

Recent findings highlighting the heterogeneity of the BBTB integrity in medulloblastoma

Medulloblastoma (MB) is the most common type of malignant pediatric embryonal tumor that forms in the cerebellum (43). Large scale genomics studies have defined MB into four major subgroups, namely Wingless (WNT), Sonic Hedgehog (SHH), Group 3 (Gp3) and Group 4 (Gp4). These subgroups have been further subdivided into a total of 13 subtypes with distinct molecular and clinical features (43–46). Standard treatment for children greater than three years of age includes surgery, radiation to the cranio-spinal axis and adjuvant chemotherapy (43, 44). WNT-driven MB displays the most favorable prognosis among the four subgroups (43, 44), in part attributed to their robust therapeutic response. CE-MRI studies clearly show variable BBTB integrity across MB subgroups. Solid enhancement indicative of increased BBTB permeability was observed in WNT MB. Heterogeneous contrast enhancement was observed in patients with SHH and Gp3 MB, indicating variable BBTB permeability among regions of the same tumor. Minimal or non-enhancing tumors with an entirely intact BBTB were characteristic of Gp4 (47–49).

Elegant preclinical studies are consistent with clinical observations, with heterogeneous BBTB permeability observed in various widely used preclinical MB mouse models. High-resolution dynamic CE-MRI analyses was recently used to evaluate the integrity and permeability of BBTB in a murine genetically engineered mouse model (GEMM) of SHH MB and patient-derived orthotopic xenograft (PDOX) models from SHH and Gp3 MB (39). BBTB integrity was highly variable in preclinical models of MB, with heterogeneous contrast enhancement

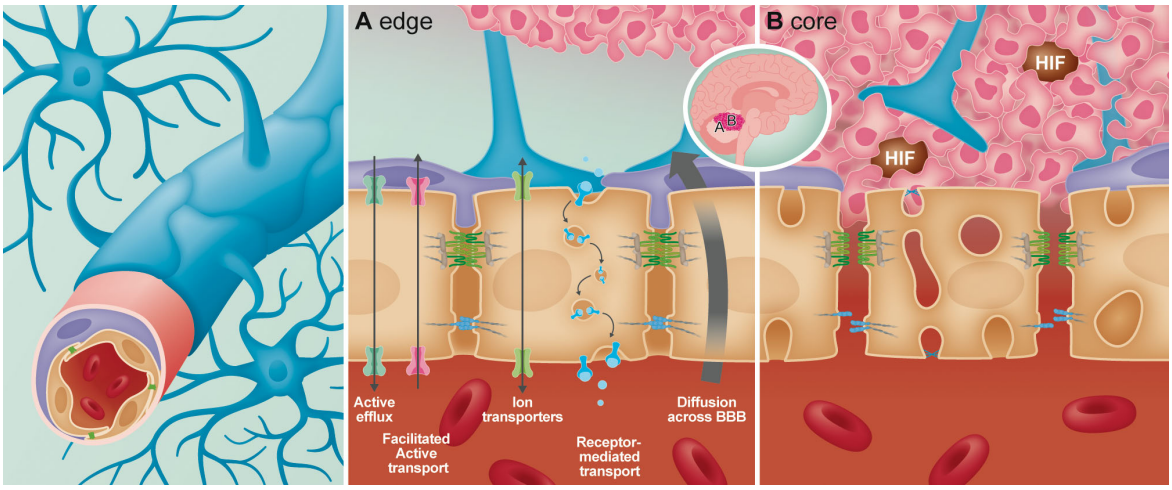

FIGURE 1
Overview of the structural and anatomical position of the cellular components forming the neurovascular unit (NVU) of the blood-brain-barrier (BBB). In the BBB the endothelial cells act as the interface between the circulating blood and the brain parenchyma. Interlocked with the endothelial cells *via* "peg-and-socket" connections are the pericytes which aid in maintaining and promoting BBB integrity. Additional components of the NVU are astrocytic endfeet that encapsulate all CNS capillaries for maintenance of the BBB. In the presence of a tumor, heterogeneity of the BBB from the edge **(A)** to the core **(B)** has been identified. **(A)** At the edge of the tumor, the BBB remains intact, with unperturbed transport across the endothelial cells (paracellular and carrier/receptor mediated transcytosis). At the core of the tumor **(B)**, permeability of the BBB increases due to loss of astrocytes and pericyte coverage. In the endothelial cells, junctional integrity diminishes *via* loss of tight and adherens junctional proteins, while endothelial fenestrations increase leading to enhanced permeability.

observed in both SHH and Gp3 PDOX MB. The invasive front of SHH PDOX tumors displayed minimal contrast enhancement, indicating clear differences in vascular integrity where tumor tissue meets the brain parenchyma. A completely intact functional BBTB was observed in SHH MB tumors initiated in an independent GEMMs despite significant tumor burden (39, 50), consistent with findings from an additional GEMM model of SHH MB (50). Together, these findings indicate that distinct biological processes govern tumor vascularization in tumors that initiate endogenously (GEMM tumors) compared to ectopic tumor cell engraftment in an adult host mouse. Recently, lineage tracing studies performed in a GEMM of SHH MB showed that Sox2-positive MB cells extend protrusions to directly ensheathe nearby capillaries, similar to astrocytes endfeet, contributing to a more intact BBTB formation and function (51). These Sox2-positive MB cells that construct the BBTB were shown to be mechanoresponsive, with Piezo2-mediated signaling regulating both the state of Sox2-positive MB cells and the BBTB. Knockout of Piezo2 resulted in a compromised BBTB, as shown by intratumoural accumulation of systemically administered 1kDa Cadaverine and 70kDa Dextran (51), and extended survival in response to etoposide treatment compared to tumor-bearing controls. Another study systemically administered 70kDa tetramethylrhodamine (TMR)-dextran to demonstrate that a GEMM model of WNT MB lack a functional BBTB (50), consistent with the solid enhancement observed in patients with WNT MB. Increased BBTB permeability has been suggested to dictate the improved therapeutic response of WNT MB (52). Indeed, functional studies have shown that tumors with a permeable BBTB from a genetically engineered mouse model (GEMM) of WNT MB responded to vincristine, which does not penetrate well into normal brain tissue (52). Disruption of WNT signaling restored BBTB function, blocked delivery of vincristine to the tumor and rendering them resistant to therapy *in vivo*. Together these studies point to the influence of tumor genotype on the development of an intact BBTB and reinforce that *in vivo* preclinical models of MB faithfully recapitulate the intratumoral and intertumoral heterogeneity of the BBTB phenotype of primary MB.

Various alterations in a number of components of the NVU is likely contributing to BBTB dysfunction in MB. The degree of BBTB permeability observed by MRI was shown to be correlated to differences in the structural and subcellular features of tumor-associated vasculature (39). Intact tumor-associated vasculature of the GEMM model of SHH MB displayed organized, linear expression of junctional markers CD31 and CLDN5, outlining a continuous vessel structure with extensive astrocytic encircling (39, 51). Basement membrane components, pericyte coverage and tight junction proteins were significantly reduced in this model upon genetic deletion of Piezo2, leading to increased leakage of fluorescent dyes into the brain parenchyma (51). In SHH and Gp3 PDOX models, the BBTB is compromised with abnormal barrier features such as disorganized endothelial cell-cell junctions, minimal astrocyte coverage and the presence of fenestrated, immature endothelium as determined by the expression of fenestrae marker Plvap (53, 54), and the loss of Glucose transporter 1 (Glut1) (53). Similar defects were observed in tumor-associated vasculature of PDOX and GEMM WNT MB, with hemorrhagic, aberrant vascular networks displaying a non-BBB immunophenotype characterized by the ectopic expression of Plvap and the loss Glut1 (52). Transmission and scanning electron microscopy confirmed fenestrated pores connecting the luminal and abluminal compartments of endothelium of tumors from GEMM WNT MB, with disruption of endothelial tight junctions also confirmed (52). These vascular changes were not observed in tumor endothelium from GEMM SHH MB. Transcriptomic analysis also confirmed that this endothelium was more similar to peripheral endothelium with the down-regulation of endothelial tight junctional

protein Cldn5 and Glut1, while endothelium from GEMM SHH MB was very similar to normal brain endothelium (52). Decreased pericyte coverage was also observed in tumors from GEMM WNT MB compared to normal coverage in GEMM SHH MB (52).

Whilst the above-mentioned studies have begun to explore functional differences in BBTB integrity and the structural and subcellular features of tumor-associated vasculature in MB, very little is known regarding the processes driving tumor vascularization. Several mechanisms of tumor vascularization have been defined including sprouting angiogenesis, intussusceptive angiogenesis, vessel co-option, vasculogenic mimicry and lymphangiogenesis (55). Histological analysis of brain tumor sections implies a role for angiogenesis and vascular mimicry in GEMM and PDOX tumor sections of SHH and Gp3 MB (39), with an earlier study identifying elevated Vascular endothelial growth factor (*VEGF*), a principal angiogenic factor, in cell line xenograft Gp3 models and Gp3 MB patients (56). Given vasculature architecture has been shown to influence therapeutic response (52, 57), further characterization of tumor vasculature and the processes driving this in MB is necessary to ensure effective treatment of this disease.

An intact BBTB represents a major hurdle in the treatment of Diffuse Midline Glioma

Diffuse Intrinsic Pontine Glioma (DIPG), more recently termed Diffuse Midline Glioma (DMG) (58), is a highly aggressive, lethal pediatric brain tumor that grows diffusely throughout the brainstem (59, 60). Surgical options are limited for DMG patients, largely due to the location of the tumors. Chemotherapy or other targeted therapies have not been shown to significantly improve survival rates for DMG patients (60, 61). One proposed explanation for the failure of systemically delivered therapies in DMG is due to the intact nature of the BBTB (59, 62, 63), as evidenced by the failure of contrast-enhancing agents to penetrate tumor tissue. Histological and molecular analyses of primary DMG, PDOX and *in utero* electroporation (IUE) mouse models, all revealed a minimal change in vascular phenotype within tumors compared to normal brain (58). The ECs displayed continuous expression junctional proteins CLDN5 and CD31, normal expression of the transporter Glut1 and did not express the pathological marker, Plvap. Extensive coverage by pericytes was also revealed and administration of 10kDa TMR-dextran in the IUE model, showing limited leakage. Together, these findings suggest that blood vessels are unaffected by the presence of DMG cells, possibly explaining why systemic therapy is not efficacious for this disease. Instead, novel delivery technologies such as small lipophilic drugs designed to cross an intact BBTB with minimal active efflux, are likely to be more successful. Another emerging technology is MRI-guided focused ultrasound (MRIg-FUS) that induces transient openings of the BBB by acoustic activation of circulating microbubbles. This technique has been shown to disrupt the BBB in a controllable manner in both animal models and in patients with high grade gliomas (64, 65). More recently, a similar approach was applied to target blood vessels in a PDOX model for DMG, revealing a significant increase in intra-tumoral doxorubicin concentrations and reduced tumor volume through MRIg-FUS (66).

The studies described here have begun to unravel the complexity of BBTB in MB and DMG and the relevance of modifying BBTB function for more effective, targeted treatment. However, before we can do this, a better understanding of the processes underlying tumor vascularization and the tumor-specific changes in NVU composition and BBTB function are urgently required. Next, we describe a range of innovative preclinical models that can be utilized to accelerate this understudied aspect of pediatric brain tumor biology.

Innovative preclinical models to interrogate pediatric brain tumor vasculature

Preclinical mouse PDOX and GEMM models of MB and DMG are widely used as the gold standard for preclinical testing of novel therapeutics (67–69). Studies utilizing these models clearly show that a greater understanding of how tumor cells interact with each other, and their surrounding microenvironment is urgently needed. Further to this, we need to better understand how tumors orchestrate structural and functional changes in their associated vasculature before we can develop effective, targeted therapies for pediatric brain tumors. Such mouse models have further uncovered the important roles for astrocytes (70), pericytes (13, 71, 72) and microglial (73) cells for BBB development and integrity. However, the complex interplay of the NVU in the context of tumor progression and therapy response remains to be determined.

In murine models, non-invasive techniques such as positron emission tomography (PET), computed tomography (CT), and more commonly MRI, have uncovered changes in the BBTB (74). However, longitudinal dynamic high-resolution imaging is required to understand the interactions between the developing tumor and its associated microenvironment. Live imaging technologies have been developed for rodent brains (75, 76), however these approaches are incredibly costly and present with a number of technical challenges due to the location of MB and DMG within the cerebellum and the pons of the brain stem respectively.

Dynamic modelling of tumor-vessel interactions in zebrafish

A vertebrate model that is rapidly developing as a robust model for cancer research is the zebrafish (77–81). A major reason for the expansion of zebrafish studies is the cost-effectiveness of the model due to the large number of offspring that can be obtained from a single mating and lower husbandry costs associated with zebrafish housing. Another great advantage is that zebrafish embryos and larvae are optically transparent and develop *ex utero*, allowing live visualization of organs and cells during zebrafish development. Although structural similarities of zebrafish and human proteins remain to be determined, whole genome sequencing has identified that approximately 80% of disease associated genes in humans are conserved in zebrafish (82). This finding combined with the ease of

genetic manipulation in zebrafish (83–85), led to the development of genetically engineered zebrafish models for a range of cancer types (86). In addition to genetic models, xenografting approaches are also widely applied to study cancer biology in zebrafish. Since the adaptive immune system in zebrafish is fully functional from 28 days post fertilization (dpf) (87, 88), mouse or human cancer cells are tolerated without inflicting an immune response prior to this stage.

The brain is challenging to image in higher vertebrates due to the presence of a thick skull, zebrafish brains are much more accessible and thus researchers can visualize and monitor tumor cell behavior in space and time. The anatomy of zebrafish brains is comparable to that of mammalian brains, with significant homology in molecular signatures and structure of distinct brain regions (89). Notable differences however do exist in terms of size and organization of distinct brain regions (90–92). In terms of the BBB, live imaging of zebrafish vascular transgenic marker lines has been applied to establish that the BBB in zebrafish begins to form at three days post-fertilization (dpf) and is fully functional at 10 dpf (93, 94). The zebrafish NVU is made up of endothelial cells, pericytes and radial glial cells (95). The radial glial cells express key astrocytic markers including Gfap, glutamine synthase and Aqp4 (95) and therefore are considered to perform orthologous roles to astrocytes (95). Transgenic marker lines, labelling distinct cell types of the NVU have been developed (96–99), allowing live visualization of the morphology, abundance, and dynamic behavior of distinct NVU cell types simultaneously.

In the context of brain cancer, zebrafish provide a powerful model to monitor dynamic interactions between tumor cells and NVU cell types, therefore determining what pathological changes might be contributing to BBTB malfunction. To date a small set of genetic zebrafish models have been established to study pediatric brain tumors (100–102). One such model that recapitulates central nervous system primitive neuroectodermal tumors (CNS-PNETs) was developed by Solin and colleagues (102), whereby TALEN-mediated genome editing was applied to inactivate retinoblastoma1 (*rb1*). When placed on a *p53* null background, these *rb1* knockout fish developed malformations of the skull and lesions on the eye. Histological analysis of the lesions revealed that the majority resembled CNS-PNET tumors and others were glial-like (102). Others have tested whether oligoneural precursor cells (OPCs) could give rise to CNS-PNETs by overactivated NRAS/MAPK signaling exclusively in these *sox10* expressing cells (101). This resulted in the development of large lesions in 6-week old zebrafish with conserved genetic and histologic hallmarks. Since hundreds of zebrafish embryos can be derived from a single paired mating it is highly suited for drug screening, especially when screening water soluble compounds that can be added to the zebrafish water. The authors utilized a screening approach to show that addition of MEK inhibitors to the fish water could effectively inhibit the growth of CNS-PNETs upon orthotopic xenografting (101). Human derived pediatric brain tumor cells have not been studied using zebrafish xenografting, however, tumor cells derived from adult brain cancers have been grafted successfully showing that human cells can utilize the zebrafish brain microenvironment to proliferate and migrate from the initial injection site (87). One challenge to utilizing zebrafish for xenografting is the optimal physiological core temperature of 28°C. However, zebrafish can adapt to changes in temperature. Previous studies have successfully grown zebrafish at temperatures of up to 36° C for xenografting purposes without notable side effects (103–106). Whether changes in temperature might invoke more subtle changes in the biological response to xenografted cells remains to be determined (107, 108).

Nevertheless, we propose that with well-established methods in place to visualize NVU cell types in the zebrafish brain (96–99) and determine BBB permeability (95), the zebrafish model is perfectly positioned to enable in depth studies that will generate new knowledge into the fundamental aspects of pediatric tumor heterogeneity, drug responses, metastatic potential, and alteration of the microenvironment.

Microfluidic tumor-vessel co-culture models

Traditional *in vitro* cell culture methods have been used in basic research for many years to study mechanisms of cancer cell growth and evaluate drug efficacy. Various pediatric brain cancer cell lines are currently commercially available, with the most widely published MB models such as D283MED, D341MED, D425MED, UW228-2 and DAOY propagated *in vitro* for decades. As seen with other widely utilised brain cancer cell lines (109), it is increasingly likely that the original molecular features and biological behaviour of tumor cells would have been lost, failing to recapitulate tumour heterogeneity. Additionally, it is well appreciated that these simplistic 2D cell culture systems do not model the spatial, cellular and chemical complexity of tumors and the associated TME (110), limiting the translational utility of these model systems.

3D spheroid models are being increasingly developed for a variety of pediatric brain cancers (111, 112) replicating elements of the tumor microenvironment such as a gradient distribution of nutrients, oxygen, pH, cell-cell and cell- extracellular (ECM) contact (113). 3D tumor spheres derived from a variety of pediatric brain cancers including PDOX models of Gp3 MB (114) and primary biopsy material from MB, Ependymoma, Glioblastoma and Astrocytoma patients (111) have been recently established. Whilst pediatric 3D tumor spheroid cultures represent an important advance for the field, they still lack several essential components of the brain specific TME such as the ECM and the diverse non-cancerous cells including endothelial cells (ECs), pericytes, fibroblasts, immune cells, astrocytes, neurons and microglia (115, 116). Advances in cellular engineering, biomaterials and biofabrication technologies have led to the development of co-culture platforms whereby 3D tumor spheroids can be grown in the context of blood vessels and biologically relevant ECM hydrogels (117). To model the brain endothelium specifically, distinct types of brain ECs have been employed to form so called 3D BBB models. Of particular interest are recent protocols that utilize induced pluripotent stem cell (iPSC) derived brain microvascular ECs (iBMECs) (118–122). This is because these iBMECs when grown with other NVU cell types have been shown to form a tight BBB with physiologically relevant barrier properties as measured by transendothelial electrical resistance (TEER) and extravasation of fluorescent dyes (118–122). The validation of structural and functional features of an intact BBB supports the utility of these models to monitor how tumor cells alter

the BBB and how this impacts the efficacy of therapeutics. Further details on the overall benefits and drawbacks of BBB culture have been reviewed elsewhere (123–125).

Although 3D BBB-tumor co-culture models have not yet been implemented to study tumor spheroids derived from pediatric brain cancer, data from adult brain tumors supports the feasibility of this approach (126, 127). For glioblastoma, tumor spheres were grown alongside an iPSC-derived BBB to test combination therapies. Vincristine and doxorubicin, two anti-cancer drugs that do not cross the BBB, were added to the 3D vessel in combination with mannitol and gintonin, to temporally open the BBB (128). Drug uptake was significantly improved within the 3D glioblastoma spheroid in combinations with mannitol and gintonin addition which induces BBB permeability (128). With platforms and applications of organ-on-a-chip models now widely accepted for a variety of diseases (129–134), it is imperative that these models are adapted and transitioned for the study of pediatric brain cancer. These innovative *in vitro* platforms in conjunction with animal models will be highly important to better understand the molecular mechanism of such ailments and providing novel therapeutics that have been thoroughly tested to target tumors in the presence of a heterogeneous BBTB.

Discussion

Ineffective drug delivery is thought to be a contributing factor underlying the failure of novel therapeutic strategies in early phase clinical studies after demonstrating significant preclinical anti-tumor efficacy. Understanding the plasticity of the BBTB by utilizing complementary pre-clinical models will help to overcome one of the biggest barriers to effective intratumoral drug penetration. This review has summarized recent discoveries that emphasize the relevance of BBTB heterogeneity in pediatric brain tumors. We propose that, in order to better understand how BBTB differences arise and what the functional consequences are for treatment, multi-disciplinary approaches that utilize innovative pre-clinical models hold great potential.

Author contributions

EM, SD-M, SS, LG and AL conceptualized the idea of this review and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Epidemiological characteristics, clinical presentations, and prognoses of pediatric brain tumors: Experiences of national center for children's health

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Background: We aimed to describe the epidemiological characteristics, clinical presentations, and prognoses in a national health center for children.

Methods: From January 2015 to December 2020, 484 patients aged 0-16 years, who were diagnosed with brain tumors and received neurosurgery treatment, were enrolled in the study. Pathology was based on the World Health Organization 2021 nervous system tumor classification, and tumor behaviors were classified according to the International Classification of Diseases for Oncology, third edition.

Results: Among the 484 patients with brain tumors, the median age at diagnosis was 4.62 [2.19, 8.17] years (benign tumors 4.07 [1.64, 7.13] vs. malignant tumors 5.36 [2.78, 8.84], p=0.008). The overall male-to-female ratio was 1.33:1(benign 1.09:1 vs. malignant 1.62:1, p=0.029). Nausea, vomiting, and headache were the most frequent initial symptoms. The three most frequent tumor types were embryonal tumors (ET, 22.8%), circumscribed astrocytic gliomas (20.0%), and pediatric-type diffuse gliomas (11.0%). The most common tumor locations were the cerebellum and fourth ventricle (38.67%), the sellar region (22.9%) and ventricles (10.6%). Males took up a higher proportion than females in choroid plexus tumors (63.6%), ET (61.1%), ependymal tumors (68.6%), and germ cell tumors (GCTs, 78.1%). Patients were followed for 1 to 82 months. The overall 5-year survival rate was 77.5%, with survival rates of 91.0% for benign tumors and 64.6% for malignant tumors.

Conclusion: Brain tumors presented particularly sex-, age-, and regional-dependent epidemiological characteristics. Our results were consistent with previous reports and might reflect the real epidemiological status in China.

KEYWORDS

children, brain tumor, epidemiology, clinical presentation, prognosis

Background

According to the Central Brain Tumor Registry of the United States (CBTRUS) report, brain tumors and other central nervous system (CNS) tumors are the most common solid tumor in the population aged 0-14 years. Malignant brain tumors are the most common cause of death in this group. It is known that brain tumors have specific site distribution and predilection age patterns. The meninges are the most common tumor site in all age groups, with meningiomas being the most common tumor histology in the 2021 CBTRUS report (1, 2). However, brain tumors in the pediatric population have different epidemiological characteristics than those in the adult population. According to the CBTRUS 2016 report, the most common brain tumor site was the cerebellum, and gliomas were the most common histological group, of which pilocytic astrocytoma accounted for the majority of brain tumors among 0- to 14-year-old children (3).

An annual CBTRUS report characterized pediatric brain tumors in the US, which also hinted at racial differences among brain tumors. However, demographic data are seldom reported in Chinese children due to the lack of a nationwide tumor registration system. The only available study is Zhou's (4) report, which summarized pediatric epidemiological characteristics with a single data source: Beijing Tiantan Hospital. However, their data were based on the World Health Organization (WHO) 2000 and were incomplete due to a lack of prognostic data. Hence, we reviewed all patients with brain tumors who received surgical treatment in Beijing Children's Hospital, National Center for Children's Health from 2015 to 2020, aiming to validate and update the epidemiological characteristics of pediatric brain tumors.

Material and methods

Data source

From January 2015 to December 2020, patients between 0 and 16 years who were diagnosed with brain tumors and underwent neurosurgery were enrolled in the study. Duplicated data generated by tumor recurrence or other treatments were deleted. Patients who were hospitalized but refused neurosurgery or data from patients without a pathological diagnosis were excluded. Demographic information and clinical information, including medical history, initial symptoms, pathology, WHO grade, tumor location, surgery date, surgery duration, ventriculoperitoneal shunt (V-P shunt), average length of hospital stay, and medical expenditures, were collected. Patients were followed up by telephone or scheduled outpatient visits. The follow-up items included adjuvant treatment programs, survival status, tumor relapse, and date of death. This work was approved by ethic committee board of Beijing Children's Hospital (IRB ID # [2021]:-E-232-Y).

Classification

Histological diagnosis was based on the 2021 WHO Classification of Tumors of the Central Nervous System (2021 WHO classification) and was divided into 12 subgroups (5). Tumor behavior was labeled according to the International Classification of Diseases for Oncology, third edition (ICD-O-3), with behavior codes of 3 for malignant tumors and 0 or 1 for nonmalignant tumors. Low-grade gliomas (LGGs) included all the gliomas of WHO 1 and 2, and high-grade gliomas (HGGs) included that of WHO 3 and 4. In addition, neuronal and mixed neuronal-glial tumors were not categorized into gliomas.

Anatomical locations of tumors

Tumor location referred to the categories of the CBTRUS report and topography code in the ICD-O-3. To make it more practical, some details were revised. The fourth ventricle and cerebellum were merged into one group, namely, the cerebellum or the fourth ventricle group. The ventricles here are referred as the lateral ventricles and third ventricle. The sellar region was used to replace the pituitary gland and craniopharyngeal duct, which included tumors from the pituitary gland and optic chiasma. Cranial nerves referred to all the cranial nerves apart from the optic chiasma or optic nerve.

Statistical analysis

Descriptive parameters, including the mean, median, counts, and proportions, were calculated with Python 3.7. The mean was used to describe the continuous variables that fit a normal distribution, and the median and quartile are used to describe the continuous variables that did not fit a normal distribution. The Chi-square test was used to test the difference for categorized variables. Kruskal-Wallis test and Mann-Whitney test were used to detect differences for continuous variables among multi-groups and two groups respectively. Kaplan–Meier analysis was used to compute the survival rate.

