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WHO classification of pediatric brain tumors: A final wedding between morphology and molecular biology?

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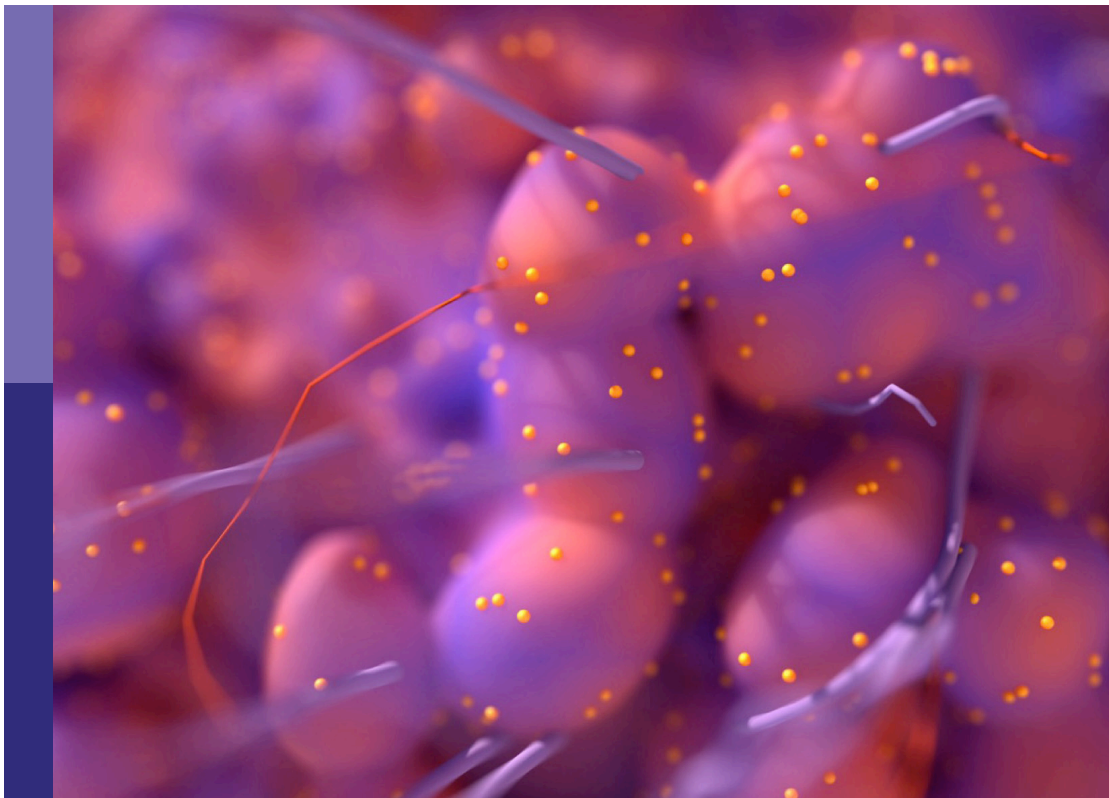

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2021 WHO classification of pediatric brain tumors: A final wedding between morphology and molecular biology?

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Editorial: 2021 WHO classification of pediatric brain tumors: a final wedding between morphology and molecular biology?

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pediatric brain tumors, WHO CNS5, CNS tumors, pediatric cancer, molecular profiling

Editorial on the Research Topic
2021 WHO classification of pediatric brain tumors: a final wedding between morphology and molecular biology?

This Frontiers Research Topic includes a collection of nine original contributions and reviews on different aspects of pediatric tumors of the central nervous system (CNS), specifically related the World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS5) (Louis et al., 2021) and their implication in diagnosis, prognosis, stratification, and target therapies on patients (Fuller et al., 2017; Fangusaro and Bandopadhayay, 2021; Guo et al., 2023; Li et al., 2023).

Skitchenko et al. identified four candidate somatic mutations potentially explaining the medulloblastoma (MB) onset in two pediatric patients and providing new biological insights into the mechanisms of tumor development. Molecular diagnostics for two WNT-MB cases without chromosome 6 monosomy or mutations in CTNNB1 and APC are described.

Vallero et al. reviewed the current literature on H3K27-altered diffuse midline glioma (DMG) and addressed questions such as when additional mutations are found, which one should we focus on in order to make the correct clinical decision. H3K27 status has become a fundamental supplement to the histological grading of pediatric gliomas but not sufficient alone to exhaustively define the complex biological behavior of DMG in children and might not represent an indication for a unique treatment strategy across all patients, irrespective of age, additional molecular alterations, and tumor location. Therefore, each DMG case should have its own unique and precise molecular characterization. The ultimate goal is to treat all patients with a personalized therapy tailored to the specific characteristics of their tumor.

In their review Cipri, Del Baldo et al. described the major molecular alterations detected in pediatric low-grade gliomas (pLGGs) and the molecular target therapy, which is feasible/available to date. Having a better understanding of tumor biology and a germline and somatic genomic approach will play a central role in the therapy strategy of pLGG for the development of increasingly tailored therapies. It cannot be underestimated that limitations still exist, regarding the adverse effects of long-term treatment.

De Martino et al. reported on two pediatric patients affected by DMG with extra-neural dissemination, both showing disease progression at bone sites and partial response of intracranial DMG to second-line treatment with craniospinal irradiation and systemic chemotherapy with irinotecan and bevacizumab regimen. Extra-neural metastasis of DMG is a rare event and no standard therapy exists. Due to its rarity, the biological mechanisms behind tumor dissemination outside the CNS of DMG have not been well-described. Although improved care of patients affected by DMG is going to lead in some cases to longer survival, extra-neural metastases in DMG were detected at diagnosis or relatively early after diagnosis.

The review of Caroleo et al. described an exceptional case of an infant carrying a germline and somatic pathogenic variant of PTEN and a germline and somatic pathogenic variant of CHEK2 who developed a MB SHH in addition to intestinal polyposis. PTEN gene variants often present in childhood with macrocephaly, developmental delay, and/or autism spectrum disorder while tumors and intestinal polyps are commonly detected in adults. PHTS is rarely associated with childhood brain tumors with only two reported cases of MB. Although the association is rare, the panel of genes to be tested in the presence of an MB SHH could be extended to PTEN. To date, the role of CHEK2 remains uncertain. The discovery of a PTEN germline mutation should induce the clinician to promptly provide genetic counseling in order to assess and monitor the occurrence of other PHTS clinical features and set up careful surveillance.

Weiser et al. explained that understanding the longitudinal overlap and glioma evolution from childhood to adulthood is an important research gap. Treatment optimization, including implementation of targeted therapies, starts with the adoption of appropriate molecular testing as part of the diagnostic work-up, for biomarker identification. Even though the molecular features vary between pediatric, adult, and—most likely—adolescent and young adult (AYA) gliomas, these tumors also share common tumorigenic pathways, including overexpression of oncogenes, activation of RTKs, epigenetic dysregulations, and increased metabolic pathways, which should be explored for introducing new therapies in age-inclusive clinical trials. To bridge this gap and offer better treatment options, exchange of expertise and close collaboration between pediatric and adult neuro-oncologists—and broader multidisciplinary clinical teams—is indispensable. Ensuring access to appropriate molecular testing to detect key biomarkers, designing age-inclusive clinical trials for gliomas and creating multidisciplinary teams, bridging the pediatric/adult divide, are some of the many actions needed and being implemented in several centers across the world. Additional factors to be considered include the socioeconomic and mental health burden that AYA patients experience.

In their publication Morgacheva et al. explained a case that highlights need for the implementation of molecular methods, especially tumor DNA methylation, in the diagnosis of CNS neoplasms in children. Pediatric CNS tumors demonstrate clinical and biological diversity and variability in the morphological picture, which can lead to misdiagnosis and wrong therapeutic strategies. Diagnostic challenges can be overcome by using novel technological diagnostic approaches such as DNA and RNA sequencing, RNA expression profiling, fluorescence *in situ* hybridization, and DNA methylation. They stated that their case demonstrates the complexity of diagnosing a CNS tumor in a pediatric patient, which was caused by a non-specific clinical and morphologic picture of the tumor itself, which twice led to misdiagnosis and a wrong therapeutic approach. An additional molecular analysis allowed them to find a potential target for precision therapy, which may be useful in the event of disease progression. In diagnostic cases, at least a complete IHC and first level molecular methods [PCR, fluorescence *in situ* hybridization (FISH)] should be used.

Cipri, Fabozzi et al. demonstrated that tropomyosin receptor kinase inhibitors, such as larotrectinib and entrectinib, have showed high efficacy in pediatric patients, also in CNS tumors carrying alterations in NTRK genes. Additional research is necessary to help us to understand better the mechanism of action of these drugs and to identify biomarkers that can help identify patients who will benefit most from therapy.

d'Amati et al. summarized the major changes in the 2021 WHO CNS5, highlighting for each entity the molecular alterations and other information that are relevant for diagnostic, prognostic, or therapeutic purposes. The rationale of this "molecular classification" is also related to the effective and experimental molecular therapies, targeting some cancer-specific genetic events. Reclassification based on molecular investigations has allowed identification of specific entities that appear homogeneous in their response to treatment and clinical outcomes. These implications highlight the necessity to adopt the new classification when considering therapeutic options (clinical trials, targeted therapies) and discussing prognosis.

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Case report: Somatic mutations in microtubule dynamics-associated genes in patients with WNT-medulloblastoma tumors

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Medulloblastoma (MB) is the most common pediatric brain tumor which accounts for about 20% of all pediatric brain tumors and 63% of intracranial embryonal tumors. MB is considered to arise from precursor cell populations present during an early brain development. Most cases (~70%) of MB occur at the age of 1–4 and 5–9, but are also infrequently found in adults. Total annual frequency of pediatric tumors is about 5 cases per 1 million children. WNT-subtype of MB is characterized by a high probability of remission, with a long-term survival rate of about 90%. However, in some rare cases there may be increased metastatic activity, which dramatically reduces the likelihood of a favorable outcome. Here we report two cases of MB with a histological pattern consistent with desmoplastic/nodular (DP) and classic MB, and genetically classified as WNT-MB. Both cases showed putative causal somatic protein truncating mutations identified in microtubule-associated genes: *ARID2*, *TUBB4A*, and *ANK3*.

KEYWORDS

medulloblastoma, exome sequence data, somatic mutation analysis, Wnt, microtubule - associated proteins

Introduction

Medulloblastoma (MB) – is a solid neuroepithelial tumor arising from the cerebellum. MB accounts for about 20% of all childhood brain tumors and 63% of intracranial embryonal tumors (1). MB is considered to arise from precursor cell populations present during an early brain development (2). Most cases (~70%) of MB

occur at the age of 1–4 and 5–9, but are also found in adults (3). Total annual frequency of pediatric tumors is about 5 cases per 1 million children (1).

WHO declares two classifications of MB according to the method of diagnosis: histologically determined and genetically determined (4). Both groups are divided into several subgroups according to the immunohistochemical and genetic features, respectively (4). For histologically determined MB there are the following subgroups: 1) classic MB; 2) Desmoplastic/nodular MB; 3) MB with extensive nodularity; 4) Large cell/Anaplastic MB; 5) MB not otherwise specified (4). In turn, the following subgroups are distinguished for genetically defined MB: 1) WNT-activated MB; 2) SHH-activated, TP53-wild-type MB; 3) SHH-activated, TP53-mutant MB; 4) Non-WNT/non-SHH MB which is commonly divided into Group 3 and Group 4 MB (4).

Of all cases of MB, about 10% are of the wingless-type (WNT) (5). WNT-MB are usually located along the brain midline with involvement of the brainstem or cerebellar bundle and cerebellopontine angle cistern (6). WNT-MB is thought to arise from progenitor cells in the inferior rhombic lip of the developing brainstem. The vast majority of WNT tumors (~90%) contain a mutation affecting *CTNNB1*, which encodes β-catenin. Mutations in the tumor suppressor gene *APC* explain the majority of WNT-cases which do not have *CTNNB1* mutations (2).

Some studies suggested the existence of two subtypes of WNT: WNTα and WNTβ. The WNTα subtype occurs mainly in children and for 98% of cases is associated with chromosome 6 monosomy, whereas the WNTβ subtype occurs in older children and adults and infrequently (29%) has monosomy (7).

Here we present molecular diagnostics for two WNT-MB cases without chromosome 6 monosomy or mutations in *CTNNB1* and *APC*.

Methods

Clinical and genetic data collection

Patients were observed at Almazov National Medical Research Center in 2020-2022. Informed consent for molecular genetic testing was provided by parents of patients. The study was approved by the institutional ethics committee (Protocol #3502-22 from 21.02.2020).

Hematoxylin-eosin staining analysis was used for the purpose of histological classification of medulloblastomas.

A panel of three staining assays: 1) beta-catenin staining, 2) filamin A, 3) GAB1 was used to obtain immunohistochemical (IHC) confirmation of the diagnosis of MB and determine its genetically defined subtype. Ki-67 was assessed as a marker of proliferation activity along with synaptophysin expression, which is used to distinguish MB from embryonal tumor with multilayered rosettes (ETMR) and most atypical teratoid rhabdoid tumors (ATRT), which can potentially mimic MB (4).

Genomic DNA samples were prepared for sequencing using Kapa Biosystems (Roche) kits. To enrich the coding part of the genome, the TruSeq Exome Capture kit (Illumina) was used. The quality of the obtained libraries was controlled using the Fragment Analyzer. Sufficiency of the DNA quantity was assessed with the qPCR. After quality control and DNA quantity estimation, the pool of libraries was sequenced on 2 lanes of the Illumina NovaSeq 6000.

Identification of putative causal variants

We assembled a list of 616 oncogenes, based on a broad list of 565 known oncogenes (8), and an overlapping set of 87 previously reported MB susceptibility genes (Sup. Materials – Susceptibility gene lists assembly; Sup. Table S1) (9–43).

Raw sequencing data in the form of FASTQ files were obtained using bcl2fastq v2.20 Conversion Software (Illumina). Germline and somatic variant calling were performed in accordance with GATK and Mutect2 best practices (44, 45).

Identified putative somatic variants were subjected to the quality filtration using the following thresholds based on GATK metrics: 1) DP>30, 2) GERMQ>90, 3) TLOD>3, 4) POPAF≥4, 5) ROQ>85.

We took extra caution in interpreting long indels. They often could be unreliably called and require a specialized approach for analysis (46, 47). Therefore, for indels greater than 10 nucleotides that could potentially be nominated as “causal” in both patients, we manually checked the alignment of the short reads with IGV. Such an approach was carried out consistently with common standards in the field (48).

All variant coordinates mentioned are based on the reference genome version of GRCh38 and are declared according to HGVS requirements (49). In assessing the functional effect of the variants found, we rely on the joint recommendations of Clinical Genome Resource (ClinGen), Cancer Genomics Consortium (CGC), and Variant Interpretation for Cancer Consortium (VICC) (Sup. Materials – Strategy for variant oncogenicity classification) (50).

To evaluate the functional importance of identified variants, we used databases of oncogenic variants. For this purpose, we used COSMIC (51) and PeCan (52) focused on pediatric oncology. Furthermore, we use PeCan’s built-in Pathogenicity Information Exchange (PIE) (53) tool, which estimates the pathogenicity of variants based on its sample cohort and additional estimates as Sorting Intolerant From Tolerant

(SIFT) (54) score, likelihood ratio test (LRT) and Combined Annotation Dependent Depletion (CADD) (55) assessments.

Results

Report of cases

The patients were a female and a male of 10 years old (hereafter **Patient #1** and **Patient #2**) presented with complaints of headache, vomiting and visual impairments. Both patients underwent MRI analysis, surgical removal of the tumors, histological and immunohistochemical analysis. An exome sequencing from the blood and tumor DNA was performed and followed by germline and somatic variant calling. The sequencing data analysis was then performed to identify the likely genetic causes for the disease.

Patient #1

A multi-spiral CT scan (MSCT) of the brain revealed a formation in the cerebellum and brainstem as well as triventricular hydrocephalus and periventricular oedema. A magnetic resonance imaging (MRI) of the brain confirmed the results of the MSCT and additionally revealed a mass in the IV ventricle of the brain (Figure 1A); MRI screen of the spinal cord showed no signs of metastasis (Figure 1B). An additional optometric exam revealed signs of optic disc stasis. The patient was prescribed dexamethasone, which had a positive effect on reducing the headaches.

After 17 days of observation, a suboccipital bone-plastic craniotomy was performed under neurophysiological monitoring, with microsurgical removal of tumors of the cerebellum, IV ventricle and brainstem.

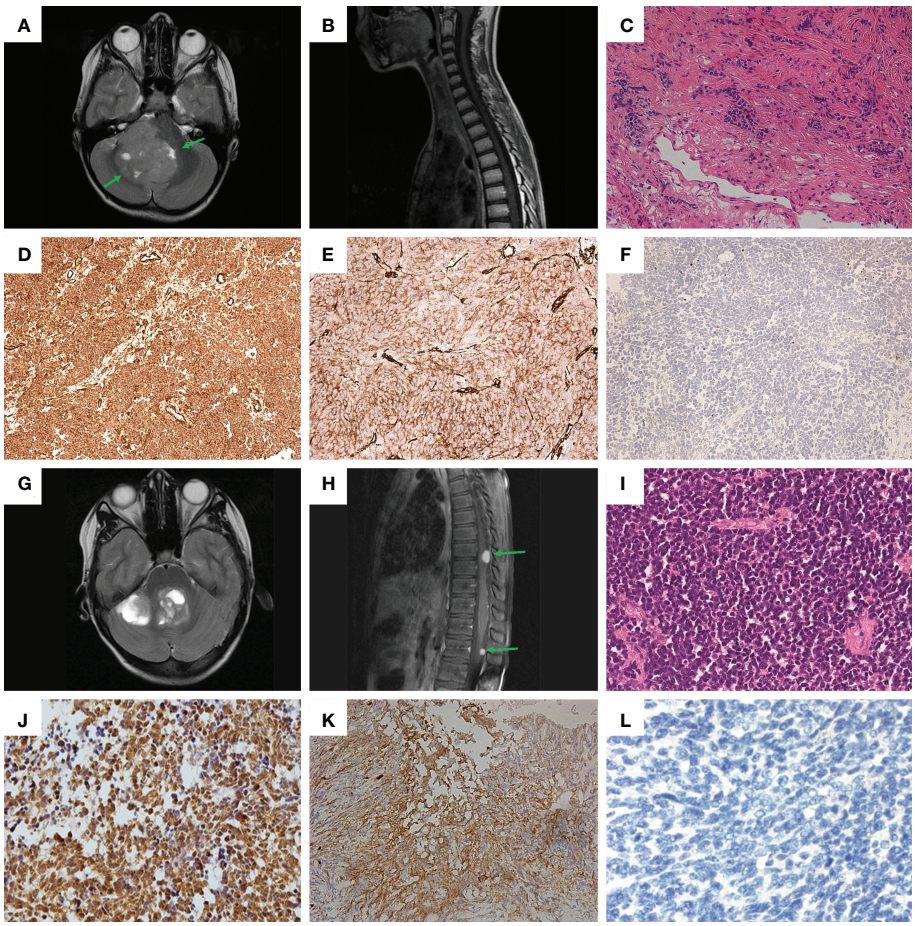

FIGURE 1
Clinical and histological characteristics. **(A, B)** – MRI screens in **Patient #1**: **(A)** brain; **(B)** spinal cord; **(C)** Hematoxylin-eosin staining of sample from **Patient #1**. **(D-F)** Immunohistochemical (IHC) staining of tumor sample from **Patient #1**: **(D)** beta-catenin; **(E)** filamin A; **(F)** GAB1. **(G, H)** MRI screens in **Patient #2**: **(G)** brain; **(H)** spinal cord. **(I)** Hematoxylin-eosin staining of sample from **Patient #2**. **(J–L)** IHC staining of tumor sample from **Patient #2**: **(J)** beta-catenin; **(K)** filamin A; **(L)** GAB1.

Further histological examination of the tumor fragments showed a highly cellular tissue sample of small cells with polymorphic hyperchromatic nuclei, with poor eosinophilic cytoplasm. Areas of nodular structure of light-colored cells were also present. The formation of Homer-Wright-type rosettes was noted. The sample was characterized by an increased number of mitoses, including atypical ones, and endothelial proliferation (Figure 1C). As a result, the tumor from **Patient #1** was assigned to the desmoplastic/nodular type of MB according to the WHO classification (4).

Immunohistochemical (IHC) analysis for the sample obtained from **Patient #1** revealed: 1) positive membrane-cytoplasmic and nuclear beta-catenin staining (Figure 1D); 2) positive cytoplasmic filamin A staining (Figure 1E); 3) negative GAB1 staining (Figure 1F). Therefore, the tumor was assigned to the WNT subtype, according to the genetically defined WHO classification (ICD-10-CM:C71.8; G97.9). Additionally, the proliferative activity of Ki-67 was assessed, which was about 25-30%, as well as synaptophysin expression (Figure S1A), which distinguished MB from ETMR and most ATRT, which can potentially mimic MB (4).

Patient #2

MRI of the brain showed formation in the IV ventricle and right hemisphere of the cerebellum and internal hydrocephalus (Figure 1G). In addition, MRI of the spinal cord showed signs of spinal metastasis (Figure 1H).

After 5 days, a partial surgical removal of a tumor of the right cerebellar hemisphere, IV ventricle, was performed.

Histological examination revealed a monotonous, dense-, small- and blue-cellular malignant tumor with rosettes and little stroma and numerous mitoses (Figure 1I). As a result, in the course of histological examination, the preparation from **Patient #2** was assigned the classical type of MB according to the WHO classification (4).

Patient #2 had the same set of IHC confirmations as **Patient #1**: 1) positive membrane-cytoplasmic and nuclear beta-catenin staining (Figure 1J); 2) positive cytoplasmic filamin A staining (Figure 1K); 3) negative GAB1 staining (Figure 1L). Thus, the results of IHC analysis suggest that the tumor should be assigned to the WNT subtype, according to the genetically defined WHO classification (ICD-10-CM: C71.8; G91.1, G96.8, G83.2). Additional IHC analysis yielded the following: 1) positive expression of synaptophysin (Figure S1B); 2) Proliferative activity Ki-67 on level 20-30%.

Molecular diagnosis

Somatic variant calls were subjected to quality filtration to ensure only high-confidence somatic mutations entered the analysis (**Methods**). The chromosome 6 monosomy was ruled out for both patients using heterozygosity analysis that indicated presence of the two copies of the chromosome 6 (Figure S2). In total there were 50 and 37 good quality somatic variants for analysis in **Patient #1** and **Patient #2** respectively (Sup. Tables S2, 3). Out of these variants, 26 and 17 were eliminated from the analysis as non-coding, 3 and 1 as inframe indels, 2 and 1 were eliminated as synonymous for **Patient #1** and **Patient #2**, respectively. Furthermore, 9 and 11 variants each with ambiguous or missing annotation were excluded from the analysis for **Patient #1** and **Patient #2**, respectively.

Initially, we focused our analysis on missense variants and protein truncating variants (PTV). In the data, there were five and two missense variants and five PTV for each **Patient #1** and **Patient #2**, respectively.

Patient #1 had only one variant in a gene from the list of MB susceptibility genes (87 genes list). For **Patient #2**, the genes from the MB susceptibility gene list did not contain any mutations.

None of the identified somatic missense variants was found in the two examined gene sets in both patients. Six of seven missense variants outside the lists of known oncogenes were eliminated as unlikely to affect any important conservative parts of the gene, as their missense deleteriousness (MPC) (56) score was ≤2 (Sup. Table S4).

Patient #1 had only one mutation in a known oncogenic gene from the analyzed list – a stop gain somatic mutation (NC_000012.12:g.45849701C>T, NM_152641:p.Gln613Ter) in *ARID2* (Figure 2A), which disrupts cell cycle regulation and has previously been identified as a MB risk gene (87 genes list) (10, 11). Variant was found to be in a close proximity to RFX DNA-binding protein domain (the domain boundary is at amino acid 601). For **Patient #1** it was the only PTV within the MB susceptibility gene list (87 genes list) and/or expanded gene list (616 genes list).

Four other PTVs found in **Patient #1** were located in *NOBOX*, *SRRM2*, *CTCF*, *RAB11FIP4*. Upon screening of these variants in IGV (57), frameshifts in *SRRM2* and *RAB11FIP4* were eliminated because of the poor mapping quality (**Methods – Identification of putative causal variants**). Frameshift variant in *NOBOX* was excluded from consideration because of its specific expression only in testis and ovarian tissues as was indicated by GTEX (58) (Sup. Table S5).

CTCF is an evolutionarily conserved gene responsible for the spatial properties of chromatin, including its accessibility to chromatin, so the frameshift indel (NC_000016.10:g.67611435_67611436insA, NM_006565:p.Thr204AsnfsTer26) (Figure 2B) in *CTCF* can potentially be considered as a secondary priority cause of MB in **Patient #1**.