Results

From 2015 to 2020, 484 individuals aged between 0-16 years were diagnosed with brain tumors and underwent neurosurgery in our center. The median age of this cohort was 4.62 [2.19, 8.17] years, with males making up 57.0% of the cohort. The median ages at diagnosis of the male and female groups were 4.79 [2.26, 8.12] and 4.55 [2.14, 8.19] years respectively, not significantly different ($p>0.05$). Benign tumors accounted for 53.1% of all brain tumors; the median age for these patients was 4.07 [1.64, 7.13] years and the male-to-female ratio was 1.09:1. Distinct from benign brain tumors, patients with malignant brain tumors (46.9%) had a median age of 5.36 [2.78, 8.84] years and a

Abbreviations: CBTRUS, Central Brain Tumor Registry of the United States; CNS, Central nervous system; WHO, World Health Organization; V-P shunt, Ventriculoperitoneal shunt; ICD-O-3, International Classification of Diseases for Oncology, third edition; MB, Medulloblastoma; AT/RT, Atypical Teratoid Rhabdoid Tumors; ET, Embryonal tumors; EP, Ependymoma; DMG, Diffuse midline glioma; CP, Craniopharyngioma; GCTs, Germ cell tumors; GE, Germinoma; NGGCTs, Nongerminomatous Germ Cell Tumors; LGGs, Low-grade gliomas; HGGs, High-grade gliomas; OS, Overall survival; PFS, Progression-free survival.

male-to-female ratio of 1.62:1. Mann-Whitney U test and chi square test found significant difference in age (p=0.008) and sex (p=0.029, chi square =4.743) among the malignant and benign groups. Figure 1 showed the overall age distribution among male and female. Overall, the average length of hospital stay was 19.18 days, and the average medical expenditure was 10903.43 $.

Clinical presentations

Overall, the most frequent initial symptom was nausea and vomiting (24.0%), followed by headache (23.4%), motor impairment (12.6%), and epilepsy (10.5%). Other symptoms, such as visual impairment, behavior change, growth or endocrinal abnormity, accounted for less than 10% of the symptoms. For posterior fossa tumors, the most frequent symptom was headache (32.4%), followed by nausea and vomiting (32.4%), and motor impairment (13.5%). Among individuals with sellar tumors, visual impairment (20.9%) was the most common symptom, followed by headache (15.5%), motor impairment (14.7%), nausea and vomiting (14.0%), and tumor growth or endocrinal abnormity (14.0%) (see Supplementary Table 1).

Patients with temporal lobe tumors experienced the longest median duration of 18 [4, 51] weeks while meningioma had the shortest duration of 2 weeks. The median duration of symptoms of patients with sellar tumors was 13 [4, 52] weeks and 4 [3, 13] weeks for patients with cerebellum or fourth ventricle tumors. Details were shown in Supplementary Table 2. According to the records, approximately 35.2% (167/475, 9 records did not have a clear description of the medical history and were excluded) of the patients were misdiagnosed in their first visit to the hospital. The top three pathology types most likely to be misdiagnosed were pineal tumors (misdiagnosis rate[cases misdiagnosed of pineal tumors/all cases of pineal tumors]: 60.0%), glioneuronal and neuronal tumors (40.5%), and embryonal tumors (40.0%). The top three tumor location most likely to be misdiagnosed were temporal lobe (48.6%), ventricles (42.9%), and cerebellum or fourth ventricular (37.2%).

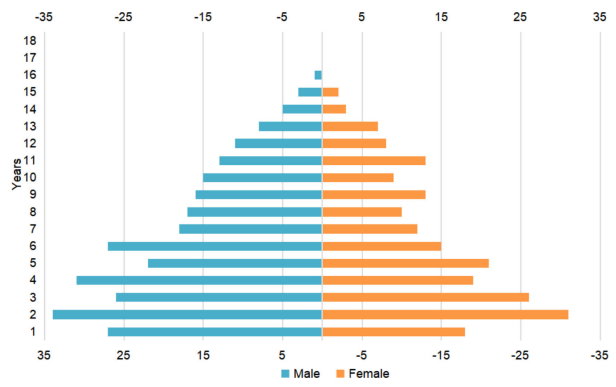

FIGURE 1
Age distribution for males and females in Beijing Children's Hospital from 2015 to 2020.

Distribution of tumor pathology

Based on the 2021 CNS WHO classification, the most common tumor type was ET (22.5%), followed by circumscribed astrocytic gliomas (20.0%), pediatric-type diffuse gliomas (11.0%) and craniopharyngioma (CP, 10.7%), see Figure 2. Among benign tumors, the three most common types were circumscribed astrocytic gliomas (37.5%), CP (21.7%), and glioneuronal and neuronal tumors (14.6%). ET (44.8%) were the most common type in the malignant brain tumor group, followed by pediatric-type diffuse gliomas (22.0%) and ependymal tumors (14.5%). Among ET, the most common pathology was medulloblastoma (MB), accounting for 82.4% of the tumors.

Distribution of tumor sites

Overall, cerebellum or the fourth ventricle was the most common site of brain tumors (38.7%), followed by the sellar region (22.9%). Details were shown in Figure 3. However, tumors in supratentorial space in sum (57.4%) still comprised more than half of all brain tumors. Benign and malignant tumors had significantly different patterns of site distribution (p<0.001). In the benign tumor group, the most common site was the sellar region (40.0%), followed by the cerebellum or the fourth ventricle (24.6%) and ventricles (17.1%). Regarding the malignant brain tumors group, the cerebellum or the fourth ventricle (52.7%) were the most common sites, with other sites sharing an even proportion.

In different regions of brain, the pathology distribution was various (see Figure 4). In the cerebellum or fourth ventricle, MB (43.7%) were the most common tumor type, accounting for near half of the total tumors. The second and third most common tumor types in this hospital were low grade gliomas (31.4%) and ependymal tumors (9.0%). In the sellar region, the three most common pathologies were CP (45.0%), low grade gliomas (37.2%).

Sex and age distribution

Across all brain tumors, the proportion of males (57.17%) was slightly greater than that of females (42.83%). The sex distribution varied greatly with different tumor pathologies, despite no significant difference was identified among different pathological types (p>0.05). Males (52.08%) and females (47.92%) shared a similar proportion of benign tumors, while malignant tumors were more common in males (62.24%) than females (37.76%). Specifically, there was no obvious sex bias for circumscribed astrocytic gliomas (Male: Female, 47.4% vs. 52.6%), Pediatric-type diffuse gliomas (50.9% vs. 49.1%), glioneuronal and neuronal tumors (51.4% vs. 48.6%) or CP (51.9% vs. 48.1%). However, a higher proportion of males than females were diagnosed with ET (61.1% vs. 38.9%), ependymal tumors (68.6% vs. 31.4%), pineal tumors (100.0% vs. 0%), GCTs (78.1% vs 21.9%), and choroid plexus tumors (63.6% vs. 36.4%) than females. Malignant tumors have slightly but significantly higher rate of male proportion compared with female (61.9% vs. 52.1%, p=0.029).

The median age varied significantly with different histological types of brain tumors (p<0.001, see Table 1). Patients with ependymal

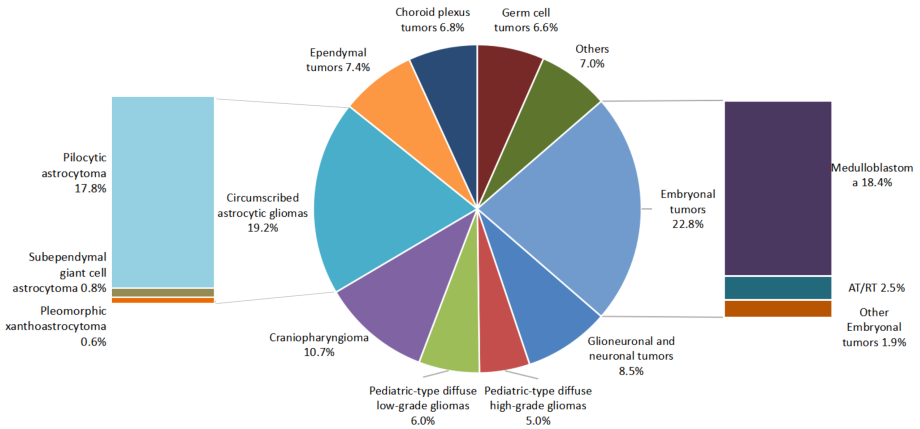

FIGURE 2
Pathology distribution of brain tumors in Beijing Children's Hospital from 2015 to 2020.

tumors (4.10[2.14, 7.60]), ET (5.93[2.96,8.87]), GCTs (9.30[4.15, 10.99]), CP (7.74[3.53,8.21]), meningiomas (11.98[7.85, 13.19]), and metastatic tumors (5.54[4.96, 6.88]) had a median age older than 3 years, while patients with pineal tumors (1.52[1.49, 1.77]) and choroid plexus tumors (0.73[0.44, 1.51]) were generally younger than 3 years of age. In ET, an age difference was noted. The median age of MB patients was 6.93[4.12, 9.75] years, while that of AT/RT patients was 2.30[1.34, 2.74] years. In all patients, malignant tumors had a older age than begin tumors (5.36[2.79, 8.84] vs. 4.08[1.64, 7.13], p<0.001).

Treatment and survival

All the patients received tumoral resection. The adjuvant therapy varies among different types of tumors, while a primary principle was followed: Generally, a gross total or extensive resection and regular outpatient surveillance were a priority for most of the brain tumors except for optic pathway gliomas and diffuse intrinsic pontine gliomas. Chemotherapy and radiotherapy were administered for all malignant tumors and optic pathway gliomas and diffuse intrinsic pontine gliomas under radiologists' and chemotherapists' suggestions. For metastatic ET, the gross total resection was also the priority followed by chemotherapy and/or radiotherapy, while the biopsy was not recommended. In addition, children under 3 years old was not recommended for radiotherapy as the serious cognitive impairment effect. And adjuvant therapy was not recommended for CP as gross total resection was encouraged in our center in the past clinical practice and almost all the patients reached gross total resection. Recent studies have shown that subtotal resection followed by proton therapy can reach a similar survival curve but with less morbidity (6–8). It is changing the surgical concept of CP. However, not all the patients followed doctors' advice after surgery. Table 2 summarized the treatment profile of these patients.

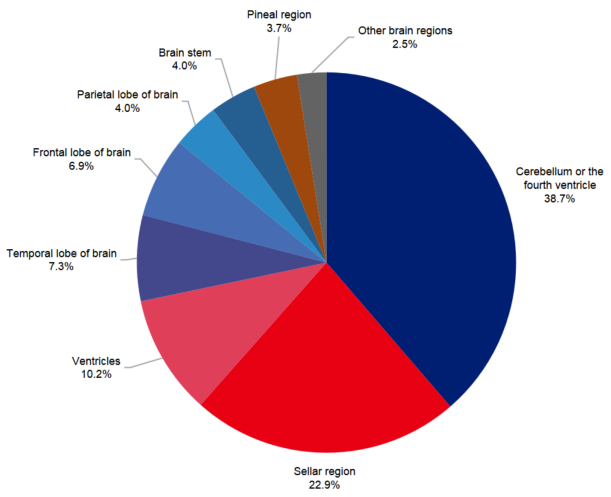

FIGURE 3
Sites distribution of pediatric brain tumors in Beijing Children's Hospital from 2015 to 2020.

All the patients were followed up for 1 to 82 months. There were 54 patients lost during the follow-up. The overall 5-year survival rate was 77.4% in the 430 patients with brain tumors, with a median survival time of 81.0 months. Patients with malignant and benign tumors had 5-year survival rates of 64.6% and 91.0%, respectively. The median survival time of patients with malignant tumors was 76.0 months. Table 2 shows survival rate of several main tumor groups. Given that more than half of the patients remained alive, it was not feasible to calculate the median survival time of patients for some tumor groups.

Discussion

Central nervous system tumors account for a quarter of all childhood cancers and are the most common solid tumors, of which brain tumors account for the majority (9–11). In the United States, brain tumors make up more than 1% of newly diagnosed cancer cases (12). Despite improved treatment in recent years, brain tumors are still the leading cause of cancer-related death among children (13). Brain tumors are heterogeneous and vary by race, sex, age and so on (14–16). Epidemiological studies in China are scarce and are based on the dated CNS WHO classification (4). Hence, it is meaningful to summarize and validate the epidemiological

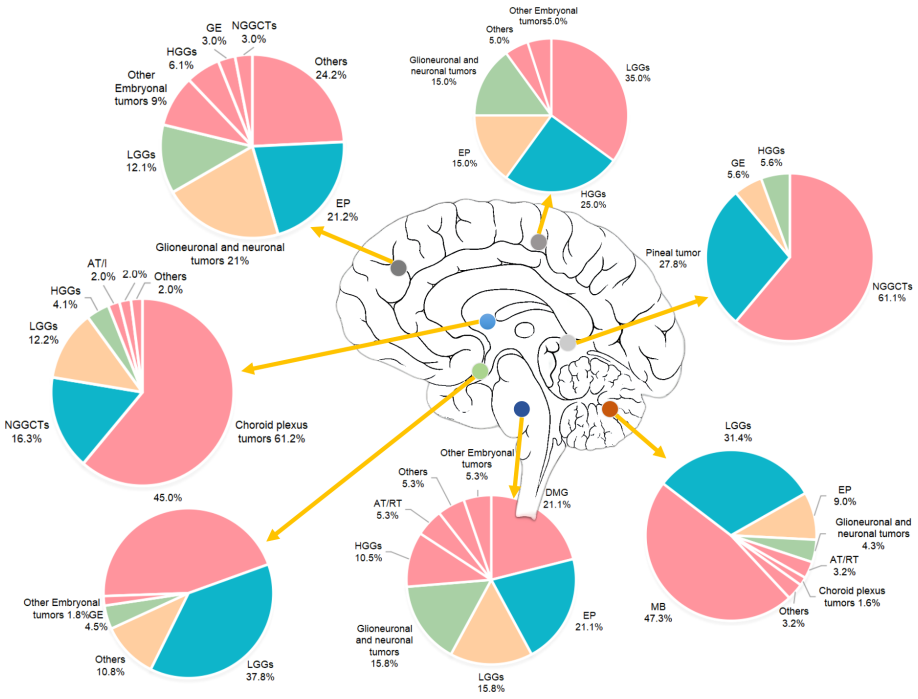

FIGURE 4
Pathology distributions in different brain regions.

TABLE 1 Sex, age and sites distribution of brain tumors.

	Males, n (%)	Median age (years)	Most frequent site
Choroid plexus tumors (N=33)	12 (63.6)	0.73 (0.44, 1.51)	Ventricles
Melanocytic tumors (N=1)	1 (100.0)	1.69 (1.69, 1.69)	Frontal lobe of brain
Tumors of pineal region (N=5)	5 (100.0)	1.52 (1.49, 1.78)	Pineal region
Mesenchymal, non-meningiothelial tumors (N=4)	2 (50.0)	2.92 (2.35, 4.59)	Other brain regions
Neuronal and mixed neuronal-glial tumors (N=37)	19 (51.4)	3.85 (1.31, 6.49)	Temporal lobe of brain
Other astrocytic tumors (N=97)	46 (47.4)	4.10 (1.80, 7.09)	Cerebellum or the fourth ventricle
Ependymal tumors (N=36)	24 (68.6)	4.26 (2.17, 7.62)	Cerebellum or the fourth ventricle
Diffuse astrocytic and oligodendroglial tumors (N=53)	27 (50.9)	5.30 (2.89, 7.69)	Cerebellum or the fourth ventricle
Others (N=13)	7 (53.8)	4.38 (3.90, 8.18)	Sellar region
Craniopharyngioma (N=52)	27 (51.9)	5.74 (3.53, 8.21)	
Embryonal tumors (N=110)	66 (61.1)	6.08 (3.14, 8.98)	Cerebellum or the fourth ventricle
Metastatic tumors (N=7)	3 (42.9)	5.55 (4.96, 6.88)	Frontal lobe of brain
Tumors of Cranial and paraspinal nerves (N=1)	1 (100.0)	7.07 (7.07, 7.07)	Cerebellum or the fourth ventricle
Germ cell tumors (N=32)	25 (78.1)	9.30 (4.15, 10.99)	Pineal region
Meningiomas (N=3)	2 (66.7)	11.98 (7.85, 13.19)	Meninges
Benign tumors (N=240)	125 (52.1)	4.08 (1.64, 7.13)	Sellar region
Malignant tumors (N=244)	151 (61.9)	5.36 (2.79, 8.84)	Cerebellum or the fourth ventricle
All tumors (N=484)	276 (57.0)	4.62 [2.19, 8.17]	Cerebellum or the fourth ventricle

TABLE 2 Treatment options and survival rate of main tumor groups.

Pathology	Radiotherapy	Chemotherapy	Median survival time (Month)	1-year survival rate (%)	5-year survival rate (%)
All tumors (N=437), n (%)	137 (34.1)	146 (40.8)	–	89.0	77.5
Malignant tumors (N=211), n (%)	137 (64.9)	146 (69.2)	76.0	81.0	64.6
Benign tumors (N=216), n (%)	12 (5.6)	33 (15.3)	–	97.5	91.0
MB (N=80), n (%)	65 (81.3)	68 (85.0)	76.0	86.7	70.5
AT/RT (N=10), n (%)	5 (50.0)	7 (70.0)	3.0	0	0
Other ET (N=8), n (%)	4 (50.0)	5 (62.5)	14.0	50.0	37.5
EP (N=34), n (%)	26 (76.5)	23 (67.6)	–	97.1	78.2
DMG (N=6), n (%)	3 (50.0)	3 (50.0)	5.0	13.1	0
LGG (N=105), n (%)	11 (10.5)	34 (32.4)	–	94.3	89.5
HGG (N=13), n (%)	8 (61.5)	10 (76.9)	27.0	62.2	17.9
GE (N=6), n (%)	4 (66.7)	4 (66.7)	–	91.7	80.0
NGGCTS (N=21), n (%)	11 (52.4)	12 (57.1)	–	89.7	89.7
CP (N=52), n (%)	0 (0)	0 (0)	–	100.0	87.7

Not all tumor types are listed in this table. Patients lost for follow up were not included in this table.
MB, medulloblastoma; AT/RT, Atypical Teratoid Rhabdoid Tumors; ET, embryonal tumors; EP, ependymoma; DMG, diffuse midline glioma; LGGs, low grade gliomas; HGGs, High grade gliomas; GE, Germinoma; NGGCTS, Nongerminomatous Germ Cell Tumors; CP, craniopharyngioma.

information again. In addition, this study collected and described other important information, such as medical expenditures, manifestations, and prognoses.

Presentations

The most frequent initial symptoms were nausea, vomiting and headache. Symptoms of motor impairment were present in only 12.59% of all children with brain tumors. The results are consistent with those of a previous meta-analysis study (17). Manifestations are associated with brain tumor locations. Visual impairment is frequently seen in patients with sellar tumors, while nausea and vomiting and headache are more common in patients with cerebellum and fourth ventricle tumors. Misdiagnosis and delay in diagnosis might occur because these common symptoms are not specific to CNS tumors; our data showed that the rate of misdiagnosis reached 32.5%. Supratentorial tumors are more insidious than infratentorial tumors. The general median duration of symptoms across all children brain tumors was 4 weeks, while children with tumors of the temporal lobe experienced the longest duration, with a median of 18 weeks. The general median symptom duration of children with posterior fossa tumors was 4 weeks. This result implies that the diagnostic capability in China has reached that of the international level (18). However, the longest symptom duration of children with posterior fossa tumors was more than 10 years. Increasing awareness of the varied and complex symptomatology that often occurs with CNS tumors in China is necessary and could help reduce misdiagnosis and achieve early diagnosis. Prompt cranial imaging examinations for children with unknown headache and nausea is necessary.

Predilection age

The number of brain tumors decreased with advancing age, which is consistent with the CBTRUS 2015 report (apart from tumors of the pituitary gland) (3). The median age of the total group was 4.62 years, with a median age of 5.36 years for the malignant tumor group, which was slightly higher than that of the benign group. This trend is consistent with a previous report (16). However, the recent CBTRUS report indicates that malignant tumors, compared with nonmalignant tumors, tend to affect younger children (1). This might be due to the sampling bias for tumor histology. Benign pituitary gland tumors tend to affect adolescents, which causes an increase in the median age of the benign tumors group; participants in this age group were not sufficiently enrolled in our study. Our data show that CP, ependymal tumors, ET, metastatic tumors, GCTs, meningiomas and cranial and paraspinal nerve tumors tend to affect older children, while choroid plexus tumors, melanocytic tumors, pineal tumors, glioneuronal and neuronal tumors, and circumscribed astrocytic gliomas tend to affect infants and toddlers. This is in accordance with previous CBTRUS reports (19).

Sex

The sex distribution across all brain tumors is almost balanced. However, in the subgroup analysis, a significant greater proportion of malignant tumors than benign tumors were present in males. This is in accordance with a previous report that malignant tumors occur much more frequently in males (14). We found that sex differences varied by histology. GCTs, ET, ependymal tumors, and choroid plexus tumors were observed in more males than females, while little sex bias was noted for gliomas, and glioneuronal and neuronal tumors and CP. These results are similar to the data of the CBTRUS report (1). These results might also inspire researchers to study the harmonic effect in the pathogenesis of brain tumors.

Location

Similar to previous studies, supratentorial tumors were more slightly common (57.4%) than infratentorial tumors (42.6%). Specifically, the three most common sites were the cerebellum or fourth ventricle, the sellar region and ventricles. This was different from the CBTRUS report (1), which showed that the three most common sites are the sellar region, cerebellum and other brain regions. Furthermore, the proportion of tumors in the cerebellum in our center was approximately two times that in the US. This might be due to that we classified tumors in the cerebellum and fourth ventricle into one group, but the CBTRUS report classifies tumors in the fourth ventricle and cerebellar tumors as two groups. To have a firm conclusion, a national wide data source is necessary in future studies. Given the difficulty in differentiating tumors of the cerebellum or the fourth ventricle in magnetic resonance imaging (MRI), we believe it is more applicable to categorize these two sites into one group. When looking at the nonmalignant and malignant groups, the site distribution pattern was different. The most common site in the malignant tumors group was the cerebellum or fourth ventricle, while the sellar region was the most common site in the benign tumors group. The pathology distribution patterns in posterior fossa, sellar region and ventricles were in accordance with previous studies (20). With these figures, our data might help clinicians have a better understanding of the differential diagnosis of pediatric brain tumors.

Pathology

Different from the existing studies, this study was based on the WHO 2021 classification. Tumors accounting for less than 5% of all brain tumors were assigned to the "others group". Above all, the most common pathology type was ET, accounting for 22.5% of all brain tumors, followed by other astrocytic tumors and diffuse astrocytic and oligodendroglial tumors. This is consistent with a previous study of the Chinese population (4). However, the CBTRUS reported that pilocytic astrocytoma is the most common pathology type (1, 3, 14, 19). This still need to be validated by more studies in future. A potential cause for this might be different approaches of classification. According to the WHO 2021, we included all ET into one group, leading to an increase in the proportion of ET while CBTRUS did not classify in this way. Distinct from a previous study in China (4), we showed that MB were more frequent than CP, and ependymal tumors were more common than GCTs, which is consistent with previous reports (19, 21, 22). We speculate that there might be sample bias in the previous Chinese report because it was not a children's hospital, the younger patients might prefer to attend a children hospital rather than a general hospital. GCTs only account for 6.7% of whole-brain tumors, which is similar to the results of an investigation in China but different from other reports in Japan, Taiwan (China) and far eastern countries with an incidence of brain tumors of 10–14%. This difference might be explained by the fact that patients with germinoma (GE) often undergo nonsurgical treatments, and the actual number of GCTs might have been significantly underestimated in this study. The WHO 2021 address the importance of the molecular scope of brain tumors and classify these tumors into different molecular groups. The medulloblastoma has been divided into four different molecular types, and they are closely related to the prognosis and treatment regimen. The current molecular classification has been used to stratify the treatment intensity. KIAA1549–BRAF fusion is found in 80% of all LGGs whereas only about 10%-20% LGGs possess BRAF-V600E mutation (23). Studies have indicated that BRAF inhibitors could lead to a partial response in patients with BRAF aberrant pilocytic astrocytoma (24). The molecular classification not only provide specific treatment, but also help stratify the patients into different risk categories (25, 26). However, it is a pity that, due to our data being retrospective collected, the molecular diagnosis was absent. Despite of this, our current clinical work flow has introduced the molecular diagnosis. We hope to report our data in future research. Besides, due to the lack of a national wide brain tumor registration system for children and few epidemiological reports, we have to explain these differences based on experience. A registration system for brain tumors is urgent and necessary in China.

Survival

It is known that the survival of patients with brain tumors varies by histology, age at diagnosis, tumor location, and so on. Our data showed that the 5-year overall survival (OS) rate of patients with benign tumors was 90.0%, consistent with previous studies. In the United States, 96% of the children aged 0–19 years with nonmalignant tumors survived 10 years after diagnosis (27). Tore Stokland (28) reported that the 5-year OS rate of children with LGGs was up to 96.4%. However, the outcomes could vary with the extent of resection. If complete surgical resection is performed, the 10-year progression-free survival (PFS) rate exceeds 85% but it drops below 50% if there is radiologically visible residual tumor (29). Sahaja Acharya (30) reported that the 10-year OS of children with LGGs reached 76.4% (high risk) ~ 95.6% (low risk). Alvaro Lassaletta (31) reported that the 10-year PFS was 27% and 60.2% for the BRAF VE600 mutation and wild-type cohorts, respectively. The OS rate of patients with CP ranges from 83% to 96% at 5 years (32) and from 65% to 100% at 10 years (33–35) and is, on average, 62% at 20 years. At present, whether age at diagnosis of CP, sex, and pathological subtype are prognostic factors for survival remains controversial (8).

The 5-year OS rate of children with malignant tumors was 64.6%, similar to previous reports (75.4%) (1). The 10-year survival rate for children aged 0–19 years diagnosed with malignant brain and other

CNS tumors was estimated at 72%, with the lowest rate (17%) attributed to glioblastoma (16). Other studies reported that less than 5.5% of glioblastoma patients survived more than 5 years (36, 37). Another malignant tumor, diffuse intrinsic pontine gliomas, generally has an OS rate of less than 1 year (38). Children with atypical teratoid/rhabdoid tumors were reported to have a four-year OS rate of 43% (39), and another study reported a 6-year OS rate of 35% (40). High-dose chemotherapy and radiation therapy were associated with better survival, while tumor metastasis, intrathecal chemotherapy and the extent of resection did not significantly affect survival (41). Overall, the prognoses of malignant tumors remain unsatisfactory, and more resources need to be introduced in this field. Due to the limitation of the sample size, we did not calculate the survival rate of the specific tumor types. We hope to perform this analysis in future studies.

To date, little information of the epidemiological characteristics of Chinese people is known. As a national center for children's health, we summarized our data and hope that our experiences will provide more information about pediatric brain tumors in China. Moreover, we acknowledge that due to the lack of a nationwide registration system for brain tumors, some inevitable bias might exist. Apart from this, children aged 15-18 years in China prefer to attend general hospitals rather than children's hospitals. Hence, the number of adolescent patients was relatively small in this study.

Conclusion

Overall, the epidemiological characteristics of brain tumors in our center presented a similar pattern to those in previous reports. Some difference was noted and are needed to be confirmed by more epidemiological studies in future.The ratio of benign to malignant tumors approached 1:1.03. Males were more vulnerable to malignant tumors. The site distribution patterns of benign and malignant brain tumors were significantly different. These demographic characteristics provide us with further understanding of pediatric brain tumors, such as sex predispositions and predilection age of onset. Our data might be able to reflect the actual situation of pediatric brain tumors in China.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethic committee board of Beijing Children's Hospital. Written informed consent for participation was not provided by the participants' legal guardians/next of kin because: This is a retrospective study and no identical participant information was included in our manuscript and our institutional ethic committee waived the request for written consent.

Author contributions

MG, WY, and YC contributed to the study conception and design. Material preparation, data collection and patient follow up were performed by JC, PY, ZY, YL, ML, HS, YJ, XP, and data analysis was performed by WY, KZ and WM. Pathology reconfirmation was performed by NZ. The first draft of the manuscript was written by WY, and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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In vivo loss of tumorigenicity in a patient-derived orthotopic xenograft mouse model of ependymoma

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Introduction: Ependymomas (EPN) are the third most common malignant brain cancer in children. Treatment strategies for pediatric EPN have remained unchanged over recent decades, with 10-year survival rates stagnating at just 67% for children aged 0-14 years. Moreover, a proportion of patients who survive treatment often suffer long-term neurological side effects as a result of therapy. It is evident that there is a need for safer, more effective treatments for pediatric EPN patients. There are ten distinct subgroups of EPN, each with their own molecular and prognostic features. To identify and facilitate the testing of new treatments for EPN, *in vivo* laboratory models representative of the diverse molecular subtypes are required. Here, we describe the establishment of a patient-derived orthotopic xenograft (PDOX) model of posterior fossa A (PFA) EPN, derived from a metastatic cranial lesion.

Methods: Patient and PDOX tumors were analyzed using immunohistochemistry, DNA methylation profiling, whole genome sequencing (WGS) and RNA sequencing.

Results: Both patient and PDOX tumors classified as PFA EPN by methylation profiling, and shared similar histological features consistent with this molecular subgroup. RNA sequencing revealed that gene expression patterns were maintained across the primary and metastatic tumors, as well as the PDOX. Copy number profiling revealed gains of chromosomes 7, 8 and 19, and loss of chromosomes 2q and 6q in the PDOX and matched patient tumor. No clinically significant single nucleotide variants were identified, consistent with the low mutation rates observed in PFA EPN. Overexpression of *EZHIP* RNA and protein, a common feature of PFA EPN, was also observed. Despite the aggressive nature of the tumor in the patient, this PDOX was unable to be maintained past two passages *in vivo*.

Discussion: Others who have successfully developed PDOX models report some of the lowest success rates for EPN compared to other pediatric brain cancer types attempted, with loss of tumorigenicity not uncommon, highlighting the challenges of propagating these tumors in the laboratory. Here, we discuss our collective experiences with PFA EPN PDOX model generation and propose potential approaches to improve future success in establishing preclinical EPN models.

KEYWORDS

ependymoma, posterior fossa, patient-derived, xenograft, molecular, pediatric cancer, brain cancer, mouse model

1 Introduction

Ependymomas (EPNs) are malignant central nervous system (CNS) tumors that can arise in the supratentorial brain, the posterior fossa, or the spinal cord. EPN occurs in both adults and children, but is more frequent in children, accounting for approximately 5% of CNS tumors in children aged 0-14 years (1). There has been little change in the treatment of EPN over recent decades, and current standard of care remains surgical resection of the tumor followed by radiotherapy where appropriate, depending on the patient's age (2). Survival rates for children with EPN remain inadequate, with a 10-year survival rate of just 67% for those aged 0-14 years (1). Moreover, long-term survivors of the most common pediatric EPN (posterior fossa A (PFA)) experience neuro-cognitive sequelae, as well as other significant late effects as a result of their treatment (3), highlighting the need for more effective and less damaging treatment options for these patients.

In addition to PFA EPN, a landmark study incorporated genetic and epigenetic analyses to identify a further eight molecular subgroups of EPN (4), with the recent description of a tenth subgroup (5, 6), which have been incorporated into the most recent edition of the World Health Organization classification of CNS tumors (7). Of these subgroups, PFA EPN is the most common subgroup affecting infants and young children and carries a dismal prognosis, with 10-year overall survival rates of approximately 56% (4). When patients are stratified by extent of resection, 10-year overall survival rates plummet further to just 32.7-45.1% for patients with subtotal resection (8). PFA EPN are considered epigenetically-altered tumors and are frequently characterized by loss of histone H3 lysine 27 tri-methylation and overexpression of *EZHIP* (also known as *CXorf67*) (9, 10). No recurrent genetic drivers have been identified for PFA EPN (11), however gain of chromosome 1q and loss of chromosome 6q have been identified as poor prognostic indicators (4, 10, 12).

Molecular classification in other brain tumor types, such as medulloblastoma, has demonstrated the value and importance of clinically stratifying and treating CNS tumors based on molecular features (13). In order to best use this information in the preclinical translational space for PFA EPN, we need to develop representative laboratory models to facilitate the identification and testing of new treatments for this disease (14, 15). The lack of clear genetic drivers for PFA EPN precludes the ability to generate genetically engineered mouse models, and thus we currently rely heavily on the establishment of patient-derived orthotopic xenograft (PDOX) models to represent this cancer in the laboratory. However, the development of these models is a challenging task, requiring specialized skills and significant time and resource input for a relatively low chance of engraftment success (16–19). Here, we describe the establishment of a PDOX model of PFA EPN that persisted for two passages *in vivo* before losing tumorigenicity. The challenges of PFA EPN PDOX model generation are discussed, as

well as potential approaches that may help drive success in the establishment and propagation of these models in the future.

2 Materials and methods

2.1 Human samples

The parents/guardians of the patient gave their informed consent before the donation of the tumor tissue for research purposes and for retrospective research access to relevant medical records and previously obtained pathology samples. Written informed consent was obtained from the minor's legal guardian for the publication of any potentially identifiable images or data included in this article. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Child and Adolescent Health Service, Western Australia (HREC: 1769/EP (PRN 0000002372) A Perth Children's Hospital Oncology Protocol for Collecting and Banking Paediatric Research Specimens; approved 21/08/2003).

2.2 Implantation of patient tumor cells and *In vivo* serial transplantation

Animal experiments were approved by the Animal Ethics Committee of the Telethon Kids Institute and performed in accordance with Australia's Code for the Care and Use of Animals for Scientific Purposes (AEC#263 approved 1/9/2013, AEC#300 approved 18/4/2016, AEC#362 approved 24/4/2020). Immunodeficient BALB/c nude mice were purchased from the Animal Resources Centre (Murdoch, Western Australia, Australia) and J:NU mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Implantation of tumor cells was performed as previously described (20). Specifically, approximately 1 hour following the patient's final surgical procedure, tumor tissue (fourth surgical sample) from the patient (ID 801806) was mechanically dissociated, filtered through a 100 µm cell strainer, and suspended in Matrigel (BD Biosciences, San Jose, California, USA). Animals received general anesthesia and pre-operative analgesia (ketamine 100mg/kg intraperitoneally, medetomidine 1mg/kg intraperitoneally) and post-operative analgesia (0.4mg/ml ibuprofen in drinking water for five days). Cells (approximately 10^6 per mouse in 2µl) were implanted into the cortex (approximately -0.45mm from bregma at a depth of 3mm; n=3) or cerebellum (approximately -6.3mm from bregma at a depth of 2mm; n=2), of five 8-week-old mice using a Hamilton syringe. The implantation process took approximately 5 minutes per mouse. Upon tumor-related morbidity, the brain was bisected at the implantation site and one half of the brain containing the tumor was kept for histology. The remaining tumor was removed, dissociated and reimplanted into the cortex of successive recipients as described above (referred to as PDOX TK-EPN862). At autopsy, no evidence of leptomeningeal spillage of tumor cells from the implantation procedure was observed, nor was there evidence of leptomeningeal metastasis of the tumor.

2.3 Histochemical staining

Tissue samples were fixed in 4% paraformaldehyde in phosphate buffered saline or neutral buffered formalin for 24 hours and embedded in paraffin. Patient and mouse PDOX tissue sections (5 µm) underwent microwave antigen retrieval in a citrate buffer before immunohistochemistry (IHC) using the following antibodies and dilutions: Olig2 (Millipore, Burlington, MA, USA, MABN50; 1:200), Tri-methyl-histone H3 (K27) (Cell Signaling, Beverly, MA, USA, 9733; 1:200), GFAP (Sigma Aldrich, St Louis, MO, USA, G3893-2ML; 1:200), Ki67 (Cell Signaling, 9027; 1:400), p53 (Cell Signaling, 2527; 1:160), synaptophysin (Cell Signaling, 36406; 1:200), EMA (Dako, Santa Clara, CA, USA, M0613; 1:100) and EZHIP (Sigma Aldrich, HPA004003-25UL; 1:200). Sections were incubated with species-specific biotinylated goat anti-IgG secondary antibodies, followed by detection with an Elite ABC kit and NovaRED peroxidase substrate, then counterstained with Gill's hematoxylin according to manufacturer's instructions (Vector Laboratories, Burlingame, California, USA). Hematoxylin and eosin (H&E) staining was performed as per standard protocols using a Leica Autostainer XL.

2.4 DNA and RNA extraction

Genomic germline DNA was prepared from peripheral blood mononuclear cells using a QIAamp DNA Mini Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany, 51304) as per the manufacturer's instructions for DNA extraction from lymphocytes. Genomic tumor DNA and RNA were prepared from fresh frozen patient and PDOX tumor samples using an AllPrep DNA/RNA Mini Kit (Qiagen, 80204) according to the manufacturer's instructions. DNA quality was determined by gel electrophoresis and spectrophotometry (Nanodrop, Thermo Fisher Scientific, Waltham, MA, USA), and quantified using fluorometry (Qubit, Life Technologies, Waltham, MA, USA, Q32851). RNA quality and quantity were determined using the LabChip GX nucleic acid analyzer (Perkin Elmer, Waltham, MA, USA) (performed by the Australian Genome Research Facility, Perth, Western Australia, Australia).

2.5 Methylation array

Genomic DNA (500–1000 ng) was treated with sodium bisulphite using the EZ DNA methylation kit (Zymo Research, Irvine, CA, USA) and bisulphite conversion was confirmed by methylation specific PCR as described previously (21, 22). Quantification of DNA methylation was performed at the Australian Genome Research Facility (Melbourne, Victoria, Australia) using the Human Methylation EPIC BeadChip (Illumina, San Diego, CA, USA) run on an Illumina iScan System (Illumina) using the manufacturer's standard protocol. Raw idat files were uploaded to an online DNA methylation-based classification of CNS tumors platform (www.molecularneuropathology.org, version 11b4 and version 12.5) (23) and basic copy number variant profiles from methylation array data analyzed using the output generated from this classifier (24). Fisher's exact test was performed using

GraphPad Prism software (version 9.4.0) to determine statistical significance between 1q gain and PDOX generation success.

2.6 Whole genome sequencing

Whole genome sequencing (WGS) data obtained from the patient germline and tumor DNA samples were analyzed as reported in (25). For the PDOX, an additional step to remove mouse reads using BBSplit version June 11 2018 (26) was done prior to the previously described method. Default parameters were used except for ambiguous2 that was set to `toss` in order to conservatively exclude ambiguously mapped reads to either the mouse or human reference genomes. WGS analysis included the identification of somatic single nucleotide variants, short indels, cytogenetic-scale and gene-level copy number and structural variants, as reported in (25). WGS dataset generated by this study are available from the European Genome-Phenome Archive under accession number EGAS00001006843.

2.7 RNA sequencing, clustering analysis and expression profiling

RNA sequencing (RNAseq) analysis and expression profiling was performed as reported in (25). Differential expression analysis was conducted using the R package edgeR. Genes were removed if the counts per million was less than 1 in 2 or more samples. Genes were considered significantly differentially expressed with an absolute fold change ($|FC|$) ≥ 2 and a false discovery rate (FDR) < 0.05. For the differential analysis between cranial metastasis and the PDOX model a further filtration was performed that removed all genes with a counts per million of 0 due to mouse infiltrating reads. Correlation analysis was performed between the different tumors and PDOX using the R package corrplot on the filtered gene set. KEGG pathway enrichment analysis was performed using DAVID with the significant differentially expressed genes from the PDOX comparing the cranial metastasis as input. Transcripts per million (TPM) expression values were used for plotting and for comparing the patient tumor and PDOX model samples against the ZERO cohort, a reference dataset containing high-risk pediatric brain tumors (25). RNAseq dataset generated by this study are available from the European Genome-Phenome Archive under accession number EGAS00001006844.

3 Results

3.1 Case report

A previously well, three-year-old male presented with a three-week history of headache, early morning vomiting, seizures and lethargy. Magnetic resonance imaging (MRI) of the brain revealed the presence of a large posterior fossa mass (71mm by 50mm) within the fourth ventricle and extending into the foramen of Luschka (Figures 1A, B). An extraventricular drain was placed to release the pressure followed by subtotal excision of the mass (first surgical sample). Histopathological assessment of the resected mass showed a highly cellular tumor with evidence of widespread perivascular pseudorosettes, moderate nuclear pleomorphism, and multifocal necrosis. Of note, no true ependymal rosettes were

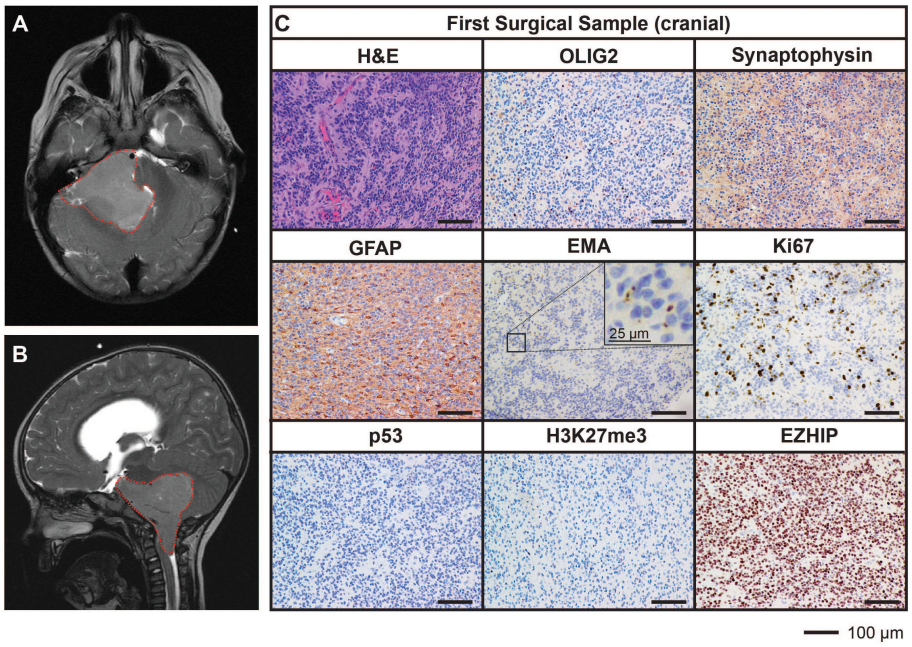

FIGURE 1
MRI and histological assessments of the first surgical sample (cranial) were consistent with the diagnosis of PFA EPN. **(A, B)** Diagnostic MR images depicting a large posterior fossa mass (*red dotted line*). **(C)** Tumor sections obtained from the first surgery were stained with hematoxylin and eosin (*H&E*) or using IHC with the antibodies indicated (*brown*) followed by hematoxylin counterstain (*blue*). Scale bars are as indicated.

identified. The proliferative index, as assessed by Ki67-positivity, was approximately 20% with 9-12 mitotic figures per 10 high power fields of view identified. Tumor cells were predominantly negative for OLIG2 and synaptophysin, positive for GFAP and demonstrated intracytoplasmic dot-like EMA staining. These characteristics are consistent with the diagnosis of WHO grade III EPN with anaplastic features. Immunostaining for p53 was negative. Tumor cells were also negative for histone H3 lysine 27 tri-methylation (H3K27me3) and expressed high levels of EZHIP, consistent with the features of PFA EPN (Figure 1C).

Following surgery, the patient suffered from severe posterior fossa syndrome and required intense rehabilitation before being clinically fit to receive radiotherapy. Postoperative imaging confirmed a residual mass (12mm by 8mm) at the right lateral lower pons with extension over the petrous ridge into the middle cranial fossa. The patient was treated as per the ACNS0831 Children's Oncology Group study protocol (27). He received two cycles of induction chemotherapy (vincristine, carboplatin, cyclophosphamide and etoposide). Imaging assessment post-induction cycles indicated further progression of the residual tumor (increase to 27mm by 10mm). A further surgical attempt achieved a partial resection. Histopathological assessment revealed the residual mass retained the features of the original tumor, however no tissue sample was available from this resection for research. The patient received 59.4 Gy of focal radiotherapy followed by four cycles of maintenance chemotherapy (vincristine, cisplatin, cyclophosphamide and etoposide).

After being in remission for 12 months following the completion of treatment, surveillance imaging revealed the presence of a solitary large drop metastasis in the terminal thecal sac between L4-S2 (Figure 2A) with stable residual intracranial disease. Complete resection of the spinal lesion was performed (second surgical sample), followed by 36 Gy craniospinal irradiation with 14.4 Gy focal boost. The histological features of the metastasis were consistent with the primary lesion (Figure 2B).

Ongoing surveillance scans 10 months after the completion of craniospinal irradiation detected another metastatic spinal lesion at T12 (Figure 3A). The patient then commenced an early phase trial protocol for recurrent malignancies [ACCT007: Rap-CV (28)] involving treatment with rapamycin, cyclophosphamide, and vinorelbine. No response was observed following two cycles of treatment, and the spinal lesion progressed to 50 mm in size. A further metastatic lesion (12 mm) in the mesial occipital region was also discovered at this time (Figure 4A). To prevent spinal cord compression, complete excision of the spinal metastasis was performed (third surgical sample), followed by 15 Gy focal radiation. The patient was further treated with fluorouracil according to another early phase clinical trial. The mesial occipital lesion continued to progress (Figure 4B) requiring complete resection (fourth surgical sample) followed by a 20 Gy focal boost to the tumor bed. The spinal and cranial lesions were both histologically consistent with previous tumor samples (Figures 3B, 4C). The patient had stable disease for four months, following which leptomeningeal metastases were detected throughout the brain and spinal cord. He was treated with one dose of gemcitabine according to an early phase trial treatment without success. The patient was provided with end-of-life care and died a short time later, four years following the primary diagnosis. A

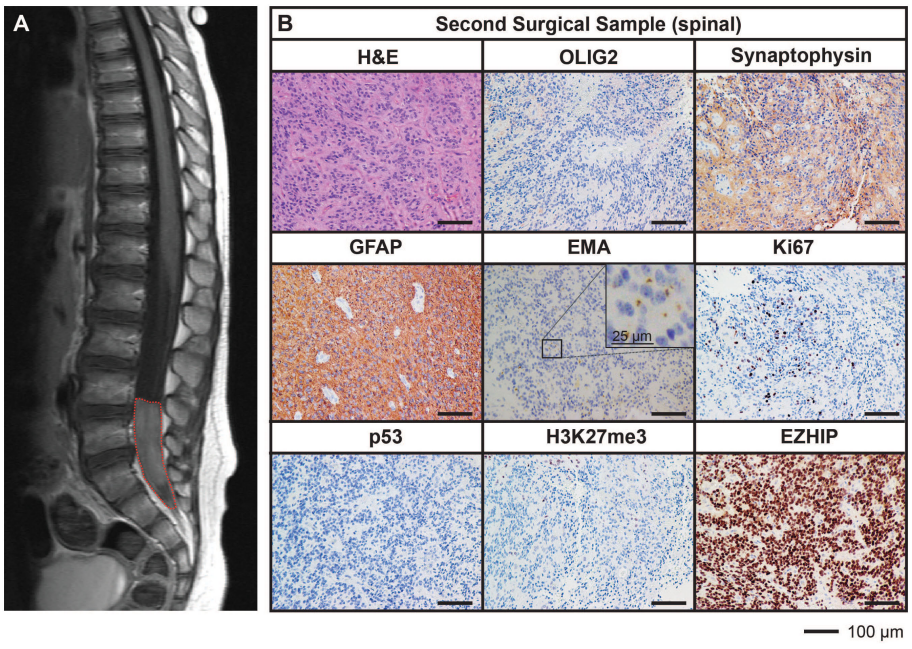

FIGURE 2
MRI and histological assessments of the second surgical sample (spinal) were consistent with the lesion being a metastasis of the primary PFA EPN. **(A)** MRI depicting a spinal metastasis in the terminal thecal sac (*red dotted line*). **(B)** The tumor tissue was stained with H&E or using IHC with the antibodies indicated (*brown*) followed by hematoxylin counterstain (*blue*). Histological findings were consistent with the features of the primary tumor. Ki67 proliferative index was estimated to be 25%. Scale bars are as indicated.

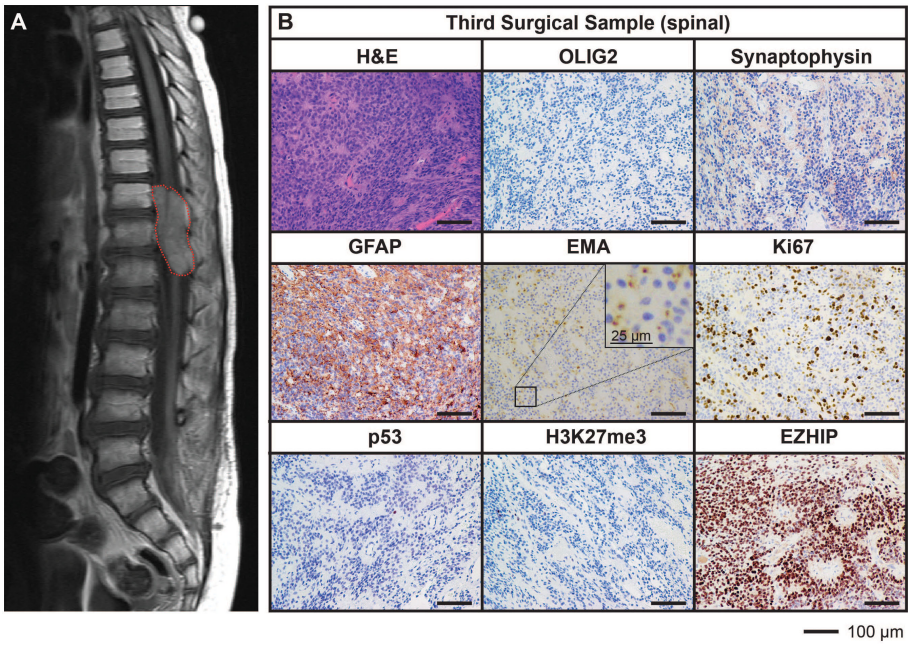

FIGURE 3
MRI and histological assessments of the third surgical sample (spinal) were consistent with the lesion being an additional metastatic tumor arising in the spine from the initial PFA EPN. **(A)** MRI depicting a large spinal metastasis (*red dotted line*). **(B)** Tumor tissue was stained with H&E or using IHC with the antibodies indicated (*brown*) followed by hematoxylin counterstain (*blue*). Histological findings were consistent with the features of the primary tumor. Ki67 proliferative index was estimated to be 25%. Scale bars are as indicated.

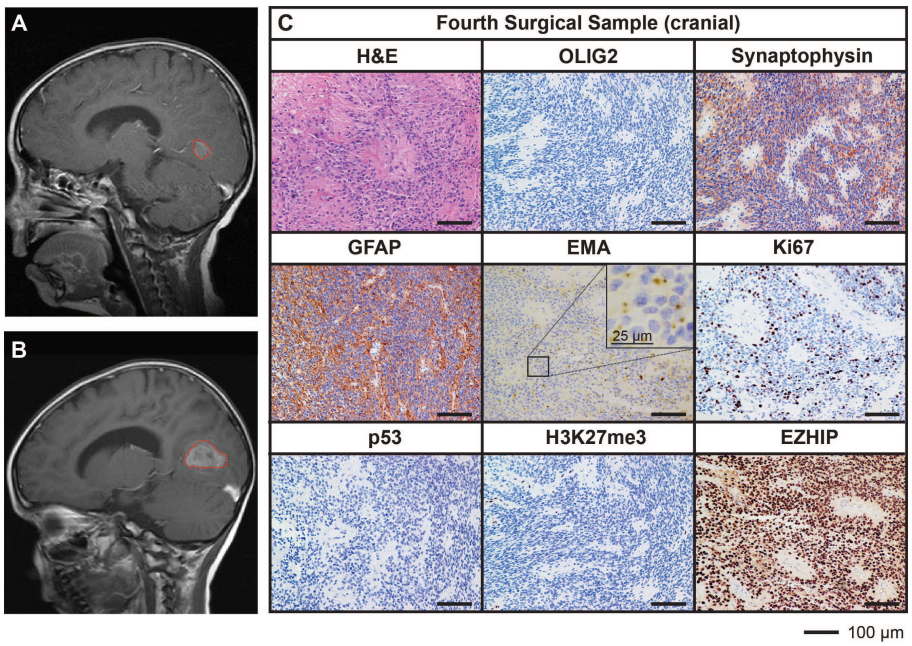

FIGURE 4
MRI and histological assessments of a cranial metastatic lesion distant from the primary PFA EPN (fourth surgical sample) has features concordant with the initial disease. **(A)** MRI depicting a metastatic tumor in the mesial occipital region (*red dotted line*). **(B)** Progression of the mesial occipital lesion (*red dotted line*) at three months following the scan shown in **(A)**. This tumor required surgical excision, from which a fourth surgical sample was obtained. **(C)** Tumor tissue was stained with H&E or using IHC with the antibodies indicated (*brown*) followed by hematoxylin counterstain (*blue*). Histological findings were consistent with the features of the primary tumor. Ki67 proliferative index was similar to previous samples (25%). Scale bars are as indicated.

summary of the treatment procedures performed and surgical samples collected is illustrated in Supplementary Figure 1.