For **Patient #2**, none of the variants were found in the 87 genes list. Next, we considered an extended list of 616 oncogenes in which the long frameshift in *MAP2K4* was detected. We performed visual control of this PTV with IGV

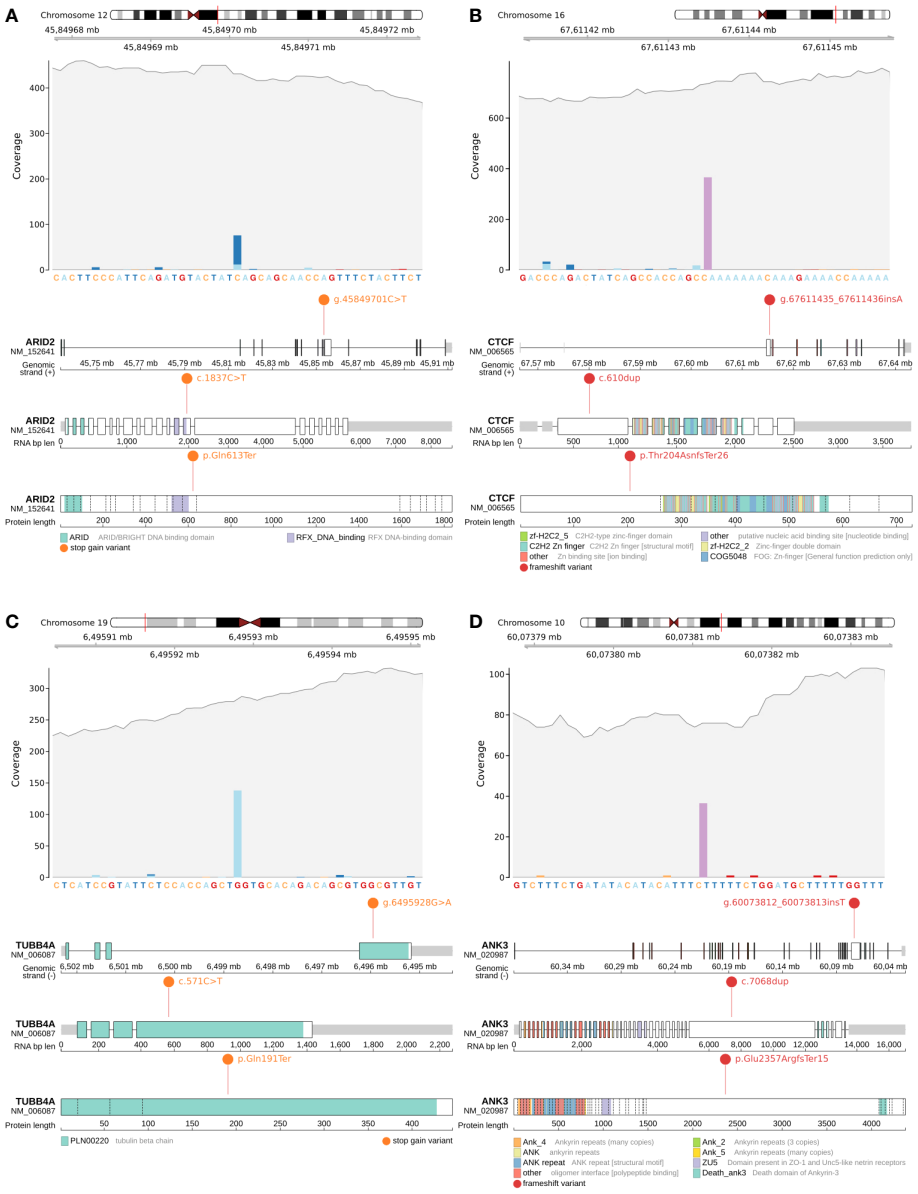

FIGURE 2
Candidate somatic protein truncating variants (GRCh38): **(A, B)** – coverage and functional effects of PTVs on DNA, RNA and protein level in Patient #1. **(A)** NC_000012.12:g.45849701C>T in *ARID2* (NM_152641:p.Gln613Ter); **(B)** NC_000016.10:g.67611435_67611436insA in *CTCF* (NM_006565:p.Thr204AsnfsTer26); **(C, D)** – coverage and functional effects of PTVs on DNA, RNA and protein level in Patient #2. **(C)** NC_000019.10:g.6495928G>A in *TUBB4A* (NM_006087:p.Gln191Ter); **(D)** NC_000010.11:g.60073812_60073813insT in *ANK3* (NM_020987: p.Glu2357ArgfsTer15).

and eliminated this candidate variant due to poor mapping quality. In a further analysis, we considered variants in all genes and found 4 PTVs in *AP003062.1*, *KLHL4*, *ANK3*, *TUBB4A*. After visually screening all 4 variants in IGV (57), we discarded 2 long frameshifts in *AP003062.1* and *KLHL4* due to poor mapping quality (Methods – Identification of putative causal variants; Sup. Table S6).

The remaining pair of PTVs were stop gain somatic mutation (NC_000019.10:g.6495928G>A, NM_006087:p.Gln191Ter) in *TUBB4A* (rs1376427129, gnomAD_AF=6.57x10^{-6}) (Figure 2C) and frameshift indel (NC_000010.11:g.60073812_60073813insT, NM_020987:p.Glu2357ArgfsTer15) in *ANK3* (Figure 2D).

Conclusively, taking into account clinical symptoms, IHC and genetic analyses the diagnosis was defined as WNT-β

medulloblastomas without chromosome 6 monosomy and no known mutations in *CTNNB1* and *APC*. Novel identified risk variants align well with the previous knowledge of *ANK3*, *TUBB4A*, *ARID2* and *CTCF* functionality in cancer but the specific variants that were identified in these patients have not been observed previously. In addition, the role of these variants in pediatric tumors of the central nervous system has not been previously reported.

Discussion

Patient #1

The *ARID2* is a highly conservative gene (pLI=1) involved in various biological processes, including the cell cycle control, regulation of transcription and modification of chromatin structure and is a known tumor suppressor gene (8). The *ARID2* gene product functions as a subunit of the PBAF (SWI/SNF-B) chromatin remodeling complex, which promotes ligand-dependent transcriptional activation by nuclear receptors. It was previously known that *ARID2* co-immunoprecipitates with α-tubulin and that *ARID2* localizes to the spindle pole during mitosis (59). Rare somatic mutations in *ARID2* can lead to severe phenotypes, including MB. For one-third of WNT-MB cases, functional annotation of the recurrently altered genes revealed somatic dysregulation of chromatin modeling genes of the SWI/SNF family, which also includes *ARID2* (10, 60). PeCan (52) did not show an exact match for the p.Gln613Ter in *ARID2* in pediatric oncology reports. However, PeCan's (52) built-in PIE classified p.Gln613Ter as "GOLD" ["truncation in gold gene (tumor suppressor)"], likewise based on LRT ("Deleterious") and CADD (CADD=38, $CADD_{raw}$=11.70) estimates. According to COSMIC, p.Gln613Ter in *ARID2*, has been reported several times in the database as a variant found in various cancer types, though not in the central nervous system (61–63). We categorize g.45849701C>T as "oncogenic" according to accumulated evidence, as suggested by Horak et al. (Sup. Materials – Strategy of variant oncogenicity classification) (50).

Considering *CTCF* as a secondary finding in **Patient 1** it is worth noting its properties of regulating chromatin spatial regulation. It is known that CTCF-binding sites often define topological associating chromatin domains (TAD) boundaries and removal of these sites can lead to a moderate upregulation of a nearby gene. Therefore, alterations in *CTCF* genotype may potentially lead to significant gene expression alterations (64–66). Variant p.Thr204AsnfsTer26 was found to have an exact match with ClinVar and was assessed as "pathogenic" (Variation ID: 280869). PeCan (52) has shown that variant p.Thr204AsnfsTer26 has already been reported several times in pediatric oncology studies of lymphoblastic leukemia and solid tumors (67–69). PIE classified p.Thr204AsnfsTer26 as "GOLD". Additionally, COSMIC shows multiple lines of evidence in studies involving various tumor types (65, 70, 71). The abundance of evidence in the database allows this variant to be identified as a cancer hotspot. *CTCF* is a very conservative gene, with almost no PTVs observed in germline DNA in large population-based cohorts (pLI=1), yet, there was no specific linkage to pediatric brain tumors reported to date. The accumulated evidence for g.67611435_67611436insA indicates that this is an "oncogenic" variant (Sup. Materials – Strategy of variant oncogenicity classification) (50).

Patient #2

In a previous survival analysis study, *TUBB4A* expression in tumors was found to be associated with MB patients survival, suggesting that *TUBB4A* may have oncogenic properties (72). Interestingly, observed PTV is found in the last exon of the gene. Previous studies indicated that in other genes, including cancer genes, such mutations result in gain-of-function effect (73–75). This is consistent with the observation of lower expression of *TUBB4A* benefiting the survival. *TUBB4A* is non-conservative gene (pLI=0.11), which could potentially reduce the effect of PTV on viability. Missense mutations in *TUBB4A* are known to affect various neurological phenotypes, including those associated with cerebellar atrophy, early infantile encephalopathy, which may be due to the selective effects of different mutations on cells and microtubule dynamics (76).

Microtubules are components of the cytoskeleton that contribute to the morphology of axons and dendrites in neurons and facilitate the transport of cell cargos. In dividing cells, microtubules of polymerized α-/β-tubulin dimers control the process of mitosis at different stages of its course, which has been previously well studied (77, 78). Microtubules are prone to constant phases of polymerization and depolymerization, and changes in microtubule dynamics can lead to errors in chromosome segregation and chromosome instability, a key feature of oncological cells (78–81).

In cancer cells, changes in microtubules dynamics, often associated with cancer-specific tubulin isotypes and tubulin post-translational modifications, are involved in metastatic cell migration, drug resistance, and tumor vascularization (81, 82). It is important to clarify that the hyperfunction of tubulin motility in mitosis is also a molecular target for numerous "antitubulin agents", which have been shown to interact with multiple sites on α- or β-tubulin and have been successfully used as chemotherapeutic agents to induce mitotic arrest and cancer cell death (83, 84).

The *ANK3* regulates the mitogen–activated protein kinase (MAPK) pathway related to extracellular matrix organization, cell motility through PTK2 signaling and somatodendritic inhibitory synapses, which determines its high conservativity (pLI=1) (85). Abnormalities in MAPK signaling are known to be associated with the process of metastasis and have long been

proposed as targets for selective therapy for oncologies, since the presence or absence of metastasis often determines the prognosis of survival (85). But, even more importantly, that brain-specific *Ank3* is linked to microtubule dynamics through a GSK3/CRMP2-dependent mechanism, which has been confirmed using mouse models (86). There is evidence that increased *ANK3* expression in cancer tissues correlates with better survival in prostate cancer, suggesting that *ANK3* is a tumor suppressor gene (87).

Early gene expression studies in the hippocampus of Ank3 +/- and Ank3+/+ mice revealed altered expression of 282 genes that were enriched with microtubule-related functions (86). *ANK3* binds microtubules directly or through the binding of microtubule-associated proteins at the plus-end stabilization cap, which prevents depolymerization and directly affects microtubule dynamics (88–90).

COSMIC and PeCan did not show an exact match with the p.Gln191Ter in *TUBB4A* and p.Glu2357ArgfsTer15 in *ANK3*, which makes it impossible to classify them as cancer hotspots. PIE has added evidence of p.Gln191Ter in *TUBB4A* oncogenicity through SIFT ("Damaging"), CADD (CADD=36, $CADD_{raw}$=10.41) and LRT ("Deleterious"). PIE did not have sufficient information about p.Glu2357ArgfsTer15 in *ANK3*. Given involvement of these variants in oncological processes, the severity of the functional effect on the protein, and the available data from the survival analysis incline us to classify g.6495928G>A in *TUBB4A* as "variant of uncertain significance" and g.60073812_60073813insT in *ANK3* as "oncogenic" (Sup. Materials – Strategy of variant oncogenicity classification) (50).

Conclusively, we identified four candidate somatic mutations potentially explaining the MB onset in two pediatric patients and providing new biological insights into the mechanisms of the pediatric tumor development.

Data availability statement

A full list of somatic mutations passing quality filtration is available in Sup. Tables S1 and S2. Patients' consents for clinical DNA sequencing do not permit free data sharing, however, reasonable requests for specific details of genotypes, not conflicting with consent could be accommodated by contacting the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Almazov National Medical Research Center institutional ethics committee (Protocol # 3502-22 of 21.02.2020). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

RK, YD, MA designed the study. YD, SS, MK, AS, DM directly supervised patients and obtained biospecimen. RK, MA, analyzed the data. RK, MA 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1085947/full#supplementary-material

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Pediatric diffuse midline glioma H3K27- altered: A complex clinical and biological landscape behind a neatly defined tumor type

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The 2021 World Health Organization Classification of Tumors of the Central Nervous System, Fifth Edition (WHO-CNS5), has strengthened the concept of tumor grade as a combination of histologic features and molecular alterations. The WHO-CNS5 tumor type "Diffuse midline glioma, H3K27-altered," classified within the family of "Pediatric-type diffuse high-grade gliomas," incarnates an ideally perfect integrated diagnosis in which location, histology, and genetics clearly define a specific tumor entity. It tries to evenly characterize a group of neoplasms that occur primarily in children and midline structures and that have a dismal prognosis. Such a well-defined pathological categorization has strongly influenced the pediatric oncology community, leading to the uniform treatment of most cases of H3K27-altered diffuse midline gliomas (DMG), based on the simplification that the mutation overrides the histological, radiological, and clinical characteristics of such tumors. Indeed, multiple studies have described pediatric H3K27-altered DMG as incurable tumors. However, in biology and clinical practice, exceptions are frequent and complexity is the rule. First of all, H3K27 mutations have also been found in non-diffuse gliomas. On the other hand, a minority of DMGs are H3K27 wild-type but have a similarly poor prognosis. Furthermore, adult-type tumors may rarely occur in children, and differences in prognosis have emerged between adult and pediatric H3K27-altered DMGs. As well, tumor location can determine differences in the outcome: patients with thalamic and spinal DMG have significantly better survival. Finally, other concomitant molecular alterations in H3K27 gliomas have been shown to influence prognosis. So, when such additional mutations are found, which one should we focus on in

order to make the correct clinical decision? Our review of the current literature on pediatric diffuse midline H3K27-altered DMG tries to address such questions. Indeed, H3K27 status has become a fundamental supplement to the histological grading of pediatric gliomas; however, it might not be sufficient alone to exhaustively define the complex biological behavior of DMG in children and might not represent an indication for a unique treatment strategy across all patients, irrespective of age, additional molecular alterations, and tumor location.

KEYWORDS

H3K27, WHO classification, diffuse midline glioma, pediatric, CNS tumors, brain cancer, pediatric neuro-oncology, WHO CNS 5

1 Introduction

The integration of genomics into the histopathology of pediatric brain tumors has changed the way we diagnose, classify, and treat brain cancer in children.

In the last two decades, our understanding of the etiology and the biological origin of several types of childhood brain tumors has profoundly improved. Genomics has enriched and supplemented traditional histopathology methodologies: DNA and RNA sequencing, RNA expression profiling, fluorescence *in situ* hybridization, and, finally, DNA methylation have been demonstrated to be valuable tools for refining and improving both the classification and diagnosis of adult and childhood brain cancers. The application of genomic and epigenomic molecular profiling techniques has unveiled a complex biological landscape behind all forms of pediatric brain cancer, revolutionizing our knowledge in the field of pediatric neuro-oncology. We have been moving from a morphology-based to a molecular-based categorization of diseases, in which we now are able to identify many subgroups of tumors characterized by different clinical behavior, prognosis, anatomical location, and age at presentation (1).

The importance of genomics and molecular features of brain tumors started to emerge in the updated fourth edition of the World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS) (2016). For the first time, the 2016 WHO CNS classification used molecular parameters in addition to histology to define many tumor entities. It encompassed new sub-classification for diffuse gliomas, medulloblastomas, and other embryonal tumors, and it defined new entities based on their unique molecular features (glioblastoma, IDH-wild-type, and glioblastoma, IDH-mutant; diffuse midline glioma, H3K27M-mutant; RELA fusion-positive ependymoma; medulloblastoma, WNT-activated and medulloblastoma, SHH-activated; and embryonal tumor with multilayered rosettes, C19MC-altered) (2).

The integration of molecular and genomic features in histology then became increasingly important in the fifth edition of the WHO classification of CNS tumors (2021) (3). As far as pediatric CNS tumors are concerned, this led to some peculiar changes in the 2021 classification: (i) there are now "pediatric-type" and "adult-type" tumor families for both low- and high-grade gliomas; (ii) several novel tumor entities of interest in pediatric age have been defined (in many cases, primarily by their molecular characteristics), as shown in Table 1 (4); (iii) molecular parameters have been integrated into tumor grading, which is a result of combined histological and molecular grading within-tumor-type; (iv) precise molecular diagnostic tools (including in some cases DNA methylation) are indicated as needed for the diagnosis of particular tumor types (3).

2 Pediatric high-grade diffuse midline gliomas, H3K27-altered, and the WHO Classification of central nervous system tumors

Pediatric high-grade gliomas (HGGs), which are among the least curable and most challenging brain neoplasms in children, have been greatly involved in such a crucial biological and histopathological revolution. For many decades, HGGs in children have been considered similar to their adult counterparts. Indeed, in recent years, several genomic studies largely showed that childhood aggressive gliomas are represented by several peculiar biological entities and are not at all the pediatric equivalents of adult malignant gliomas (5). In WHO CNS 2021, pediatric HGGs are formally distinguished from adult HGGs, emphasizing their biological differences. In the pediatric HGG family, four different HGG types are identified: diffuse midline glioma, H3K27-altered; diffuse

TABLE 1 Glioma types of clinical interest in children and adolescents, as per the 2021 WHO Classification of Tumors of the Central Nervous System, Fifth Edition.

Gliomas of clinical interest in children and adolescents	New entity (2021 CNS WHO)	Genetic/molecular alterations
Pediatric-type diffuse low-grade gliomas		
Diffuse astrocytoma, MYB- or MYBL1-altered	x	MYB, MYBL1
Angiocentric glioma		MYB
Polymorphous low-grade neuroepithelial tumor of the young	x	BRAF, FGFR family
Diffuse low-grade glioma, MAPK pathway-altered	x	FGFR1, BRAF
Pediatric-type diffuse high-grade gliomas		
Diffuse midline glioma, H3 K27-altered	refined	H3 K27, TP53, ACVR1, PDGFRA, EGFR, EZHIP
Diffuse hemispheric glioma, H3 G34-mutant	x	H3 G34, TP53, ATRX
Diffuse pediatric-type high-grade glioma, H3-wild-type, and IDH-wild-type	x	IDH-wild-type, H3-wild-type, PDGFRA, MYCN, EGFR
Infant-type hemispheric glioma	x	NTRK, ALK, ROS, MET
Circumscribed astrocytic gliomas		
Pilocytic astrocytoma		KIAA1549-BRAF, BRAF, NF1
High-grade astrocytoma with piloid features	x	BRAF, NF1, ATRX, CDKN2A/B
Pleomorphic xanthoastrocytoma		BRAF, CDKN2A/B
Subependymal giant cell astrocytoma		TSC1, TSC2
Astroblastoma, MN1-altered		MN1

Newly defined entities are marked in the second column. Typical genetic alterations are listed in the third column for each tumor type. Adapted from (3, 4). NB: tumors that are exclusively found in adults, although present in the 2021 WHO CNS classification, are not listed in this table. Glioneuronal tumors and ependymomas, although of pediatric interest, are not listed.

hemispheric glioma, H3G34-mutant; diffuse pediatric-type high-grade glioma, H3-wild-type, and IDH-wild-type; and infant-type hemispheric glioma (3).

Among pediatric HGG, diffuse midline gliomas (DMG) encompass an apparently homogeneous group of aggressive central nervous system (CNS) neoplasms that can arise in the brainstem (including the formerly defined "diffuse intrinsic pontine gliomas" or DIPG), the thalamus, the cerebellum, the gangliocapsular region, the cerebellar peduncles, the third ventricle, the hypothalamus, the pineal region, and in the spinal cord (6). They represent around 20% of all pediatric CNS tumors, with around 200-300 cases per year in the United States (7). DMGs, and DIPGs in particular, are leading causes of solid tumor death in children; overall, their prognosis has remained extremely poor, and for many years, no significant improvement has been achieved in their treatment (8). The majority of DMGs occur in children aged 5 to 10 years, without any gender predilection (9). Dissemination at diagnosis is possible but rare; secondary metastases are more frequent, being reported in 13% of cases, and can present as intraparenchymal, ventricular, or leptomeningeal (10, 11).

Magnetic resonance imaging (MRI) is the gold standard in the diagnosis of DMG and, in particular, of DIPG, in which typical findings include a T1- and T2-hyperintense lesion involving >50% of the pons and high perfusion and restricted diffusion sequences (Figure 1) (12–14). Routine biopsy in DIPG remains under debate and is mainly restricted to cases with an atypical imaging appearance (15). A typical DIPG diagnosis may be made based on MRI and clinical criteria only: multiple cranial neuropathies, long tract signs (hyper-reflexia, clonus, increased tone, presence of a Babinski reflex), and ataxia (12). Positron emission tomography (PET) imaging might also find its role as an integrative diagnostic tool in DMG (16).

Current treatment strategies for DMG encompass focal intensity-modulated radiation therapy (IMRT) to the primitive tumor (usually 54–60 Gy in 1.8–2 Gy fractions, given over 6 weeks) and variable lines of chemotherapy. Nonetheless, despite the various attempts at new treatment approaches described so far, the prognosis remains poor (8). Re-irradiation, which represents the only effective treatment for recurrent disease, can lead to symptom relief or neurological improvement in the majority of patients and slightly prolong survival after relapse but remains a palliative and not a curative option (17–21).

Somatic mutations in histone 3 (H3) gene variants H3F3A and HIST1H3B, encoding histone H3 variants H3.3 and H3.1,

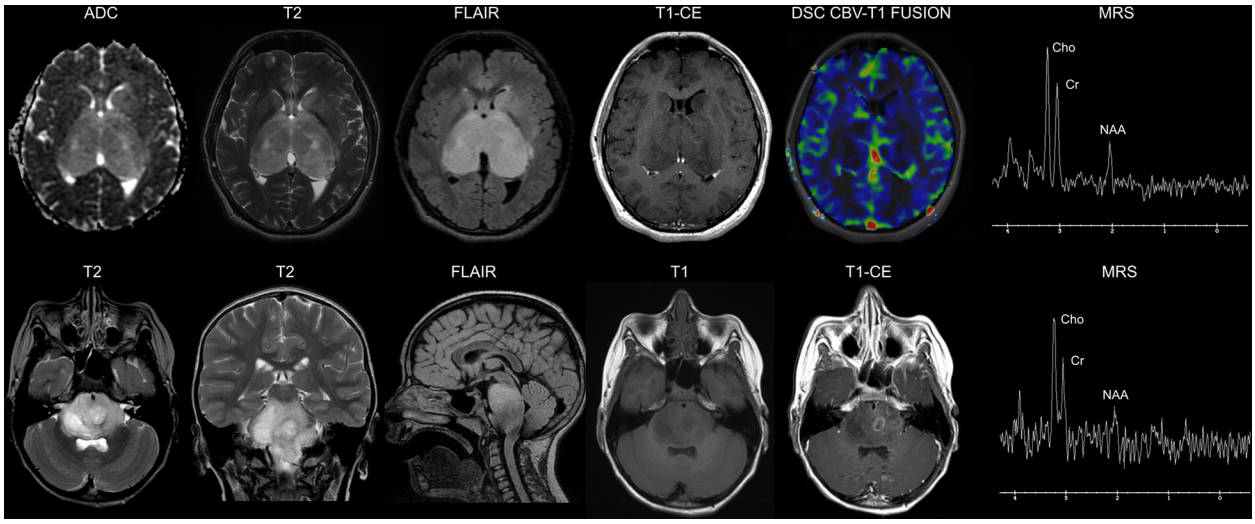

FIGURE 1
Neuroimaging findings in diffuse midline gliomas H3K27-altered. Upper row: 16-year-old male. Diffuse Midline Glioma, EGFR-mutant. Brain axial Apparent Diffusion Coefficient (ADC) map, T2-weighted, Fluid Attenuated Inversion recovery (FLAIR) and Contrast-Enhanced (CE) T1-weighted images show a bi-thalamic infiltrating and expansile lesion with increased diffusivity and a lack of contrast enhancement. There is concomitant infiltration of the left striatum. Dynamic Susceptibility Contrast (DSC) Cerebral Blood Volume (CBV) perfusion-weighted imaging map fused with T1-weighted imaging shows low perfusion of the lesion. Single voxel Magnetic Resonance Spectroscopy (MRS) with an echo time of 144 ms shows a prominent increase in the Cho/NAA ratio. **Lower row**: 7-year-old male. Diffuse Intrinsic Pontine Glioma (H3.3 K27-mutant). Brain axial and coronal T2-weighted, sagittal FLAIR, and axial T1-weighted images show a diffusely infiltrating lesion involving the pons. The axial CE T1-weighted image shows a left paramedian focal area of ring enhancement. Single-voxel MRS with an echo time of 144 ms shows a marked increase in the Cho/NAA ratio.

respectively, collectively referred to as H3K27M (p.Lys27Met), have been detected in the majority of biopsied DIPG and in general in DMG. An H3.2 variant has also been documented (22, 23). The K27M mutant variant causes a global reduction in levels of H3 lysine 27 trimethylation (H3K27me3). In normal cells, trimethylation is mainly established by the H3K27-specific histone methyltransferase enhancer zeste 2 (EZH2) within the Polycomb Repressive Complex 2 (PRC2). Thus, H3K27M results in hypomethylation and ultimately leads to an epigenetic dysregulation of cellular processes due to the inactivation of PRC2, through an interaction between EZH2 and the mutant histone (24, 25).

In the previous 2016 WHO classification, diffuse midline glioma (H3K27M-mutant) was defined as an infiltrative midline high-grade glioma with predominantly astrocytic differentiation and a K27M mutation in either H3F3A or HIST1H3B/C (2).

In 2018, the consortium cIMPACT-NOW (the "Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy"—Not official WHO), which aims to link the WHO classification effort and the daily work of practicing physicians, clarified one important key-point regarding the diagnosis of "Diffuse Midline Glioma, H3K27M-mutant" (as defined in the WHO 2016 classification), stating that the term Diffuse Midline Glioma, H3K27M–mutant should be used to identify tumors that are diffuse (i.e., infiltrating), midline, gliomas (with the expression of glial markers, particularly Olig2) and H3K27M-mutant, and should not be applied to other tumor types (e.g. non-diffuse gliomas) that are H3K27M-mutant. While at first H3K27M mutations were documented exclusively in DMG, appearing as an exclusive molecular hallmark of such disease, these mutations were later reported in other brain tumors. Nonetheless, the detection of these mutations now seems to confer a strong clinical prognostic value only when they occur in the setting of diffuse midline gliomas (26).

Immunohistochemistry (IHC) is useful for identifying mutations and, in particular, for diagnosing H3K27M-mutant diffuse midline gliomas. IHC is inexpensive, and several studies have reported significant associations between H3K27M protein expression and the H3K27M mutation (27). The assay is widely available nowadays, but its results need to be carefully interpreted; positivity needs to be identified as nuclear staining in neoplastic cells rather than cytoplasmic staining in macrophages and/or microglia. H3K27me3 immunoreactivity is mutually exclusive with H3K27M positivity in most cases, so the loss of H3K27me3 expression should always be analyzed and evidenced in conjunction with H3K27M positive immunohistochemistry (Figure 2) (26). It has to be noted that some nomenclatures use the designation K28 rather than K27 to identify the affected lysine residue (28).

H3K27M mutation status can also be assessed by other methods beyond IHC, including Sanger sequencing, next-generation sequencing (NGS), droplet-digital polymerase chain

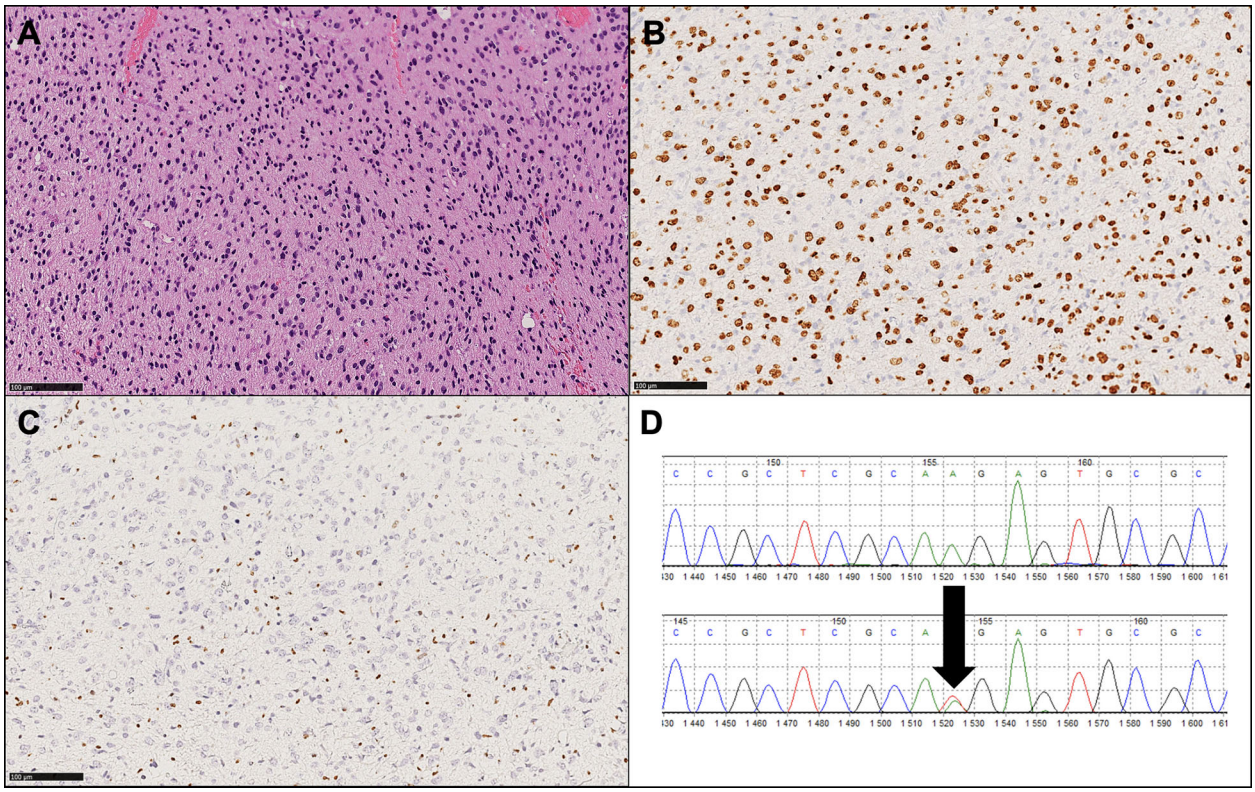

FIGURE 2
Histopathological and molecular findings of a representative diffuse midline glioma, H3 K27-altered (WHO 2021). **(A)** Hematoxylin and eosin image (original magnification: 100X) showing a diffuse, infiltrative glioma with astrocytic morphology. **(B)** Diffuse expression of OLIG2, a glial marker, is consistent with this tumor type. **(C)** Loss of H3K27me3 is present and exemplifies a mandatory diagnostic feature. **(D)** Sanger sequencing output showing a K27M mutation (arrow), the most frequent molecular alteration observed in this tumor type.

reaction, and pyrosequencing. Indeed, it has been found that DMG can contain sub-clonal, mosaic-pattern H3K27M mutations, and it has been shown that in some cases, tumor cells displayed cytoplasm positivity or lymphocyte immunopositivity and have been later confirmed to be H3 wild-type by Sanger sequencing. Therefore, in some instances, further sequencing is needed to detect the status of H3K27M. It has been shown that IHC can reach almost 100% sensitivity, while Sanger sequencing has 100% specificity. Thus, while IHC is an efficient method for routine use, a combination of IHC and Sanger sequencing (or NGS) is strongly advisable since it can virtually provide 100% sensitivity and specificity for the definition of H3K27M status (29).

Furthermore, it has been recently further demonstrated that in addition to the K27M mutation, other molecular changes can be found in pediatric DMG, namely overexpression of the EZH inhibitory protein (EZHIP) and alterations in the epidermal growth factor receptor (EGFR).

EZHIP overexpression, resulting in H3K27me3 global reduction, has been first observed in posterior fossa type-A ependymomas (30). After having observed that rare cases of DIPG and DMGs lacked a histone H3 mutation, Castel et al. identified nine out of 241 cases (3.7%) displaying a typical infiltrating DIPG histopathology and H3K27me trimethylation loss that, however, lacked K27M positivity by immunohistochemistry (IHC). By analyzing EZHIP expression in DMG, they then identified its systematic overexpression. Importantly, such EZHIP overexpression can be detected by IHC, and Castel et al. ultimately proposed that these EZHIP/H3-WT tumors might be considered similar to K27M mutated DMGs, extending the spectrum of DMG with PRC2 inhibition beyond the H3K27M mutation (31).

More recently, Mondal et al. described the existence of a subset of diffuse gliomas, with mainly thalamic or bithalamic origin that show frequent epidermal growth factor receptor (EGFR) gene amplification and/or mutation and loss of H3K27me3. Loss of trimethylation seems to be mediated by either the H3K27 mutation or EZHIP overexpression (32, 33). The authors concluded that loss of H3K27me3 should then be considered a common feature of three different molecular classes of pediatric DMG: (i) the "typical" DMG with H3K27M mutation, (ii) the DMG with EZHIP overexpression (which additionally shows a high frequency of ACVR1 mutations),

and (iii) the mainly bithalamic diffuse gliomas that present H3K27M or EZHIP overexpression together with strong enrichment for EGFR alterations (33).

Taking into account these recent discoveries, which are of paramount biological importance, the 2021 WHO classification of CNS tumors (fifth edition) adopted the revised designation "diffuse midline glioma, H3K27-altered" to include subtypes of DMG with an alternative mechanism for the loss of H3K27 trimethylation (EZHIP overexpression DMG, EGFR mutant DMG), in addition to the most common H3K27M mutation (3). The subclassification of pediatric DMGs according to the 2021 WHO CNS Classification is resumed in Table 2.

3 Other tumors with H3K27 mutations

Over the past few years, the same H3K27M mutation has been identified in several tumor types that are not diffuse midline gliomas (26); in particular, it has been reported in ependymomas, pilocytic astrocytomas, pediatric diffuse astrocytomas, and gangliogliomas.

3.1 Ependymoma

In 2017, Ryall et al. showed that while K27M mutations can be found, they are extremely rare in posterior fossa type A (PFA) ependymomas, identifying only one case out of 151 harboring the K27M mutation and stating that routine evaluation of K27M mutations in PFA ependymomas is of limited utility and unlikely to have any prognostic role (34). Indeed, more recent studies suggested that PFA ependymomas might be driven by epigenetic changes in DNA and histone methylation, and that while the K27M mutation is actually rare in PFA ependymomas, a global loss of H3K27me3 can be typically observed in PFA ependymomas. Such lower levels of H3K27me3 in PFA ependymomas are due to the overexpression of EZHIP ("enhancer of zeste homolog inhibitory protein"), a protein that might work as a potential tumor driver in PFA and that mimics K27M mutated histones, functioning as an intrinsic inhibitor of PRC2 function (35). Several reports described elevated EZHIP expression in DMG cases that lack H3 mutations, which supports the fact that EZHIP expression and H3K27M mutations are mutually exclusive and are encountered in reverse proportions: 3% versus 97% and 96% versus 4%, respectively, in DMG and PFA ependymomas (30, 31, 36). Interestingly, neither histopathologic distinctions nor outcome differences have been found between PFA EZHIP-overexpressing ependymoma and H3 K27M-mutant ependymoma (37). Nonetheless, investigating further the role of epigenetic changes, loss of H3K27 trimethylation, and EZHIP overexpression in PFA might hopefully lead to a better understanding of the genesis of such tumors and the identification of potential drug targets.

3.2 Non diffuse – pediatric low-grade astrocytoma and ganglioglioma

Pilocytic astrocytoma (PA) is the most common brain tumor in children. It is a well-circumscribed tumor with slow growth and is classified as a grade I tumor by the World Health Organization. Malignant transformation (MT) of low-grade gliomas (LGG) is a very unusual event in the pediatric population (38). The H3 K27 mutation in PA is considered to be very unusual, but some reports in the literature tend to suggest a longer survival than K27M DMG. Hochart et al. described the case of a child with spinal pilocytic astrocytoma that had been surgically removed and remained off-therapy without treatment for 10 years. The tumor relapsed 10 years later as a glioblastoma. The exclusive presence of an H3.3- K27M mutation was found in the primary tumor (PA), while both K27M and TP53 mutations were detected in the relapsed tumor (glioblastoma). It might be hypothesized that the H3.3-K27M mutation was the first oncogenic hit, while the TP53 mutation, as the second hit, was responsible for the malignant transformation (39). Jones et al. described a patient with pilocytic astrocytoma and H3.3- K27M in association with somatic NF1 and FGFR1 mutations (40). In a study from 2020, it was shown that patients with H3K27M mutant LGG had significantly lower survival than the wild-type group (median OS, respectively, 17.1 months vs. more than three

TABLE 2 Subclassification of pediatric-type diffuse midline gliomas, H3 K27-altered. Adapted from (4).

Diffuse midline glioma, H3.3 K27-mutant	H3.3 pK28M/I (K27M/I) mutation, often co-occurring with TP53/PPM1D mutation and PDGFRA alteration
Diffuse midline glioma, H3.1 or H3.2 K27-mutant	H3.1 or H3.2 pK28M (K27M) mutation, often co-occurring with PIK3CA, PIK3R1 or PTEN mutations, and ACVR1 mutation
Diffuse midline glioma, H3-wild-type with EZHIP overexpression	EZHIP overexpression
Diffuse midline glioma, EGFR- (and H3 K27-) mutant	EGFR mutation (insertion/deletion within exon 20 or p.A289T or p.A289V mutation), often co-occurring with TP53 mutation

years), suggesting that in histologically classified LGG, H3K27M mutant tumors should be treated more aggressively (41).

4 A complex biological and clinical picture behind a unifying definition

Although the WHO classification offers a clear and net definition of pediatric DMGs (H3K27-altered), many studies have demonstrated some heterogeneity within this unique entity, both from a biological and clinical point of view.

Many different reports seem to indicate that the presence of the H3K27M mutation works as an independent negative prognostic marker in DMGs (22, 23, 34, 41, 42).

Nonetheless, other variables in biology, anatomy, and age could potentially help identify different prognostic subcategories of DMGs with slightly different clinical behavior, leading to a stratification of patients according to different risk factors.

4.1 Biological variables

4.1.1 H3 mutation subtypes

Some authors hypothesized that different subtypes of H3 mutation might impact OS in DMG; in 2015, Castel et al. described differences in clinical behavior according to different subtypes of H3K27M-mutant DMG: HIST1H3B (H3.1) mutant gliomas displayed better prognosis and better response to treatment than H3F3A-mutant (H3.3) gliomas. It also has been observed that these two groups had different onset ages (younger in H3.1) and locations along the midline (the H3.1 mutation is almost exclusively seen in the brainstem; the H3.3 mutation is more evenly found along the midline) (43). In another study, it has been observed that the H3.3-K27M mutation is present in almost 60–70% of DIPG and is associated with a short OS (median 11 months). The other variants (H3.1 and H3.2) have a relatively longer OS (median 15 months) and a lower risk of metastasis spread (44). Similar results have been described in a study on long-term survivors of DIPG: H3.1-K27M is associated with a longer median OS than H3.3-K27M (45). A very comprehensive systematic review and meta-analysis by Vuong et al. in 2022 included 26 studies with 102 H3.1-mutant DMGs and H3.3-mutant DMGs. H3.1-K27M mutation confers a better prognosis than H3.3-K27M mutation in children, while in the adult population, H3.3-mutated tumors are associated with better survival (46).

4.1.2 Concomitant molecular alterations

The biological picture of DMG has been enriched and made more complex by the finding of several additional molecular alterations that have been described alongside mutations in H3 and beyond the already cited over-expression of EZHIP and alterations or mutations in EGFR. In fact, different authors have shown that DMG exhibits p53 mutations in almost 50% of cases and amplifications or activating mutations of platelet-derived growth factor receptor alpha (PDGFRA) in 35% of cases (47–49). FGFR1 mutations have also been well described, mainly reported in the thalamus, while PDGFRA alterations are more frequent in the pons (50, 51). Mutations of activin receptor type 1A (ACVR1) can also be detected in 21–32% of DMG patients, and they are significantly associated with young age, prolonged survival, and the H3.1 variant (52). Rarer mutations have been reported in a minority of patients: PPM1D, PIK3CA, PIK3R1, PTEN, and ATRX (33, 53, 54). The prognostic meaning of all such additional molecular alterations in DMGs is still largely undefined, although some initial indications have emerged: in a retrospective study of 94 adults and 70 pediatric cases of diffuse midline glioma, age above 18 years (P=0.007), loss of ATRX expression (P=0.032), and Ki-67 index ≤5% (P=0.039) represented independent favorable prognosticators for longer survival across the entire cohort of H3K27M-mutant DMGs (55), while P53 overexpression has been identified as a negative prognostic factor for overall survival by multivariate analysis in another study (56).

4.1.3 BRAF co-mutations

Particular interest has been focused on the presence of BRAF co-mutations: several cases (at least 15 to 20) of H3K27M/BRAFV600E double mutant gliomas have been described in many reports (mainly gangliogliomas, thalamic gliomas, and diffuse supratentorial gliomas), and sometimes such cases showed long survival (42, 51, 57–61). This seems to suggest the presence of a biological overlap between histologically defined low- and high-grade gliomas and may be associated with a better prognosis than expected, compared to BRAF wild-type and H3K27-mutant DMGs.

4.2 Anatomical variables: Debulking and tumor location

4.2.1 Debulking

Although the literature is not conclusive, the extent of surgical resection might have prognostic importance in tumors that are at least partially resectable. Karremann et al., in their cohort of 85 pediatric DMGs, observed that survival did not depend on the extent of tumor resection in H3K27M mutated tumors, while it positively influenced the prognosis in H3K27-wild-type midline gliomas with extended resection >90% (42). On the contrary, as far as pediatric thalamic gliomas are concerned, the HERBY Trial results evidenced that in 42 patients with thalamic-based DMG, 28 had DMG *H3K27* mutant tumors, with no differences in outcome compared with other DMGs. However, participants who underwent major

debulking or total or near-total resection had longer overall survival (OS): 18.5 months vs. 11.4 months (14). Of note, since H3.1-mutant DMGs are primarily located in the pons and thalamus, the rate of tumor resection for these tumors is lower as compared to H3.3-mutated DMGs (46).

4.2.2 Tumor location

A study by Wang et al. (comprising both children and adults) showed that the H3K27M mutation might have a different prognostic impact based on anatomical location. K27M tumors had a poorer prognosis in infratentorial gliomas compared with the corresponding H3 wild-type tumors (mainly in the brainstem and spinal cord; P <.0001). However, the OS of patients with supratentorial gliomas did not significantly differ between K27M-mutated and H3 wild-type tumors. Furthermore, patients with spinal H3K27M–mutant DMG demonstrated to have a better chance at survival than patients with brainstem DMG (median, 13.2 months vs. 6.6 months), although no statistically significant difference has been recorded. Finally, patients with H3K27M–mutant gliomas in unusual anatomical locations (cerebellum, corpus callosum, lateral ventricle, frontal lobe, and temporal lobe) had a better prognosis compared with those with corresponding tumors in the brainstem (62). Similar results have been recorded by Vuong et al., who tried to stratify patients with H3K27 DMG among more than 800 patients (children and adults). They found that patients with thalamic and spinal cord tumors had significantly better survival than patients with brainstem tumors (46). Furthermore, unlike thalamic tumors, the presence of the H3K27M mutation in DIPG is a much weaker prognostic indicator: wild-type DIPG (approximately 15% of all biopsied cases) has the same unfavorable prognosis as H3K27M-mutant DIPG (63).

4.3 Patient's age as a prognostic variable

Different observations seem to point out that DMGs do not behave the same in children and adults, and in particular that the finding of H3K27 alterations, which has a strong prognostic value in children, is more uncertain as a marker of a worse outcome in adults (when compared to H3 wild-type tumors).

The general characteristics of adult H3K27M-mutant gliomas are very similar to those reported in the pediatric population. As in children, H3K27M mutations are found mainly in midline tumors, suggesting their role as oncogenic alterations in progenitors implicated in the development of midline structures. Nonetheless, location frequency varies between children (in whom H3K27M-mutant gliomas are mainly pontine) and adults (in whom H3K27M tumors seem more frequently located in the thalamus and the spine) (58). In adults and children, these H3K27-mutant DMGs also seem to be associated with a poor prognosis, although no significant difference has been observed between the median survival of H3K27M-mutant and IDH/H3 wild-type gliomas (64). Also, the report by Ebrahimi et al. in 41 DMG (12 pediatric and 29 adult cases) reported that H3K27M mutations are associated with a poorer prognosis in pediatric patients compared to wild-type tumors, while in adult patients these mutations do not significantly influence survival (65). Some authors even documented that in adult patients with DMG, survival may be similar or unexpectedly improved in H3 K27M-mutant tumors compared to wild-type midline gliomas (66).

On the opposite side of the epidemiological spectrum of age, it has been reported that very young children with DMG (less than three years old) might have a significantly longer OS than older patients (42, 67).

5 Discussion

In the medical process of diagnosing and treating patients with cancer, the roles of the pathologist and the physician are often considered to be distant.

Pathologists are primarily committed to obtaining a diagnosis with adequate timing and maximal accuracy to correctly identify the pathology and its sub-types. Moreover, pathologists have a particular interest in disclosing the biological and molecular features of each tumor, since these alterations play a crucial role in the categorization of a disease.

Physicians, on the other hand, are primarily focused on treating patients successfully by administering effective therapies in a timely manner and avoiding toxic or unnecessary treatments. As oncologists, they are interested in the biological and molecular characteristics of the tumor, which can help define the best therapeutic strategy, especially in a modern setting of targeted and personalized medicine.

Any nosological classifications of diseases should be conceived and refined to guide pathologists in making correct and precise diagnoses with a high concordance rate and a low risk of diagnostic error. At the same time, classifications must function as practical tools for clinicians: the histological types and sub-types should, where possible, correlate with the clinical behavior of the disease, the prognosis, and the age of patients to guide physicians in providing the most appropriate therapies.

The 2021 WHO Classification of CNS Tumors (fifth edition) achieved the goal of being a precise and detailed descriptive categorization of diseases while also providing indications with strong clinical and practical value in many ways. This ambitious goal has been pursued in a variety of ways: terminological simplification, often aimed at avoiding misunderstandings (as in the case of the grade written in Arabic rather than Roman numerals); the separation of some pediatric tumors from those of adults (to avoid clinicians being forced to deal with the sub-classifications of entities that they never meet in their clinical practice); and the integrated use of molecular biology and

genetics in the definition of nosological entities, more than the mere evaluation of the morphological characteristics of tumors (26).

The WHO classification has been updated and revised in just a few years (between 2016 and 2021) by accepting and integrating the suggestions of cIMPACT-NOW (a consortium created to bridge the gap between the categorization needs and the clinical contextualization of diseases) (26) into the newer version. As far as the main object of this review is concerned, it is important to note that the 2021 classification, taking into account the observations of cIMPACT-NOW, has changed the name of the entire category of tumors: from the original name of 2016 ("diffuse midline glioma, H3K27M-mutant"), it has changed in 2021 to the more generic term "diffuse midline glioma, H3K27-altered," in order to include the new biologic sub-variants that have been described between 2018 and 2021 (EZHIP and EGFR variants) (Figure 3) (3).

The newly named tumor type "Diffuse midline glioma, H3K27-altered" (within the "Pediatric-type diffuse high-grade glioma" family) tries to ideally represent a perfect integrated and homogeneous diagnostic entity in which age (pediatric), location (midline), histology (diffuse glioma), and genetics (H3K27 alteration) are clearly defined, apparently identifying unequivocally a precise and definite tumor entity.