3.2 PFA EPN tumor cells successfully engrafted in mice but serial propagation was unsuccessful

Tumor cells from the fourth surgical sample were implanted into the brains of five immunodeficient mice. Three of these five mice developed brain tumors (two from cortical implants and one from cerebellar implants) generating a PDOX model termed TK-EPN862. Upon the development of tumor-related morbidity in these animals, tumor tissues were harvested and serially transplanted into the cortex of a further 11 immunodeficient mice. Of these secondary implant recipients, two mice developed a brain tumor. Upon serial implantation of these tumor cells into the cortex of five further mice (tertiary implant recipients), no further tumors grew, resulting in the loss of the PDOX model (Figure 5A). Attempts to resurrect the TK-EPN862 model by orthotopically implanting cryopreserved cells were unsuccessful.

The median time to morbidity across all tumor-related deaths was 262 days. Asymptomatic mice were either euthanized for reasons unrelated to tumor growth (such as rectal prolapse) or at the predetermined experimental endpoint in accordance with animal ethics requirements (defined as 365 days following implant).

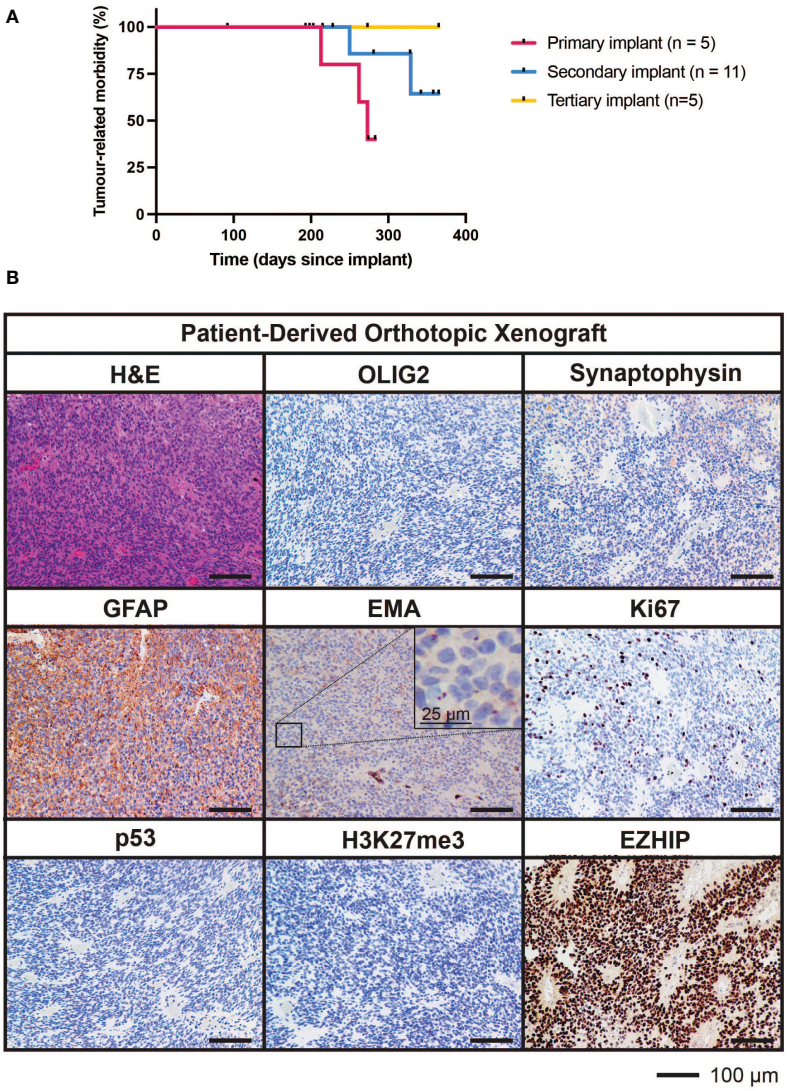

FIGURE 5
Survival characteristics and histological features of TK-EPN862. **(A)** Time to morbidity in the TK-EPN862 PDOX model. Mice were implanted with tumor cells from the fourth surgical sample (primary implant, *pink*), or serially transplanted with TK-EPN862 cells (secondary implant, *blue*; tertiary implant, *yellow*). Mice euthanized for non-tumor-related reasons were censored (black vertical dash). **(B)** H&E staining (top left) or IHC for the indicated antibodies (*brown*) demonstrate that TK-EPN862 xenografts recapitulated the histological features of the matched surgical sample. Sections are counterstained with hematoxylin (*blue*) and the sizes of the scale bars are indicated.

3.3 TK-EPN862 histologically recapitulates the matched patient tumor

Histological assessment of tumor tissue from the TK-EPN862 PDOX (Figure 5B) demonstrated that the tumors growing in mice recapitulated many features of the patient tumor from which they were derived. Similar to the matched patient surgical sample (Figure 4C), TK-EPN862 tumors were highly cellular with evidence of perivascular pseudorosettes. Immunostaining of TK-EPN862 tumor cells was consistent with the features of EPN, including negative staining for OLIG2 and synaptophysin, positive staining for GFAP, and intracytoplasmic dot-like positivity for EMA. TK-EPN862 tumor cells were also negative for p53 and had a proliferative index of approximately 20%. Consistent with the features of PFA EPN, tumor cells from TK-EPN862 were negative for H3K27me3 and expressed high levels of EZHIP (Figure 5B). These histopathological features were maintained across *in vivo* passages of TK-EPN862.

3.4 TK-EPN862 molecularly classifies as PFA EPN

Methylation profiling of the first surgical sample from patient 801806 indicated it classified clearly as a PFA EPN (calibrated score >0.99 using the Molecular Neuropathology 2.0 classifier versions 11b4 and 12.5) (Supplementary Table 1). The metastatic surgical samples examined (two spinal lesions and one distal cranial lesion) also robustly classified as PFA EPN (calibrated score > 0.99 using the Molecular Neuropathology 2.0 classifier versions 11b4 and 12.5), irrespective of where the tumor recurred, consistent with the findings of others (4, 29). Additionally, the TK-EPN862 PDOX classified as PFA EPN (calibrated score > 0.98 using the Molecular Neuropathology 2.0 classifier versions 11b4 and 12.5), demonstrating faithful recapitulation of the original patient tumor in the mouse (Supplementary Table 1).

PFA EPN can be further divided molecularly into nine subtypes (PFA-1a-f and PFA-2a-c), each with distinct survival outcomes (10). Additional analysis of this patient's disease using the most recent version of the Molecular Neuropathology 2.0 classifier (v12.5), which includes these subclasses, further classified all surgical and PDOX samples as PFA-2 EPN (calibrated score > 0.99). While the primary (first) surgical sample and the third surgical sample (spinal) were unable to be further subclassified, possibly due to normal tissue contamination, the second surgical sample and the PDOX robustly classified more specifically as PFA-2b (calibrated score >0.9). The fourth surgical sample from which the PDOX was derived also best classified as PFA-2b (calibrated score = 0.89) (Supplementary Table 1). Of note, PFA-2 tumors are associated with a higher rate of distant relapse compared to PFA-1 tumors (10), consistent with the features of this case.

3.5 Chromosomal abnormalities in the patient tumors increased with disease progression

Copy number profiling using methylation data revealed gains of chromosomes 7, 8 and 19 across all surgical samples and in the TK-EPN862 PDOX tumor, supporting the notion that the secondary and subsequent tumors were metastases of the primary tumor, rather than *de novo* occurrences. Whole chromosome gains, including chromosomes 8 and 19 as observed in this case, are more common in PFA-2 EPN compared to PFA-1 EPN (10), and are consistent with the molecular classifications of the patient and PDOX tumors (Supplementary Table 1). In the fourth surgical sample and the matched PDOX, an additional loss of chromosomes 2q and 6q were observed, suggesting progressive genomic instability of the tumor (Figure 6). Gain of 1q, which is associated with more aggressive disease and poorer outcome in PFA EPN (4, 30), was not observed, consistent with the low frequency of this alteration in PFA-2b EPN (10).

WGS of the fourth surgical sample and matched PDOX confirmed the chromosomal abnormalities observed in the copy number plots (gain of chromosomes 7, 8 and 19 and loss of chromosomes 2q and 6q depicted in the third circle of the CIRCOS plots in Figure 7). In addition, loss of chromosome 16 and gain of 17q were observed in the PDOX by WGS (Figure 7B), however, no cancer-relevant genes in these locations were found to be significantly over- or under-expressed compared to the matched surgical sample by RNAseq. No single nucleotide variants of clinical significance were identified in either sample, consistent with the low mutation rates observed in PFA ependymomas (11). In particular, the absence of histone H3 K27M mutations [which are observed solely in PFA-1 EPN and absent from PFA-2 EPN (10)] are consistent with the molecular classification of these tumors as PFA-2 EPN.

3.6 Gene expression patterns were maintained across primary and metastatic tumors

In an effort to investigate if there were gene expression differences in the metastatic samples compared to the primary tumor that may provide new knowledge about relapsed EPN, we performed RNAseq on the primary tumor (first surgical sample), one subsequent spinal metastasis (third surgical sample), and the cranial recurrence (fourth surgical sample). There was insufficient high-quality RNA available from the second surgical sample to perform RNAseq on this tumor. Gene expression analysis showed little variance between the primary tumor, spinal metastasis and cranial recurrence, with correlation coefficient values above 0.95 between all sample comparisons (Figure 8A), despite the marked chromosomal losses observed in the fourth surgical sample compared to earlier samples. These data suggest that this PFA EPN predominantly retained its pattern of gene expression across metastases in different compartments of the CNS.

Comparing the primary tumor with one of the spinal lesions, eight genes were differentially expressed (Figure 8B and Supplementary Table 2), with *IFITM1* and *ZFHX4* being notable due to their described roles in cancer metastasis (31, 32). Expression levels of *IFITM1* increased 16.6-fold in the spinal metastasis compared to the primary cranial lesion (Supplementary Table 2). *IFITM1* is associated with glioma cell proliferation, migration and

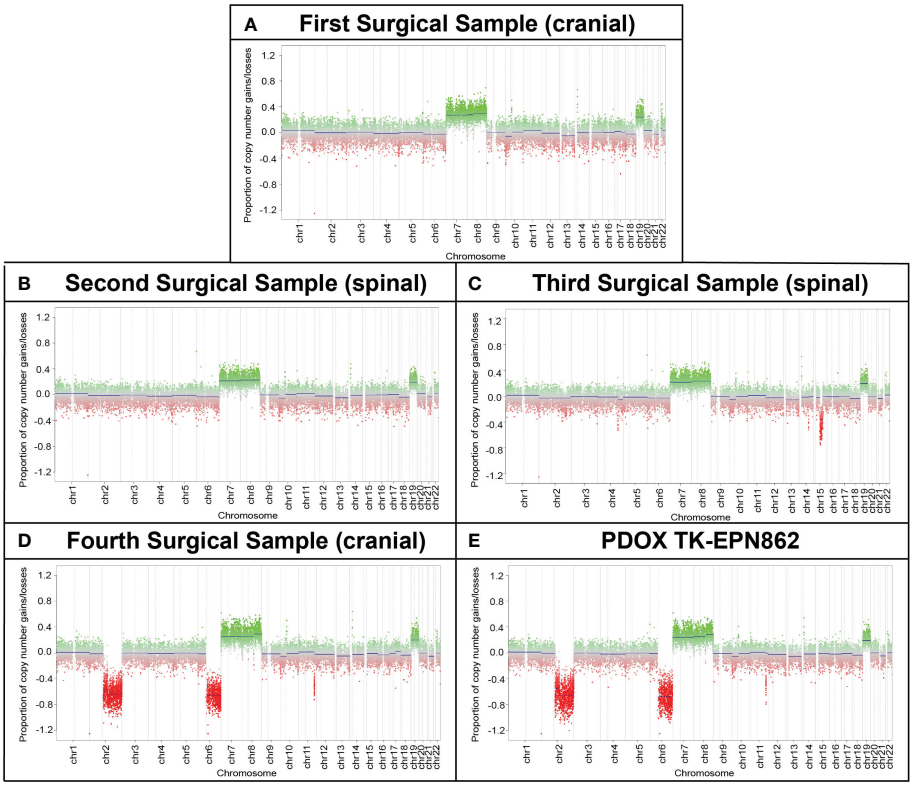

FIGURE 6
Longitudinal copy number analysis indicates the acquisition of additional genomic changes with disease progression. Copy number estimates (generated by MolecularNeuropathology.org using DNA methylation array data) for chromosomes 1 to 22 showing gains/amplifications (*green*) or losses (*red*) for **(A)** the primary cranial tumor (first surgical sample) and **(B, C)** two spinal metastases (second and third surgical samples). **(D)** The cranial metastasis (fourth surgical sample) and **(E)** TK-EPN862 PDOX (derived from the fourth surgical sample), exhibited the same chromosomal gains as samples 1-3, with additional losses of chromosomes 2q and 6q observed.

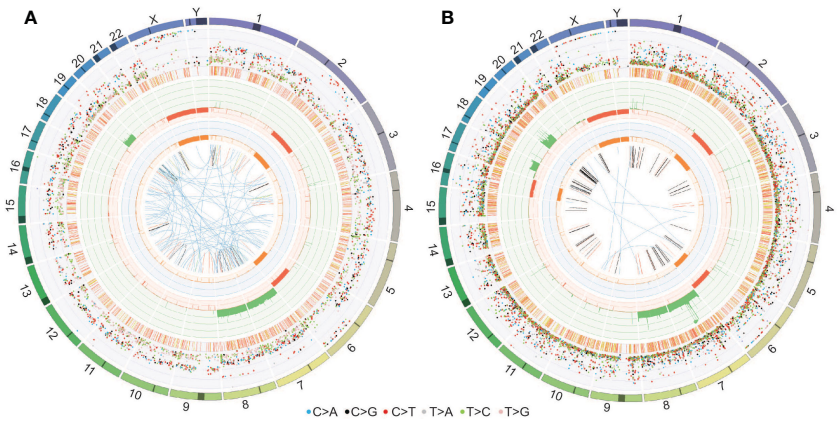

FIGURE 7
CIRCOS plots for **(A)** the fourth surgical sample and **(B)** the PDOX TK-EPN862 tumor confirm the chromosomal gains and losses observed in the copy number plots. Key to the CIRCOS plots: Outermost circle indicates the chromosomes, where darker shading represents large gaps in the human reference genome (e.g., centromeres). Second circle (*grey shading*) shows the somatic variants. These are divided into an outer ring of single nucleotide variants where each dot represents a single variant colored as shown with allele frequencies (corrected for tumor purity and scaled from 0-100%) and an inner ring of short insertions and deletions (*yellow* and *red*, respectively). Third circle (*red* and *green shading*) shows all observed tumor purity-adjusted copy number changes (losses and gains indicated in *red* or *green*, respectively; scale ranges from 0 (complete loss) to 6 (high level gains)). Fourth circle (*orange* and *blue shading*) represents the observed 'minor allele copy numbers' across the chromosome, ranging from 0 to 3. The expected normal minor allele copy number is 1. Values below 1 are shown as a loss (*orange*) and represents a loss of heterozygosity event, whilst values above 1 (*blue*) indicate amplification events of both alleles at the indicated locations. Innermost circle displays the observed structural variants within or between the chromosomes. Translocations are indicated in blue, deletions in red, insertions in yellow, tandem duplications in green and inversions in black.

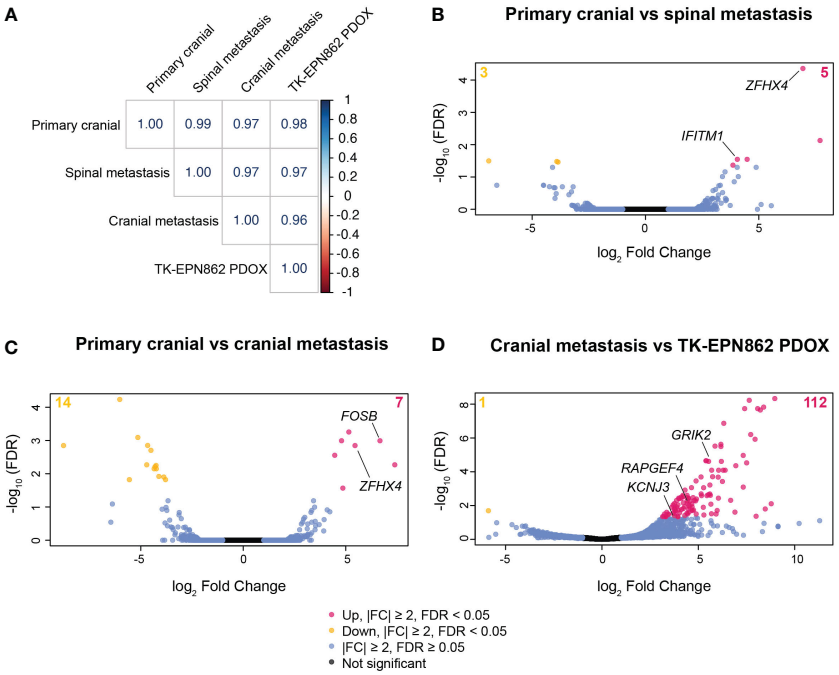

FIGURE 8
Gene expression patterns are highly conserved between the primary tumor, spinal metastasis, cranial recurrent lesion and the TK-EPN862 PDOX model. **(A)** Correlation matrix showing the correlation coefficient values for each sample comparison shown. **(B–D)** Volcano plots depicting differentially expressed genes between the samples stated on each plot. *Pink* dots and *yellow* dots represent genes that are statistically significantly over- or under-expressed, respectively, between the samples described (|FC| ≥ 2 and FDR < 0.05), *blue* dots represent genes that have |FC| ≥ 2 and FDR ≥ 0.05, and *black* dots represent genes that are not significantly differentially expressed (|FC| ≤ 2 and FDR ≥ 0.05).

invasion (31), and so may have played a role in the metastatic process in this lesion. Additionally, the expression of *ZFHX4* increased over 120-fold in the spinal metastasis (Supplementary Table 2). Higher expression of this gene may have contributed to the progression of this tumor as *ZFHX4* has been associated with poor survival and metastasis in ovarian cancer (32) and is reported to play a role in the maintenance of tumor-initiating cells in glioblastoma (33).

When comparing the primary tumor and the cranial recurrence, 21 genes were significantly differentially expressed (|FC|≥2, FDR<0.05; Figure 8C and Supplementary Table 3), with expression of the proto-oncogene *FOSB* increased over 100-fold in the cranial recurrence compared to the primary tumor. *FOSB* has been reported to be highly expressed in glioma tissue compared to normal brain and is associated with glioma cell proliferation, migration, and invasion (34). The high expression levels of *ZFHX4* observed in the spinal metastasis were also observed in the cranial recurrence (43.8-fold increase compared to the primary tumor), reinforcing the possibility this gene may have played a role in the metastatic progression of this disease (Supplementary Table 3).

We next aimed to compare the transcriptome of TK-EPN862 with the matched lesion from which it was derived (the fourth surgical sample). Transcriptome analysis revealed 113 differentially expressed genes between the PDOX and the cranial metastasis (Figure 8D and Supplementary Table 4). We then performed KEGG pathway analysis using this gene list in order to elucidate specific biological pathways that may be altered in the PDOX. This revealed that most of the significantly altered genes were associated with pathways expressed in normal brain tissue (Supplementary Table 5). Additionally, three genes (*GRIK2*, *KCNJ3* and *RAPGEF4*) located on chromosomes 2q or 6q were highly overexpressed in the PDOX model, which was unexpected given the loss of 2q and 6q demonstrated by copy number estimates in both samples. Taken together, this suggests that the differential gene expression patterns observed are most likely due to normal mouse brain contamination, rather than alterations arising in the tumor cells post-engraftment.

Following this, we evaluated expression levels of *EZHIP* as a marker of PFA EPN (10), using the ZERO cohort of high-risk pediatric brain tumors as a reference dataset (25). As expected, PFA EPN within the reference cohort expressed high levels of *EZHIP* (Figure 9; green dots). High expression of *EZHIP* was observed in the first, third and fourth surgical samples, as well as in TK-EPN862 (Figure 9; red and yellow dots, respectively), which correlates with the high levels of EZHIP protein expression observed by IHC (Figures 1, 3-5). The lower RNA expression level of the PDOX compared to the matched patient tumor (fourth surgical sample) is most likely due to the contaminating normal mouse brain tissue as discussed above.

3.7 1q gain may be an important predictor of PFA EPN PDOX establishment success

EPNs are challenging tumors to propagate in the mouse, with other laboratories publishing low success rates with this tumor type compared to other malignant CNS tumors (16, 18). Our combined

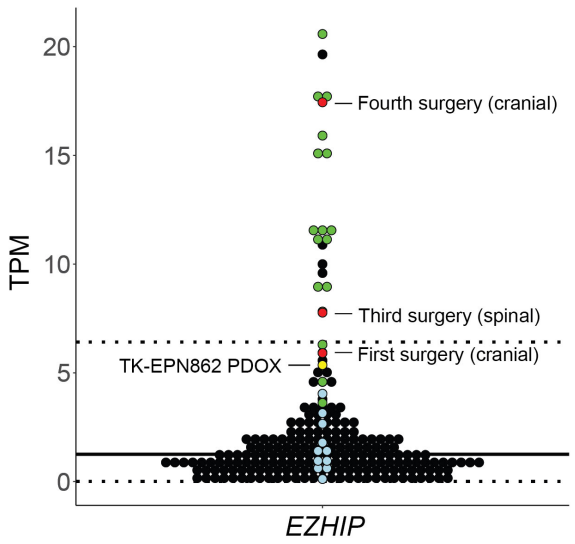

FIGURE 9
Gene expression levels (y axis: transcripts per million, TPM) for *EZHIP* in the first, third and fourth surgical tumor samples and matched PDOX TK-EPN862 tumor. The patient tumors (*red*) and TK-EPN862 (*yellow*) are compared to a reference cohort containing high-risk pediatric brain tumors. PFA EPN (*green dots*), other non-PFA EPNs (*blue dots*), and all other brain tumors (*black dots*) from the reference cohort are shown. Solid black line shows the mean TPM of the reference cohort, and dotted line shows the TPM values that are two standard deviations away from the mean.

data on attempts at establishing EPN PDOXs from the Telethon Kids Institute (Perth, Australia), Fred Hutchinson Cancer Centre (Seattle, USA) and Children's Cancer Institute (Sydney, Australia) show that collectively only five out of 36 attempts (5/36; 13.8%) were successful beyond two passages *in vivo* (excluding pending attempts that have not yet had the opportunity to be propagated beyond two passages). Indeed, 25 of all attempts (25/39; 69.2%) failed to establish at all from the primary implant (Supplementary Table 6). Furthermore, of the five successful models, three of these have begun to demonstrate loss of tumorigenicity at later *in vivo* passages, further highlighting the challenges of creating EPN PDOX models.

As the development of these models requires significant time and resource input for a relatively low chance of engraftment success, we sought to identify any biomarkers that may be indicative of increased likelihood of PFA EPN PDOX generation success. Despite the aggressive nature of the tumor in the patient presented in this report, the tumor and the matched PDOX did not demonstrate 1q gain, which is associated with poorer outcomes and more aggressive disease in PFA EPN (4, 30). Of the published PFA EPN PDOX models with molecular data, 1q gain was reported in all but one of these models (16, 18, 35), raising the possibility that 1q gain may be associated with an increased likelihood of a PFA EPN PDOX successfully establishing. To investigate this, we performed DNA methylation array to determine 1q status on the tumors from all historic attempts to establish a PFA EPN PDOX model in the laboratories of Telethon Kids Institute (Perth, Australia) and Children's Cancer Institute (Sydney, Australia). A lack of primary patient material precluded analysis on unpublished samples from the Fred Hutchinson Cancer Center cohort. Molecular classification as PFA EPN were confirmed for all tumors using the Molecular Neuropathology 2.0 classifier (v11b4 and v12.5). A successful PDOX model was defined as having been successfully propagated beyond two passages *in vivo*. Including data from published PDOX models, we found that 10/11 successful PFA EPN PDOX models had 1q gain, compared to only 1/7 attempted PFA EPN PDOX models that failed to establish (Table 1 and Supplementary Figure 2), and that this difference was statistically significant ($p = 0.0025$). Although the sample size is small owing to the rarity of this specific subtype, these data suggest that 1q gain may be an important predictor of PFA EPN PDOX establishment success.

4 Discussion

PFA EPN is one of the deadliest brain cancers in children. Here, we describe the case of a patient that presented with a cranial PFA EPN that later metastasized multiple times to the spine. The cancer then recurred at a distal site in the brain before the patient succumbed from widely disseminated metastatic disease through the CNS. Transcriptome analyses demonstrated significant similarity between the primary tumor and the spinal and cranial metastases, suggesting these recurrent lesions had not genetically diverged from the primary lesion. The most notable genes that were significantly overexpressed in the metastases compared to the primary tumor (*IFITM1*, *ZFHX4* and *FOSB*) are associated with glioma proliferation, migration and invasion (31, 34), and maintenance of glioblastoma tumor initiating cells (33), suggesting they may have contributed to the progression and metastasis of this disease. This is in contrast with a recent study, where expression of a different subset of genes including *NOTCH*, *EPHA2* and *SUFU* were reported to be significantly altered in metastases of pediatric PFA EPN compared to the primary tumor (29). Longitudinal primary and relapse samples from pediatric PFA EPN patients are very rare, with Zhao and colleagues (29) reporting on just five patients with matched primary and metastatic tumors over a 13-year period. Consequently, it is possible that the differences in genes reported may be due to the small sample size examined in each study, highlighting the need for further research in a larger number of longitudinal patient samples.