Such a well-defined pathological categorization has a significant impact on the entire pediatric neuro-oncology community. Clinicians usually value precisely classified and clearly defined nosologic entities, especially when they identify diseases that are characterized by a homogeneous prognosis and univocal treatment. However, the identification of the H3K27 alteration in a case of DMG carries in and of itself a very high risk of simplification in everyday clinical practice. In fact, it might in many cases override the histological, radiological, and clinical peculiarities of each individual patient. For a pathologist, the detection of any H3K27 alteration in the presence of a DMG overrides the need for grading the tumor, which has previously been a heavy responsibility for pathologists because incorrect grading could result in a radical change in the treatment strategy

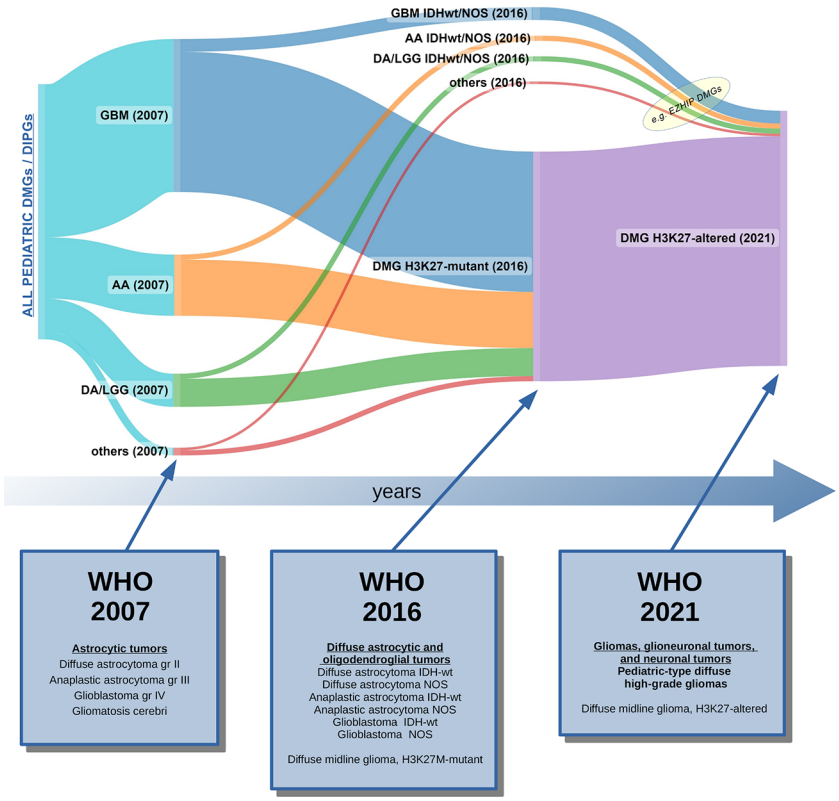

FIGURE 3
Graphic representation of how pediatric DIPGs and DMGs have been classified over the last 15 years, based on the current and the past WHO Classifications of CNS tumors (2, 3, 68). In 2007, DIPG/DMG was not recognized as a distinct entity: data from autopsies and rare biopsies showed that DIPG/DMGs were histologically classifiable as glioblastomas (GBM gr. IV), or less frequently, anaplastic astrocytomas (AA gr. III), low-grade gliomas (mainly diffuse astrocytomas, LGG-DA gr. II), or other rarer histotypes (69). In 2016, the majority of cases were classified as DMG, H3K27M-mutant tumors, although some non-K27M-mutant DMG still remained unclassified. The 2021 WHO CNS Classification unified all cases of pediatric DMGs in which a H3K27 alteration was found (loss of H3 K27me3 trimethylation). Legend: GBM, glioblastoma; AA, anaplastic astrocytoma; LGG, low-grade glioma; DA; diffuse astrocytoma; IDHwt, IDH wild-type; NOS, not otherwise specified; DMG, diffuse midline glioma; DIPG, diffuse intrinsic pontine glioma.

(for example, labeling a pediatric diffuse glioma as "high-grade" would have paved the way for radiotherapy, whereas labeling it "low-grade" would have maybe authorized an initial watch-and-wait strategy). Nonetheless, clinicians are now warned to consider all H3K27-altered diffuse midline gliomas as malignant, incurable diseases, and thus they are inclined to treat aggressively all patients with such a diagnosis, regardless of age, duration of symptoms, neurological deficits at presentation, tumor location, and the presence of concomitant mutations beyond H3K27.

We have been learning that all tumors labeled as H3K27-altered DMG globally share the same dismal prognosis: they are aggressive gliomas, not amenable to radical surgery, that respond only to radiotherapy and just for a limited period of time, and then progress lethally in more than 90% of patients within one year (8). Various medical approaches using neoadjuvant or post-irradiation chemotherapy have been tested over the decades, but none has demonstrated that it is able to substantially improve OS or PFS, sometimes resulting in increased toxicities and the need for hospitalization (70–72).

However, is it truly this straightforward? Should we really treat each patient with an H3K27-altered DMG in the same manner?

In actuality, the diagnosis of H3K27-altered DMG will easily lead physicians to communicate the same dismal prognosis to all patients with such a diagnosis, regardless of their age, the location of their tumor, or the presence of other concomitant gene mutations or alterations. Most likely, each patient will be given front-line radiotherapy in the hopes of having the longest possible post-radiotherapy free-of-symptoms honeymoon, after which they will be either enrolled in some promising early-phase clinical trial or receive metronomic chemotherapy and/or palliative re-irradiation.

We do agree with the core of the mainstream message: H3K27-altered DMG are almost invariably aggressive tumors for which there is no effective treatment other than palliative radiotherapy.

Nonetheless, this last sentence contains the two concepts that we would like to primarily emphasize in our review: (i) first, H3K27-altered DMGs are "*almost*" always aggressive tumors, but there are very few cases in which, unexpectedly, some patients have long survival; (ii) second, H3K27-altered DMG "*currently*" have no effective treatment, but we believe that restless investigations by clinicians and biologists will likely soon change again the way we classify these tumors, and hopefully the way we learn to treat and cure them.

5.1 DMG are almost invariably aggressive tumors

H3K27 mutations seem to have a strong prognostic value only in diffuse midline gliomas, while in non-diffuse gliomas, non-midline gliomas, or tumors that are not gliomas, their clinical importance is much lower. In fact, the detection of H3K27 alterations in pilocytic glioma, ganglioglioma, and ependymoma does not have the same strong role in characterizing the tumor's malignancy and biological and clinical behavior as it does in DMGs. It is then very hard to determine to what extent the malignant potential of DMGs is attributable to their intrinsic location (and thus their inoperability and scarce druggability), to their diffuse nature (and so their propensity to infiltrate the normal surrounding brain tissue), or to the biological aggressiveness given by the H3K27 mutation (23).

The prognosis of H3K27 DMG is not identically and homogenously dismal across ages; very young children with DIPG/DMG fare better, and in adult patients, the role of H3K27M as a prognostic indicator is far from being clear and definite. Thus, as pediatric oncologists, when we talk to parents of a child with DMG, perhaps we should modify our communication about prognosis and life expectancy in very young children (because they can have longer than usual survival) or late adolescents (because their tumors may occasionally resemble more those of adults rather than children). Furthermore, we should be aware that in such cases, rare but precious examples of unexpectedly long survival can happen, so we should focus our clinical skills on eagerly trying to transform our patient into one of these fortunate outliers.

Another factor that seems to influence survival is tumor location: while typical intrinsic pontine DMGs are inoperable and in most cases rapidly progress after radiotherapy, other tumors are amenable to partial surgery (partially exophytic tumors, thalamic, cerebellar, and spinal tumors, for example). Although the impact of partial resection in children with H3K27-altered tumors is not as clear as in adults with malignant glial tumors, surgery might sensibly improve prognosis, especially in patients with thalamic and spinal DMGs.

The presence of rare long-term survivors among patients with H3K27-altered DMG (and formerly with DIPG or other midline tumors) has been reported in the literature, both in single case reports and in population studies. In a study of over a thousand DIPG cases by Hoffmann et al., approximately 10% of the patients survived more than two years after diagnosis. Such long-surviving patients more commonly presented at ages <3 or >10 years; they had longer symptom duration and less commonly presented with cranial nerve palsy, ring enhancement, necrosis, and extra-pontine extension; the HIST1H3B mutation also seemed more likely to be found in long-term survivors (45). Indeed, such observations, although representing a major and valuable contribution to the research field of DMG, must be cautiously interpreted, as pointed out in a specific commentary (73): the possibility of enrollment bias or variations in the standard of care between countries and institutions might influence the interpretation of results.

Even more interesting is the presence, in certain reports, of very long-term survivors of DIPG and DMG (e.g., patients

surviving more than five years from diagnosis). Such patients, in the current setting, are to be considered real "outliers," and are reported to account for around 2.5-6.9% of all patients with DIPG (45, 74, 75). Although H3K27 alterations do define this category of DMGs in children, the role of the pathologist must not be minimized as being the person who just writes down a diagnosis that is disclosed by the H3 analysis. Assessment of H3 mutation status alone, especially by the use of IHC alone, is not sufficient to distinguish the more frequent "typical" H3K27M-mutant DMG from the EZHIP or EGFR-altered DMG. Moreover, many additional molecular alterations (PDGFR, ATRX, P53, ACVR1, BRAF, and many others) may be found in DMG alongside H3 mutations that can refine the diagnosis and sometimes change the prognosis. Furthermore, defining the subcategory of histone mutation (H3.1 vs. H3.3) can be of clinical interest since it has been demonstrated to have an influence on survival.

The global biological picture has been made even more complex by the recent demonstration that DMGs are characterized not only by a wide range of inter-tumoral genetic variability but also by a relevant intra-tumoral genomic heterogeneity, with the coexistence of genetically distinct subclones in each tumor, as seen by whole genome and exome sequencing (76, 77). Other techniques, such as single-cell mass cytometry, yielded similar results, revealing significant inter- and intra-tumoral heterogeneity at the protein level (78). H3K27-altered DMG is probably made of multiple, genotypically, and phenotypically distinct subpopulations of tumor cells: this may result in resistance to therapy and exacerbate clinical malignancy. Differences have also been detected across multiple tumor samples collected throughout the brain at autopsy, revealing branching evolutionary trajectories within the same tumor. In rare cases, researchers found distinct low-grade and high-grade components in the same tumor specimen, with key oncogenic mutations in one region but not the other (76).

Indeed, the complete molecular characterization of each DMG case would be of paramount importance for the individual patient and future patients. Although at present this might have limited clinical relevance, a complete molecular characterization of each case of DMG may be critically important in the future, especially if relevant and durable responses to targeted therapeutic approaches are evidenced (33). Such molecular alterations become even more important when coupled with non-uniform histological features: as an example, they can be ancillary in guiding decisions if a tumor shows morphological aspects of diffuse glioma together with features of a glioneuronal tumor. Cases presenting H3K27 alterations that are either "not-so-diffuse", "not-so-midline" or "not-so-glioma" tumors should be thoroughly examined from a molecular point of view (DNA and RNA NGS, and methylation if possible) in search of additional alterations (e.g., BRAF V600 mutations); furthermore, in such cases, a centralized pathological revision or second opinion is always needed before attributing a definitive category to the tumor.

5.2 H3K27-altered DMG currently has no effective treatment

Despite all past and present efforts to find a cure for DIPG and malignant DMG, the vast majority of patients with H3K27-altered DMG will not survive the disease.

This is why conventional treatment for such tumors (currently encompassing radiotherapy and variable subsequent schemes of low-dose medical therapy with little or no impact on survival) is sometimes thought of as a front-line palliative approach. In such a setting, one of the most important aspects of the care of children and adolescents with DMG is avoiding unnecessary toxic treatments, useless hospitalization, and invasive diagnostic procedures (17).

The possibility of using MRI as a widely accepted gold standard for diagnosis in DIPG, in conjunction with the risk of performing a biopsy on intrinsic pontine lesions, has sparked a long and unresolved debate over the need for performing a biopsy in DIPGs versus treating patients on the basis of a radiological diagnosis alone over the last decades. A tumor biopsy is not required for DIPG diagnosis and is only unavoidable in cases of atypical radiological features, although sometimes it is a mandatory requirement for inclusion in a clinical trial (48, 79–81).

Numerous clinical trials have been testing new therapeutic approaches with DMG-targeted drugs. Candidate drugs and compounds include monoclonal antibodies, small molecules, tyrosine kinase inhibitors, angiogenesis inhibitors, and more. Some recent non-intensive approaches suggest slight advantages over the standard of care (frontline radiotherapy alone): there are some encouraging reports on the use of nimotuzumab, a humanized anti-EGFR antibody, with similar outcomes to more intensive chemotherapy regimens, with a lower burden of toxicity and no need for prolonged hospitalization; its use is described in particular in combination with vinorelbine and radiation and re-irradiation by the Milan group (82). The use of personalized, biopsy-based targeted therapies has been investigated, and in some reports, it seemed to produce a slight improvement in prognosis, and low toxicity (81). The use of adoptive T cell therapy is also a promising approach that has been recently tested preclinically and clinically in the context of DMG: a few clinical trials are currently recruiting patients for the use of CAR-T cells in DIPG, and the very first results are encouraging (83–85). Moreover, the use of intratumoral infusion of oncolytic viruses followed by radiotherapy has also been reported (86). The description of the rationale and results of such new therapeutic approaches is beyond the scope of this

review. That said, so far, none of the recent clinical trials with published results has demonstrated a relevant impact on improving survival (8), although many of them are still ongoing and many others are not active yet. Much hope and scientific effort are being invested in those modern approaches. Such strategies find their theoretical foundation in the identification of one or more molecular abnormalities in the tumor tissue. As such, these approaches support and motivate a biopsy assessment of the tumor to discover potential therapeutic targets. Ethical concerns about the decision to biopsy all patients are still legitimate, especially in centers where a biopsy is not routinely performed outside the setting of a clinical trial. Nonetheless, the role of biopsy has been gradually reconsidered in recent years, as it is the only way for biology-driven translational research to lead us to an understanding of the mechanisms underlying DMGs and the possible development of more promising clinical trial studies and targeted therapies. Thanks to the development of modern surgical techniques, the procedural risk of biopsies in DIPG has lowered over time, and many report biopsy as a relatively safe technique in experienced centers (60, 79, 87, 88).

In the general setting of pediatric DMGs, independent of their localization, the possibility to obtain extensive information on each and every tumor is of paramount importance in collecting the maximum amount of information about this aggressive disease. The molecular characterization of DMGs may reveal itself to be important not only for the broader aim of determining future treatment strategies but also for the treatment of individual patients. In fact, several studies reported that some patients have benefited from molecularly driven personalized therapies based on the extensive genomic analysis of their tumors (60, 68, 81, 87).

6 Conclusion

The WHO classification of central nervous system tumors has rapidly evolved over the last few years. In 2007, diffuse intrinsic gliomas of childhood were not even cited as a separate entity (69); by 2021, the classification had completely changed, separating pediatric HGG and LGG from other gliomas of adulthood and defining the tumor type as "diffuse midline glioma, H3K27-altered" (3). It is therefore plausible to predict that within a few years there may again be some changes in the classification, which would redefine the way we categorize DMG and malignant gliomas in children. Most likely, new discoveries and research will also provide additional information on the significance of histone mutations in gliomas, perhaps to the point of changing their biological significance and prognostic role.

When interpreting nosological classifications of diseases, it's important to be aware that these are based on current knowledge and scientific discoveries, which are constantly changing and being updated. In clinical practice, as pediatric pathologists, and oncologists, we must therefore act on a case-by-case basis, adapting the indications that the WHO 2021 CNS classification provides and taking into account the clinical and demographic characteristics of every single patient, together with the radiological, histological, biological, and molecular characteristics of their tumor.

Especially when we take care of patients with diseases that are universally characterized by poor survival and few curative therapies, such as H3K27-altered DMG, we must always be alert to identify exceptions, and we must almost spasmodically search for "outliers" among our patients to tailor a specific treatment to them and offer an otherwise minimal chance of cure.

It is our duty to capitalize on the strengths and innovative, modern information offered by the 2021 WHO CNS classification in the management of pediatric patients with H3K27-altered DMG. Nonetheless, we must give each DMG case its own unique and precise molecular characterization. The ultimate goal is to treat all patients with a personalized therapy tailored to the specific characteristics of their tumor, if possible within a clinical trial of molecular medicine, in order to obtain innovative hope for a cure despite the presence of the H3K27 alteration that characterizes the poor prognosis of pediatric DMGs.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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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Unlocking the power of precision medicine for pediatric low-grade gliomas: molecular characterization for targeted therapies with enhanced safety and efficacy

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In the past decade significant advancements have been made in the discovery of targetable lesions in pediatric low-grade gliomas (pLGGs). These tumors account for 30-50% of all pediatric brain tumors with generally a favorable prognosis. The latest 2021 WHO classification of pLGGs places a strong emphasis on molecular characterization for significant implications on prognosis, diagnosis, management, and the potential target treatment. With the technological advances and new applications in molecular diagnostics, the molecular characterization of pLGGs has revealed that tumors that appear similar under a microscope can have different genetic and molecular characteristics. Therefore, the new classification system divides pLGGs into several distinct subtypes based on these characteristics, enabling a more accurate strategy for diagnosis and personalized therapy based on the specific genetic and molecular abnormalities present in each tumor. This approach holds great promise for improving outcomes for patients with pLGGs, highlighting the importance of the recent breakthroughs in the discovery of targetable lesions.

KEYWORDS

pediatric low-grade glioma, brain tumors, neuro-oncology, molecular diagnostic, clinical trials, targeted therapies, risk stratification, glioma

1 Introduction

Pediatric low-grade gliomas (pLGGs) are one of the more frequent pediatric brain tumors accounting for about 30-50% of central nervous system (CNS) tumors of pediatric patients. They carry a favorable prognosis with an overall survival (OS) at 10 years greater than 90%. In a minority of cases an aggressive behaviour is described (1, 2).

To date, complete resection is the most favourable outcome measurement of the patients, but it is not easy to conduct for deep or infiltrative lesions (3), and for progressive residual disease adjuvant chemotherapy or radiation were historically performed (4–12). However, we did not forget that the side effects are far from negligible (5, 13–16). Pediatric LGGs comprise of a heterogeneous group of tumors, and recently molecular studies led to a better clarification and classification of pLGGs, and which paved the way for promising new therapeutic strategies.

Many types of tumors are included under the umbrella of pLGGs. Historically, these types of neoplasms have been classified on the basis of histology, but today we know that the same histologies can underlie different entities and histological classification alone is no longer useful (17). The molecular characterization advancements have revealed that appear similar under a microscope can have different genetic and molecular characteristics, so the new classification system divides pLGGs into several distinct subtypes based on these characteristics, rather than solely on their histological appearance. Better knowledge of the molecular characteristics, technological advances, and new applications in molecular diagnostics of pLGGs have helped overcome these challenges (18).

The updated 2021 World Health Organization (WHO) Classification of Tumors of the CNS has reflected the focus on the integration of histopathological and molecular characteristics to facilitate a more accurate diagnosis (19). In the new classification of pLGGs places a strong emphasis on the molecular characterization of these tumors for significant implications on the prognosis, diagnosis, management, and finally development of personalized treatment (19).

This classification describes three families of tumors that encompass pLGGs and glioneuronal tumors (GNTs) (Table 1), which are now defined by their driver molecular alterations rather than by histopathological features alone: “Glioneuronal and neuronal tumor”, “Circumscribed astrocytic gliomas” and “Pediatric type diffuse low-grade gliomas” (17, 19).

In this review, we described the major molecular alterations detected in pLGGs and the molecular target therapy available to date.

2 MAPK/ERK and PI3K/AKT/mTOR signaling pathway alterations in pediatric low-grade gliomas

2.1 MAPK/ERK and PI3K/AKT/mTOR signaling pathway in physiological conditions

In the Mitogen-Activated Protein Kinase/extracellular signal-regulated kinases (MAPK/ERK) signaling pathway (Figure 1), stimulation of receptor tyrosine kinases (RTKs) in physiological conditions causes MAPK activation. The activation of Ras enabled the activity of the serine/threonine-protein kinase B-raf, which homodimerizes or heterodimerizes by phosphorylating and triggering mitogen-activated protein kinase kinase (MEK1 and MEK2), which in turn phosphorylates and trigger ERK 1 and ERK2. Finally, the latter boost dedifferentiation, proliferation and cell survival by scalable transcriptional asset within the nucleus; consequently, downstream activation of ERK causes feedback inhibition of the upstream pathway (20–23).

The activation of the phosphatidylinositol 3-kinase/protein kinase B/mechanistic target of rapamycin (PI3K/AKT/mTOR) pathway (Figure 1) is mediated by transmembrane receptor tyrosine kinases of growth factors (24). The Phosphatidylinositol 3-kinase (PI3K) is triggered from the bond of oncogenes or growth factors (24). PI3K transfom phosphatidylinositol-4,5-phosphate (PIP2) to phosphatidylinositol-3,4,5-phosphate (PIP3) (24). The

TABLE 1 WHO 2021 classification for pLGG/low-grade GNTs (19).

Pediatric-type diffuse low-grade gliomas	Circumscribed astrocytic gliomas	Glioneuronal and neuronal tumors
1. Diffuse astrocytoma, MYB- or MYBL1-altered 2. Angiocentric glioma 3. Polymorphous low-grade neuroepithelial tumor of the young (PLNTY) 4. Diffuse low-grade glioma, MAPK pathway-altered	1. Pilocytic astrocytoma 2. Pleomorphic xanthoastrocytoma (PXA) 3. Subependymal giant cell astrocytoma (SEGA) 4. Choroid glioma	1. Ganglioglioma 2. Desmoplastic infantile ganglioglioma/desmoplastic infantile astrocytoma 3. Dysembryoplastic neuroepithelial tumor 4. Diffuse glioneuronal tumor with oligodendroglioma-like features and nuclear clusters 5. Rosette-forming glioneuronal tumor 6. Papillary glioneuronal tumor 7. Myxoid glioneuronal tumor 8. Diffuse leptomeningeal glioneuronal tumor (DLGNT) 9. Gangliocytoma 10. Multinodular and vacuolating neuronal tumor 11. Dysplastic cerebellar gangliocytoma (Lhermitte-Duclos disease) 12. Central neurocytoma 13. Extraventricular neurocytoma 14. Cerebellar liponeurocytoma

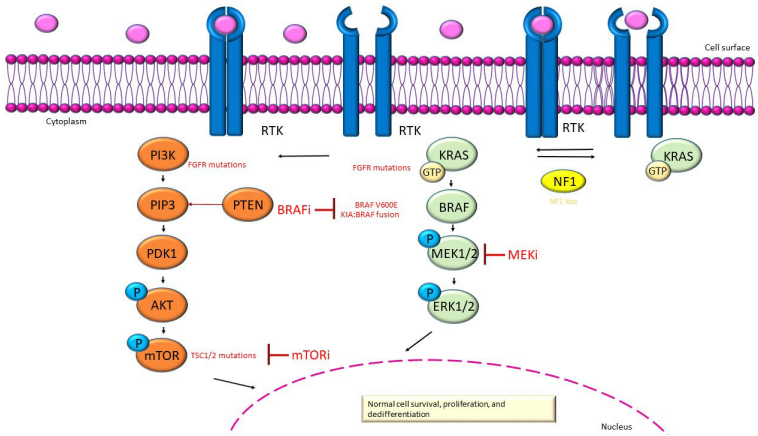

FIGURE 1
MAPK/ERK (green) and PI3K/AKT/mTOR (orange) signaling pathway. In red show the inhibitors and the alterations in gene that are causative to dysregulation of these pathways in pLGGs.

lipid Phosphatase and tensin homolog (PTEN) has the function of countering the build-up of PIP3 and enroll to the membrane protein kinase B (PKB or Akt) and phosphoinositide-dependent kinase 1 (PDK1), which are phosphorylated and triggered (24). The molecular complexes mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) have both a catalytic subunit mTOR which is negatively regulates by the heterodimer of tuberous sclerosis proteins TSC1 (hamartin) and TSC2 (tuberin) [a GTPase-activating complex (GAP) to Rheb (homolog of Ras enriched in the brain)], in contrast the activation of the PI3K pathway, AKT phosphoryl TSC2 and disable the TSC1/TSC2 complex (25–27). Mechanistic target of rapamycin (mTOR) can even be triggered by the MAPK pathway via RAS/MEK/ERK (28).The phosphorylation of TSC2 by ERK and ribosomal S6 kinase (RSK) can induce mTORC1 activation; instead, RSK can target the mTORC1 complex by directly promoting the kinase activity of the complex (28). Aberrant activation of mTOR may be related to various mutations that activate the mTOR pathway, such as alterations at mTOR negative regulators or mTOR pathway components (28). PI3K activation facilitates the activation of mTORC1 and mTORC2. Activation of mTORC1 downstream of PI3K and protein- kinase B (AKT) promotes cell survival, growth and proliferation. Moreover, mTORC2 increases cell proliferation and survival through regulation of protein kinases, including AKT, which provides significant motivation for further studies on therapeutic targeting of mTOR complexes in cancer, as mTOR plays an important role in tumor progression (29).

2.2 *BRAF* alterations and targeted therapy

Within pLGG a notorious troublemaker has been identified: the B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) gene. This gene encodes a protein from the RAF family that is responsible for regulating the MAPK/ERK pathway (30). Pediatric LGGs often harbor alterations in the BRAF gene, such as the p.V600E point mutation and the translocation between *BRAF* and *KIAA1549*. These alterations result in a hyperactive protein that wreaks havoc on the MAPK pathway, leading to uncontrolled cell division and tumorigenesis (31–41).

Most of sporadic pLGGs are characterize by *BRAF* mutations (2, 42). A three-class system was defined based on the result of *BRAF* mutations on the activity of the encoded protein. RAS-independent as monomers represent the class I mutations RAS-independent as dimers belong to class II mutations, and RAS-dependent with altered kinase activity are class III mutations (24).