From the cranial recurrence, we generated and characterized a PFA EPN PDOX model, TK-EPN862, that faithfully recapitulated the matched patient tumor from which it was derived. Despite the aggressive nature of the tumor in the patient, the PDOX was unable to be maintained past two passages in mouse brain before losing tumorigenicity. In all but one PFA EPN PDOX models published with molecular data, high expression levels of *EZHIP* and 1q gain were reported (16, 18, 35). The one model that did not have 1q gain harbored additional alterations including mutations in *APOB*, *CDKN1B* and *CDKN2C*, potentially driving tumorigenicity (18). *EZHIP* overexpression at both the RNA and protein level is characteristic of PFA EPNs, with the exception of the PFA-1f subtype (10). Overexpression of *EZHIP* inhibits polycomb repressive complex 2 function, resulting in the global reduction of H3K27me3 in PFA EPN (37), and is mutually exclusive with

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TABLE 1 1q gain is associated with an increased likelihood of establishment success of PFA EPN PDOX models.

Institute	Sample ID	Primary implant established in mouse	Status*	1q status	Other genetic alterations reported	Publication
Fred Hutchinson Cancer Center	EPD-210FH	Y	Successful	1q gain	Chr 6q loss. Chr 10q loss. Chr 11q loss. Chr 12p loss. Chr 17 gain. Chr 22q loss.	(16, 36)
Fred Hutchinson Cancer Center	EPD-710FH	Y	Successful	1q gain	Chr 10 loss.	(16, 36)
Children's Hospital Colorado/University of Colorado	MAF-811_XC	Y	Successful	1q gain	High expression of *EZHIP*. Chr 6 loss. Chr 22 loss.	(35)
Children's Hospital Colorado/University of Colorado	MAF-928_XC	Y	Successful	1q gain	High expression of *EZHIP*. Chr 6 loss.	(35)
St Jude Children's Research Hospital	SJEPPF-15-8710	Y	Successful	1q gain	High expression of *EZHIP*. Low H3K27me3 methylation. Chr 6q loss. Chr 10q loss.	(18)
St Jude Children's Research Hospital	SJEPPF-16-02472	Y	Successful	1q gain	High expression of *EZHIP*. Low H3K27me3 methylation. Chr 9 gain.	(18)
St Jude Children's Research Hospital	SJEPPF-16-08404	Y	Successful	Balanced	High expression of *EZHIP*. Low H3K27me3 methylation. *APOB* mutation. *CDKN1B* and *CDKN2C* mutations. Chr 6q loss. Chr 16q loss.	(18)
St Jude Children's Research Hospital	SJEPPF-16-09238	Y	Successful	1q gain	High expression of *EZHIP*. Low H3K27me3 methylation. *RAG1* mutation. Chr 16q loss. Chr 22q loss.	(18)
Baylor College of Medicine	0614EPN	Y	Successful	1q gain		(29)
Baylor College of Medicine	2002EPN	Y	Successful	1q gain		(29)
Baylor College of Medicine	4423EPN	Y	Successful	1q gain		(29)
Telethon Kids Institute	801806/TK-EPN862	Y	Failed	Balanced	High expression of *EZHIP*. Low H3K27me3 methylation. Chr 2q loss. Chr 6q loss.	Model described in this report
Telethon Kids Institute	857224	N	Failed	Balanced	No whole arm chromosomal alterations found.	Unpublished
Telethon Kids Institute	861048	N	Failed	1q gain	Chr 16q loss.	Unpublished
Telethon Kids Institute	861756	N	Failed	Balanced	No whole arm chromosomal alterations found.	Unpublished
Telethon Kids Institute	903149	N	Failed	Balanced	No whole arm chromosomal alterations found.	Unpublished
Telethon Kids Institute	906462	N	Failed	Balanced	No whole arm chromosomal alterations found.	Unpublished
Children's Cancer Institute	P001001	N	Failed	Balanced	High expression of *EZHIP*.	Unpublished
Children's Cancer Institute	P002103	Y	Pending	1q gain	High expression of *EZHIP* and *SMYD3*. Low expression of *CDKN1A:SH2B3*.	Unpublished
Children's Cancer Institute	P012301	Y	Pending	1q gain	High expression of *EZHIP*, *HSP90AA1*, *ABL2*, and *VEGFA*.	Unpublished

1q status of all attempts to establish PFA EPN PDOX models from Telethon Kids Institute and Children's Cancer Institute as well as published data are shown. Other reported genetic alterations including chromosome (Chr) loss or gain are described. *Status categories are as follows: Successful - sustained propagation of PDOX beyond two passages *in vivo*; Failed - PDOX did not propagate from the primary implant or failed to propagate beyond two passages *in vivo*; Pending - PDOX still being established and has not yet been propagated past two passages *in vivo*.

histone H3 K27M mutation (38). In TK-EPN862 and the matched patient tumor, we observed high expression of *EZHIP* RNA and protein, and the associated low levels of H3K27me3 detected *via* IHC, with a lack of histone gene mutations, consistent with a diagnosis of PFA EPN. However, whilst high levels of *EZHIP* expression were observed in TK-EPN862, there was no evidence of the 1q gain consistently reported in successful PDOX models of PFA EPN. Indeed, in combination with published data, retrospective analysis of our attempts to establish PFA EPN PDOX models demonstrated that PFA EPN tumors that harbor 1q gain are more likely to lead to successful PDOX establishment than tumors that do not (Table 1). In support of this theory, Zhao and colleagues (29) recently demonstrated that 1q gain in primary PFA EPNs is consistently maintained upon orthotopic xenograft, supporting a role of 1q gain in the tumorigenicity of this disease. As 1q gain is associated with poorer outcomes and more aggressive disease in PFA EPN (4, 30), it is possible that the lack of this alteration (in the absence of other oncogenic alterations) in TK-EPN862 contributed to its inability to be serially transplanted *in vivo* beyond two passages.

Although chromosome 1q was unaltered, TK-EPN862 PDOX and its matched patient tumor harbored a number of large-scale copy number alterations including gains in chromosomes 7, 8 and 19, and loss of 2q and 6q. A recent study of 240 pediatric PFA EPN reported that while gain of either chromosome 7 (12/240), 8 (15/240) or 19 (12/240) were observed in 5-6% of PFA EPN tumors, few demonstrated concurrent gain of all three chromosomes (2/240), and loss of 2q was rarely observed (2/240) (4). A more recent analysis showed that whole chromosome gains, including gain of chromosomes 8 and 19 were more common in PFA-2 EPNs (as is the case described here) compared to PFA-1 EPNs (10). These findings suggest that these alterations may be recurrent in this specific subset of PFA EPN, although their significance in the development or progression of these tumors remains unclear. Whilst whole chromosome 7 gain has been associated with an increased risk of recurrence in pilocytic astrocytomas (39), this link in PFA EPN has not yet been demonstrated. By contrast, 6q loss was more frequently observed in PFA EPN (25/240) in the Pajtler et al. (4) analysis and has been associated with recurrence in PFA tumors and poor prognosis independent of 1q gain (10, 12, 40, 41).

No clinically significant mutations were present in TK-EPN862 or the matched patient tumor. Unlike some other brain tumor types, PFA EPNs are often genetically silent and lack hallmark gene amplification or specific recurrent mutational events (11, 42). Instead, PFA EPNs tend to demonstrate global changes in the epigenome, with widespread loss of histone H3 K27 tri-methylation being the major tumor driver (9, 10). Efforts to mimic such events in the laboratory to genetically engineer mouse models of PFA EPN is challenging. This is in contrast to the development of mouse models for supratentorial EPN, where expression of the *ZFTA-RELA* fusion is strongly tumorigenic (42–44). Overexpression of *EZHIP* in mouse hindbrain progenitor cells has been shown to generate tumors that resemble EPN in the mouse (45); however, this required additional genetic alterations not common in PFA EPN.

Given these challenges, PDOX models would be incredibly valuable for PFA EPN translational research; however, as our study highlights, the success rate of establishing such models in the laboratory is low. Others have also noted lower success rates for this tumor subtype compared to all other CNS malignancies attempted, including medulloblastoma, primitive neuroectodermal tumors, atypical teratoid/rhabdoid tumors, and high-grade gliomas (16, 18), demonstrating how difficult these models are to generate. Even in laboratories that have had success generating EPN PDOX models (16), the gradual loss of tumorigenicity with subsequent *in vivo* passages is not uncommon, highlighting the challenges of generating PDOX models of this particular brain tumor type.

One possible reason for the lack of PDOX success for PFA EPN is the potential role of the tumor microenvironment, which is becoming increasingly important in our understanding of these cancers. Preliminary data suggest that PFA EPN cell proliferation and tumor progression may be driven by a cycle of continual and unresolved "wound repair", initiated by hypoxia or myeloid cell interactions that trigger epithelial-mesenchymal transition (46). Indeed, Michealraj et al. (47) demonstrate that primary cultures of PFA EPN grow best in hypoxic conditions (1% oxygen), where they have a higher establishment rate, proliferate more, and have reduced markers of cellular senescence and apoptosis. Hypoxia also plays a critical role in the characteristic hypomethylation of lysine 27 on histone H3 in PFA EPN (47). This group went on to report that hypoxia gene expression signatures are at their peak in the murine fetal hindbrain microenvironment at the same point in development when the cells of origin for PFA EPN arise, specifically embryonic days (E) 10 and 16 in the mouse (47–49). Additionally, the metabolic phenotype of mouse hindbrain at E16 closely resembles that observed in PFA EPN (47).

In this study, we exclusively used adult immune-deficient mice to propagate PDOXs. Based on the findings of Michealraj et al. (47), we hypothesize that implantation of patient-derived PFA EPN cells into embryonic mouse brains at approximately E16 may improve PDOX success, as this coincides with conditions in which the microenvironment is most supportive of PFA EPN growth. Whilst the use of immune-compromised strains is common for PDOX modelling, there have been reports of successful intracranial implantation of patient-derived glioblastoma cells into immune-competent E12.5 mice (50). Although the number of tumor-bearing brains progressively decreased after birth, tumors persisted in some mice at P28, highlighting the exciting potential of this technique. If established, an embryonic PDOX model in an immune-competent mouse such as that described in Hoffmann et al. (50) would also allow investigation of immune cell interactions in the development and treatment of PFA EPN.

In conclusion, PFA EPN is the most common and the deadliest subclass of EPN in children, with high rates of recurrence. There is a pressing need for more effective treatments for these patients. PDOX models facilitate a better understanding of the biology of the disease and allow for preclinical testing of novel therapies, with the hope of translation to the clinic and improved outcomes for patients. The development of PDOX models of PFA EPN is urgently needed and very challenging. We have extensively characterized a PDOX model of PFA EPN that persisted *in vivo* for two passages before losing tumorigenicity. Comparison with successful models developed across six independent laboratories suggests that 1q gain, predictive of tumor aggression and poor outcome clinically, may be

an indicator of likely PDOX generation success. Additionally, we postulate that implantation of patient-derived tumor tissue into the brains of embryonic mice may increase the chances of success, as the microenvironment is most supportive of PFA EPN tumor growth at this stage of development.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repositories and accession numbers can be found below: EGA archive, WGS: EGAS00001006843, RNAseq: EGAS00001006844.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Child and Adolescent Health Service, Western Australia (HREC: 1769/EP (PRN 0000002372) A Perth Children's Hospital Oncology Protocol for Collecting and Banking Paediatric Research Specimens. Written informed consent was obtained from the minor's legal guardian for the publication of any potentially identifiable images or data included in this article. The animal study was reviewed and approved by the Animal Ethics Committee of the Telethon Kids Institute and performed in accordance with Australia's Code for the Care and Use of Animals for Scientific Purposes.

Author contributions

Conceptualization, JW, NG, RE. Methodology, JW, HH, CM, MW, MC, CW, MT, EG, RE. Software, MC, CM, MW. Formal analysis, JW, MH, CM, MW, PB, PA, LC, RE. Investigation and validation, JW, HD, HH, CM, MW, PB, PA, LC, CW, MB, JB, KR, MT, EG, JD, SL. Resources, RE, NG, PE, MC, SL, JD, MT, EG, DZ. Data curation, JW, HD, CM, MW, PB, PA, LC, MC, PE, RE. Writing—original draft preparation, JW, HD, MH, RE. Writing—review and editing, all authors. Visualization, JW, HD, MH, RE, CM, MW, PB, PA, LC. Supervision, NG, RE, MC, PE. Project administration, RE, JW. Funding acquisition, NG, RE, MC, PE, DZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1123492/full#supplementary-material

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A new era for optic pathway glioma: A developmental brain tumor with life-long health consequences

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Optic pathway and hypothalamic glioma (OPHG) are low-grade brain tumors that arise from any part of the visual pathways frequently involving the hypothalamus. The tumors grow slowly and present with features driven by their precise anatomical site, their age at presentation and the stage of growth and development of the host neural and orbital bony tissues. Up to 50% of optic pathway glioma arise in association with Neurofibromatosis type 1 (NF1), which affects 1 in 3,000 births and is a cancer predisposition syndrome. As low-grade tumors, they almost never transform to malignant glioma yet they can threaten life when they present under two years of age. The main risks are to threaten vision loss by progressive tumor damage to optic pathways; furthermore, invasion of the hypothalamus can lead to diencephalic syndrome in infancy and hypopituitarism later in life. Progressive cognitive and behavioural dysfunction can occur, as part of NF1 syndromic features and in sporadic cases where large bulky tumors compress adjacent structures and disrupt neuro-hypothalamic pathways. Persistently progressive tumors require repeated treatments to attempt to control vision loss, other focal brain injury or endocrine dysfunction. In contrast tumors presenting later in childhood can be seen to spontaneously arrest in growth and subsequently progress after periods of stability. These patterns are influenced by NF status as well as stages of growth and development of host tissues. The past two decades has seen an expansion in our understanding and knowledge of the clinical and scientific features of these tumors, their modes of presentation, the need for careful visual and endocrine assessment. This influences the decision-making surrounding clinical management with surgery, radiotherapy, chemotherapy and most recently, the potential benefit of molecularly targeted drug therapy. This article, based upon the authors' clinical and research experience and the published literature will highlight advances in approach to diagnosis, the established role of vision loss as justification of treatments and the emerging evidence of endocrine and neurological consequences that need to be incorporated into judgements for case selection for therapy or observation. Consideration is given to the current state of biological evidence justifying current trials of new therapies, the genetic studies of the NF1 gene and the potential for new approaches to OPHG detection and treatment. The outstanding health system priorities from the perspective of children, their parents and health system commissioners or insurers are discussed.

KEYWORDS

optic pathway hypothalamic glioma, childhood, treatment selection, health outcomes, vision loss, endocrine late effects

Introduction

Optic pathway hypothalamic glioma (OPHG) are a group of low-grade developmental tumors of the brain that can arise anywhere along the visual pathways from the optic nerves to the optic radiations as well as involving the adjacent hypothalamus and surrounding limbic structures. These tumors classically present in early childhood (under the age of eight years). Up to 50% are associated with the inherited cancer predisposition syndrome neurofibromatosis type 1 (NF1), which usually presents earlier in life at less than five years of age. From the perspective of children with NF1 up to 20% can present with OPHG. Overall, sporadic and NF1 associated OPHG account for 3%–5% of childhood brain tumors. They seldom metastasise within the central nervous system and almost never systemically. Long term survival into adulthood can be expected in over 80%. NF1, as a genetic cancer pre-disposition state, places individuals at increased risk of specific low-grade and malignant tumors throughout life. These lifetime risks influence treatment selection justifying minimal use of radiotherapy and avoidance of DNA mutating drugs such as alkylators, wherever possible. Furthermore, the risk of vision loss requires careful justification for the use of drugs with toxicities linked to hearing damage or other neurological toxicities (1, 2).

The detection and management of OPHG pose significant challenges for the wide variety of practitioners seeing children (3). Their deep midbrain, central location makes the majority unsuitable for surgical resection, without the risk of significant visual, endocrine and/or cognitive and behavioural consequences (4). Scientific progress in the past decade has identified targetable cellular growth pathways, which have opened up the opportunity for trials of innovative therapies (5). This article will address the following questions:

- How do OPHG present clinically and can we accelerate diagnosis?
- How do you select children for treatment and monitor its benefit and toxicity?
- What are the risks of vision loss
- What are the risks of neuro-endocrine deficiencies?
- How will the new clinical knowledge influence clinical practice?
- What are the trial questions under current study?
- What are the outstanding questions from patients and families and health care providers?
- What is the emerging biological evidence for current and future trials?
- What are the outstanding questions from the patients' and families' perspectives?

How do OPHG present clinically and can we accelerate diagnosis?

OPHG can present with:

- signs and symptoms of impaired visual function due to optic nerve damage the nature of which is related to the precise anatomical site of nerve involvement along the visual pathways. Nystagmus due to poor visual acuity or focal mid brain abnormality can occur;
- acute hydrocephalus requiring urgent cerebro-spinal fluid (CSF) diversion, particularly when the tumor or an associated cyst fills the third ventricle;
- proptosis due to retrobulbar optic nerve tumor displacing the eye forward;
- disturbances of growth and sexual development patterns due to disruption of afferent and efferent hypothalamic signalling;
- diencephalic syndrome due to hypothalamic tumor involvement in the first two years of life causing an extreme form of metabolic disturbance characterised by impaired weight gain with preserved growth in length /height, hyperactivity, hypermetabolism, persistent vomiting and an eye movement disorder (See **Figures 1A, 1B**).

Many of these presentations occur in the first five years of life from effects of growing tumor affecting the hypothalamic control of endocrine, metabolic and neuro-behavioural functions affecting longitudinal growth, weight gain, sexual development, cognitive and emotional functioning. Identification of a child with these presenting symptoms or signs requires parents (6), carers and practitioners to be aware, vigilant and curious to select children in a timely way for the key diagnostic tests for tumor diagnosis. Subsequent neurodevelopmental and endocrine assessments are required to delineate the degree of hypothalamic disorder.

OPHG and NF1

Where NF1 has been established as a diagnosis by family history or observation of classical café au lait patches and other features of NF1; regular visual surveillance together with growth, puberty and developmental monitoring in the first five years of life, is recommended (1, 2). Brain imaging is increasingly being used as a screening/surveillance test to detect those at risk of progressive growth abnormalities and visual loss, especially if compliance with vision testing is sub-optimal. The benefits of screening with brain imaging in NF1, remains to be proven as many structural abnormalities of the optic pathways fail to progress and lead to vision loss, furthermore spontaneous tumor regression can occur. On the other hand, children can present with large tumor with minimal symptoms on surveillance. These situations parallel the challenge of detecting neuroendocrine signalling disturbance in NF1 in early life (7, 8), where GH excess syndromes in NF1 (6) which appear to spontaneously evolve to GH deficiency are increasingly reported in the youngest infants. Specific mutations within the NF gene are now recognised to be associated with the risks of optic nerve glioma development at specific developmental stages (1, 9) (See **Figures 1C, 1D**).

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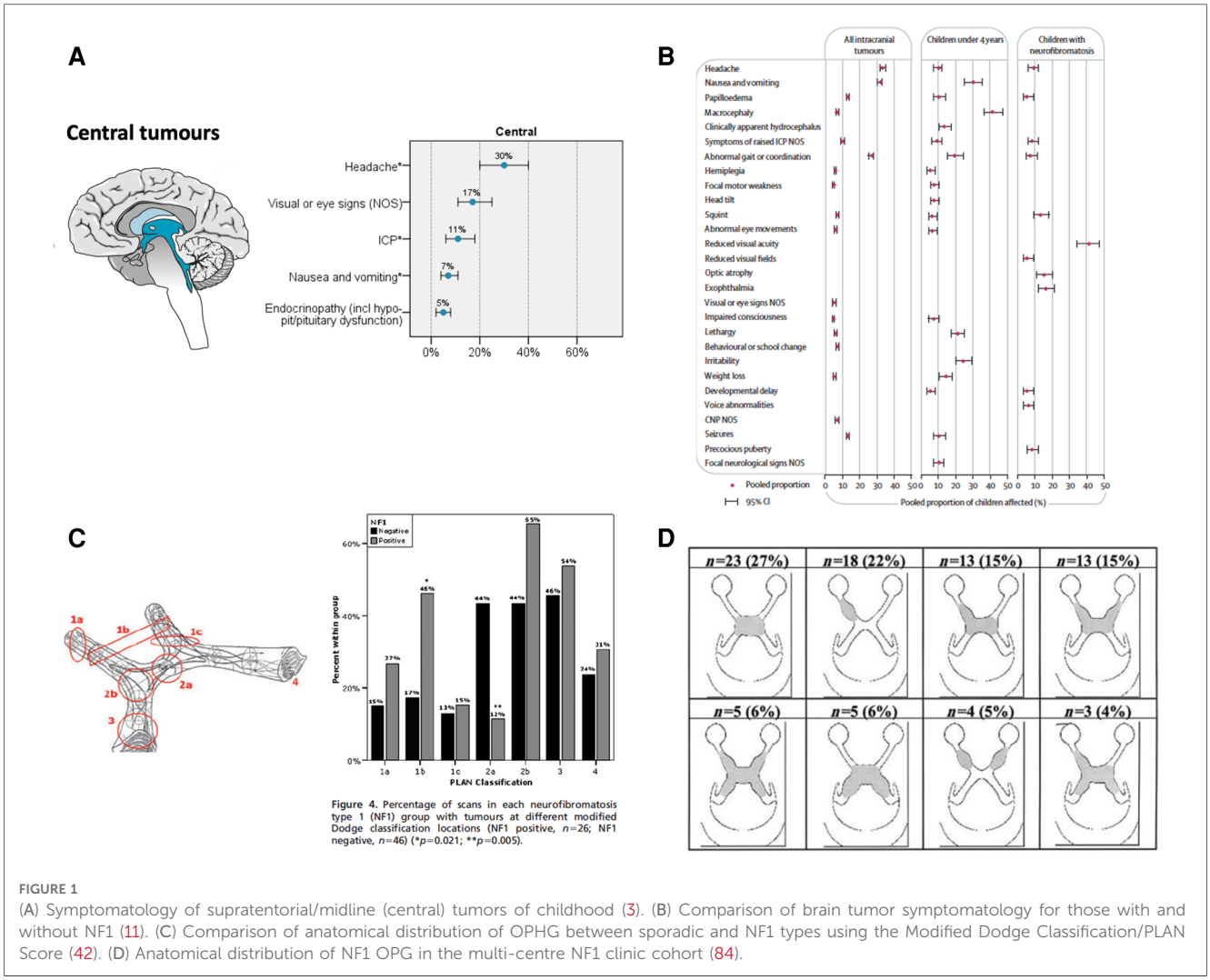


Figure 4. Percentage of scans in each neurofibromatosis type 1 (NF1) group with tumours at different modified Dodge classification locations (NF1 positive, $n=26$; NF1 negative, $n=46$) (*$p=0.021$; **$p=0.005$).

FIGURE 1
(A) Symptomatology of supratentorial/midline (central) tumors of childhood (3). (B) Comparison of brain tumor symptomatology for those with and without NF1 (11). (C) Comparison of anatomical distribution of OPHG between sporadic and NF1 types using the Modified Dodge Classification/PLAN Score (42). (D) Anatomical distribution of NF1 OPG in the multi-centre NF1 clinic cohort (84).

It has recently been established that raising awareness of early signs and symptoms of brain tumor in childhood amongst the public and health professionals can accelerate diagnosis of brain tumors; though growth/puberty abnormalities and thirst dysregulation remain poorly recognised symptoms by practitioners and the public (10). The HeadSmart programme identified age-stratified and NF status-stratified symptom checklists which have been published (11) and trialled with the public and health professionals. They have been shown to be acceptable for selection and rejection of patients for brain scanning. Their widespread use in the hands of the public and professionals has been associated with accelerating diagnosis of childhood brain tumor in the UK national health systems (3, 12, 13). However, tumors in the central region of the brain including OPHG, currently have the longest total diagnostic interval (3). Taken together, a clinical diagnosis of NF1 presents an opportunity for enhanced precision in predicting the risk, or early detection, of OPHG as a pathway to select young children for sight-preserving and neuro-endocrine evaluation strategies at an early stage.

How do you select children for treatment and monitor its benefit and toxicity?

Typically, the results of the brain scan make the diagnosis. The co-existence of clinical features of NF1, assessment of visual function, growth parameters, neurodevelopmental, endocrine and metabolic status/risk provide the key elements for consideration of treatment or observation. The European trials used a standardised age and NF1 stratified algorithm for case selection of medical treatments and radiotherapy (**Figures 2A, 2B**).

Multi-disciplinary team assessment

It is recommended that all cases should be considered by the paediatric neuro-ophthalmic and neuro-oncology multi-disciplinary team and to these should now be added neuroendocrine and neurodevelopmental expertise (14). A key element of the clinical consideration is the role of neurosurgery

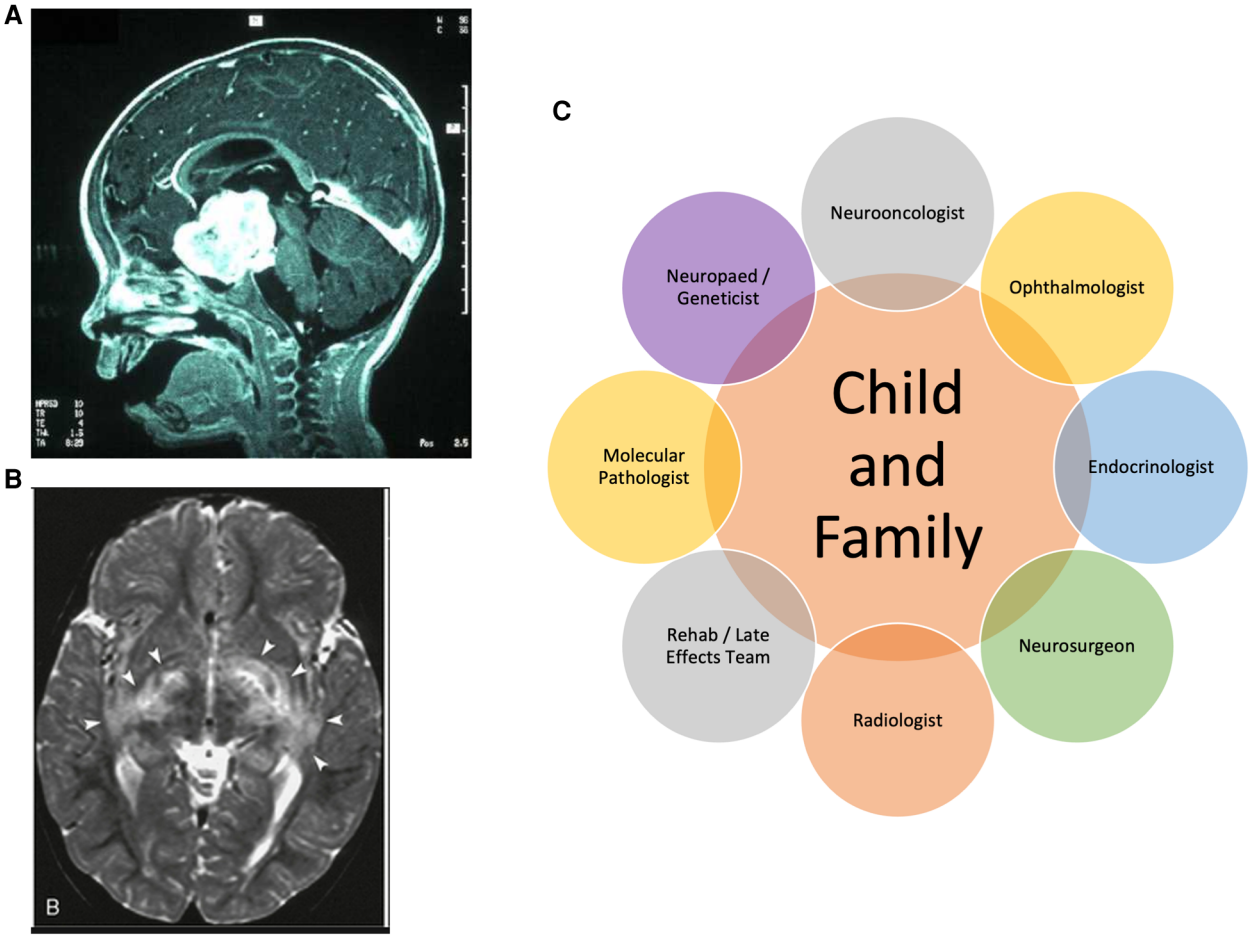


FIGURE 2
MRI scan of typical (A) sporadic hypothalamic and (B) multi-focal NF1 OPHG involving posterior radiations; (C) clinical specialisms involved in the OPHG multidisciplinary team.

for biopsy, management of raised intra-cranial pressure and consideration of tumor debulking (see below). A range of genetic mutations have been described converging on the MAPK/ERK regulatory pathway and contributing to functional activation of the pathway. The overwhelming majority are low grade histology with molecular characteristics defined in the recent WHO classification including what used to be described as pilocytic astrocytoma (PA), pilomyxoid astrocytoma, diffuse low-grade glioma and an adult variant of anaplastic pilocytic astrocytoma; rarely, higher grade gliomas occur in this anatomical region and need to be identified (5, 15).

Historically, multi-disciplinary teams had not specified that ophthalmologists, endocrinologists, neurodevelopmentalists or geneticists should be mandatory members for case discussion. As the treatments evolve under clinical trials, visual outcomes are now specified as primary outcome measures, requiring ophthalmologists to be central to decision-making and outcome measurement. It can be anticipated that, for children who often demonstrate occult endocrine presentations or evolving consequences of both disease and therapies, lifelong endocrine follow up will be required (16). Similarly, specialist genetics clinics now often manage children with NF1, especially where there are complex features and detailed genotyping offers risk assessment for OPHG development. The clinical perspectives of these disciplines are of great importance given the multiple problems associated with NF1 across all ages (2). The specialists with particular expertise in non-surgical therapy are the paediatric oncologists and radiotherapists whose role is to weigh the potential benefit of their anti-tumor therapies against the genetic and age-stratified risk of vascular (moya moya), endocrine, neurological toxicities and the risk of second tumors. The high survival rates for OPHGs make these judgements of particular importance (**Figure 2C**).

Selecting cases for observation vs. treatment

Diencephalic syndrome: There is general agreement that infants presenting with diencephalic syndrome due to hypothalamic astrocytoma require drug treatment directed at reducing the tumor's metabolic activity and continued growth (17). Chemotherapy with vincristine and carboplatin or vinblastine monotherapy has been extensively used and the parameters of the hypermetabolic syndrome [in which GH excess may play a

part] can be expected to be reversed by such treatments (17, 18). Serious neurological toxicity has been reported where tumor response is dramatic (19). As nutritional failure is a presenting feature, intensive nutritional management is needed in parallel with anti -tumor treatments (20). Even with attempts to treat such cases, the metabolic and neurological challenges are such that brain injury, spontaneous haemorrhage, surgical complications or acute neuroendocrine disruption can lead to life threatening complications. For those who survive lifelong neurobehavioural, neuroendocrine disturbances, as well as visual, hypothalamic and developmental consequences, can be expected.

Indications for (immediate) surgical intervention

Modern clinical practice requires tumor tissue to be examined histologically and molecularly. In NF1, it is still justifiable to omit biopsy if there is any risk of surgery adding to vision loss, endocrine or neurological toxicity. In sporadic cases, biopsy is needed to ascertain both histological and molecular phenotype, especially if a child is to be entered in a clinical trial using targeted therapy. Management of hydrocephalus is also indicated where appropriate. The selection of cases for consideration of resection/debulking of hypothalamic tumors is an area of particular debate (4, 21, 22). While a significant proportion of the tumor infiltrates optic pathways and the hypothalamus, and is therefore unresectable without further harm, most OPHGs also contain exophytic components and cystic elements. Resection of exophytic tumor into the third ventricle or frontal lobes may be effective at reducing tumor size rapidly with minimal surgical risk. Cystic components exert high mass effect and are not generally responsive to chemotherapy. Drainage or fenestration of large cysts, or implantation of an indwelling reservoir, may be useful in supporting the benefits of chemo- and radiotherapy. Some tumors also have large posterior extensions, leading to symptomatic brainstem compression. In practice, the main difficulty lies in identifying the normal hypothalamic tissue radiologically and intra-operatively. Intra-operative MRI is useful to obtain a tailored resection with maximal safety (23). Although some series have advocated early and extensive resections, it is not clear that clinical outcome is improved in the long term (24, 25). The balance of risks between a large operative procedure that may itself cause hypothalamic injury but substantially reduce tumor bulk, and the long-term compressive effects of a large tumor on central structures is not known and needs further study. Similarly, the timing of major surgical interventions, and specifically whether surgery should be considered early after diagnosis or only after radiation and/or several cycles of chemotherapy have failed, is unclear. Specialist and multidisciplinary post-operative care and continuous endocrine and neurodevelopmental rehabilitation is needed after surgical resection. A recent institutional series of OPHG identified surgery of whatever type to be associated with risks of posterior pituitary endocrine failure in nearly 60% of cases (21, 26).

Proptosis: This presentation occurs with tumors arising in the optic nerve in the retro-orbital space. When presenting in the first two years of life, vision may be threatened or lost. If optic atrophy is present, vision recovery with chemotherapy will be limited by the established loss of nerve function. If vision is preserved and the main consequences are cosmetic, then differential growth of the orbit and tumor may reduce the severity of the proptosis in the first five years of life. The only surgical option is resection of the optic nerve for cosmetic reasons, if the eye is blind and the proptosis is disfiguring, leaving the eye *in situ*.

Chemotherapy

The young children with OPHG (<5 years) have been offered treatment with chemotherapy as primary treatment over the past three decades. The drugs used have focused predominantly upon two drug classes: platinum agents: carboplatin/cisplatin and vinca alkaloids: vincristine/vinblastine (17, 27, 28). They were selected for their low mutagenic toxicity profiles, they can be administered as a day case in fractionated doses and have predictable toxicities. Intravenous administration is required for both drug classes and is associated with the risks of bone marrow suppression with neutropaenia, immunodeficiency, thrombocytopaenia and the need for blood transfusion. Carboplatin was found to be associated with significant drug reactions in up to 20% when given over prolonged periods (29, 30). Renal and auditory toxicities are important to watch for, but infrequent with carboplatin; they are predictable and more common with cisplatin. Vincristine is much less toxic to the bone marrow than vinblastine and was primarily selected for early trials for this reason. However, its prolonged pharmacological half-life (~5 days) causes cumulative peripheral and autonomic neuropathy when used on a weekly schedule. There have been reports of vision loss associated with such neuropathies (31, 32). Using a drug, administered in neuropathic doses, to reverse a neuropathy seems unwise, just as is the use of ototoxic drugs where vision is already compromised/threatened. Adopting a four-weekly schedule to minimise the risk of vincristine neuropathy would seem a reasonable precaution. Monotherapy with carboplatin has comparable outcomes (33) for tumor control. Monotherapy with vinblastine is less neurotoxic but more marrow toxic (34) than vincristine. There is increasing experience in the use of monotherapies as primary therapy in OPHG. It is unclear whether speed of tumor response is comparable to combination therapies. Reports of irinotecan and bevacizumab in relapsed patients has been associated with improvements in vision (17). The optimal duration of therapy has not been determined. The use of these drug regimens ranging from 12 to 18 months have been reported. The age at treatment onset may be a key variable, given the tendency for tumor to spontaneously arrest in growth after 5–8 years of age. Tumor regrowth during adolescence is reported but not fully studied. Tumors have usually not been reported as progressive during adulthood. How these active and quiescent periods reflect

age- and maturation-dependent key growth periods driven by hypothalamic hormone/neural signalling seems an important, and as yet, unexplored, future research question.

Radiotherapy and its consequences

Radiotherapy has been used and has a stronger track record for controlling visual deterioration than chemotherapy but is known to cause impairments of local tissue growth of skull and brain tissue especially in very young children (35, 37). Radiotherapy is largely contra-indicated in NF1 because of the risk of secondary malignant tumor development within radiation fields (36). Radiotherapy involving the hypothalamic structures and adjacent carotid arteries carries additional risks of moya moya phenomenon of the carotid arteries (39–41). The risk of second tumors is lower for sporadic cases than for cases associated with NF1. Both carry the risk of (meningioma) and malignant tumor development such as Glioblastoma Multiforme (GBM). In NF1 the exaggerated risk of malignant peripheral nerve sheath tumors after radiotherapy is well recognised (38).The development of proton therapy with its more contained fields of treatment offers reduced risk of off-target radiation dosing with as yet unknown benefit on cognitive or endocrine function (37). Current practice is to defer radiotherapy until after one or more drug treatments have been tried, and been seen to have failed (37).

What are the risks of vision loss?

Visual development: OPHG presentation during infancy and in pre-school age children is at a time where vision testing can restricted by their [in]ability to cooperate with visual acuity and field testing. Children's vision is in the process of developing to maturity during specific age- and time- dependent windows, and the brain's capacity to interpret the quality of image they experience which changes as their brain matures. This limits precision of early baseline vision assessment. Precise anatomical classification of tumors on imaging offers a prediction of the risk of bilateral vision loss (42) (See **Figures 1C, 1D**). Optical coherence tomography, measuring retinal fibre layer thickness, is being evaluated as a tool to detect early signs of optic nerve injury and its correlation with risks of, and actual, vision loss in young children (43). MRI studies of visual tracts with fractional anisotropy are also under evaluation as an imaging tool to predict visual loss (44).

Can vision be improved or saved?

A recent systematic review failed to identify sufficient published information to reliably report the impact of treatments on visual outcomes (45). This has been studied in limited cohorts of children with NF1 and reported by US and European investigators (46, 47). The conclusions are influenced by the way their study cohorts were recruited. The US study was a multi-institution study cohort. It had a lower median age at diagnosis and reported only patients who were treated. The European study was trials-based and had an older median age and an observation arm and reported outcomes after "immediate therapy" and "therapy after observation" (48) (See **Figure 3**). Taken together, the following conclusions about visual outcomes can be drawn. Case selection at diagnosis has a big impact on visual outcomes. The European trial cohort had greater

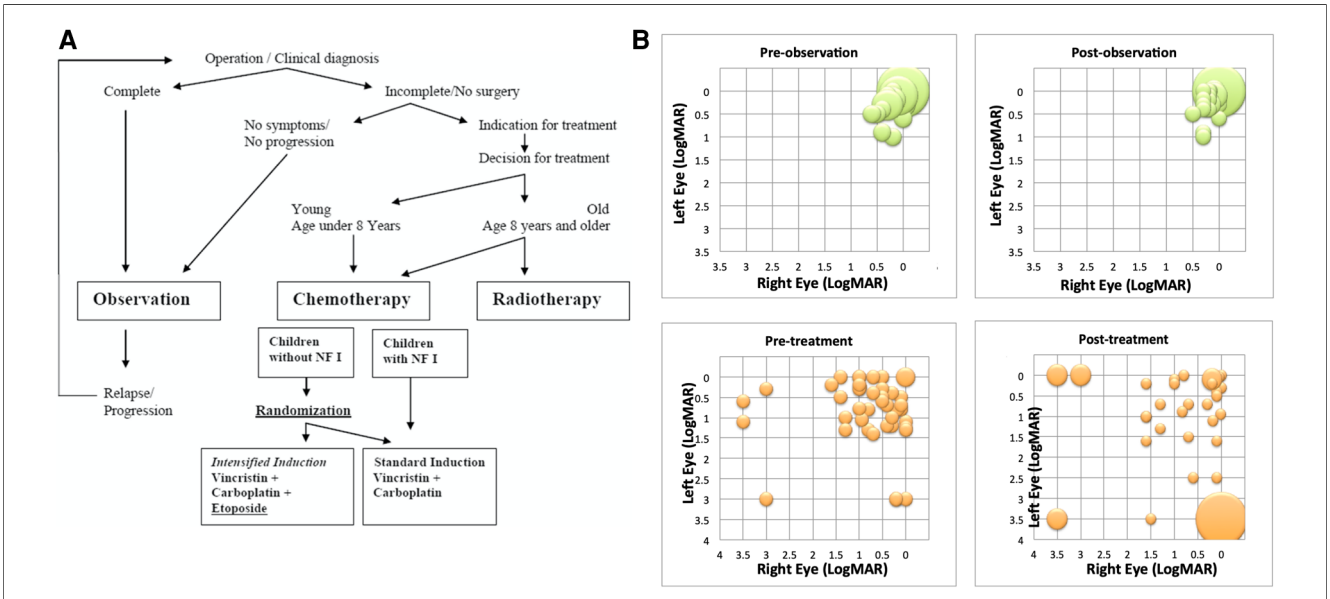


FIGURE 3
(A) Patient selection criteria for observation vs. treatment in SIOP LGG 2 (004 randomised trial (17). (B) Comparison of LogMAR visual acuity results from SIOP LGG 2004 workshop comparing pre- and post- bilateral visual acuity for observation (top green graphs) and treatment (lower orange graphs) with vincristine and carboplatin in patients with NF1 (46).

standardisation of case selection for treatment vs. observation than the institutional cohort where all reported, were treated. Despite these differences both studies showed overall, only 20%–30% of children experience improvement in vision with chemotherapy treatments. About 40%–50% experience stability of vision, whilst the remainder experienced deterioration in vision despite therapy. There was no clear correlation between imaging evidence of tumor response and visual outcomes. Those who are observed initially and seen to lose vision under observation, have a better chance of subsequently retrieving vision with therapy, compared to those treated immediately with more advanced vision loss and symptomatology at presentations. Specifically, those presenting with bilateral vision loss, multiple visual symptoms and optic atrophy seldom experience improved vision after therapy. The neurophysiological explanation for these observations has focused upon the rarity of spontaneous regression, whether the tumor is truly congenital and whether neuronal loss is related to local pressure effect or loss of trophic signalling between neurones and glia (49). Bevacizumab has been reported to improve vision in patients being seen to lose vision under observation (50). Standardisation of methods for measuring and recording imaging and vision outcomes have been developed to standardise selection of patients for treatments (2). Currently, the primary concern about vision loss due to tumor progression is used to justify commencement of treatments, a powerful motivating factor in the minds of parents. To date, apart from diencephalic presentations, endocrine status and late outcomes have not been widely used as a trigger for considering treatment or observation within trials.

Seeking evidence to support selection of cases for observation vs. treatment

An international consensus survey was conducted using clinical and imaging information from children with OPHG associated with NF1 who were entered into SIOP LGG 2004 trial (51) (See **Figure 4**). These cases were presented in a questionnaire format to experienced international physicians ($n = 98$) from the full range of specialities involved in the design of clinical trials of therapy for OPHG. For each case they were offered the opportunity to observe, treat or randomise within a trial from a matrix of 25 cases structured by anticipated risk of tumor progression determined by unilateral or bilateral visual loss, age of the child and anatomical characteristics of the tumor. This consensus survey and its qualitative analysis of supporting comments identified that there was more than 70% agreement (consensus) on the selection of 14 out of 25 cases for observation or treatment. In 11/25 scenarios, however, the respondents did not reach consensus and considered them suitable for a randomised comparison of observation vs. treatment to determine the best course in future practice. The respondents identified the importance of as much detail as possible about the visual and neurological status of children in the period leading

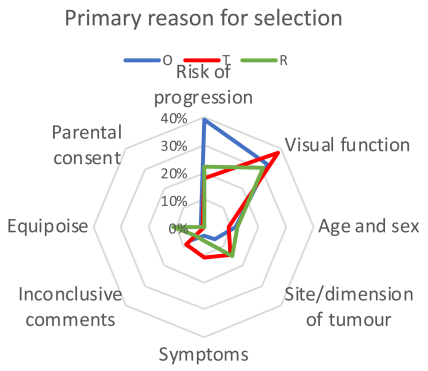
A

Age group / VA	0-2		2-5		5-10		10+	
Both eyes LogMAR ≤ 0.5	PLAN 3/4 (-)	PLAN 3/4 (+)	PLAN 3/4 (-)	PLAN 3/4 (+)	PLAN 3/4 (-)	PLAN 3/4 (+)	PLAN 3/4 (-)	PLAN 3/4 (+)
One eye LogMAR > 0.5	PLAN 3/4 (-)	PLAN 3/4 (+)	PLAN 3/4 (-)	PLAN 3/4 (+)	PLAN 3/4 (-)	PLAN 3/4 (+)	PLAN 3/4 (-)	PLAN 3/4 (+)
Both eyes LogMAR > 0.5	PLAN 3/4 (-)	PLAN 3/4 (+)	PLAN 3/4 (-)	PLAN 3/4 (+)	PLAN 3/4 (-)	PLAN 3/4 (+)	PLAN 3/4 (-)	PLAN 3/4 (+)

B

Age group / VA	0-2		2-5		5-10		10+	
Both eyes LogMAR ≤ 0.5	O	?	O	?	T	?	?	O	? (2)	O	?
One eye LogMAR > 0.5	T	T	T	?	?	T	?	PLAN 3/4 (+)
Both eyes LogMAR > 0.5	T	PLAN 3/4 (+)	T	T(2)	T	?	PLAN 3/4 (-)	PLAN 3/4 (+)

C



D

	O	T	R
Risk of progression	39%	18%	22%
Visual function	33%	38%	31%
Age and sex	12%	9%	12%
Site/dimension of tumour	5%	14%	15%
Symptoms	3%	11%	5%
Inconclusive comments	7%	9%	4%
Equipoise	0%	0%	11%
Parental consent	1%	1%	0%
TOT	100%	100%	100%

FIGURE 4
(**A**) A matrix of patient characteristics including visual acuity (LogMAR scores for one/both eyes), PLAN stage ¾ +/– (optic radation involvement) and age at diagnosis, (**B**): consensus (>70%) voting for 25 NF1 OPHG patient histories reported within the matrix identifying cases selected for initial observation (**O**), treatment (**T**) or? randomisation (?). (**C**) Spider plot of primary reason for consensus judgement for O,T & R. (**D**) Table of clinical reasons supporting strategy selection for O,T & R (51).

up to diagnosis and strategy selection, further supporting the justification for observation before treatment.

What are the risks of neuro-endocrine deficiencies?

A single institution cohort of children with OPHG ($n = 166$), studied over 30 years has reported a 20-year overall survival (OS) of 81.0%, and progression-free (PFS) and endocrine event-free survival rates (EEFS) of 47.2 and 20.8%, respectively. Growth Hormone deficiency (GHD) affected 40.3%, followed by central precocious puberty (CPP, 26.0%), gonadotropin (GnD; 20.4%), TSH (13.3%), and ACTH (13.3%) deficiencies (16, 26). These develop hierarchically. Central precocious puberty (CPP) was associated with future gonadotrophin deficiency. Posterior pituitary dysfunction occurred in 57.9% after surgery involving biopsy or shunt procedures and was associated with 6/13 deaths in the whole cohort. In this cohort, half (50.2%) of surviving children were worryingly obese, with later risks of metabolic syndrome, and other life-limiting consequences including type 2 diabetes. Endocrine deficits ascribed to radiotherapy ranked growth hormone deficiency as the greatest risk followed by ACTH deficiency, insulin resistance and gonadotrophin deficiency. Endocrine Event Free Survival (EEFS) declined up to 15 years after diagnosis, with hypothalamic involvement of tumor being implicated more than radiotherapy in early onset endocrinopathy. GHD surprisingly increased in later treatment eras when radiotherapy was used less frequently (26).

90 children in this cohort were diagnosed aged <3 years and followed for 40 years, they are reported separately (16). Endo-metabolic dysfunction was reported in 58.7%, the main factor contributing to this risk was a clinical presentation with diencephalic syndrome, followed by tumor involvement of hypothalamus, the use of radiotherapy and surgery. These studies suggest a biphasic pattern of detecting endocrinopathy; at diagnosis, as a consequence of tumor damage, and after treatment, as a result of delayed damage from the tumor's continued impact and/or its treatment.

How will the new clinical knowledge influence clinical practice?

This information about endocrine outcomes is newly described and needs to be integrated with multi-disciplinary decision-making, outcome assessment and discussion of the benefits and risks of therapy as well as targeted individualised endocrine remediation in clinical practice. OPHG clinical complexity poses major challenges to parents and their children seeking advice for the best options (52), illustrated mathematically by a multi-state model analysis of a large trial cohort (52). The developmental framework of childhood and adolescence makes decisions at different developmental windows and ages, influenced by stages of brain growth and pubertal maturation, physical characteristics of skeletal growth, as well as social and neuro-psychological maturational competence. This is made more complex by the child's and their family's experience of visual-impairment and/or the clinical complexities of panhypopituitarism. The multi-disciplinary considerations are heavily determined by which specialists are involved in the discussion with the family, their experiences and their own beliefs (53) A model is proposed in **Figure 5**.

To date, there is no formal scoring system where the potential for, and importance of, preservation of visual, endocrine or neuro-behavioural outcomes can be weighed against each other with different treatment approaches. This complexity is a major challenge to communication between physician and the child and family seeking advice on the "best interests" for the child in this disease. It is difficult for parents to find an equitable emotional balance between their perception of risks of mortality vs. risk of lifelong disability for the wide variety of outcomes for their child. Mortality is a categorical risk at a moment of diagnosis, feared by the parent, whilst disability is a qualitative risk over a lifetime. It is frequently a shared experience by the developing individual and their "supporters and advocates".

What are the trial questions under current study?

Developments in the application of novel technology to this disease are occurring. There has been an explosion in biological understanding of tumor tissue biology in childhood. There is a global emphasis now placed upon the need to optimise diagnostic pathways for children with cancers as part of the WHO Cure All Strategy (54). This strategy seeks to influence health systems from all economic categories of countries to level up outcomes for children with cancer globally, justified by a health economic capacity to triple the impact of any investment on health outcomes (55). For this to be realised in brain tumors, a strong focus on reducing neuro-disability with its economic consequences is required. OPHGs represent one of the commonest groups of tumors with clearly defined disabilities of acquired vision loss, endocrine, neurological and developmental deficits with lifelong consequences. Consequently, they offer opportunities for risk stratified approaches to new therapies seeking to reduce disability outcomes.

What are the outstanding questions from patients and families and health care providers?

Experience with the HeadSmart programme in the UK identified the impact of raising awareness amongst the public and professional communities to accelerate diagnosis of childhood brain tumor within a national health system (3, 56). Similar projects have now been launched in several countries. In high income countries (HICs), OPHG have been identified as one of the most common treatable cause of vision loss in children (57), justifying special consideration for accelerating

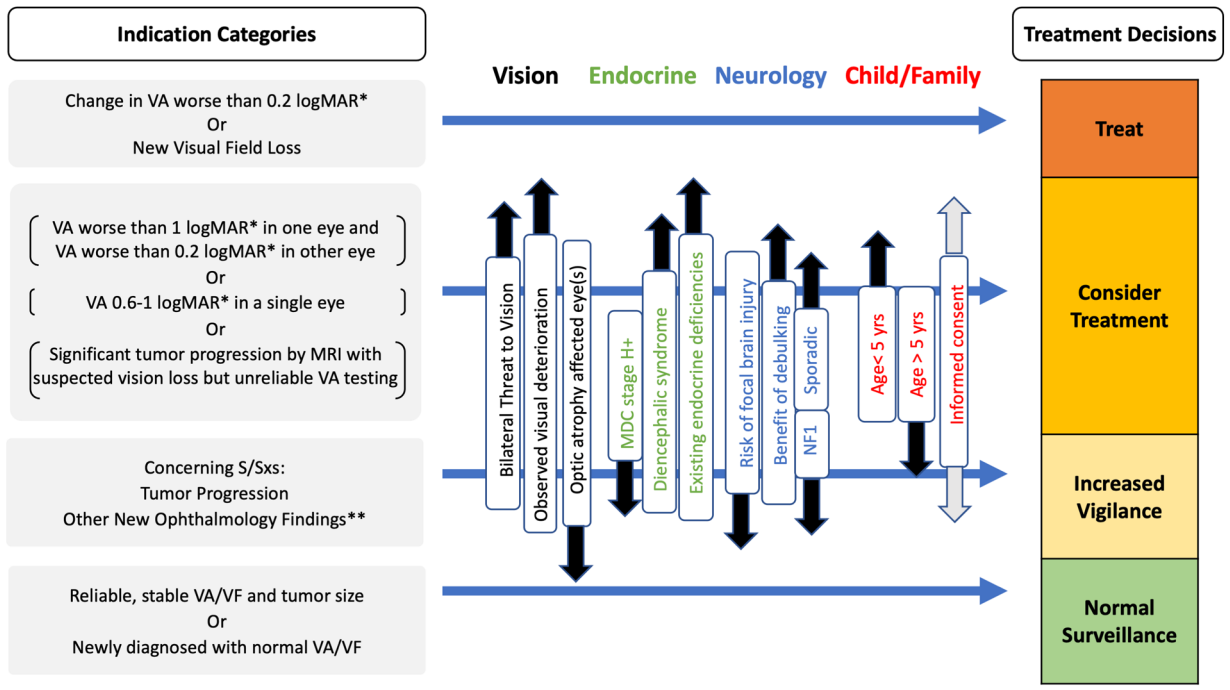


FIGURE 5
Evidence-based multi-disciplinary factors to be considered for selection of treatments (surgery, chemotherapy or radiotherapy) vs. observation in OPHG of infancy and childhood in OPHG Adapted from (3, 46, 79).

diagnosis. The neuro-paediatricians diagnose and manage OPHGs with geneticists, neuro-ophthalmologists, brain imaging specialists and endocrinologists, who all work with specialist teams to screen, diagnose and manage the neurotoxic and endocrine consequences of these tumors. As time passes and the child becomes an adult, the need for lifelong rehabilitative neurobehavioural and endocrine/follow-on clinics to transition successfully into adult services. System models exist but their further development (58) requires the health economic data to justify their incorporation into adult service models of public or private health service commissioners or insurers (59).