Class I mutations, which include the mutation on 600 codon of *BRAF*, hyperactivate kinases through promotion of MEK/ERK activation regardless of the protein dimerization (for example with Raf has low effect) and activation of RAS (24). In fact, inhibition of upstream ERK feedback has any impact on class I mutations because, although *BRAF* p.V600E dimerization stays Ras dependent and is blocked by upstream ERK response, but it can yet turn on the pathway like monomer (43, 44). A point mutation c.1799T>A causes the replacement of valine with glutamic acid at codon 600 (p.V600E) within the gene's activation region. The occurrence of *BRAF* p.V600E in non-pilocytic pLGGs varies significantly depending on the tumor's histology and location. Ganglioglioma (25-45%) and pleomorphic xanthoastrocytomas (40-80%) frequently exhibit the variant, while it is less commonly observed in pilocytic astrocytoma (PA) (5-10%) and GNTs (5%) (45–52). Combining histological and molecular data helps to achieve a more precise diagnosis. For example, identifying *BRAF* p.V600E, along with the detection of a mildly and minimally atypical glial proliferation without eosinophilic granular bodies and Rosenthal fibers (RFs), enables categorizing the tumor as a "low-grade diffuse glioma" (19). In a retrospective study, 17% of children with LGGs carried the BRAF p.V600E variant and presented a 10-year progression-free survival (PFS) rate of about 27% versus a 60% rate for those without the same variant (53). This trend is confirmed by several studies (53–57). However, almost one-third of patients who experienced complete resection relapsed, indicating that *BRAF* p.V600E is the most interfering phenotype than other mutation known in patients with pLGGs (53). A

progression-free survival of 5-year are reported in a study on children with low diencephalic astrocytomas carried *BRAF* p.V600E (22%) versus the children without BRAF mutations (52%) (58). The *BRAF* p.V600E variants were more frequently detected in pLGGs that transform into high-grade gliomas (59). Several studies have demonstrated that 25% of patients with pLGGs exhibit BRAF p.V600E in conjunction with deletions of *CDKN2A*, which probably operates as a second hit, altering the regulation of cell cycle (38, 45, 53, 60–62). Tumors with *BRAF* p.V600E and a *CDKN2A* deletion represent a separate subtype of pLGGs which are inclined to change into HGG (59). Reports show that both these mutations are related with oncogene-induced senescence escape and poorer OS and PFS (38, 45, 62). Therefore, pLGGs with *CDKN2A* deletions, particularly those with p.V600E or possible high-grade histological characteristics, should be considered high-risk tumors requiring close clinical follow-up (63). Finally, some studies have reported rare cases of *BRAF* missense variants at the p.V600 residue, in which valine is replaced with other amino acids such as lysine (p.V600K), aspartic acid (p.V600D), or arginine (p.V600R). Desmoplastic infant astrocytomas/gliomas exhibit the p.V600K variant, while the BRAF p.V504_R506dup variant was reported in cases with PA. Supratentorial lesions are more frequently associated with *BRAF* p.V600E, while cerebellar lesions more commonly present *KIAA1549-BRAF* (47, 51, 64).

Class II mutations involve *BRAF-KIAA1549* fusion and other gene fusions. They trigger both intermediate and high kinase activity, requiring dimerization of the protein to activate the MEK/ERK pathway (24). The *KIAA1549-BRAF* fusion is a great slice of gene fusions involving BRAF in pLGGs, accounting for a whopping 30-40% of cases (65). The *KIAA1549* gene belongs to the mysterious UPF0606 family and we are still trying to understand what it does (66). *KIAA1549-BRAF* fusion is a major player in a variety of CNS tumors. It is particularly prevalent in infratentorial and midline PAs, although it shows up less often in supratentorial tumors (34, 38, 67–72). Interestingly, early studies have proven that fusions that involved these genes are correlated to tandem duplication that creates a brand-new oncogenic fusion. This rearrangement messes with domain at the N-terminal regulatory region of the BRAF protein, which in turn causes RAS/MAPK pathway altered regulation (35, 36, 73). But despite these complicated genetics, one thing is clear: the presence of the *KIAA1549-BRAF* fusion is associated with better OS and PFS in pLGGs that cannot be fully removed and do not tend to progress too quickly (53, 69, 70, 72). Unfortunately, in cases where the tumor is located in a difficult-to-reach part of the brain, progression is more likely (53).

Other alterations in addition to the *BRAF-KIAA1549* fusion, such as *CDKN2A* deletions, and tumor location may alter the outcome of the patient (45, 74).

Other rearrangements that activate the RAS/MAPK pathway and involving *BRAF* are the *MKRN1* (Makorin Ring Finger Protein 1), *SRGAP* (SLIT-ROBO Rho GTPase Activating Protein 2), *GIT2* (GIT ArfGAP 2), *FAM131B* (Family with Sequence Similarity 131 Member B), *RNF130* (Ring Finger Protein 130), *CLCN6* (Chloride Voltage-Gated Channel 6), *GNAI1* (G Protein Subunit Alpha I1), and *FXR1* (FMR1 Autosomal Homolog 1) mergers involving deletion of BRAF N-regulatory domain (34, 74–76). These non-canonical fusions in particular manifest in older children and adolescents, frequently in brainstem lesions and hemispheres, and are also observed in a series of rare histological profiles (67, 75–77).

Class III mutations are found to be linked to poor or no kinase activity and need both the activation of upstream RAS and dimerization with CRAF to further induce induction of MER/ERK pathway activation (24). In literature are reported a few cases of *BRAF* p.D594G and p.G466V mutations (78).

Finally, *BRAF* p.V600E mutations and *BRAF* fusions enable molecular characterization of nearly 2/3 of pLGGs (2).

BRAF inhibitors (BRAFi), including vemurafenib, dabrafenib, and encorafenib, are drugs that selectively bind to mutated B-Raf proteins and block the activation of MEK by inhibiting the MAPK/ERK cascade signaling (79). Clinical studies have demonstrated that vemurafenib and dabrafenib first-generation BRAFi are highlyeffective in treating children with LGGs, with numerous case reports showing complete responses (52, 80–89). However, these inhibitors have been found to activate the signaling pathway of RAS/MAPK when used in tumors with the fusions that involved KIAA1549 and BRAF or BRAF *wild-type* (wt) (90, 91). To address this issue, "paradox-breaker" secondo generation agents have been developed that do not activate the RAS/MAPK pathway (92). Ongoing clinical trials are investigating the use of the dual combination of BRAFi and MEK inhibitors (MEKi) to treat BRAF p.V600 mutation-positive gliomas (Table 2) (93–98). There are also emerging new class II BRAF inhibitors, such as TAK-580, that look promising in treating LGGs (116). Overall, BRAF inhibitors offer a remarkable therapeutic option for pLGGs, particularly in pediatric patients where traditional treatment methods may have long-term effects on brain development.

In addition, MEKi have emerged as a potential treatment strategy for pLGG patients and ongoing clinical trials are examining the use of several drugs such as selumetinib in treating of young patients with recurrent or refractory LGGs (characterized by the presence or absence of *BRAF* V600E mutations or *BRAF-KIAA1549* fusion); trametinib for pediatric neuro-oncology patients with refractory tumor and activation of the MAPK/ERK pathway causative by a KIAA 1549-BRAF fusion; and a study of MEK162 for children with LGGs characterized by a *BRAF* truncated fusion (KIAA1549 and similar translocations) (Table 2) (99–102, 105, 108–110).

2.3 *FGFR1* alterations

The subunits of the RTKs, which are crucial in transmitting the MAPK signal, are encoded by genes pertaining to the Fibroblast Growth Factor Receptor (*FGFR*) family (*FGFR1-4*) (117). Fibroblast Growth Factor Receptor 1 (*FGFR1*) alterations are common in pLGGs (40, 64, 76), with p.N546K and p.K656E being the most frequent mutations observed in 5-10% of patients, while *FGFR1* TKD duplication is detected in 2-23% of tumors. *FGFR1* mutations have been identified in various pLGGs, including PA with an unfavorable prognosis, although none of these changes is histologically specific (64, 76, 118, 119). Fusion genes involving *FGFR*'s N-terminal domain and other genes such as *TACC1*

TABLE 2 List of clinical trials for pLGG using targeted therapy.

Drug	Trial ID	Phase	Target	Information	Reference
BRAF Inhibitors	NCT02684058	Phase II	Children and Adolescent Patients With BRAF V600 Mutation Positive Low-Grade Glioma (LGG) or Relapsed or Refractory High-Grade Glioma (HGG)	Pediatric Study With Dabrafenib in Combination With Trametinib in Patients with HGG and LGG	(93)
	NCT01748149	Early Phase I	Children with recurrent or refractory gliomas containing the BRAFV600E or BRAF Ins T mutation	Vemurafenib in Children With Recurrent/ Refractory Gliomas	(94)
	NCT03429803	Phase I	/	This research study on the drug Tovorafenib/ DAY101 (formerly TAK-580, MLN2480) as a possible treatment a low-grade glioma that has not responded to other treatments	(95)
	NCT02428712	Phase II	Adolescent patients with advanced BRAF- mutated tumors	A Study of FORE8394 as a Single Agent in Patients With Advanced Unresectable Solid Tumors	(96)
	NCT01677741	Phase I/IIa	Children and Adolescent Subjects With Advanced BRAF V600-Mutation Positive Solid Tumors	A Study to Determine Safety, Tolerability and Pharmacokinetics of Oral Dabrafenib In Children and Adolescent Subjects	(97)
	NCT02034110	Phase II	BRAFV600E mutation	Efficacy and Safety of the Combination Therapy of Dabrafenib and Trametinib in Subjects With BRAF V600E- Mutated Rare Cancers	(98)
MEK Inhibitors	NCT01089101	Phase II	Presence or absence of BRAF V600E mutations or BRAF KIAA1549 fusion	Selumetinib in Treating Young Patients With Recurrent or Refractory Low-Grade Glioma	(99)
	NCT03363217	Phase II	NF1 LGG with KIAA 1549-BRAF fusion -Progressing-refractory glioma with activation of the MPAK/ERK pathway who do not meet criteria for other study groups	Trametinib for Pediatric Neuro-oncology Patients With Refractory Tumor and Activation of the MAPK/ERK Pathway	(100)
	NCT02639546	Phase I/II	/	Safety and Pharmacokinetics of Cobimetinib in Pediatric and Young Adult Participants With Previously Treated Solid Tumors (iMATRIXcobi)	(101)
	NCT02285439	Phase I/II	Children with LGG characterized by a BRAF truncated fusion (KIAA1549 and similar translocations) Children with NF1 and LGG Children with tumors involving the Ras/Raf pathway not included in strata 1 or 2	Study of MEK162 for Children With Low-Grade Gliomas	(102)
	NCT03871257	Phase III	Patients must have neurofibromatosis type 1 (NF1) based on clinical criteria and/or germline genetic testing * Patients must be newly diagnosed or have previously diagnosed NF-1 associated LGG that has not been treated with any modality other than surgery	A Study of the Drugs Selumetinib Versus Carboplatin/Vincristine in Patients With Neurofibromatosis and Low-Grade Glioma	(103)
	NCT04166409	Phase III	Newly Diagnosed or Previously Untreated Low-Grade Glioma (LGG) Not Associated With BRAFV600E Mutations or Systemic Neurofibromatosis Type 1 (NF1)	A Study of the Drugs Selumetinib vs. Carboplatin and Vincristine in Patients With Low-Grade Glioma	(104)
	NCT04576117	Phase III	Patients with BRAF rearranged LGG and patients with non-BRAF rearranged LGG	A Study to Compare Treatment With the Drug Selumetinib Alone Versus Selumetinib and Vinblastine in Patients With Recurrent or Progressive Low-Grade Glioma	(105)
	NCT04201457	Phase I/II	o LGG with BRAF V600E/D/K mutation; o LGG with BRAF duplication or fusion with any partner or LGG with NF1.	A Trial of Dabrafenib, Trametinib and Hydroxychloroquine for Patients With Recurrent LGG or HGG With a BRAF Aberration	(106)
	NCT02124772	Phase I/II	Children and Adolescents Subjects With Cancer or Plexiform Neurofibromas and Trametinib in Combination With Dabrafenib in Children and Adolescents With Cancers Harboring V600 Mutations	Study to Investigate Safety, Pharmacokinetic (PK), Pharmacodynamic (PD) and Clinical Activity of Trametinib in Subjects With Cancer or Plexiform Neurofibromas and Trametinib in Combination With Dabrafenib in Subjects With Cancers Harboring V600 Mutations	(107)
	NCT04485559	Phase I			(108)

(Continued)

TABLE 2 Continued

Drug	Trial ID	Phase	Target	Information	Reference
			Participants with LGG who have had surgery alone are not eligible. Participants with neurofibromatosis type 1 (NF-1) are eligible but must have available tissue per study requirements neurofibromatosis (NF) status will be collected	Trametinib and Everolimus for Treatment of Pediatric and Young Adult Patients With Recurrent Gliomas (PNOC021)	
	NCT05180825	Phase II	Patients with a determination of a negative BRAFv600 mutation by immunohistochemistry and/or molecular methods and patients without NF1	Pediatric Low Grade Glioma – MEK inhibitor TRIal vs Chemotherapy (PLGG - MEKTRIC)	(109)
	NCT03975829	Phase IV	Patients who received monotherapy of either of dabrafenib or trametinib Patients who received combination of dabrafenib and trametinib	Pediatric Long-Term Follow-up and Rollover Study	(110)
mTOR Inhibitors	NCT01158651	Phase II	Children with NF1 progressive LGG	Everolimus for Children With NF1 Chemotherapy-Refractory Radiographic Progressive Low Grade Gliomas (NFC-RAD001)	(111)
	NCT00782626	Phase II	Exclusion criteria: presence of NF1 by clinical examination or by genetic testing	Everolimus (RAD001) for Children With Chemotherapy-Refractory Progressive or Recurrent Low-Grade Gliomas	(112)
	NCT01734512	Phase II	/	PNOC 001: Phase II Study of Everolimus for Recurrent or Progressive Low-grade Gliomas in Children	(113)
NTRK Inhibitors	NCT02650401	Phase II	Primary brain tumors with NTRK1/2/3 or ROS1 gene fusions; gene fusions are defined as those predicted to translate into a fusion protein with a functional TRKA/B/C or ROS1 kinase domain, without a concomitant second oncodriver as determined by a nucleic acid-based diagnostic testing method Extracranial solid tumors (including NB) with NTRK1/2/3 or ROS1 gene fusions; gene fusions are defined as those predicted to translate into a fusion protein with a functional TRKA/B/C or ROS1 kinase domain, without a concomitant second oncodriver as determined by a nucleic acid-based diagnostic testing method	A Phase 1/2, Open-Label, Dose-Escalation And Expansion Study Of Entrectinib (Rxdx-101) In Pediatrics With Locally Advanced Or Metastatic Solid Or Primary CNS Tumors And/Or Who Have No Satisfactory Treatment Options	(114)
IDH1 Inhibitors	NCT04164901	Phase III	Patients (>/= 12 years) Residual or Recurrent Grade 2 Glioma with confirmed IDH1 (IDH1 R132H/C/G/S/L mutation variants tested) or IDH2 (IDH2 R172K/M/W/S/G mutation variants tested) gene mutation status disease	Study of Vorasidenib (AG-881) in Participants With Residual or Recurrent Grade 2 Glioma With an IDH1 or IDH2 Mutation (INDIGO)	(115)

(Transforming Acidic Coiled-Coil Containing Protein 1), *KIAA1598* (Shootin 1), *TACC2* (Transforming Acidic Coiled-Coil Containing Protein 2), *TACC3* (Transforming Acidic Coiled-Coil Containing Protein 3) and *KIAA1598* (Shootin 1) characterize pLGGs (120).

All these changes lead to FGFR1 self-phosphorylation, are correlated to the up alteration of the RAS/MAPK pathway and PI3K/AKT/mTOR pathway (76). *FGFR1* alterations' clinical manifestations are not yet fully understood and can be the product of more alterations in the genes that are mentioned earlier (64, 76, 119).

2.4 Other alterations in pLGGs and targeted therapy

The neurotrophic receptor of tyrosine kinase (NTRK) family and the *ALK* gene have significant roles in the development and fuctions of the CNS (81–88). In pLGGs, *NTRK* gene fusions including *NTRK1/2/3*, *SLMAP-NTRK2, TPM3-NTRK1, RBPMS-NTRK3* and *ETV6-NTRK3* are rare (64, 76, 121, 122). *ALK* alterations are also uncommon in pLGGs, but the fusions that involved *CCDC88A* and *PPP1CB* with *AKT* being the more prevalent and resulting from chromothripsis (123–125). These changes cause tumorigenesis by modifying the RAS/MAPK and PI3K/AKT/mTOR pathways via abnormal NTRK kinase domain dimerization or ectopic expression of the product fusion involved (125–129). Alterations in *NTRK* gene are rare in pLGGs, while they are common in adult cancers and this has enabled the development and testing of drugs already approved by the FDA. Entrectinib was approved for treatment of solid tumors when patients carrier a *NTRK* gene fusion and larotrectinib for both population of patients with solid tumors who carrier a fusion that involved TRK without a mutation known as related to acquired resistance, who are metastatic or in whom surgical excision may cause significant morbidity and who have no suitable treatment options or progressed after therapy (130–134).

In pediatric gliomas, in particular, both entrectinib and larotrectinib showed potent antitumor effects (135–137). These findings resulted to a phase I/II study presently ongoing in children to assess entrectinib in primary tumors of CNS (114).

To date there is a lack of data on the role of larotrectinib in primary CNS tumors, as few case reports have been published in particular on pediatric high-grade gliomas (pHGGs) and clinical trials have not yet been completed (138–143).

Finally, another rarely reported alteration in pLGGs, involves IDH1 whose role in these types of pediatric cancers is unclear to date (64, 144). A study of patients with LGGs and mutation in IDH1 found excellent short-term survival, but with a 5-year PFS of less than 43% and mortality after 10 years (145). To date, Vorasidenib (Ag-881), a new inhibitor against IDH1 and IDH2 mutation with high brain penetration, show a good results in clinical trial on adult patients with LGG (above/equal to 18 years of age) and IDH1 mutations (146–150). Consequently, Ag-881 was tested in a phase III clinical trial (INDIGO) in patients up/equal to 12 years of age and with residual or recurrent grade 2 Glioma who carried an IDH1 R132H/C/G/S/L or IDH2 mutation (115).

Another IDH1 inhibitor is FT-2102, used specifically in the treatment of myelodysplastic syndromes and AML, was tested in the adult population with solid tumors and gliomas in which mutation in IDH1 was found (151, 152). Other studies are on going in the adult population (153).

2.5 Cancer predisposition syndrome associated with pLGG: from alterations involving the RAS/MAPK and mTOR signaling pathway to targeted therapy

Alterations involving the RAS/MAPK pathway in pLGG pathogenesis have been studied in patients with Neurofibromatosis type 1 (NF1), of which 10-15% develop low-grade gliomas (154–156).

About 20% of patients with NF1 develop pLGGs (157): they often present with optically induced tumors that are not biopsied, NF1-pLGGs are asymptomatic and indolent, do not require any treatment, and in some cases regress without treatment; however, in case of clinical deterioration (more frequently vision loss), the first line of therapy used is chemotherapy (158–161). In addition, some studies have repositioned NF1-pLGGs patients with other additional genetic alterations of the RAS/MAPK pathway (162). Seventy-five percent of NF1-pLGGs carried a genetic mutation in one or more genes that are involved in biological process (162). Finally, in pLGGs involving *NF1*-associated alterations, *BRAF* variants are rare (68, 162).

Target therapies have also been attempted and described in patients with NF1. MEKi have emerged as a potential treatment strategy for pLGG patients who are unresponsive to BRAFi, such as those with *KIAA1549-BRAF* or NF1-pLGG (163). Ongoing clinical trials are exploring efficacy of treatment with selumetinib, trametinib, cobimetinib, and binimetinib in young patients with refractory pLGGs (Table 2) (99–102). Phase I/II trials on selumetinib have demonstrated its stability or reduction of tumor size in pediatric patients with NF1-associated and sporadic form of pLGGs, with similar results observed in a study of children with progressive/recurrent PA (Table 2) (164, 165). Phase III clinical trial are currently exploring the efficacy of selumetinib as a frontline therapy for both NF1-associated and NF1-non-associated pLGGs (Table 2) (103, 104).Trametinib and binimetinib have also shown promise in small studies, with trametinib appearing effective as a single drug or in compound with dabrafenib (107, 166–171). Broader studies are required to assess the tolerability of MEKi in pLGG patients (159). Overall, MEKi showed a promising therapeutic alternative for pLGGs, particularly for children with NF1-associated tumors without *BRAF* gene alterations (172).

of the vaste majority of children with tuberous sclerosis have a germline pathogenetic variant in tuberous sclerosis genes (*TSC1* or *TSC2*), that increase the risk of developing subependymal giant cell astrocytomas,subependymal nodules and cortical tubers, as some pathogenetic variants in these genes lead to mTOR pathway activation (173). Subependymal giant cell astrocytomas are led by mTOR activation; mTORi are active drugs that may induce the regression of the tumor in children affected by these tumors (173). Finally, germline mutations in genes (more than 10) involved in the RAS-MAPK pathway are causative of Noonan syndrome (NS), an autosomal dominant congenital condition (174). Noonan Syndrome is correlated to develop a brain tumors (174). Our group described 13-year-old patient with NS who developed a cerebellar PA, an optic pathway glioma (OPG) and a left temporal lobe glioneuronal neoplasm. A pathogenetic variant in the *PTPN11* gene was found and the molecular characterization of the GNT revealed elevated levels of phosphorylated mTOR (pMTOR) (175). Tyrosine phosphatase adaptor protein is encoded by *PTPN11* gene and it is involved, as reported before, in the RAS/MAPK pathway (78). Additionally, PTPN11 overexpression alone does not significantly activate the RAS/MAPK pathway, and further alterations like mutations in the FGFR1 gene, which activate the PI3K/AKT/mTOR pathway, are required (154).

mTOR was found to be excessively activated in pLGGs associated with syndromic conditions like TS and NF1 and this prompted to the support for the use of mTORi such as everolimus in clinical treatment alternative strategy (161, 176–181). Studies have highlighted that inhibiting the mTOR pathway is a promising therapeutic strategy for pLGG, and experimental evidence is emerging that suggests mTOR pathway activation may be a feature of most pLGGs (111, 173, 182–185). Everolimus has been successful in treating subependymal giant cell astrocytoma, a subtype of pLGG, and has demonstrated seizure control and tumor volume reduction in TS patients with SEGAs (173, 183, 186–188).

Our group suggested using everolimus for patients with RASopathies and brain tumors that have overactive mTOR signaling, and a phase II study is ongoing for recurrent or progressive pLGGs children. Everolimus has also been shown to provide a significant therapeutic alternative to immediate surgery in TSC patients, allowing for the postponement of a neurosurgical resection (Table 2) (112, 113, 175, 189). Moreover, we reported the first use of everolimus in children with pLGGs who were chemo- and radiotherapy-naïve (190). The results showed a lack of progression with a manageable toxicity profile, providing preliminary support for everolimus as a therapy for pLGG (190).

Overall, more studies are needed to develop innovative therapies for pLGG patients based on oncological mechanisms related to tumor development.

3 Discussion

Pediatric LGGs represent 30-50% of CNS childhood tumors; their prognosis greatly differed between tumor clusters and is dictated by a variety of factors, which include age at diagnosis, localization, and extension of resection surgery. pLGG represent a chronic disease and despite the treatments available to date, associated long-term morbidity remain of paramount importance.

Surgery is currently the standard of care, and children who undergo gross tumor resection (GTR) often do not demand additional action other than regularly follow-up. In a cohort of 518 patients, the 5-year PFS rate for children who have undergone GTR was high (94%) with an OS rate of 99%; any degree of residual tumor predicted a worse PFS, with up to 44% of patients with limited residual disease progressing within 5 years (3). However, a cluster of patients who are not susceptible to GTR subset exists, primarily because of tumor location. Although the low-grade biological malignancy of these tumors, products patients with both unresectable and clinically progressive disease masses receive either chemotherapy or radiotherapy, experiencing the toxicities associated with these regimens in the short and long term. The principal advances in treatment of traditional chemotherapy for LGG include carboplatin and vincristine, TPCV (thioguanine, procarbazine, lomustine, and vincristine), and weekly vinblastine monotherapy (191, 192). These conventional chemotherapeutic approaches used in pLGG patients are associated with side effects such as myelosuppression, alopecia, and less frequently ototoxicity (carboplatin) and decreased fertility potential (procarbazine) (193, 194). In addition, bevacizumab is another promising approach as it has shown improvements in the treatment of OPGs (195). Radiotherapy, a historical standard of care and time-tested efficacious therapy, has long been abandoned as a primary treatment for pLGGs. Radiation-induced late effects can be particularly devastating and include vasculopathy, stroke, endocrinopathy, cognitive impairment, and secondary malignancies (196). The decision to avoid radiotherapy in pLGG is that progression of disease may be of little consequence when there are multiple systemic treatment options available, OS remains excellent, and the risks of radiotherapy-associated late effects, particularly secondary malignancy, outweigh any potential benefits of improved progression-free survival (197). Young patients are mostly susceptible, in fact, the actual cut-off age for radiation therapy is moved beyond 12 years (15).

Recently, advancements in how we have gained an insight into pLGG biology have sparked a new promising treatment in the field of pediatric neuro-oncology. Multiple investigations repeatedly affirmed that the great majorities of the pLGG exhibit alterations in their drivers that are commonly found to lead to the dual activation of the MAPK pathway and to downstream mTOR pathway (76). In addition, novel technologies in NGS have allowed the discovery of additional new altered drivers, including *FGFR* (76). Following these findings, in the last few years, have been developed several drugs targeting the pLGG MAPK and mTOR pathway (79–102, 116, 138–143, 154–156).

Dabrafenib and vemurafenit were demonstrated to have an outstanding efficacy on pLGG mutant BRAF p.V600E patients in early phase clinical trials. Vemurafenib is a small competing drug that selectively recognizes the ATP-binding domain of the BRAF p.V600E mutant. It has been proven efficacious in the management of metastatic melanoma, a malignancy frequently mutated for BRAF. This drug's function has more recently been shown to be successful in BRAF p.V600E mutated pediatric malignant astrocytomas, while less data is currently available on its use in LGG patients, of which there are more studies on the combination of dabrafenib and trametinib (Table 3) (88, 106, 198–200, 205). The efficacy of the vemurafenib treatment in our small cohort of patients affected by pLGG is promising, with a rate of response of about 60% (88).

Furthermore, *BRAF*-fusions in pLGGs drive resistance/escape mechanisms to targeted inhibitors. For example, *KIAA1549-BRAF* has innate resistence to first-generation BRAFi vemurafenib as well as paradoxically triggered by PLX4720 treatment resulting in faster growth of tumor (90), while it shows a strong response to clinically available MEKi (e.g., trametinib) (206). Several studies showed that trametinib seems a appropriate choice in refractory as well as in progressive pLGG with *KIAA1549-BRAF* fusion and suggested that warrants further investigations in case of progression (Table 3) (167, 168, 171). The data of the study on progressive pLGGs lend weight to the class MEKi efficacy in pLGGs and the necessity of a randomized upfront trial of trametinib over current chemotherapy standard regimens (171). A phase 2 trial on patients with refractory/progressing LGG (NF1 patients and patients who carried KIA11549-BRAF fusion) and treated with trametinib, will investigate the molecular biological mechanisms that drive tumor development and progression, and the involvement of these mechanisms in resistance to therapy (100). Bouffet et al. showed the results of a phase II trial in which was compared the ORR in patients with pLGG who carried BRAF p.V600E mutation treated with both dabrafenib and trametinib (47%) or standard chemotherapy (CV) treatment (11%) (170).

In addition, in a cohort of both children and young adults treated for refractory tumors that have mutations or fusions resulting in activation of the MAPK pathway showed restricted selumetinib efficacy, suggesting that the mutation status of the pathway alone is sufficient to provide a predictor of the response to monotherapy with selumetinib for those tumors (207). In contrast, a phase II clinical trial on selumetinib among pediatric patients with relapsed and refractory LGG demonstrated impressive outcomes in sporadic OPG and hypothalamic LGG patients, with 24% partial response rate and 56% of patients showing long-term stability (104). In Table 3 are showed results of selumetinib on pLGG (165, 181, 201, 202).

With the discovery that many relapsed/refractory pLGGs have activation of mTOR pathway more treatment options may be possible for patients, including everolimus, a brain-penetrant drug already approved by the FDA for the treatment of SEGA in children (Table 3) (184, 190, 203, 204, 208–210). In our published experience, everolimus is a feasible treatment for p.V600E *wild-*

TABLE 3 List of results of targeted therapy in pLGG.

Study	N Patients	Additional information on patient population	Results	Reference
Children with LGGs and treated with vemurafenib	n=7	BRAF p.V600E	1 CR, 3 PR, 1 SD, 1 PD. In addition, in 1 patient, the follow-up is too short to establish the clinical response.	Del Bufalo et al., 2018 (88)
Children with recurrent or progressive brain tumors treated with vemurafenib	n=19	BRAF p.V600E	1 CR, 5 PR and 13 SD	Nicolaides et al., 2020 (198)
Children with pLGGs or PHGGs treated with dabrafenib or vemurafenib	n=67	56 of 67 pts have pLGGs and carried BRAF p.V600E	80% of pLGGs with BRAFi had a OS 3-year PFS was 49.6% in pLGGs with BRAFi vs 29.8% treated with CV	Nobre et al., 2020 (199)
Children with LGGs or plexiform neurofibroma with refractory tumor treated with trametinib.	n= 105	60 pts with PLGG and 45 pts with PN	53 pts with PLGG were evaluable. 1 CR, 7 PR, 17 minor response (MR), 23 SD and 5 PD	Perreault et al., 2022 (100)
Children with recurrent/ progressive LGGs treated with trametinib	n= 10	4 pts carried KIAA1549-BRAF fusion, 4 pts carried NF1 mutation, 1 pt carried FGFR mutation and 1 pts carried CDKN2A loss	2 PR, 2 MR and 6 SD	Manoharan et al., 2020 (167)
Children with sporadic PA treated with trametinib	n=6	5 pts carried KIAA1549-BRAF fusion; 1 carried hotspot FGFR1/NF1/PTPN11 mut	2 PR, 3 MR	Kondyli et al., 2018 (168)
Children with progressive LGGs treated with trametinib	n=18	8 KIAA1549:BRAF-fusions, 3 NF1 alterations, 1 BRAF V600E mutation and 1 FGFR1 K654Q mutation, 5 not detected	6 PR, 2 MR and 10 SD as best OR. DCR was 100% under therapy. Responses were observed in KIAA1549:BRAF- as well as neurofibromatosis type 1 (NF1)-driven tumors. PD was observed in 3 pts after interruption of trametinib.	Selt et al., 2020 (171)
Children with LGGs treated with trametinib or dabrafenib plus trametinib	n=139	91 pts carried BRAF p.V600 mut and treated with trametinib; 48 pts treated with dabrafenib + trametinib	In 47 pts with pLGGs ORR were 15% (trametinib) vs 25% (dabrafenib plus trametinib).	Bouffet et al., 2023 (107)
Children with LGGs treated with drabrafenib plus trametinib	N=110	73 pts carried BRAF p.V600 mut and treated with D+T and 37 pts treated with CV	ORR (CR+PR) was 47% with D+T vs 11% with CV. 12-mo PFS were 67% D+T vs 26% CV	Bouffet et al., 2022 (170)
Children with LGGs treated with drabafenib with trametinib	n=110	73 pts carried BRAF pV600E and treated with D+T vs 37 pts treated with CV	ORR was 46.6% in pts treated with D+T vs 10.8% with CV DOR was 23.7 months (D+T) vs not estimable (CV) PFS was 20.1 months(D+T) vs 7.4 months (CV)	FDA (200)
Children with BRAF aberration or NF1 associated recurrent, refractory, or progressive LGG treated with Selumetinib	n=50	25 PA pts with BRAF aberration and 25 pts with NF1 associated with pLGG	36% of PS patients had a sustained PR vs 40% of NF1 pts	Fangusaro et al., 2019 (165)
Children with recurrent optic pathway and hypothalamic low-grade glioma without NF1 treated with selumetinib	n=25	BRAF p.V600E or KIAA1549-BRAF fusion	6 pts (24%) had PR, 14 (56%) had SD and 5 (20%) PD 2-y PFS was 78 ± 8.5%. 19 pts were evaluable for visual acuity: which improved in 4 pts 21%, was stable in 13 68% and worsened in 11%. 26% had improved visual fields and 74% were stable.	Fangusaro et al., 2021 (181)
Children with recurrent or refractory LGG treated with selumetinib	N=66	25 pts with non-NF-1 and non-optic pathway recurrent/refractory PA; 25 pts with NF-1-associated LGG; 16 pts with non-NF-1 optic pathway/hypothalamic LGG	5 (32%) pts with non-NF-1 and non-optic pathway recurrent/refractory PA carried BRAF aberrations had PR with 2-year PFS (66+/-11%). 10 (40%) pts with NF-1-associated LGG had PR (2-y PFS of 96+/-4%). 2 (12.5%) pts with non-NF-1 optic pathway/hypothalamic LGG had a PR (2-y PFS of 65+/-13%).	Fangusaro et al., 2017 (201)
Pediatric patients with non-NF1-associated, non- OPG and non-pilocytic recurrent/ progressive LGG, treated with selumetinib	n= 23	LGG carried BRAF p.V600E or BRAF-KIAA1549 fusion 13 tumours with BRAF fusion and 11with BRAF p.V600E	5 pts (22%) with PR, 12 (52%) with SD and 6 (26%) had PD with a 2-year PFS of 75 + 9%.	Fangusaro et al., 2022 (202)

(Continued)

TABLE 3 Continued

Study	N Patients	Additional information on patient population	Results	Reference
Children with LGGs treated with everolimus	n=10	mTOR-pmTOR pathway overexpression	SD in 7 patients, PR in 1 and PD in 2 patients.	Cacchione et al., 2021 (190)
Children with recurrent and provessive LGG treated with everolimus	n=65	BRAF alteration in 36/65 pts	PFS is 63% for total cohort; PFS is 64% for the activated and 61% for the non-activated PI3K/Akt/mTOR pathway pts. In 52 pts the central imaging review revealed 1 PR, 1 CR, 33 SD and 17 progressive disease at the end of study therapy.	Mueller et al., 2020 (203)
Children with recurrent, radiographically progressive LGGs and treated with everolimus	n=23	/	2 PR, 10 SD without CR, 11 PD 2-y PFS was 39 ± 11%, 3-y PFS was 26 ± 11%, and 5-year PFS was 26 ± 11%; 2 pts died of disease. The 2-y, 3-y and 5-y OS were all 93 ± 6%.	Wright et al., 2021 (204)
Children with pLGGs treated with dabrafenib, everolimus, trametinib and vemurafenib.	n=55	dabrafenib (n=15), everolimus (n=26), trametinib (n=11), vemurafenib (n=3).	EFS from targeting therapy initiation were: 62.1% for 1-year EFS 38.2% for 3-year EFS 31.8% for 5-year EFS	Tsai et al., 2022 (205)

type non-TSC pLGG patients (210). Interestingly, everolimus has been shown to synergize with carboplatin in preclinical models *in vitro* and *in vivo* by suppressing the conversion of glutamine and glutamate into glutathione (211). The PI3K-AKT-mTOR signaling cascade has been considered the major escape mechanism for BRAF-fusion. Jain and colleagues have shown that combinatorial targeting using MEKi and mTORi for *BRAF*-fusion-driven tumors is effective in overcoming such emergent resistance to single-agent therapy, highlighting preclinical rationales for using MEKi and mTORi. Very limited experience exists for combination therapies. However, in *BRAF* WT cells, everolimus and AZD6244 (MEK1/2 inhibitor) have proven to be superior compared to respective monotherapies (212).

To date, due to the results obtained from the various trials, the Children's Oncology is investigating the possibility of the first-line treatment with MEKi, both as a single agent and in combination with chemotherapy in children with pLGG and relapsed cases.

Currently, the combination therapy development for pLGG patients is under investigation, and in particular, to date, the benefits of personalized therapies based on the administration of a single drug or with multiple combination drugs in non-resectable pLGG patients are unknown. In pLGGs patients, PNOC021 is the first study evaluating the combination of an mTORi (everolimus) and a MEKi (trametinib) to see if there is a possibility of achieving a safe MTD for this combination therapy strategy.

Tergeted drugs have less side systemic effects, however their fairly recent use precludes yet a comprehensive characterization regarding their long-term effects. The majority of short-term adverse events of targeted therapies are temporary and easily manageable, including creatine phosphokinase (CPK) elevation, cutaneous, cardiologic and ocular sequence and toxicities (164, 213). Long-term impact of BRAF, MEK, and mTOR inhibitors on mental and growth consequence in children remain uncertain due to the short follow up described to date. Of note, a limitation of target therapy remains the rebound effect of tumor growth at treatment suspension.

4 Conclusion

This literature review shows that targeted therapy is a feasible approach for pLGGs. Advances in cancer therapies including chemotherapy, radiation therapy, and surgery have significantly improved cancer treatment and outcomes for patients. However, these treatments can lead to a number of toxicities, which are related to a negative impact on their long-term health as well as quality of life. A greater understanding of tumor biology and a germline and somatic genomic approach will play a central role in the therapy strategy of pLGG for the development of increasingly tailored therapies. Limitations still exist regarding the adverse effects of long-term treatment.

Author contributions

AM and AC conceptualized the work. SC, GB and FF wrote the manuscript. AM and AC contributed to the finishing of the work and revised it critically for important intellectual content. All authors finally approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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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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Extra-neural metastases in pediatric diffuse midline gliomas, H3 K27-altered: presentation of two cases and literature review

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Introduction: Pediatric diffuse midline gliomas (DMG), H3 K27- altered, are the most aggressive pediatric central nervous system (CNS) malignancies. Disease outcome is dismal with a median survival of less than one year. Extra-neural metastases are an unusual occurrence in DMG and have been rarely described.

Methods and results: Here, we report on two pediatric patients affected by DMG with extra-neural dissemination. Their clinical, imaging, and molecular characteristics are reported here. An 11-year-old male 5 months after the diagnosis of diffuse intrinsic pontine glioma (DIPG) developed metastatic osseous lesions confirmed with computed tomography (CT) guided biopsy of the left iliac bone. The patient died one month after the evidence of metastatic progression. Another 11-year-old female was diagnosed with a cerebellar H3K27- altered DMG. After six months, she developed diffuse sclerotic osseous lesions. A CT-guided biopsy of the right iliac bone was non-diagnostic. She further developed multifocal chest and abdominal lymphadenopathy and pleural effusions. Droplet digital polymerase chain reaction (ddPCR) on pleural effusion revealed the presence of H3.3A mutation (c.83A>T, p.K28M). The patient died 24 months after the diagnosis of DMG and 3 months after the evidence of metastatic pleural effusion.

Discussion: Extra-neural metastasis of DMG is a rare event and no standard therapy exists. An accurate and early diagnosis is necessary in order to develop a personalized plan of treatment. Further research is needed to gain further insights into the molecular pathology of DMG, H3K27- altered and improve the quality of life and the final outcome of patients with this deadly disease.

KEYWORDS

pediatric, diffuse midline glioma, H3 K27, metastases, high grade glioma, brain tumors

1. Introduction

Pediatric diffuse midline gliomas (DMGs), H3 K27-mutant, are a rare group of malignancies, first introduced in the 2016 World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS) with loss of H3p.K28me3 (K27me3) and usually an H3 c.83A>T p.K28M (K27M) substitution in one of the histone H3 isoforms (CNS WHO grade 4). In the 2021 WHO Classification of Tumors of the CNS, the DMGs have been renamed to "diffuse midline glioma, H3 K27- altered" to include additional molecular changes (such as aberrant overexpression of EZHIP, or an EGFR mutation) that also result in H3 K27 alterations (Louis et al., 2016, 2021). The preferential location is the brainstem or the pons [the latter named diffuse intrinsic pontine glioma (DIPG)], or bithalamic, whereas DMGs in adolescents and adults predominantly arise unilaterally in the thalamus or in the spinal cord (Louis et al., 2021). On magnetic resonance imaging (MRI), DIPGs classically have their epicenter in the pons and typically involve >50% of its surface, often asymmetrically, with frequent encasement of the basilar artery (Steffen-Smith et al., 2014). There may be an exophytic component and/or infiltration into the midbrain, the cerebellar peduncles, and the cerebellar hemispheres. Thalamic tumors may be unilateral or bilateral, the latter being more frequent in the *EGFR*-mutant subtype (Broniscer et al., 2018). Although epidemiological data remain scant for DMG, the incidence of DIPG is estimated to be 0.54 cases per 1 million person-years overall and 2.32 cases per 1 million person-years in people aged ≤20 years, with no sex predilection (Mackay et al., 2017). DIPG represents 10%−15% of all pediatric brain tumors and 75% of all pediatric brainstem tumors. Thalamic DMGs are rarer, representing 1%−5% of pediatric brain tumors (25% of thalamic tumors) (Ryall et al., 2016). To date, there is no known specific genetic susceptibility for DMG, but exceptionally, DMGs may occur in the setting of a cancer predisposition syndrome such as Li–Fraumeni syndrome or mismatch repair deficiency. Independently from the location, the prognosis of DMG is poor, with a 2-year survival rate of <10% (Mackay et al., 2017). Large autopsy-based studies of DIPG have described leptomeningeal metastasis in 40%

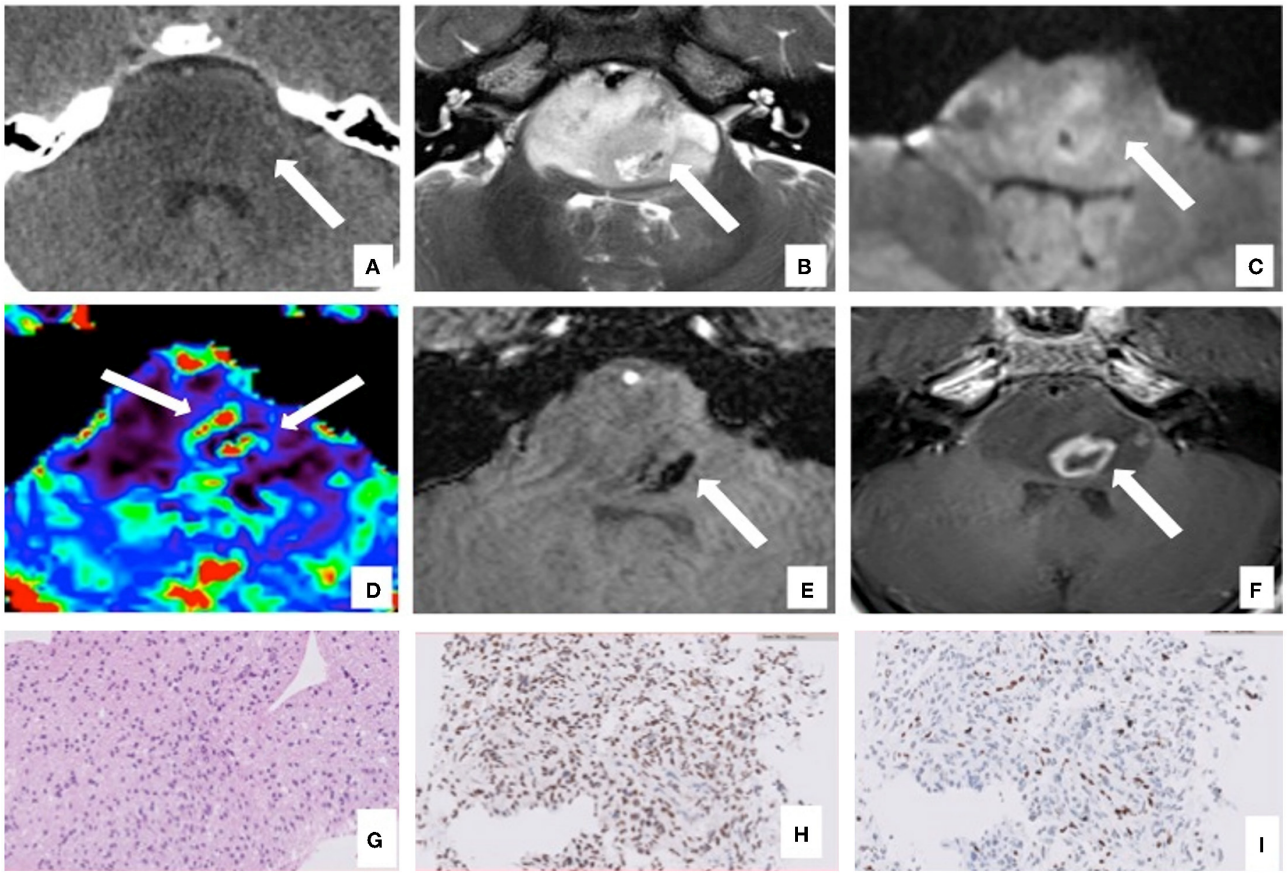

FIGURE 1
Brain computed tomography (CT), magnetic resonance imaging (MRI), and pathology of the primary site of case 1. The axial CT images show a diffuse low-density centered in the enlarged pons with the flattening of the fourth ventricle and surrounding structures [**(A)**, arrow]. Axial MRI images confirm the lesion characterized by a homogeneous high signal on T2 image **(B)** involving more than 50% of the pons with limited restricted areas on diffusion-weighted imaging [DWI, **(C)**]. The MRI also showed areas within the lesion characterized by high cerebral blood volume (CBV) values on perfusion sequences [**(D)**, arrows]. The lesion presents few necrotic components on the left side [**(B, F)**, arrow] with areas defined by a low signal on susceptibility weighted imaging (SWI) sequences due to hemosiderin deposition [**(E)**, arrow] and peripheral enhancement on T1 post-contrast sequences [**(F)**, arrow]. The T2W images also demonstrated the basilar artery encasement [**(B)**, arrowhead]. Histological examination confirmed the radiological diagnosis of DIPG, showing an infiltrative glial cell proliferation [H&E, **(G)**], which displayed the expression of H3K27M mutation **(H)** and the loss of H3K27me3 protein expression **(I)**.

of cases, as well as diffuse spread to involve the thalamus, the cervical cord, and even the frontal lobe (Buczkowicz et al., 2014). To date, extra-neural metastases in patients with DMG, H3 K27-altered, have been reported in 12 cases (Megan et al., 2018; Stephens et al., 2019; Bhatt et al., 2020; Handis et al., 2021; Li et al., 2021; Mohiuddin et al., 2021; Al Sharie et al., 2022; Lazow et al., 2022; Silva et al., 2022; Aftahy et al., 2023). In this study, we report two cases of pediatric DMG with extra-neural metastasis carrying H3.3K27 mutation: one patient was found to have osseous and bone marrow metastases and the second one showed multiple bone lesions, multifocal chest and abdominal lymphadenopathy, and metastatic pleural effusion.

2. Case reports

2.1. Case 1

An 11-year-old boy was presented in June 2020 with a 1-week history of diplopia due to VI cranial nerve (CN) deficit, headache, and asthenia. Brain computed tomography (CT) scan revealed an enlarged pons characterized by a diffuse hypodense alteration (Figure 1A). Subsequently, MRI of the brain and the spine was performed. The MRI demonstrated the presence of an infiltrative mass involving more than 50% of the pons. MRI features were characteristic and consistent with the diagnosis of DIPG (Figures 1B–F). The MRI excluded other brain and spine lesions referred to metastasis. The patient underwent a stereotactic biopsy of the lesion in accordance with the institutional protocol without complications. Histological examination confirmed the radiological diagnosis of DIPG, showing an infiltrative glial cell proliferation, with tumor cells displaying loss of H3K27me3 and expression of H3K27M-altered protein (Figures 1G–I). In line with the immunohistochemical results, molecular analysis (polymerase chain reaction [PCR] and direct sequencing) documented the presence of H3F3A mutation (c.83A>T, p.K28M); neither activin receptor 1 (ACVR1) nor B-raf proto-oncogene (BRAF) mutations were identified. The patient started a 12-week induction regimen with vinorelbine and nimotuzumab, followed by local radiation therapy (volumetric modulated arc therapy [VMAT]) with 54 Gy in 1.8 Gy per fraction from the third week (Massimino et al., 2014; Massimino, 2022). Five months after the diagnosis of DIPG, the patient presented low back pain, bilateral lower extremity weakness and headache, suggestive of clinical progression. Brain and whole spine MRI demonstrated extensive leptomeningeal enhancement throughout the brain and spinal cord. Enhancing lesions throughout the vertebrae were also noted (Figures 2A–C). Because of the extent of the disease, whole-body CT was performed and revealed numerous osteoblastic lesions involving the vertebrae, sternum, and pelvis. A left iliac bone CT-guided biopsy was performed revealing bone metastases of DIPG (Figures 3A–C). The immunohistochemical evaluation of the malignant cells revealed an expression for GFAP and H3K27M in association with H3K27me3 loss (Figures 3D, E). The clinical course of the disease was rapidly progressive and fatal. The patient died 1 month after the evidence of the metastatic progression.

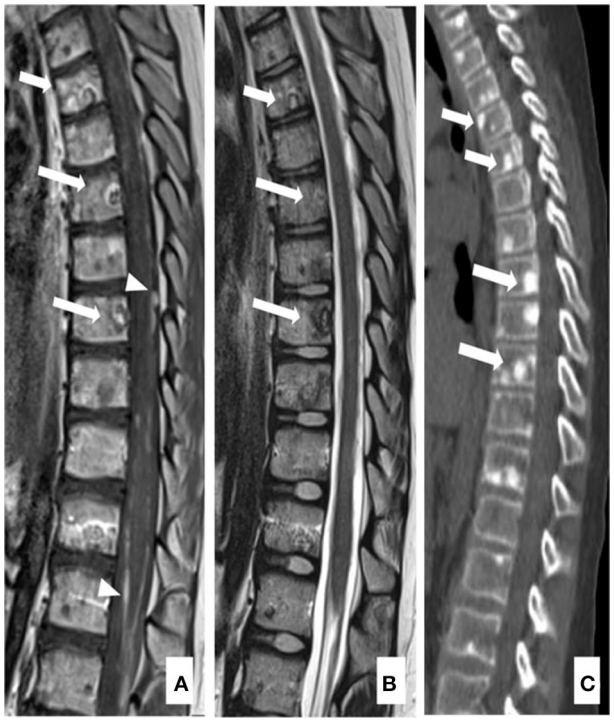

FIGURE 2
Computed tomography (CT) and magnetic resonance imaging (MRI) of bone metastases of case 1: MRI and CT exams, at 5 months after the diagnosis of DIPG. T1W after gadolinium administration **(A)** and the T2W **(B)** MRI sagittal images show diffuse leptomeningeal peri medullary contrast enhancement nodules [**(A)**, arrowheads] and multiple and diffuse nodules of all the vertebrae with irregular and partial post-contrast enhancement on T1 images [**(A, B)** arrows]. The reformatted sagittal CT images confirm these hyperdense, osteoblastic, rounded lesions involving the vertebrae [**(C)**, arrows].