How will the emerging biological evidence influence current and future trials?

The past 2 decades has seen the biology of pilocytic astrocytoma (PA) explored in detail. Nearly 100% of pilocytic astrocytoma have mutations involving the MAPK/ERK signalling pathway regulation, where BRAF kinase alterations are considered to be the characteristic hallmark. The most common rearrangement is a fusion between KIAA 1549 and BRAF genes which occurs in 70% of PAs; the next most common are inactivating NF1 alterations and oncogenic $BRAF^{V600E}$. Others reported less frequently are other BRAF fusions, FGFR1 mutations or fusions, NTRK2 fusions and oncogenic KRAS mutations. They all activate the MAPK/ERK pathway, making PA a single pathway disease, ideal for therapeutic targeting (5).

Targeting the NF1 gene

A recent review identified that clinical examination of patients combined with molecular analyses is beginning to reveal *NF1* genotype-phenotype correlations - such findings will help define novel functions of neurofibromin, its interactions with the tissue microenvironment and hormonal milieu. Sustained research, driven by access to patient samples for the development of patient and cell-specific models reflecting the human disease will drive cellular pathway analysis and the identification of therapeutic targets and biomarkers suitable for pre-clinical testing. A range of novel strategies are already under consideration including synthetic lethal screening (using CRISPR libraries), immune profiling for immunotherapy and generation of novel biomarkers for NF1-associated tumors. Gene therapy approaches focus on antisense oligonucleotides (ASOs) and nonsense suppression, whereas potential correction of mutations *via* gene editing offers a possibility of restoring endogenous *NF1* gene function, thereby providing a long-term solution for NF1 patients (60).

New trials of therapies

Drugs targeting MEK inhibition (MEKi) have been selected for testing in NF1- and BRAF-altered paediatric low-grade gliomas (pLGG) and for PAs in particular. The MEKi Selumetinib showed promising results in phase I and II trials (61–64). Similarly, the MEKi Trametinib is under trial for recurrent NF1-associated and BRAF-fusion pLGGs (65). Another trial (NCT 03871257) is

investigating Selumetinib in conjunction with vincristine/carboplatin in a front line setting for NF1-mutant pLGG. The European LOGGIC trial will be the first prospective randomised 2-arm study of pLGGs harbouring an active RAF mutation, comparing an oral pan-RAF inhibitor tovorafinib (DAY101) vs. standard of care carboplatin/vincristine or vinblastine monotherapy as first line treatment (65, 66). Most recently, results of the prospective randomized phase II trial (NCT02684058) of the combination of a BRAF inhibitor dabrafenib (dab) and the MEKi trametinib (tram) as first line therapy for $BRAF^{V600E}$-mutant pLGG identified that the "dab + tram" combination increased overall response rate and clinical benefit rate and prolongs progression free survival when compared with carboplatin and vincristine. These encouraging results and the tolerable safety profile suggest that "dab + tram" may be a promising first-line systemic treatment option for this patient population.

A preliminary consensus for treatment selection

A recent proposal for a consensus mapped the molecular relationship between the tumor's anatomical location, the age of the child and histological characteristics of the tumor tissue. They identified 3 groups and justified clinical approaches ranging from adopting either a conservative approach, or being pro-active or identifying cases justifying more aggressive approaches. They did not map their stratifying factors onto late neurological, endocrine or neuropsychological/ behavioural outcomes or indeed data concerning pre-diagnostic intervals. The consensus therefore is tumor-centered and not patient-centered and may be considered simplistic as it disregards the clinical experience of survivorship, summarised in this review (67). Despite this criticism, the biological research that has identified the wide range of molecular targets offers real hope of effective therapies that are in the process of translation through clinical trials. It is imperative, at this time, that the missing elements of this consensus are given careful consideration as not all problems will be solved by the new drugs being developed for many reasons. Furthermore, health services and translational research directed at neuroprotection already may offer opportunities to apply novel approaches for minimising adverse consequences affecting survivorship. Evidence already exists which demonstrates the potential for the role of bevacizumab in preserving and improving visual outcomes at the time of tumor progression (68); topically applied nerve growth factor has been shown to restore optic nerve function (69) in children with OPHG; preliminary research is reported where brain stem-cell therapy is being investigated for brain injury repair (70) as well as evidence of rising health service awareness of the need for early symptom awareness and specific services to support children after acquired brain injury (58, 71).

What are the anticipated developments?

These trials of new therapies are specifying clinical outcomes such as vision assessments as a primary outcome measure(s). Based upon the new reports of endocrine and neuro-behavioural outcomes for children with OPHG and the uncertainties of how to select patients for treatment vs. observation, we conclude that further studies are needed to dissect the impact of tumor progression vs. consequences of treatment on these additional health outcomes (16). A trial design selecting patients for randomisation between initial observation vs. initial therapy is justified by the existing uncertainty for when to treat or observe children using vision loss and endocrine outcomes as primary outcomes. It could be highly informative and integrated with trials of new tumor agents as part of pre-treatment registration. Such an approach would generate valuable evidence to reduce the current levels of uncertainty as to who to treat and who to observe. Taken together, the potential for these translational trials and health system interventions raise hope that it will be possible to reduce the impact of OPHG upon the late consequences of this disease and its treatment (72).

Anticipated developments

- *Accelerating diagnosis* by raising awareness of the risk and the classical neuroendocrine and intracranial pressure presentations of OPHG as well research to target populations for screening or surveillance. The opportunity exists to use a combination of NF status and clinical growth, visual, developmental biomarkers for case selection for vision testing or scanning. If applied successfully it could tackle the prolonged pre-diagnostic interval that is a characteristic feature of tumors arising around the middle of the brain and optic tracts (3, 73). This approach is justified by clinical and legal arguments used to justify compensatory awards to individuals identified as suffering additional observed disability as a consequence of diagnostic delay (74).
- *Standardised approaches to visual acuity testing* will permit more reliable assessments of visual performance as part of treatment selection and outcomes assessments in practice and trials (46, 47, 75).
- *Innovation in brain and retinal imaging* of OPHG has produced a *refined anatomical classification* of OPHG with more detailed functional descriptors (42). Diffusion Tensor Imaging is being used to explore the possibility of *predictive scoring system for vision loss* (77). *Optical coherence tomography* offers measurements of *retinal fibre layer thickness* as an objective measure of nerve loss as part of visual outcome monitoring, as well as the opportunity to understand the relationship between retinal nerve injury and tumor location, tumor size and growth across the optic tracts (43).
- *Introduction of risk stratification for early and developing neuroendocrine and neurodevelopmental* deficits which combine to emerge as so-called late consequences as part of cost-benefits of treatment vs. observation decision making within clinical trials and outcome studies (26).
- The *role of surgery is under scrutiny* for tumors arising in different locations. Optic nerve tumors are no longer considered to be a risk for chiasmatic extension. Surgeons are working towards *a consensus for attempted surgical resection* as part of safe surgery approaches. Such strategies need to offer low risks of endocrine

and neurological/ neurodevelopmental toxicity (14) and should be followed by targeted rehabilitation.

- *Proton therapy* offers reduced risks by enhanced precision of radiation field planning. Research into the lifelong benefits and risks is needed (37)
- *Trials of novel tumor targeted agents* used alone or in combination offer more precisely biologically targeted treatments aimed at changing the damaging effects of tumors on neuronal and hypothalamic functioning (77). Research into the relationship between tumor shrinkage, vision preservation and neuro-endocrine outcomes is needed.
- *Research targeting the biology of the tumor micro-environment in sporadic and NF1 associated tumors,* given the developmental features governing tumor growth and senescence (5, 78, 79). Research into the interaction between tumor cells and neuronal functioning (80) or immune mechanisms that may influence tumor microenvironment in all stages of tumor development (81–83) is required to explain clinical phenomena.
- *Treating brain injury with neuronal protection or restorative therapies* (69, 70).

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Evaluating preclinical evidence for clinical translation in childhood brain tumours: Guidelines from the CONNECT, PNOC, and ITCC brain networks

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Clinical outcomes for many childhood brain tumours remain poor, despite our increasing understanding of the underlying disease biology. Advances in molecular diagnostics have refined our ability to classify tumour types and subtypes, and efforts are underway across multiple international paediatric neuro-oncology consortia to take novel biological insights in the worst prognosis entities into innovative clinical trials. Whilst for the first time we are designing such studies on the basis of disease-specific biological data, the levels of preclincial evidence in appropriate model systems on which these trials are initiated is still widely variable. We have considered these issues between CONNECT, PNOC and ITCC-Brain, and developed a framework in which we can assess novel concepts being brought forward for possible clinical translation. Whilst not intended to be proscriptive for every possible circumstance, these criteria provide a basis for self-assessment of evidence by laboratory scientists, and a platform for discussion and rational decision-making prior to moving forward clinically.

KEYWORDS

pediatric, CNS, preclinical, models, translational, *in vitro*, *in vivo*

Introduction

Despite remarkable advances being made in the treatment of numerous paediatric cancers over the past 40 years (1), tumours of the central nervous system remain the biggest cause of cancer-related death in children and young adults (2). For many entities, survival rates have remained unchanged for decades, and represent a major unmet clinical need (3, 4). Previous generations of clinical trials were necessarily based on an incomplete appreciation of the unique biology of childhood brain tumour entities, and a lack of preclinical evidence in appropriate model systems to show that they were likely to be effective. The failure of these studies, therefore, can now been seen as not unexpected (5). In recognition of this devastating societal impact, over the past 10-15 years partnerships between patients/families, clinical, translational and laboratory-based scientists worldwide have dramatically improved access to tissue and funding for childhood brain tumour research, which coupled with the rapid advances in next-generation sequencing and other molecular profiling techniques, has revolutionized our understanding of the underlying biology of a plethora of childhood brain tumours (6–9).

In the recent 2021 5th Edition of the WHO CNS Classification of CNS tumours, a large proportion of entities were recognized as being of paediatric 'type', or occurring largely in the children and young adult populations (10). Underscoring this delineation is the integrated diagnostic approach which includes key distinguishing biological data, and the appreciation of distinct drivers of the childhood disease types and the subtypes within (11). For many of these tumours, we now have both novel targets for therapeutic development, and a framework by which these children may be stratified for clinical trial enrollment, which will lead to better response assessment in a molecularly defined context. There is still substantial uncertainty, however, around the amount and type of preclinical data that is needed to develop trials that are more likely to succeed compared to their predecessors. The key tenet for moving a concept into the clinic is a strong biological rationale, with support from robust preclinical data in appropriate model systems (12). Until recently, these have been difficult to achieve, which coupled with unselected patient populations has likely contributed to the lack of success of clinical trials for children with brain tumours. As several international paediatric neuro-oncology clinical trials consortia have emerged to address the clinical issues, we now need international consensus in developing more robust preclinical platforms to provide data packages that can be reviewed objectively and systematically prior to clinical implementation.

International childhood brain tumour consortia

The present article is a result of discussions between three international paediatric neuro-oncology clinical trials consortia. Each has a slightly different focus, approach, tumour-type or discipline expertise, and geographical footprint. The groups work non-competitively to ensure access to the most promising trials in the most appropriate environments, and have a degree of overlap in key personnel and centres worldwide.

CONNECT

CONNECT (the COllaborative Network for Neuro-oncology Clinical Trials) is a collaborative of 18 international sites across North America, Europe, UK, and Australia, with expertise in paediatric brain tumour research and clinical trials. Its purpose is to conduct scientifically rational pilot studies to assess feasibility and early efficacy of incorporating promising novel agents to established frontline therapeutic regimens in children with newly-diagnosed, high-risk brain tumours. CONNECT serves as a clinical research organisation providing concept and protocol development, data and study management, drug shipping, and all operational support. It has a diverse portfolio of trials in different childhood brain tumour entities, partnering with multiple drug companies and foundational supporters. The clinical network is supported by an active Preclinical Group, whose goal is to provide scientific assessment of novel concepts brought to the consortium for clinical translation, and to assemble collaborative research teams to provide additional experimental data as warranted.

PNOC

PNOC (Pacific Pediatric Neuro-Oncology Consortium) is an international clinical trial consortium with 22 sites in the US as well as sites in Switzerland, Israel, Netherlands, Canada and Australia with recent expansion into Germany, Egypt and India. The mission of PNOC is to develop biology driven trials and expand access to innovative therapies globally for children and young adults with brain tumors, Development of clinical trials is supported by disease specific working groups composed of clinical, translational, imaging and basic science experts spanning key entities such as high grade glioma/diffuse midline glioma; ependymoma, germinoma, medulloblastoma and craniopharyngioma amongst others. Data collected as part of PNOC trials – such as imaging and genomic data – is shared in real-time with the research community through collaboration with the Children's Brain Tumor Network (CBTN).

ITCC Brain

ITCC Brain is the CNS tumor-specific working group of ITCC (Innovative Therapies for Children with Cancer), a consortium of over 60 expert pediatric oncology centers and 25 leading research laboratories from across Europe. ITCC Brain aims to provide a framework for bringing together biologists and clinician scientists generating cutting-edge basic and translational research findings, with clinicians in large early-phase clinical trial centers, in order to accelerate the translation of novel science into effective new treatments for children with brain tumours. ITCC Brain has a portfolio of investigator-initiated trials as well as providing support for industry-led studies, and is working to expand this portfolio

through well-planned studies based on strong preclinical data. The group also works closely within the larger ITCC organization to participate in entity-agnostic, biomarker-driven studies; and the group also benefits from other ITCC-led initaitives such as the ITCC-P4 pre-clinical platform [ref] and an upcoming platform for integrating data across international pediatric precision oncology progams.

Guidelines for new concepts

Prior to the initiation of a clinical trial, we believe a robust process should be in place to critically review extant preclinical data which support the concept, as well as the strategy for clinical implementation. The intention here is not to produce binary 'go/no-go' decisions, but rather to assess whether a threshold of evidence has already been passed for which the clinical need mandates the concept moving forward. This may necessarily be different for distinct target patient populations, and if certain data are felt to be lacking, constructive and realistic suggestions should be made as to how to build confidence in the approach. Independent reviewers will be asked to judge any new concept proposal based on clinical significance, trial design including embedded correlative studies (*e.g.* CNS penetration; molecular profiling; subtype responses *etc.*) as well as feasibility in the context of competing trials and available patient population to conduct the proposed study. A strong biological rationale is required, with preclinical evidence benchmarked against specific idealized guidelines, with justification for any criteria not explicitly met. In this perspective, we will focus mostly on the preclinical aspects, but stress that these data dovetail with a careful, and early inclusion of the following clinical considerations:

Clinical significance

The concept should address a clear unmet need, and represent a novel therapeutic development. This could be in the upfront setting, when an effective standard of care (SOC) has not been developed, for tumours with an extremely short overall survival, such as paediatric-type diffuse high-grade glioma (PDHGG), in particular diffuse midline glioma (DMG); subtypes of medulloblastoma (especially Group 3/4 or SHH, TP53-altered), atypical teratoid/rhabdoid tumours (ATRT), embryonal tumours with multilayered rosettes (ETMR) and others. This could also include tumours for whom the current SOC is associated with long-term burden in terms of quality of life (QOL), such as craniopharyngioma, ependymoma *etc.*

Trial design

The concept should clearly outline the primary, secondary and exploratory aims as well as endpoints. There ought to be a valid statistical design, with innovative models to be encouraged to maximise the information gained from a minimal number of patients. It should be clearly stated how correlative studies will be used to interpret successes and failures, with inclusion of plans for access to tumour tissue (including type of material, and regulation of storage and availability for follow-up studies), genomic profiling (either as part of the study or per SOC), and digitized histology and radiology (with access and governance details). Plans for CSF and plasma/serum collection (if feasible), and details of functional (cognitive outcomes; vision, endocrine, QOL dependent on disease subtypes) and imaging endpoints ought to be provided. There should be a strategy for obtaining post-treatment tumour tissue including autopsy collection protocols, and plans for appropriate analysis of such tissue. In addition, over the last few years efforts have been made to harmonize clinical trial endpoints across consortia which will allow for more direct comparison between different study therapies. Harmonizing correlative study endpoints and biological correlates will further inform cross trial comparison within a specific disease context.

Data sharing

Data should be made accessible in real time to the research community without compromising clinical trial endpoints. This is of critical importance across our consortia (and others) to ensure rapid dissemination of both positive and negative data, application of important lessons learned, and provide a means for cross-validation of results, improving ongoing trial design, and identifying appropriate patient populations for trial inclusion beyond traditional research silos.

Feasibility

Documentation should be provided where other compounds in the same mechanism of action class have already been evaluated clinically (or preclinically) for the given or related indications. There ought to be a plan for access of the relevant patient population within the footprint of the clinical trials consortium to which the application is submitted; there should also be an evaluation of other competing trials within the consortium's portfolio, and that of other consortia.

Principles of preclinical assessment

We strongly recognize that there is no 'one size fits all' approach to the process of assessment, and that each concept should be judged on its own merits on the basis of the specific clinical need of the patient populations proposed, and the feasibility of generating the idealized preclinical data package in such a context. We therefore indicate signposts for what a strong concept proposal should ideally include, and have identified five principles that could guide assessment of a data package presented for consideration (Table 1).

Firstly, there should be a clearly defined target population identified, based upon the mechanism of action of the

TABLE 1 *Principles of preclinical assessment.*

Clearly defined target population(s) based upon mechanism of action
Efficacy in multiple relevant models *in vitro* and *in vivo*
Safety assessment of off-tumour target expression, particularly for immunotherapies
Data showing penetration into tumour tissue at clinically relevant doses
Demonstration of on-target effect at clinically achievable doses and availability of predictive biomarkers

Guidelines for consideration of a preclinical data package for clinical translation.

investigational agent(s). In some instances, this will be relatively wide, and may span tumour entities and genotypes, whilst in others a highly restricted set of patients may be targeted. In terms of preclinical assessment, distinction is not made on this basis, so long as convincing rationale is provided. Secondly, evidence of efficacy of the agent in multiple relevant preclinical models, both *in vitro* and *in vivo*, is sought. The term 'relevant' here is to be contexualised by the investigators and reviewers, and may depend on disease biology and/or the agent being tested. There is complete recognition that multiple models may not be available for all tumour types or subgroups; here explanation and justification need simply be provided for both the number and identity of models chosen. Thirdly, there should be an assessment for the therapeutic window and potential safety issues by reporting target expression in the non-tumour compartments, through analysis of either novel or published data. This is of particular relevance for agents targeting wild-type targets, and for immunotherapies. Fourthly, data should be provided showing penetration of the agent into tumour tissue at clinically relevant doses; again this could be newly-generated data by the investigators or from the literature/drug company internal data, with recognition that extrapolation of doses from *in vitro* assays is imperfect (13). Finally, there should be demonstration of an on-target effect of the agent at clinically achievable doses in a relevant model system. Assays developed to assess this should also be evaluated for their ability to serve as predictive biomarkers for trial inclusion and/or post-hoc response assessment.

With such overarching principles driving the initial consideration of the suitability of a new concept being 'ready' for clinical translation, we provide specific assessment criteria in respect of *in vitro* and *in vivo* evidence that would aid prioritization of ideas. As previously stated, it is recognized not all circumstances will allow for all criteria to be met; where they cannot, the guidelines are meant to serve as discussion points rather than reasons for exclusion (Figure 1).

Specific *in vitro* criteria

- The agent to be tested should be potent in the models tested, with evidence of a clear cellular effect in terms of cell viability, cell death, cell differentiation or other appropriate end-points. This may be demonstrated in terms of effects observed (IC_{50}/GI_{50} *etc.*) at sub-micromolar concentrations and/or showing a greater than two-fold statistically significant differential sensitivity in models representing the target population compared to (i) an appropriate 'normal' cell type and/or (ii) other disease subtypes and/or (iii) other disease entities.
- Assays should be carried out in multiple appropriately-accredited models representing the heterogeneity of the target population(s). Where available for a given entity or subtype *etc.*, this should be carried out in at least n=4-6 distinct models, with phenotypic/genotypic data for each provided. Where available, both patient-derived and genetically-engineered models are desirable for a given target.
- There should be evidence of target modulation at doses producing a cellular effect by an appropriate assay, western blot, ELISA, mass spectrometry, *etc.*) in at least n=2 models. Although not a prerequisite for preclinical assessment, indications should be given as to the applicability of such an assay that is translatable to the clinical setting a predictive biomarker.
- For immunotherapies, evidence should be provided of target antigen-specific tumour cell lysis, including where possible of (primary) tumour cells with endogenous target antigen expression. Here, level of target expression reflective of that of primary tumours should be taken into account given that immunotherapeutics commonly have a target density threshold for efficacy (14).
- For combination studies, there should be evidence of at least additivity, or better formal synergy, of the agents to be combined by one or more appropriately designed assays, including but not restricted to the Chou-Talalay median effects model (15), BLISS independence score (16), isobologram (17) *etc.*). This should be carried out in at least n=2 models, where possible.
- Collaborative studies across laboratories are encouraged in order to demonstrate reproducibility, as well as maximise resources and expand the number of models available. Where data is pooled in such a way, at least n=1 of the models/assays should be consistently assessed across all partner laboratories in order to assess comparability.

Specific *in vivo* criteria

- If clinical data from human studies is unavailable, demonstration of drug penetration into the relevant normal brain and/or tumour tissue of appropriate model organisms. These experiments should be carried out at tolerable doses resulting in concentrations at least greater than the *in vitro* IC_{50}/GI_{50} values, assessed by direct measurement (using assays such as LC-MS, MALDI-TOF, *etc.*) and/or appropriate biomarker modulation (*e.g.* western blot, immunohistochemistry, *etc.*).

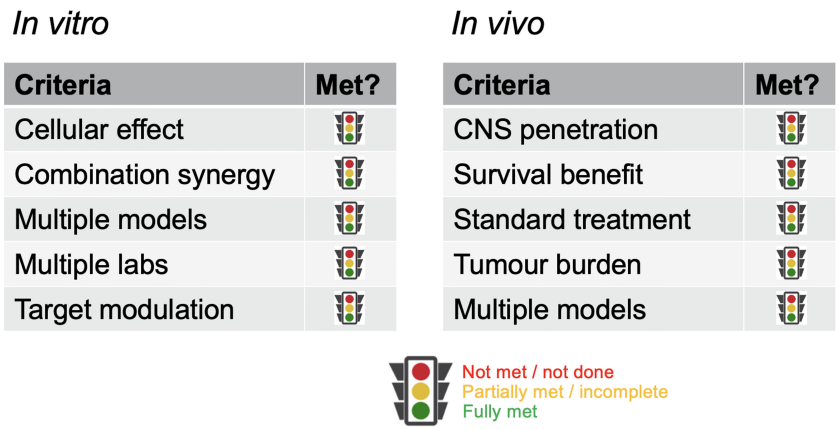

FIGURE 1
Criteria for critical review. Key parameters against which data packages should be assessed. Rather than a metrics-based scoring system, a fully justified benchmarking against each category should be provided.

- For immunotherapies, if clinical data from human studies is unavailable, demonstration of homing to and penetration into tumour tissue of appropriate model organisms at tolerable doses should be provided. Presence of the immunotherapeutic agent at tumour sites can be demonstrated by immunostaining using e.g. anti-idiotype antibodies or detection of linked marker genes).
- There should be demonstration of a statistically significant survival benefit of treated animals, typically >20% prolongation of the median survival over vehicle (or control biologic)-treated controls. For combination studies, in addition, a statistically significant survival benefit for the combination of >10% of the median survival over the most effective single agent should be seen.
- As for *in vitro*, assays should be carried out in multiple appropriately-accredited where available. This should be undertaken at least n=3 distinct models representing the target population(s), and grown in the relevant orthotopic location where feasible. Where available, both patient-derived and genetically-engineered models, with at least n=1 in an immunocompetent background, are desirable. These could be carried out in models in the same or different species, with the latter encouraged.
- There should be evidence of a statistically significant reduction in tumour burden on treatment provided, assessed by an appropriate assay (*e.g.* MRI, biolumine scent imaging, ddPCR *etc.*). As with *in vitro*, indications should be given as to clinical translation of any predictive biomarkers.
- Priority will be given to treatments which can be shown to provide a survival advantage greater than SOC treatments for a given patient population, in a preclinical trial mimicking the appropriate clinical protocols (18). This could include addition and/or comparison to a standard radio/chemotherapy regimen, including surgical resection where practicable, as well as 'mouse hospital' designs of multiple individual patient-derived models at, *e.g.* n=1 mouse each (19).