2.2. Case 2

An 11-year-old girl with no relevant family history was presented to our emergency room in March 2020 with a 1-month history of vertiginous syndrome and sporadic vomiting. On examination, horizontal nystagmus and ataxia were documented. An urgent non-enhanced brain CT revealed a large and heterogeneous hypodense mass located within the fourth ventricle, which was slightly dilated (Figure 4a). She was therefore admitted to our hospital. Subsequently, a contrast-enhanced MRI of the brain and whole spine was performed. The MRI confirmed the presence of an infiltrative mass located in the fourth ventricle extending into the left lateral recess (Figures 4b–f). There were no other brain and spine lesions referred to metastases. After multidisciplinary discussion, neuronavigation and occipital craniotomy with tumor resection with direct cortical and subcortical stimulation were performed under general anesthesia. Compared to the first MRI study, MRI scanning within 24 h after surgery documented total resection. Microscopy on tissue sections showed a heterogeneous malignant neoplasm with palisading necrosis and extensive perivascular proliferation. Tumor cells ranged in size from small to medium size, with irregular hyperchromic nuclei and eosinophilic, scarce, or clear cytoplasm arranged in sheets and at the perivascular site (Figure 4g). On

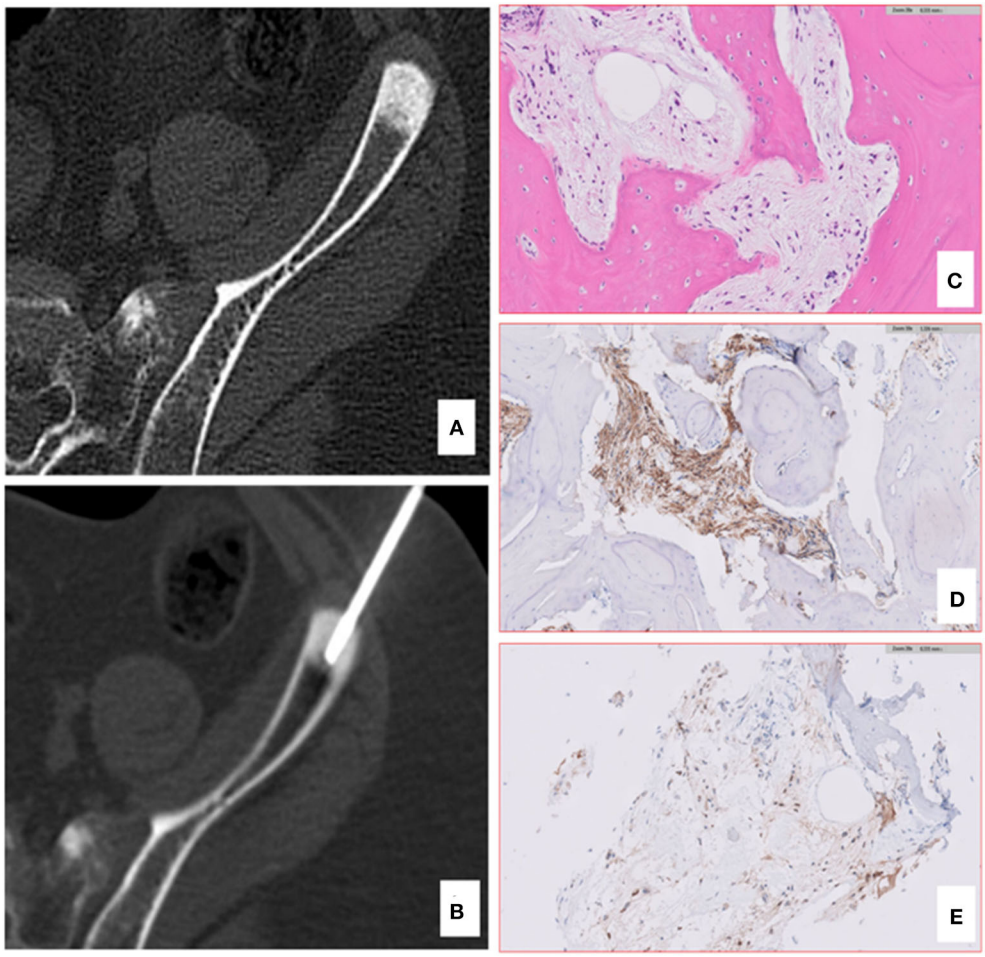

FIGURE 3
Computed tomography (CT) and pathology of iliac bone lesion of case 1. A bone biopsy was performed using an 11 gauge coaxial bone needle **(A, B)**. The lesion was so stiff that the first needle bent, and a second approach was needed. The bone histology confirmed the skeletal metastases of DIPG **(C)**, showing the presence of a bland spindle cell proliferation infiltrating the lamellar bone with positive IHC staining for GFAP **(D)** and H3K27M-altered protein **(E)**.

immunohistochemical examination, neoplastic cells were positive for vimentin, integrase interactor 1 (INI-1), glial fibrillary acidic protein (GFAP), microtubule-associated protein 2 (MAP2), histone chaperone protein ATRX, and epithelial membrane antigen (EMA). The protein P53 was not expressed. The Ki-67 proliferative index was approximately 40%. The protein H3.3K27me3 was absent, and the expression of H3K27M-altered protein was found (Figures 4h, i). Thus, the pathology was consistent with a diagnosis of pediatric DMG. Whole-exome sequencing (WES) did not reveal targetable mutations. One month after admission, our patient started a 12-week induction regimen with vinorelbine and nimotuzumab, followed by local radiation therapy (VMAT) with 54 Gy in 1.8 Gy per fraction from the third week (Massimino et al., 2014). Three months later, cerebral recurrence involving septum pellucidum, ependyma of lateral ventricles, and leptomeninges was revealed; therefore, she underwent craniospinal radiotherapy (36 Gy in 1.8 Gy per fraction) which was followed by second-line treatment with irinotecan and bevacizumab (IB) for 15 months. A brain and spine MRI scan after the completion of her second radiotherapy showed a partial response of the lesion of the septum pellucidum and of the nodules in the ependyma, absence of leptomeningeal enhancement, and appearance of vertebral lesions (Figures 5a-d). A total body CT revealed diffuse sclerotic vertebral osseous lesions suspected of metastases involving the vertebrae (Figure 5e), ribs, sternum, pelvis, proximal humeri, and proximal femurs. A positron emission tomography with 2-deoxy-2-[fluorine-18]fluoro-D-glucose integrated with computed tomography (^{18}F-FDG PET/TC) showed mild diffuse bone hypercaptation; however, multiple biopsies of the lesions were non-diagnostic (Figure 5e). The patient presented good clinical condition, except for mild chronic low back pain responsive to medical treatment. Subsequent MRIs showed brain response but progression of the bone lesions. In October 2021, a brain MRI documented left hemispheric cerebellar recurrence associated with hydrocephalus, and the patient underwent subtotal tumor resection. Next-generation sequencing (NGS) and WES of the tumor confirmed the presence of H3F3A mutation (c.83A>T, p.K28M) and the absence of targetable mutations. At the NGS, additional mutations were found: NBN LOH; NBN deletion; ATR c.5739-14_5739-6delinsT; FGFR4 p.(G388R) c.1162G>A; PTPN11 p.(A72V) c.215C>T. Subsequently, she started third-line treatment with etoposide and temozolomide; however, the clinical course of

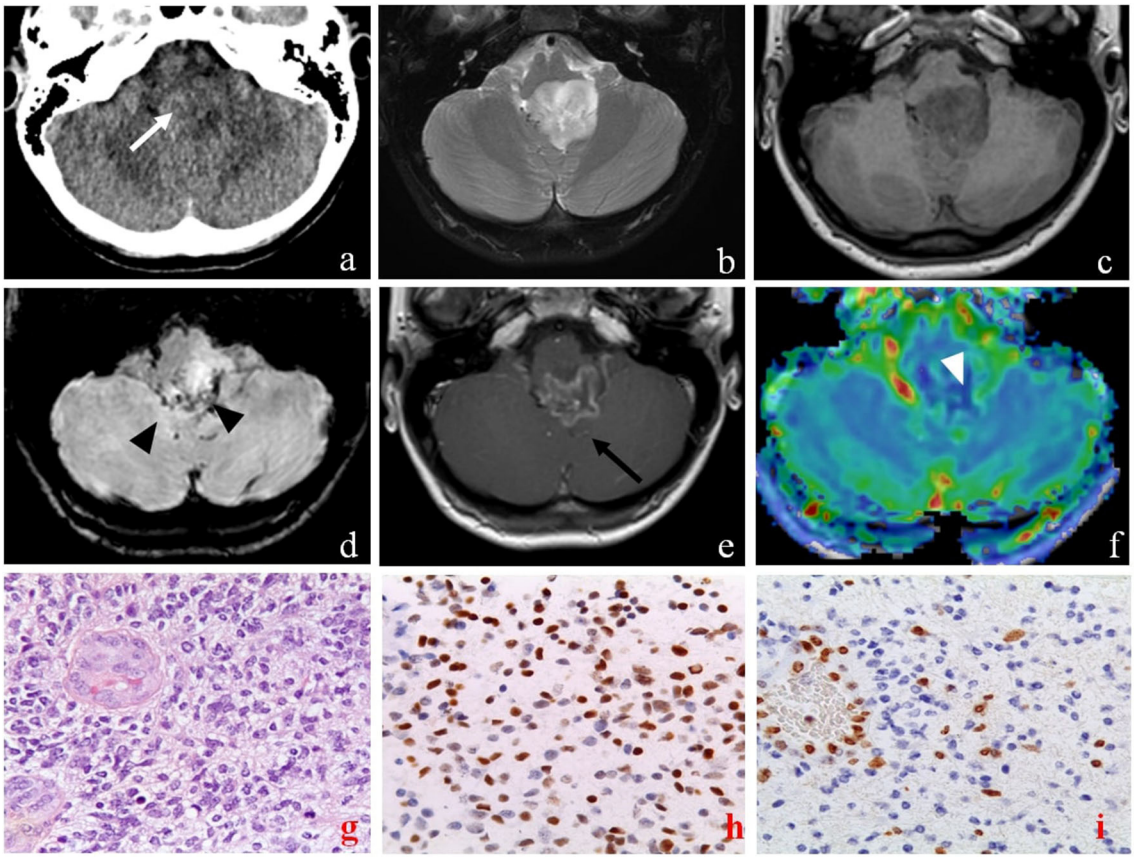

FIGURE 4
Brain computed tomography (CT), magnetic resonance imaging (MRI), and pathology of primary sites of case 2. The axial CT images show a diffuse low-density lesion centered in the fourth ventricle [**(a)** white arrow]. Axial MRI images confirm the lesion characterized by an inhomogeneous high signal on T2-weighted **(b)** and low signal on T1-weighted **(c)** sequences, extending into the left lateral recess of the fourth ventricle, and compressing the medulla and the cerebellar tonsils. The lesion presents some areas defined by low signal on susceptibility weighted imaging sequences (SWI) due to hemosiderin deposition [**(d)**, black arrowheads], and peripheral enhancement on T1 post-contrast sequences [**(e)**, black arrow]. The MRI also showed peripheral areas of the lesion characterized by high CBV values on perfusion sequences [**(f)**, white arrowhead]. Pathology of primary sites (X40) showed: infiltrative, cellular neoplasm with mitoses and microvascular proliferation [H&E **(g)**]; intense nuclear staining for H3 K27- mutant protein **(h)**; loss of H3 K27me3 expression in tumor cells with retention in endothelial cells **(i)**.

the disease was slowly progressive. In January 2021, she further developed multifocal chest and abdominal lymphadenopathy and pleural effusion (Figures 6A–C). The pleural fluid analysis did not reveal any cancer cells. However, the droplet digital PCR (ddPCR) performed on pleural effusion identified H3.3A mutation (c.83A>T; p.K28M) confirming the diagnosis of extra-neural metastases. Given the ongoing clinical deterioration, palliative treatment was initiated, and the patient eventually died 24 months after the diagnosis and 3 months after the evidence of metastatic pleural effusion.

3. Literature review

A total of 12 cases of extra-neural metastases in DMG have been reported in the literature (Table 1). We excluded from our search cases reported without biopsy or those affected by high grade glioma (HGG), not H3.3 K27-altered. Patients' ages were from 4 to 36 years with a median of 15.5 years (range 4–36) and were predominantly female patients ($n = 9$, 75%). Primary DMGs were mostly in the brain ($n = 8$, 66.7%) and, more rarely, in the spinal cord ($n = 4$, 33.3%) (Megan et al., 2018; Stephens et al., 2019; Bhatt et al., 2020; Handis et al., 2021; Li et al., 2021; Mohiuddin et al., 2021; Al Sharie et al., 2022; Lazow et al., 2022; Silva et al., 2022). The extent of surgical resection was subtotal in four patients (33.3%) (Megan et al., 2018; Stephens et al., 2019; Mohiuddin et al., 2021). Cerebrospinal fluid (CSF) diversion was performed in four patients (33.3%) because of hydrocephalus (Stephens et al., 2019; Mohiuddin et al., 2021; Al Sharie et al., 2022; Silva et al., 2022). Details about cancer treatment were not available in two cases (Megan et al., 2018). Almost all patients underwent adjuvant radiotherapy ($n = 9$, 75%) and chemotherapy ($n = 8$, 66.7%) (Stephens et al., 2019; Handis et al., 2021; Li et al., 2021; Mohiuddin et al., 2021; Lazow et al., 2022; Silva et al., 2022). Because of rapidly declining clinical status, one patient did not initiate any treatment and died of cardiorespiratory failure 2 weeks after presentation (Bhatt et al., 2020). Histological examination was performed in 11 patients (91.7%) (Megan et al., 2018; Stephens et al., 2019; Bhatt et al., 2020; Handis et al., 2021; Li et al., 2021; Mohiuddin et al., 2021; Al Sharie et al., 2022; Lazow et al., 2022; Silva et al., 2022; Aftahy et al., 2023). In one patient, the diagnosis was made on CSF

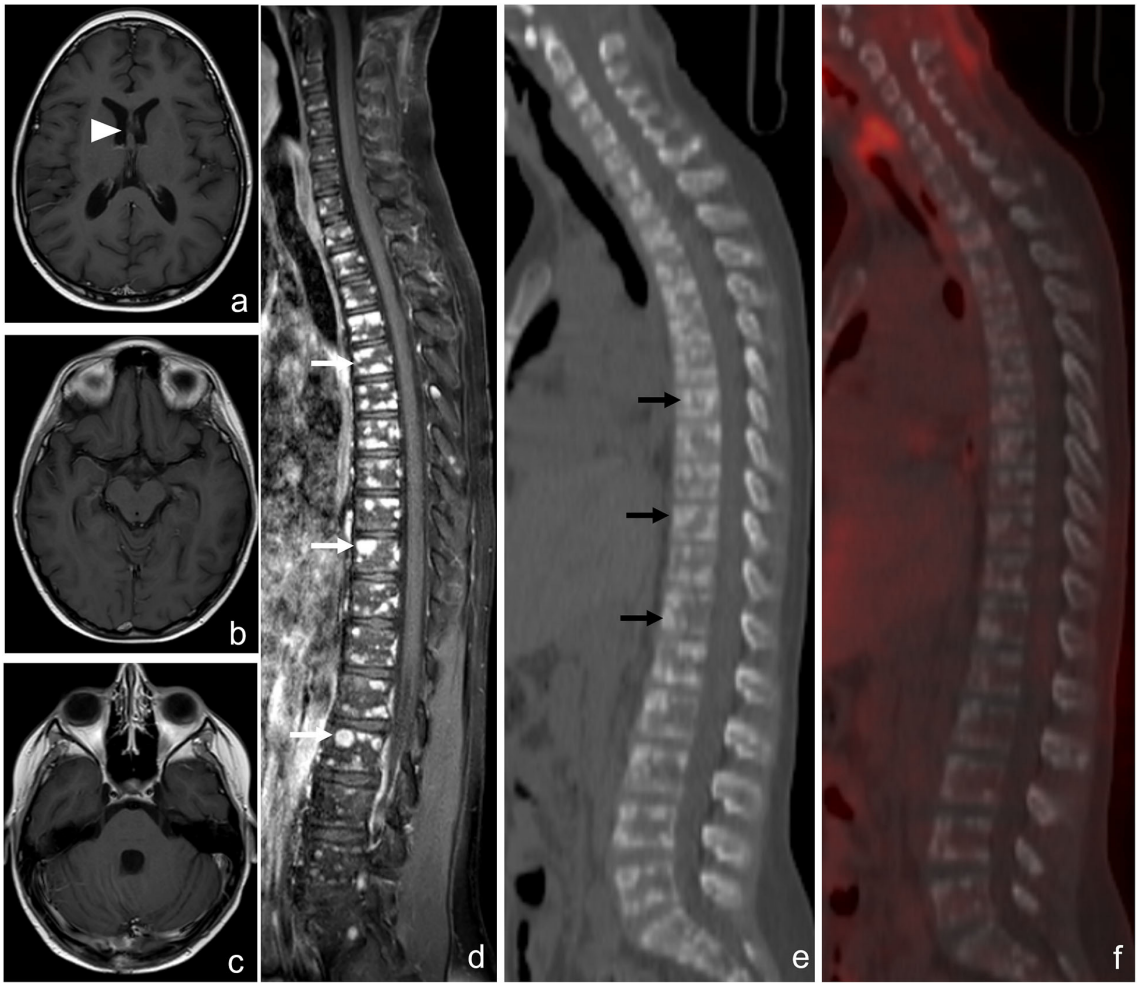

FIGURE 5
Brain and spine magnetic resonance imaging (MRI) scan and ^{18}F-FDG PET-CT after the completion of the second radiotherapy of case 2. Axial contrast-enhanced T1-weighted MRI images show the partial response of the lesion of the septum pellucidum [**(a)**, arrowhead] and the absence of leptomeningeal enhancement **(b, c)**. Spine MRI shows multiple and diffuse nodules of all the vertebrae with irregular contrast enhancement on the T1 image [**(d)**, white arrows]. The reformatted sagittal CT **(e)** and ^{18}F-FDG PET-CT **(f)** images confirm hyperdense rounded lesions involving the vertebrae [**(e)**, black arrows] with mild diffuse hypercaptation.

by NGS, which revealed a HIST1H3B mutation (Li et al., 2021). At diagnosis, brain and/or spinal metastases were present in eight patients (66.7%), while tumor spread outside the CNS was present in seven patients (58.3%) (Megan et al., 2018; Stephens et al., 2019; Bhatt et al., 2020; Handis et al., 2021; Li et al., 2021; Mohiuddin et al., 2021; Al Sharie et al., 2022; Lazow et al., 2022; Silva et al., 2022; Aftahy et al., 2023). Extra-neural metastases were detected after the diagnosis in the remaining five cases (41.7%) (Megan et al., 2018; Stephens et al., 2019; Bhatt et al., 2020; Handis et al., 2021; Li et al., 2021; Mohiuddin et al., 2021; Al Sharie et al., 2022; Lazow et al., 2022; Silva et al., 2022; Aftahy et al., 2023). In eight patients (66.7%), DMG metastasized in a single extra-neural site (seven bones, one peritoneum) (Megan et al., 2018; Stephens et al., 2019; Bhatt et al., 2020; Handis et al., 2021; Li et al., 2021; Mohiuddin et al., 2021; Al Sharie et al., 2022; Lazow et al., 2022; Silva et al., 2022; Aftahy et al., 2023). In the remaining cases ($n = 4$, 33.3%), multiple extra-neural locations were detected involving bones, lymph nodes, lungs, soft tissue, peritoneum, lungs, and pleura (Mohiuddin et al., 2021; Lazow et al., 2022; Silva et al., 2022). Extra-neural metastases were treated with palliative locoregional radiotherapy in only one patient (8.3%) and with systemic chemotherapy in two patients (16.7%) (Mohiuddin et al., 2021; Silva et al., 2022; Aftahy et al., 2023). At the last follow-up, all patients died with a median overall survival (OS) from primary DMG diagnosis of 9 months (range, 0.5–15), and a median OS from extra-neural metastases occurrence of 6 months (range, 0.5–16) (Megan et al., 2018; Stephens et al., 2019; Bhatt et al., 2020; Handis et al., 2021; Li et al., 2021; Mohiuddin et al., 2021; Al Sharie et al., 2022; Lazow et al., 2022; Silva et al., 2022; Aftahy et al., 2023).

4. Discussion

Metastases of pediatric DMG outside the CNS are extremely rare. Since "diffuse midline glioma, H3K27M- mutant" was introduced as a distinct entity in the 2016 edition of the WHO classification of tumors of CNS, a total of 12 cases (3 M) aged 4–36 years of extra-neural metastases have been reported in the literature (Table 1). We presented an 11-year-old boy with DIPG

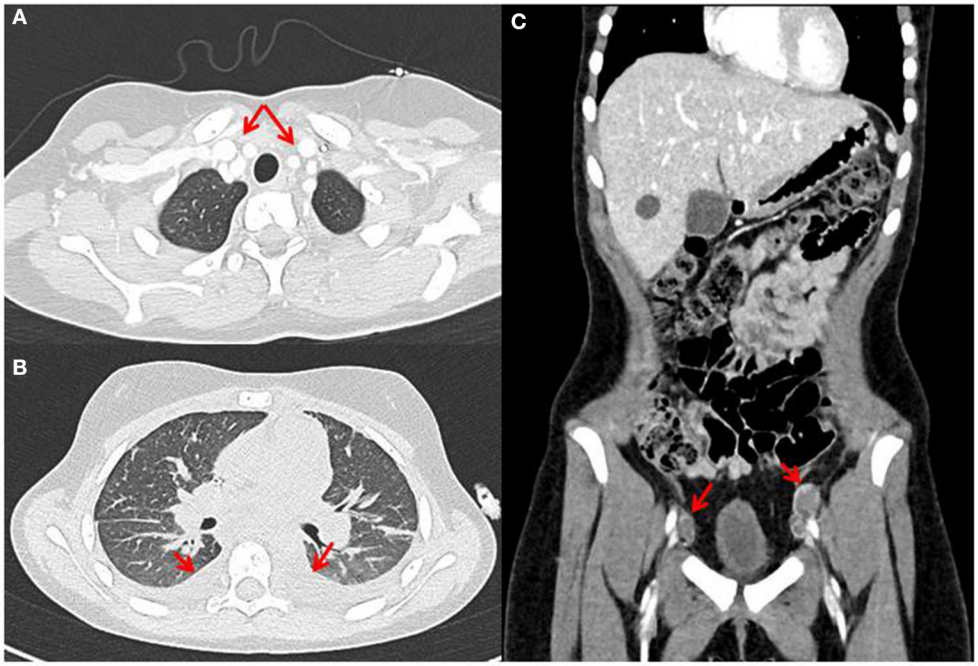

FIGURE 6
Radiographic features of diffuse midline glioma (DMG), H3K27M-altered (case 2), with multifocal chest **(A)** and abdominal **(B)** lymphadenopathy and pleural effusion **(C)**.

and an 11-year-old girl with cerebellum DMG who developed extra-neural metastases a few months after diagnosis, despite cancer treatment consisting of radiotherapy and concomitant nimotuzumab and vinorelbine (Massimino et al., 2014; Massimino, 2022). Localization of DMG in midline sites such pineal region, hypothalamus, and cerebellum is exceptional (Solomon et al., 2016; Meyronet et al., 2017; Nakata et al., 2017). In a series of 47 diffuse midline gliomas with histone H3K27M mutation, only one 9-year-old boy presented with a tumor arising in the cerebellum (Solomon et al., 2016). Similarly, in a retrospective series of 164 cases with molecularly confirmed H3K27M-mt DMGs, the cerebellum location was identified in only two patients (Zheng et al., 2022). Interestingly, Wang et al. (2018) showed that patients with H3K27M-mutant gliomas in unusual anatomical locations (cerebellum, corpus callosum, lateral ventricle, frontal, and temporal lobe) had a better prognosis compared with those with corresponding tumors in the brainstem. Recently, Hazaymeh et al. (2022) showed that patients affected by glioblastoma multiforme (GBM) undergoing gross total resection exhibited a significant survival benefit compared to their counterparts without gross total resection. Our cases 1 and 2 developed osseous metastases 5 months and 6 months, respectively, since the initial diagnosis. Osseous and bone marrow metastases of our case 1 were documented by CT-guided biopsy of the left iliac bone, and the patient died 1 month later. It is interesting to note that bone metastases are common sites of extra-neural involvement in high-grade gliomas (HGGs), such as DMG and GBM. This predilection of bone may come from both tumor-derived and extracellular niche-derived cues. For example, many hematopoietic stem cell proteins are expressed by GBM cells, including stromal cell-derived factor 1 alpha (SDF-1α), C-X-C chemokine receptor type 4 (CXCR4), osteopontin (OPN), and cathepsin K (CATK) (Hira et al., 2018). Glioblastoma cells are also able to recruit bone marrow-derived progenitor cells providing a perivascular support role regulated by vascular endothelial growth factor (VEGF) (Burrell et al., 2014). In addition, CXCR4, OPN, CATK, and CD44 are induced by hypoxia-inducible factor-1α and VEGF, two proteins known to increase glioma aggressiveness and invasion (Colwell et al., 2017). In addition to bone metastases, our case 2 developed lymph nodes and pleura involvement. The diagnosis of multiple extra-neural metastases was more difficult since bone and lymph node biopsies and pleural fluid analysis were negative for cancer cells. The diagnosis was concluded due to positive ddPCR for H3.3A mutation on pleural effusion. Recently, Wolter et al. (2022) reported on the application and validation of a set of molecular assays for glioma diagnostics based on ddPCR, enabling the detection of diagnostically relevant glioma-associated mutations in the isocitrate dehydrogenase (IDH)1, IDH2, H3-3A, BRAF, and protein kinase C alpha (PRKCA) genes, as well as in the telomerase (TERT) promoter and other relevant copy number alterations. Recently, Massimino et al. identified and validated a prognostic marker based on the expression of 13 circulating microRNAs in serum that can shed light on a patient's risk of progression (Iannó et al., 2022). Due to its rarity, the biological mechanisms behind tumor dissemination outside the CNS of DMG have not been well-described. Although improved care of patients affected by DMG is going to lead in some cases to longer survival, extra-neural metastases in DMG were detected at diagnosis or relatively early after diagnosis. Certainly, the absence of routine surveillance for

TABLE 1 Diffuse midline glioma, H3 K27a studies reporting extra-neural metastases.