Application

Within our consortia, elements of these principles have been generically applied since initiation, but not in a systematic way. By formalizing standards, we aim to achieve two things. The first is to provide an unbiased methodology for assessing concepts brought forward from multiple sources and across disparate entities and therapeutic targets, such that cross-review between the various collaborative groups can be undertaken to the same criteria. Such a harmonized but flexible approach also seeks to provide investigators with a clear set of guidelines against which they may judge their own extant data, and help to plan additional experimental work. Inherent in this is a desire to encourage and facilitate data sharing to avoid unnecessary duplication of effort. The second critical goal is to provide a framework for discussion of novel concepts, rather than a strict metrics-based exercise. A key point is that that concepts may come from many different sources, and certainly external to any of our consortia (or others). It is the hope that having such guidelines would encourage researchers not otherwise connected to such groupings to self-evalaute their own data prior to engagement with clinical trials groups, but not in any exclusionary way; the hope is to stimulate discussion and not to restrict good ideas being brought forward at any stage.

We recognize the present limitations inherent in certain fields which make adherence to certain points impossible, and aim to highlight these caveats for frank conversation as to their importance relative to the other evidence presented, and clinical need of the target population. In this way, we also hope to flag areas that are in need of further development by the field. It should also, however, hold to account other areas in which the criteria could be, but are often not, routinely met. An example is in the desire for demonstration of efficacy and on-target effects *etc.* in multiple

disease models. We appreciate that for certain high-risk childhood brain tumour entities such as ETMR and ATRT they may be limited (20, 21), or for others like Group 3/4 medulloblastoma they may be imperfect representations of the human disease (22). For others such as DMG however, large panels of patient-derived cells, PDXs and GEMMs are widely available, and single cell line studies are hard to justify (23–25). In all cases, we anticipate an iterative process whereby the criteria provide a checklist for early discussion, template for initial benchmarking, and a guidebook for eventual translational decision-making.

Limitations and challenges

The proposed criteria are intended as positive and achievable, with the goal of leading to therapies that are more likely to be successful in the clinic (26, 27). They are meant to encompass the most common treatment concepts brought forward to our consortia, and will likely need refinement to include more innovative modalities. We do not expressly provide specific proposals for how to assess novel drug delivery methodologies, for example, including such disparate approaches as nanoparticles (28), convection-enhanced delivery (29) and focused ultrasound-mediated opening of the blood-brain barrier (30), *etc.* Another emerging area which may require distinct end-points clinically, and therefore unique criteria preclinically, is that of cancer neuroscience (31). We do not explicitly lay out a framework for assessing the modulation of cancer cell – neuron interactions, nor what our expectations should be in the preclinical context for such agents to have a beneficial effect in patients. The same could be said to be true of other microenvironment modulation strategies, such as targeting tumour-associated immune cells or angiogenesis (32). Here, novel model systems such as *ex vivo* tumour explant or organotypic models which recapitulate the complex tumour milieu are being developed to provide information complementary to that of current models (33, 34). Further refinements to our *in vivo* strategy will likely come to include a more thorough consideration of the age of animals used for such studies, to better replicated the developmental context in which these tumours arise (35), as well as evaluating therapies in both male and female models, given the sex-related biological differences which are beginning to emerge (36). Moreover, as we aim to develop therapies which can spare children and young adults from the toxic effects of chemotherapy and radiation, we would need to include additional means to assess how we improve QoL measures and control for the late effects of therapies (27).

As our biological understanding of paediatric CNS tumours increases, and we subclassify them into ever-more subtypes with distinct drivers warranting unique therapies (37), we face a challenge both in terms of generating preclinical data and moving these concepts into clinical trial. Idealised criteria in which we hope to see evidence of efficacy in multiple models, *in vitro* and *in vivo*, makes little sense for ultra-rare, newly-defined subtypes, and patient numbers for such entities mean traditional clinical trial designs are unlikely to recruit sufficient numbers, even with international co-operation. It will be a challenge for the community to determine to what extent we can relax or refine our standards to assess novel concepts in these entities, and how they may be robustly and safely tested in patients. We need also to be cogniscent that positive results in model systems do not necessarily predict for success in clinical trial (38–40). Although we assume that the patient-centric and biologically-driven models we have recently developed will be a substantial advance on what went before, this is as yet unproven. Careful credentialling of the models (and assays) required as part of the criteria are inherent in generating preclinical data which will eventually prove effective in the clinic, and need constant assessment and benchmarking in the manner of the criteria we apply here to the data generated with them.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

CJ, KS and SM jointly conceived, wrote and edited the article; all other authors wrote and edited the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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A novel preclinical model of craniospinal irradiation in pediatric diffuse midline glioma demonstrates decreased metastatic disease

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Background: Diffuse midline glioma (DMG) is an aggressive pediatric central nervous system tumor with strong metastatic potential. As localized treatment of the primary tumor improves, metastatic disease is becoming a more important factor in treatment. We hypothesized that we could model craniospinal irradiation (CSI) through a DMG patient-derived xenograft (PDX) model and that CSI would limit metastatic tumor.

Methods: We used a BT245 murine orthotopic DMG PDX model for this work. We developed a protocol and specialized platform to deliver craniospinal irradiation (CSI) (4 Gy x2 days) with a pontine boost (4 Gy x2 days) and compared metastatic disease by pathology, bioluminescence, and MRI to mice treated with focal radiation only (4 Gy x4 days) or no radiation.

Results: Mice receiving CSI plus boost showed minimal spinal and brain leptomeningeal metastatic disease by bioluminescence, MRI, and pathology compared to mice receiving radiation to the pons only or no radiation.

Conclusion: In a DMG PDX model, CSI+boost minimizes tumor dissemination compared to focal radiation. By expanding effective DMG treatment to the entire neuraxis, CSI has potential as a key component to combination, multimodality treatment for DMG designed to achieve long-term survival once novel therapies definitively demonstrate improved local control.

KEYWORDS

diffuse midline glioma, craniospinal irradiation, patient-derived xenograft, metastatic disease, pediatric

Introduction

Diffuse midline gliomas (DMG) are a highly aggressive subtype of pediatric high-grade glioma. These tumors are universally fatal, with a median overall survival of less than 1 year. They account for 10-20% of pediatric central nervous system (CNS) malignancies and are the leading cause of pediatric CNS tumor-related death (1).

Focal radiation therapy (RT) remains the only therapeutic approach that has been shown definitively to lengthen DMG survival, although it usually only delays progression of the tumor by a few months. There has been minimal improvement in the prognosis of DMG due to the failure of previous clinical trials (2). Focal RT has been the standard RT field because focal progression in the pons or other primary site is usually responsible for death. However, DMG demonstrates high rates of leptomeningeal dissemination at diagnosis, during treatment, and at autopsy (3, 4). With recent or current early trials of techniques allowing localized, primary tumor-directed delivery of chemotherapy agents, including convection-enhanced delivery (CED) (5) and focused ultrasound with microbubbles (6), the potential for prolonged local control of DMG is becoming more realistic; however, some patients are now experiencing metastatic progression while their primary tumor is responding to these treatments. Thus, controlling metastatic DMG has become a pressing challenge in the fight to achieve long-term survival in this disease. Craniospinal irradiation (CSI) is a sensible modality to consider, since DMG is sensitive to RT, and CSI is crucial to survival in multiple other pediatric brain tumors with metastatic potential, such as medulloblastoma and atypical teratoid/rhabdoid tumor.

In the present study, we aimed to investigate CSI as a therapeutic approach to control metastatic disease across the neuraxis in an orthotopic patient-derived xenograft (PDX) model of DMG. We hypothesized that we could feasibly study DMG preclinically using a PDX model, and that CSI would limit metastatic disease in this model compared to focal RT, although it would not extend survival alone.

Methods

PDX model and radiation treatment

Patient-derived mouse models of DMG were generated using immunocompromised Foxn1^{nu}/Foxn1^{nu} mice. 200,000 luciferase-expressing BT245 DMG cells were injected intracranially into the right pons of 6–8 week-old mice as previously described (7). Tumor-bearing mice were randomized to one of three treatment groups: no radiation, pontine-directed focal radiation, or CSI with a focal pontine boost. A relatively large group size of 12 was chosen based on known incomplete penetrance of metastatic tumor development in the model. 23 days after tumor cell injection, mice began the assigned radiation treatment. The focal group received 4 Gy radiation to the pons on four consecutive days, while the CSI group received two days of 4 Gy CSI and two days of 4 Gy pons-directed radiation. These regimens were chosen in discussion with the radiation oncology team, led by Dr. Karam, to be feasible in mice and representative of the fairly even CSI and boost RT doses used in pediatric patients. Radiotherapy was performed using the X-RAD SmART image-guided irradiator (Precision X-Ray Inc., Branford CT) at 225kVp, 20mA with 0.3 mm Cu filter. Mice were anesthetized with isoflurane and positioned supine in a custom 3D-printed holder. Craniospinal irradiation was delivered in two pairs of opposing lateral beams, using a custom Cerrobend collimator. Radiation dosimetry was performed using a combination of Monte-Carlo simulations from SmART-ATP treatment planning software (SmART Scientific Solutions, Maastricht, The Netherlands) and Gafchromic EBT3 dosimetric film (Ashland Global, Wilmington, DE). Mice were monitored for onset of weight loss, mobility, and neurological symptoms and were sacrificed when these became apparent. At necropsy, tumors, brains, and spines were formalin fixed and hematoxylin/eosin (H&E) stained. The experiment was repeated for validation.

Animal imaging (Bioluminescence and MRI)

Tumor growth was monitored weekly by bioluminescence imaging (BLI) on luciferase-expressing cells using IVIS Spectrum (PerkinElmer, Waltham, MA). At the end of the study, the animals with the BLI-confirmed tumors underwent a multiparametric MRI session consisting of a high-resolution, T2-weighted turboRARE 3D-MRI (for tumor localization and volume, in-plane resolution 52 microns), a FLAIR MRI (for inflammation and edema), and diffusion-weighted imaging (DWI, for tumor cellularity and parenchymal edema) protocols (8). All MRI was performed on a Bruker 9.4 Tesla BioSpec MRI scanner (Bruker Medical, Billerica, MA) using a mouse head phase-array coil. All MR acquisition and image analysis was performed using Bruker ParaVision NEO v.3.1. software.

Analysis of tumors and metastases

Mouse survival was measured by the Kaplan-Meier method and compared by log-rank test. The sizes of primary tumors, regional metastasis, and spinal metastasis were analyzed by a pediatric neuropathologist blinded to treatment group. Primary tumors and each area of metastases (brain leptomeninges, lateral ventricles, and spinal cord) were graded on a 0 (no evidence of tumor) to 3 (severe metastatic involvement) scale.

Results

DMG model and RT fields

For our assessment of radiation fields in controlling DMG metastasis, we chose the BT-245 DMG model, which originated from a pediatric thalamic DMG collected at initial resection (7, 9).

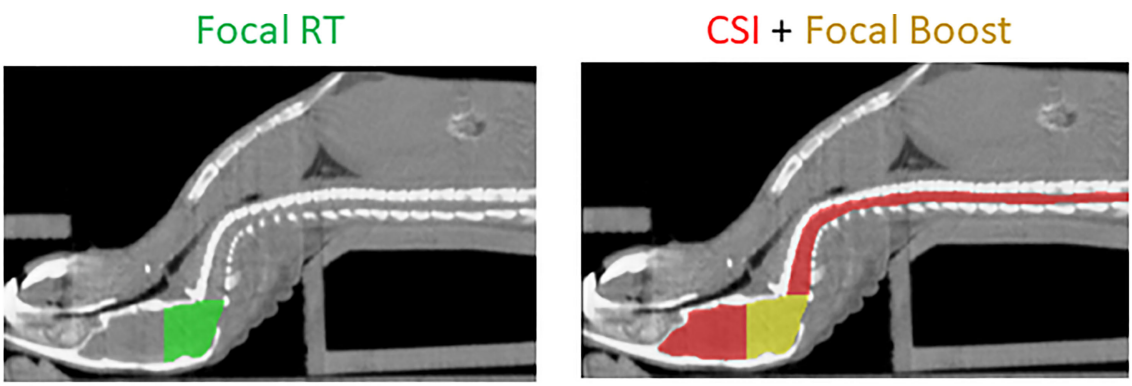
We built a custom 3D-printed platform to facilitate murine focal pontine and CSI+boost radiation delivery while avoiding RT to other thoracic and abdominal organs (Figure 1).

Treatment, tumor monitoring, and survival

23 days after tumor cell injection, when the first mice had developed signs of tumor by BLI, mice were randomized to one of three RT conditions and began treatment: no RT, focal pontine RT 4 Gy x4 days, and CSI 4 Gy x2 days + boost 4 Gy x2 days. Details of the radiation dose mapping and delivery are shown in Supplemental Figure 1. 12 mice were randomized to each treatment group. Mice were monitored weekly by whole-body BLI (Figure 2A) and by brain and spine MRI at a single time point approximately one week after the end of the treatment period (Figure 2B). Representative BLI images show steady primary tumor growth in the no RT mouse followed by spinal metastatic progression and death. In the focal RT group mouse, the primary tumor responds initially to RT, but metastatic disease develops, with growth of both primary tumor and spinal metastases before death. In the CSI+boost mouse, the primary tumor improves with radiation but eventually recurs, without the development of metastases. Representative MRI images show abnormal signal consistent with brain leptomeningeal and ventricular metastatic disease in the focal RT mouse but not in the CSI+boost mouse. Quantitative MRI endpoints for DMG treatment response are presented in Figure 2C. Metastatic invasion into the ventricles was partially prevented in the CSI+boost animals ($p<0.0001$ compared to no RT and $p=0.006$ compared to focal RT by t-test). In both the focal RT and CSI+boost groups, intracranial lesions revealed apparent diffusion coefficients (ADC from DWI) that were slightly higher as compared to the no-RT group, indicating lower cellularity/proliferation index after RT. Mice receiving focal RT and CSI +boost both showed prolonged survival in comparison to no RT mice ($p=0.01$ for each group vs. no RT), but there was no survival difference based on RT field (Figure 2D). This finding indicates that mice were likely dying of local tumor progression and not metastatic disease.

Histopathological assessment

Mouse brains and spines were collected at necropsy and assessed for tumor involvement by a pediatric neuropathologist (Dr. Gilani) blinded to treatment group. Areas of metastatic tumor involvement on H&E staining are shown in Supplemental Figures 2A, B, along with an MRI image showing correlation of MRI and histopathological findings. Dr. Gilani graded primary (pontine) tumor volume and metastatic tumor involvement on a 0 (no tumor) to 3 (severe tumor involvement) scale (Figure 3A). While not all mice developed metastatic tumor, mice from the no



	Day 1	Day 2	Day 3	Day 4	Total pons dose delivered
Focal	4 Gy Focal	4 Gy Focal	4 Gy Focal	4 Gy Focal	16 Gy
CSI + Boost	4 Gy CSI	4 Gy CSI	4 Gy Focal	4 Gy Focal	16 Gy

FIGURE 1
Planning CTs demonstrating positioning of the mouse on the custom platform and RT fields for the two RT groups, along with the RT schedule for both groups.

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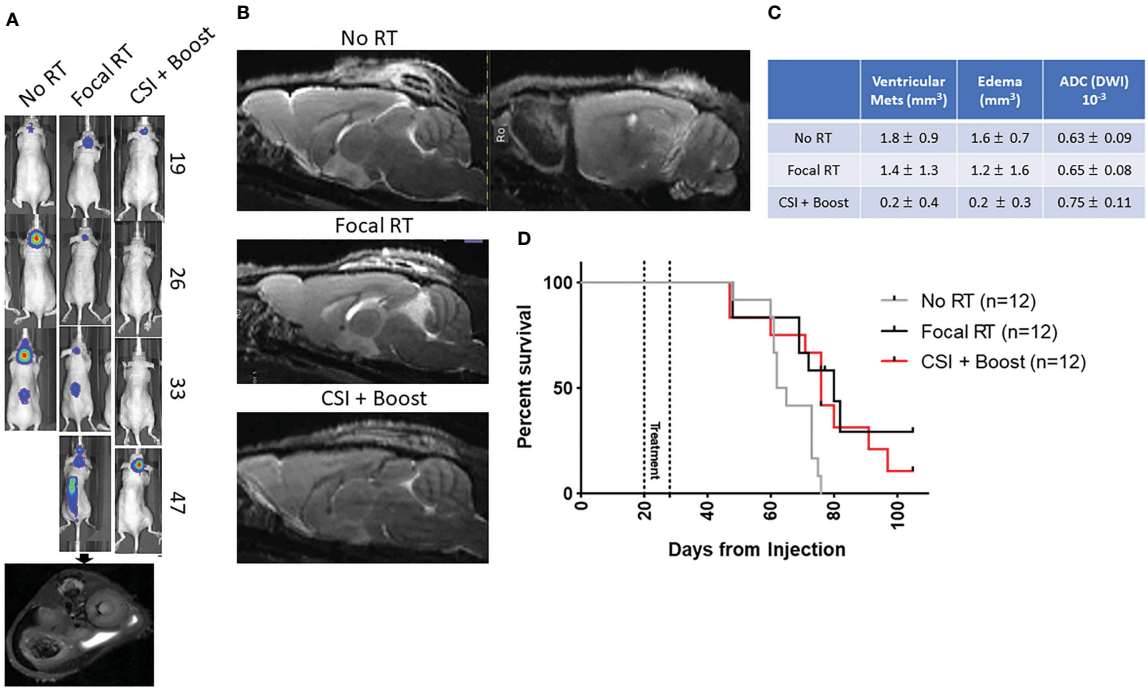

	Ventricular Mets (mm^3)	Edema (mm^3)	ADC (DWI) 10^{-3}
No RT	1.8 ± 0.9	1.6 ± 0.7	0.63 ± 0.09
Focal RT	1.4 ± 1.3	1.2 ± 1.6	0.65 ± 0.08
CSI + Boost	0.2 ± 0.4	0.2 ± 0.3	0.75 ± 0.11

FIGURE 2
(A) Representative BLI for each group with day from tumor injection on the right, with a representative MRI confirming spinal tumor spread; **(B)** Representative sagittal T2 MRI images for the no RT (with T1 as well), focal RT, and CSI+boost groups show abnormal leptomeningeal and ventricular signal in the no RT/focal RT mice brains with no apparent metastases in the CSI+boost mouse brain; **(C)** Quantitative MRI endpoints for ventricular metastatic lesion volumes (T2-MRI), edema volumes (FLAIR, DWI), and apparent diffusion coefficients (DWI) showing mean values +/- standard deviation; **(D)** Kaplan-Meier curve for the three RT groups show prolonged survival for focal RT and CSI+boost groups compared to no RT (p=0.01 for both) but no difference in survival between the groups receiving RT (p=0.48).

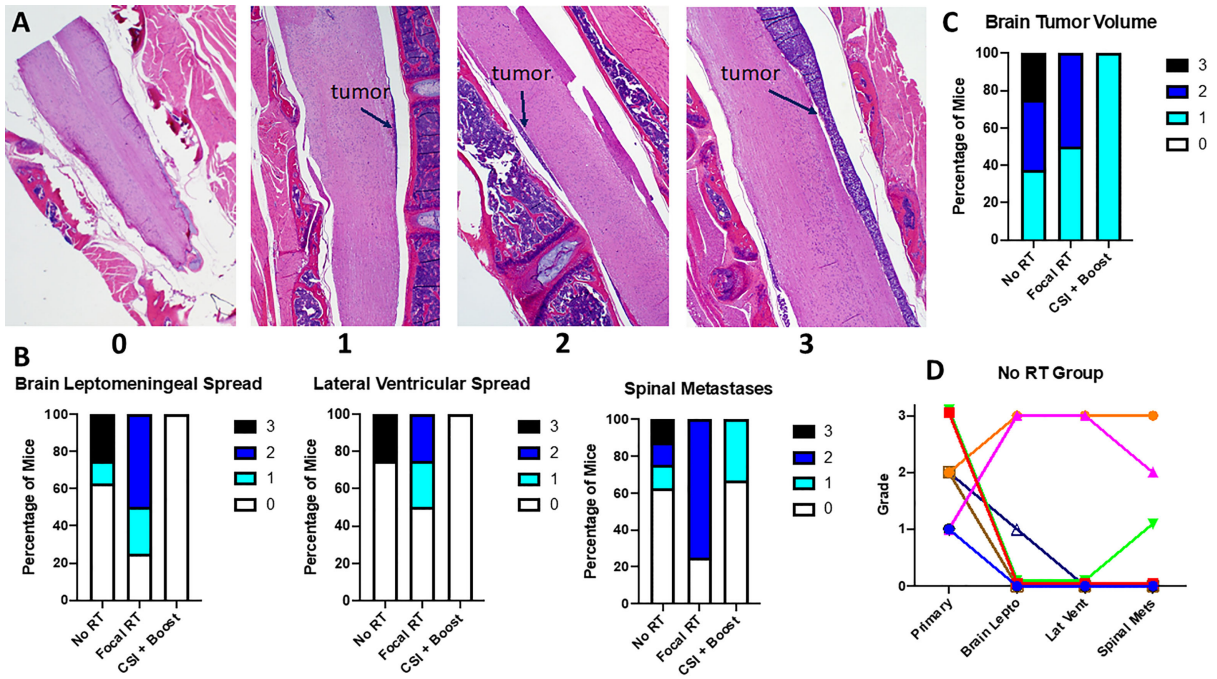

FIGURE 3
(A) Sagittal spinal cord images show examples of each gradation of metastatic disease; **(B)** Grading of metastases across locations for all mice by treatment group; **(C)** Grading of primary tumor volume for all mice by treatment group; **(D)** Correlation between primary tumor volume and metastatic gradation at each site measured for the no RT group; each color/shape represents a different individual mouse.

RT and focal RT groups were more likely to have metastatic involvement overall and more severe metastases across all areas assessed (Figure 3B). While there were also differences in primary tumor volume between treatment groups (Figure 3C), primary tumor grade did not appear to correlate with metastatic tumor grade in the no RT group (Figure 3D).

Discussion

Using a murine orthotopic xenograft model of DMG, we identified CSI as a therapeutic modality capable of limiting metastatic disease throughout the CNS compared to focal RT, the current standard-of-care therapy in DMG. This brief report represents the first preclinical assessment of CSI in DMG and raises this treatment modality as a potential contributor to a combination approach in this disease. This approach is necessitated by a developing "good" problem in DMG, that more prolonged local tumor control is potentially becoming more feasible with the introduction of experimental treatment modalities allowing focal delivery of chemotherapy, leading to the emergence of clinically apparent disease dissemination in some patients as their survival has been prolonged.

In our study, mice in the focal RT and no RT groups showed similar levels of metastatic disease. Since the only difference between these groups was pontine RT, this suggests that metastatic tumor was seeded throughout the CNS early in the disease process instead of continuing to disseminate from the primary tumor, as in this case we would expect decreased metastatic disease with focal RT. BLI tended to show spinal metastases only later, however, which matches what has been seen in patients: metastatic DMG is less frequent by MRI at diagnosis than later in treatment (3) but can often be detected at diagnosis by more sensitive assays like circulating tumor DNA, the use of which is experimental but emerging in this disease (4). The timing of treatment in this model, at first detection of abnormal signal by imaging, is necessary in mice given the need for sacrifice at symptom development, but it presents a potential issue in the translation of our findings to patients, who do not present until developing symptoms. However, the findings from our work and human studies (4), both showing metastatic tumor present from first detection of the primary tumor, suggest that CSI delivered at the time of diagnosis would be effective.

Weaknesses of the current study include the use of only one radiation dosing schedule and one model, which harbors both H3K27M and *TP53* somatic mutations; *TP53* mutation is actually associated with radiation resistance in DMG (10), so we might expect an even more profound impact of radiation in *TP53*-wild type tumors, and future work on CSI should explore a variety of DMG models. Another weakness is the inability to be sure that the pattern of metastatic disease development in this model matches that seen in patients. However, the injection of cells directly into the pons (as opposed to the ventricle), the variability of metastatic disease development, and the findings on histopathology all suggest accurate modeling of true metastases akin to those seen in patients. Strengths of the study include the multiple assessments of metastatic disease by dual imaging modalities and histopathology, the grading of metastases by a neuropathologist blinded to treatment group, and the novel approach of delivering CSI to mice through a custom-made platform to minimize toxicity.

As expected, CSI did not produce a survival benefit over focal RT in our study, as mice still died of their primary tumor with radiation alone. This again matches what has been seen in patients: RT alone is a palliative measure only in DMG that can only delay tumor progression, including in the few documented patients in which CSI has been given for metastatic tumor (11, 12), especially since even maximal CSI doses are lower than doses that can be delivered to the primary tumor. However, these studies did indicate temporary efficacy against these metastases, and combined with our findings, suggest that CSI has potential as a component of combination, multimodality therapy against DMG to control the tumor dissemination likely to become clinically apparent as treatment measures directed at the focal tumor become more effective at prolonged local control. Our methods and findings provide the groundwork for CSI to be incorporated into future preclinical studies and clinical trials. Combination approaches (13) addressing both the primary tumor through focal RT and targeted delivery of chemotherapy and/or immunotherapy (14, 15), as well as disseminated tumor through CSI and systemic chemotherapy and/or immunotherapy, have the potential to produce long-term control of DMG.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, University of Colorado Anschutz Medical Campus.

Author contributions

ALG conceptualized and planned the project, with contributions from RV and KD. ALG, AK, BC, AO, and SK created the radiation plan. AK, BVC, and AO carried out the experiments. NJS led animal imaging (BLI and MRI). AG led histopathological analysis. AK, AG, and ALG led data analysis, assisted by JD, PF, RL, and HC. EB, AK, and ALG wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1105395/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) Screenshot from SmART-ATP treatment planning software showing dose distribution for focal irradiation. Approximate tumor location is shown in green; **(B)** Dose-volume histogram for spine, brain, and pons, based on simulated focal irradiation; **(C)** Illustration of the two pairs of opposing lateral beams used for CSI on the fluoroscopy image on an x-ray image of a mouse on the custom platform; **(D)** Simulated dose distribution for opposing lateral beams through the spine, showing roughly uniform dose for all soft tissue in the field. Note that the distribution simulated for spinal irradiation is not for the exact collimator used to treat these mice; a dose-rate correction factor for the custom collimator based on Gafchromic film dosimetry was used to determine the appropriate beam times from the simulation.

SUPPLEMENTARY FIGURE 2
(A) Example images showing metastatic deposits in various areas of the brain on histopathology, with an axial MRI showing correlating leptomeningeal metastases from the same mouse; **(B)** Example images showing metastatic tumor at different levels of the spine on sagittal sections

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