Pt	Age (years)/ sex	Diagnosis	Site of tumor	CNS metastases	Timing of appearance since diagnosis	Extra-CNS metastases	Timing of appearance since diagnosis	Surgery	Radiotherapy	Chemotherapy	OS (ms)	References
1	11/M	DMG H3F3A K27mt (WES)	Left lateral ventricle, hypothalamus, fornices, and left midbrain	None	–	Bones	3.5 months	Subtotal resection	NA	NA	9	Megan et al., 2018
2	12/F	DMG H3F3A K27mt (WES)	Tectal/pineal gland	Spinal cord	At diagnosis	Bones	5.5 months	Subtotal resection	NA	NA	13	Megan et al., 2018
3	15/F	DMG H3K27mt (IHC)	Spine (T12-L1, conus medullaris)	Multifocal brain	At diagnosis	Bones, bone marrow	At diagnosis	Biopsy of the thoracic spinal mass	None	None	0.5	Bhatt et al., 2020
4	4/F	DMG H3F3A K27mt (IHC, NGS)	Brainstem	Spine	1 month	Peritoneum	14 months since diagnosis	Subtotal resection + VPS	First line Focal RT:54 Gy + CSI (+ boost to S1 metastasis) : 50.4 Gy At progression Skull base-mid lumbar spine: 50.4 Gy Cranial disease:35 Gy S1 disease :15 Gy	First line Temozolomide	15	Stephens et al., 2019
5	36/F	DMG HIST1H3B K27mt (NGS)	Pons	CSF	At diagnosis	Bones	At diagnosis	None (liquid biopsy for diagnosis)	First line Focal RT:54 Gy	First line Temozolomide	13	Li et al., 2021
6	20/F	DMG H3F3A K27M (NGS)	Right thalamus	Intracranial and spinal leptomeninges	4–6 months	Bones	4–6 months	Subtotal resection	First line Focal RT:60 Gy At progression Proton RT Midbrain-spinal axis:36 Gy + Boost to spinal disease: cumulative dose of 45 Gy	First line Temozolomide At progression Bevacizumab (10 mg/kg) every 2 weeks 2. Panobinostat 30 mg/3 days a week every other week in combination with bevacizumab	11	Mohiuddin et al., 2021
7	17/F	DMG H3F3A K27M (NGS)	Left hippocampus extending into the left posterior midbrain	Intracranial and spine leptomeninges	At diagnosis	Chest, abdomen, and pelvis lymph nodes, lung, pleura, liver, and omental fat stranding	4 months	Biopsy of the brain lesion + VPS	First line CSI: 39.6 Gy + Focal boost:18 Gy	First line Temozolomide	5	Mohiuddin et al., 2021

(Continued)

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TABLE 1 (Continued)

Pt	Age (years)/ sex	Diagnosis	Site of tumor	CNS metastases	Timing of appearance since diagnosis	Extra-CNS metastases	Timing of appearance since diagnosis	Surgery	Radiotherapy	Chemotherapy	OS (ms)	References
8	16/F	DMG H3F3A K27M (NGS)	Spine (T7-L3)	Intracranial pial and parenchymal disseminations	At diagnosis	Bones	At diagnosis	Open biopsy of spinal lesion	First line Whole cranial and vertebral axis	First line Vincristine (1.5 mg/m^2 once daily), procarbazine (100 mg/m2 once daily), lomustine (100 mg/m^2 once daily), cyclophosphamide (1000 mg/m^2 once daily)	5	Handis et al., 2021
9	8/F	DMG H3K27-altered with EZHIP overexpression (NGS)	Pons, thalamus, and bilateral temporal lobes	None	–	Muscle, peritoneum, infratemporal fossa, and along the lumbosacral nerve roots	At diagnosis (muscle, brachial plexus, lumbosacral nerve roots) 4 months (peritoneum)	Biopsy of the lateral rectus muscle + biopsy of the temporal lobe + VPS	First line Proton RT CSI (+right orbit) : 52.2 Gy + Brachial plexus : 46.5 Gy	None	NA	Silva et al., 2022
10	12/F	DMG H3F3A K27M (NGS)	Periventricular white matter, temporal structures, optic chiasm, brainstem, septum pellucidum, cerebellum	Spine	At diagnosis	Bones, lungs	At diagnosis (bones) Later (lungs)	Biopsy of thoracic spinal lesion + Bone biopsy	First line CSI : 45 Gy	At progression Cabozantinib 40 mg/m^2 daily for 28-day cycles	9	Lazow et al., 2022
11	19/M	DMG H3K27 altered	Spine (D11-L1)	Intracranial and spine leptomeninges	At diagnosis	Bones	At diagnosis	Biopsy of bone lesion + VPS	First line D11-L1: 5.4 Gy CSI: 39.6 Gy Boost to the suprasellar mass :14.4 Gy	First line Temozolomide	7	Al Sharie et al., 2022
12	24/M	DMG H3K27mt (IHC)	Spine (C5-D7)	Brain and spine	At diagnosis	Bones (vertebrae, sternum)	At diagnosis	Biopsy of brain lesion + bone biopsy	First line CSI (included vertebrae) : 36 Gy	First line Temozolomide	3	Aftahy et al., 2023
13	11/M	DMG H3K27M (IHC, NGS)	Pons	Brain and spinal leptomeninges	5 months	Bones	5 months	Biopsy of pons	First line Focal RT: 54 Gy	First line Nimotuzumab (150 mg/m^2) and Vinorelbine (20 mg/m^2) every week	6	Present case
14	11/F	DMG H3K27M (IHC, NGS)	Cerebellum	Septum pellucidum, lateral ventricles, leptomeninges	6 months	Bones, chest, abdomen, and pelvis lymph nodes pleura	6 months (bone) 23 months (lymph nodes and pleura)	Total resection of the cerebellar mass	First line Focal RT: 54 Gy At progression CSI: 36 Gy CFP: 28 Gy	First line Nimotuzumab (150 mg/m^2) and Vinorelbine (20 mg/m^2) every week At progression Bevacizumab (10 mg/kg) + Irinotecan (125 mg/m^2) every 2 weeks 2. Etoposide + Temozolomide	24	Present case

M, male; F, female; DMG, diffuse midline glioma; mt, mutant; a, altered; IHC, immunohistochemistry; NGS, next-generation sequencing; RT, radiotherapy; CSI, craniospinal irradiation; Gy, gray.

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extra-neural metastases in the staging of DMG may contribute to the underreporting of metastatic disease. Although recent studies have suggested that CSF shunting could be a risk factor for disseminated disease, a recent comparative outcome multivariate analysis showed that the ventricular route is not a likely pathway for the spread of leptomeningeal disease or for distant tumor recurrences (Mistry et al., 2019). Increasing evidence from clinical and experimental studies suggests that surgical trauma caused by biopsies or resections may potentially lead to tumor progression and metastatic disease (Alieva et al., 2018). On hospital admission, both our patients underwent neurosurgical procedures: case 1 a stereotactic biopsy of the pontine lesion and case 2 a complete surgical resection of cerebellum mass. Recently, Massimino et al. showed that pediatric patients affected with pediatric DMG undergoing biopsies had more dissemination ($P = 0.04$) and less local progression (Massimino et al., 2014). Alieva et al. (2018) showed, in a retrospective analysis of GBM patients, an increase in tumor volume after biopsy. In mice, the cellular mechanisms mediating this response are dependent on inflammation, especially on the CCL-2-dependent recruitment of macrophages, which can be blocked by treatment with dexamethasone. The immune system may also have a role in tumor extra-neural dissemination. In patients affected by GBM, multiple hypotheses have been postulated as why, such aggressive tumors, only rarely exhibit metastases outside the brain. The main pathophysiological ideas are the "seed vs. soil" hypothesis (Mohme et al., 2017) that describes the preference of metastatic tumor cells to grow inside the brain where the local microenvironment is favorable, and the "peripheral immunosurveillance" hypothesis, which holds that the activated peripheral immune system is able to eliminate GBM tumor cells that left the immune protected brain microenvironment. However, the discovery of circulating tumor cells (CTCs) in up to 20% of GBM patients has renewed interest in this discussion. However, it remains unclear why the CTCs do not form extra-neural metastases at the expected frequency. The occurrence of extracranial GBM metastases in recipients of organ transplantation from donors diagnosed with GBM points to a decisive role of the immune system in containing extracranial growth (Jimsheleishvili et al., 2014; Nauen and Li, 2014). Recently, Mohme et al. (2020) described a case of extracranial metastases from GBM during immunological remission of the intracerebral tumor with checkpoint inhibition. They postulated that the combination of functional impairment of the peripheral immune system, as reflected by a steady increase of exhaustion markers and the occurrence of metastasis with an increased mutational burden, enabled the extracranial dissemination and disease progression, while intracranial GBM could be controlled by checkpoint inhibition (Mohme et al., 2020). Similarly, our case 2 showed disease progression at bone sites and partial response of intracranial DMG to second-line treatment with craniospinal irradiation and systemic chemotherapy with irinotecan and bevacizumab regimen. At the time, neither an international nor European consensus chemotherapy regimen was universally agreed upon for DMG treatment, and the Phase 2 Children's Oncology Group (COG) study ACNS0126 demonstrated that single-agent TMZ during and after radiotherapy failed to improve pediatric HGG survival compared to historical controls (Jakacki et al., 2016). In Italy, the standard treatment of pediatric H3K27M-a DMG includes radiotherapy, concomitant nimotuzumab and vinorelbine, and re-irradiation at relapse (Massimino, 2022). Recent studies support the bevacizumab regimen showing superior survival compared to historical-agent regimens and nearly all other published treatment strategies (Hummel et al., 2016; Lu et al., 2019). However, further research is needed to prove the safety and efficacy of bevacizumab in children and adolescents affected by DMG, H3 K27-altered.

5. Limitations

Our study have some limitations, primarily due to the paucity of extra-neural DMG metastases in the literature. All the studies included in this review are case reports or case series. None of our patients completed genetic testing for germline mutations; therefore, familial predisposition cancer syndrome cannot be excluded.

6. Conclusion

Extra-neural metastasis of DMG is a rare event. The present cases emphasize the need to consider unusual localization of pediatric brain cancers, especially because early diagnosis and active treatment may be crucial to improve prognosis and survival. Even if no standard therapy exists to treat extra-neural metastasis of DMG, it is mandatory to establish promptly an accurate and specific diagnosis in order to develop a personalized plan of treatment. Moreover, the biopsy of the primary and metastatic sites should be considered for any pediatric patients with DMGs due to the important prognostic implications as well as to develop more effective treatment strategies. Considering the rarity of extra-neural metastasis of DMGs, international registries and collaborative multicenter studies are warranted to gain further insights into the molecular pathology of DMG, H3K27-altered and improve the quality of life and the final outcome of patients with this deadly disease.

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

Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

LD and LQ: conceptualization and methodology. LD, MP, AC, GS, GSC, and PS: data curation. MM and AM: formal analysis.

LQ, LD, SP, GS, GC, ME, MM, AM, and NN: investigation. GC: resources. MM and LQ: supervision. LD, ME, GSC, AC, and CR: writing—original draft. SP, AM, NN, GC, and MM: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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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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SHH medulloblastoma and very early onset of bowel polyps in a child with *PTEN* hamartoma tumor syndrome

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Phosphatase and tensin homolog (*PTEN*) hamartoma tumor syndrome (PHTS) is a cancer predisposition syndrome characterized by an increased risk of developing benign and malignant tumors, caused by germline pathogenic variants of the *PTEN* tumour suppressor gene. *PTEN* gene variants often present in childhood with macrocephaly, developmental delay, and/or autism spectrum disorder while tumors and intestinal polyps are commonly detected in adults. PHTS is rarely associated with childhood brain tumors with only two reported cases of medulloblastoma (MB). We report the exceptional case of an infant carrying a germline and somatic pathogenic variant of *PTEN* and a germline and somatic pathogenic variant of *CHEK2* who developed a MB SHH in addition to intestinal polyposis.

KEYWORDS

cancer predisposition syndrome (CPS), pediatric, PTHS, medulloblastoma (MB), intestinal polyp, *PTEN* hamartoma tumor syndrome

1. Introduction

Phosphatase and tensin homolog (*PTEN*) hamartoma tumor syndrome (PHTS) is a rare neurocutaneous syndrome caused by germline pathogenic variants of the *PTEN* tumor suppressor gene (Hendricks et al., 2020; Isik et al., 2020; Kim et al., 2020) that cause an increased risk of developing benign and malignant tumors of the thyroid, breast,

endometrium, skin, and brain. In addition to cancer susceptibility, PHTS features include macrocephaly, autism spectrum disorder, atypical neurodevelopment, benign thyroid lesions, and dermatologic findings (trichilemmomas, papillomas). PHTS may be considered a non-classical brain tumor polyposis syndrome, as central nervous system (CNS) manifestations are a rare component of the patient's clinical burden (Kim et al., 2020). It is a rare disease with an estimated prevalence of 1/200.000, but it is probably underestimated because most patients are not recognized as such (Nelen et al., 1999; Ngeow and Eng, 2015; Karczewski et al., 2020). Approximately 50% of PHTS cases are inherited in an autosomal dominant manner, with the remainder of cases having a *de novo* mutation; in approximately 80% of case mutations of the *PTEN* gene affects the germline (Kim et al., 2020). All types of pathogenic variants (loss-of-function, deletions, missense, and promoter abnormalities) have been reported with no clear genotype–phenotype correlation (Smith et al., 2019). *PTEN* gene mutations show age-related penetrance (Lachlan et al., 2007): in childhood, they are often associated with macrocephaly, developmental delay (DD), and/or autism spectrum disorder, less commonly with thyroid lesions, while the development of tumors and intestinal polyps are rare, being more frequently detected in adult individuals (Heald et al., 2010; Hansen-Kiss et al., 2017; Ciaccio et al., 2019; Macken et al., 2019).

Medulloblastoma (MB) is a heterogeneous tumor that represents about 10% of CNS malignancies in children between 0 and 14 years of age (Millard and De Braganca, 2016). There are four MB subgroups (Sonic Hedgehog or SHH, WNT, group 3, and group 4), which are associated with specific transcriptional, epigenetic, and clinical characteristics (Vladoiu et al., 2019). However, the molecular details of each subgroup are not fully understood to date (14). Recently, it has been shown that the SHH subgroup is most frequently (approximately 20–40%) associated with germline mutations (*BRCA2*, *PALB2*, *PTCH1*, *SUFU*, and *TP53*; Waszak et al., 2018; Garcia-Lopez et al., 2021). Currently, MB cases are rarely described in individuals with Cowden syndrome (Waszak et al., 2018; Tolonen et al., 2020), a condition included in the PHTS spectrum. Instead, cases associated with other CNS tumors such as dysplastic gangliocytoma, meningioma, pineal tumor, oligodendroglioma, and glioblastoma have been reported in patients with this condition (Kim et al., 2020).

Susceptibility to develop intestinal polyps is one of the most distinctive features of PHTS, involving up to 95% of PHTS patients throughout life (Heald et al., 2010). Bowel polyps may be found from the stomach to the colon, and histology may include hamartomatous polyps (most common), ganglioneuromas, adenomas, and inflammatory polyps (Heald et al., 2010). This clinical manifestation is similar to Juvenile Polyposis Syndrome (JPS): hamartomatous polyps are indistinguishable (Schreibman et al., 2005) but tend to occur in adulthood (Lachlan et al., 2007; Heald et al., 2010). While the increased risk for the development of breast and thyroid cancers is well documented, the development of hamartomatous polyps does not lead to an increased risk of colorectal cancer. Heald et al. documented that colorectal cancer occurred in 7.1% of cases of their series (Heald et al., 2010).

Here we report the first pediatric case of PHTS with both a germline and somatic variant in *PTEN* and in *CHEK2*, who presented a significantly early onset of MB SHH (15 months), in addition to a remarkably early picture of hamartomatous intestinal polyposis.

2. Materials and methods

The patient and his legal guardians conferred informed consent for the study. A centralized review of histological characterization was performed. Molecular genetics studies were performed on genomic DNA extracted from peripheral blood using a next-generation sequencing (NGS) panel including medulloblastoma and cancer predisposition genes (*APC*, *BRCA2*, *PALB2*, *PTCH1*, *PTCH2*, *SUFU*, *PTEN*, *TP53*, *CHEK2*, and *GPR161*), according to the manufacturer's protocol (Twist Bioscience, CA, USA). The presence of deletions and duplications in *PTCH1* and *SUFU* genes on peripheral blood was also excluded by multiplex ligation-dependent probe amplification (MLPA) according to the manufacturer's protocol (MRC Holland, Amsterdam, Netherlands).

3. Case report

The patient is a Caucasian male, referred to the Bambino Gesù Children's Hospital at 15 months of age after the removal of a cerebellar mass, histologically compatible with MB at another center. He is the firstborn child to unrelated parents. His family history is free of neurocognitive developmental alterations, his father has intestinal polyposis, his paternal grandfather and uncle died of intestinal cancer; his paternal grandmother died of pancreatic cancer. He was born at 39 weeks of gestational age after an uneventful pregnancy. His birth weight (3,700 gr, 60 percentile, +0.79 SD) and height (50 cm, 20 percentile, −0.9 SD) were normal, while his head circumference was above normal (38 cm, 98 percentile, +3.0 SD). On arrival to the hospital, at the age of 15 months, he presented with macrocephaly (+3.0 SD) and psychomotor delay with major weaknesses related to language skills as detected by Griffiths Developmental Scales.

The surgical removal was fraught with difficulty, despite neuroimaging suggested a superficial, almost extra-axial lesion. The tumor was in fact very hard and bled profusely, to the point of reminding more of a hemangioblastoma, with a complex pattern of intratumoral vessels, than of an MB, which was moreover completely isodense at the pre-operative computed tomography (CT) scan. Complete resection was confirmed by postoperative magnetic resonance imaging (MRI) (Figure 1). Cerebrospinal fluid was free of neoplastic cells.

The histological examination revealed an embryonic neoplasm characterized by the presence of nodular and internodular areas. The nodular areas showed elongated aspects and consisted of neurocytic-type cells immersed in a fibrillar stroma. In the internodular areas, the cells were markedly hyperchromic with frequent mitosis. The immunohistochemical investigation showed a pattern coherent with MB SHH. The cells were positive for synaptophysin in the nodular areas; positivity was observed for GAB1, YAP1, and Filamin A. The proliferation index evaluated with Ki67 was high in the internodular areas (about 30%).

Molecular genetic characterization by NGS was performed on genomic DNA extracted from circulating leukocytes of the patient and unaffected parents to check for the presence of germline variants in high-risk cancer-predisposition genes. None of the genes typically associated with MB (*APC*, *BRCA2*, *PALB2*, *PTCH1*, *SUFU*, and *TP53*) (Waszak et al., 2018) were found to be mutated. Sequence analysis showed a germline heterozygous variant c.79 T > A in the *PTEN* gene

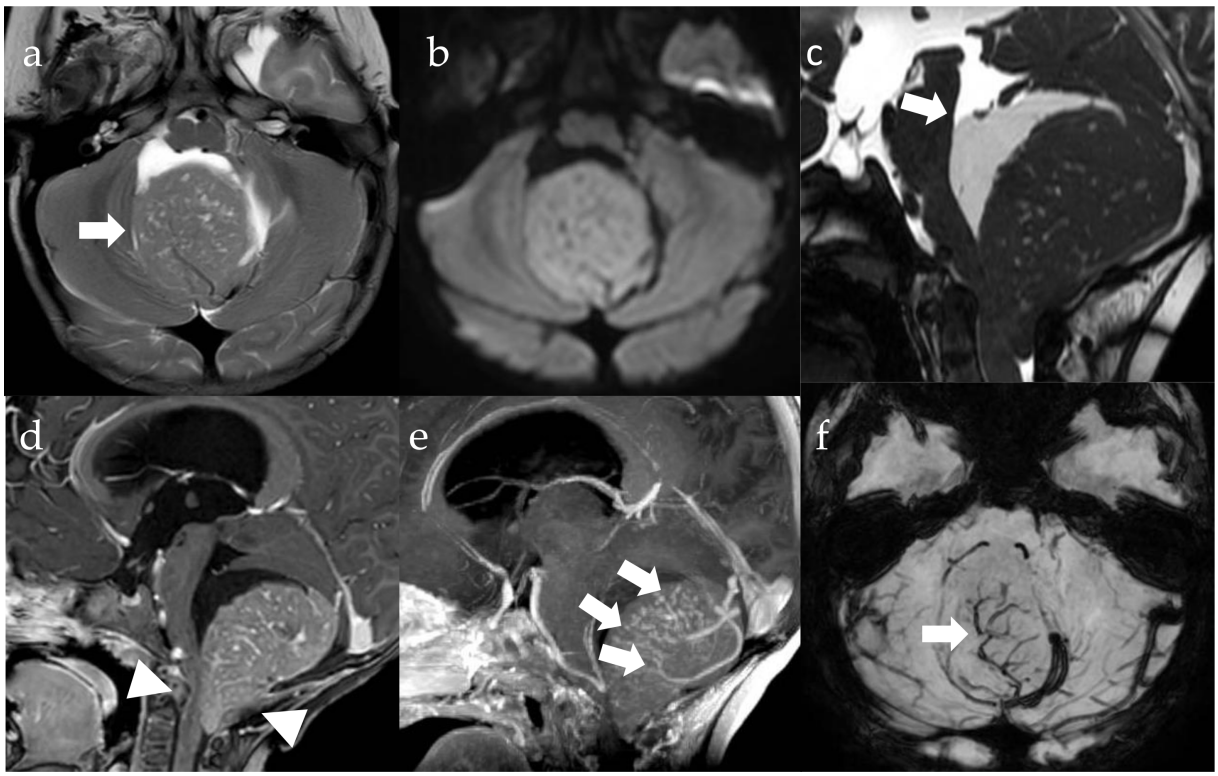

FIGURE 1
MRI imaging. Pre-operative axial T2w **(A)**, DWI (diffusion weighted imaging, **B**) and SWI (susceptibility weighted imaging, **F**) images, sagittal CISS (three-dimensional constructive interference in steady state, **C**) Gd T1w **(D)** and MIP (maximum intensity projection, **E**) images. There is a well-circumscribed lesion in the posterior fossa (**A**, arrow), which is centered on the cisterna magna and pushing the vermis cranially, with growth into the fourth ventricle and extension through the foramen of Magendie onto the posterior aspect of the upper cervical cord (**D**, arrowheads). The tumor is isointense to the cerebellar cortex on T2 **(A)** and shows restriction of diffusivity **(B)** due to high cell density along with high nuclear-to-cytoplasmic ratio **(B)**. There is significant contrast-enhancement **(D)** and intralesional vessels (**E,F**, arrows). Cystic components are appreciable and appear larger in the cranial portions of the lesion (arrow, **D**).

(NM_000314.6) determining the missense change p.Tyr27Asn (rs746128825), previously reported in association with PHTS (Ngeow et al., 2014). This single-base substitution affects the last nucleotide position of the exon 1 and could be a splicing variant. However, further RNA studies are needed to test this hypothesis but are not feasible at present due to sample unavailability. This variant can be classified as pathogenic according to the ACMG criteria (PP3, PP2, PM2, PM1, PM5 and PS2).

Segregation analysis performed on the parents confirmed the *de novo* nature of the variant.

Genetic analysis also revealed the presence of a germline heterozygous variant c.507delT (p.Phe169LeufsTer2, rs587780183) in the *CHEK2* gene (NM_007194.3). The analysis of this gene was recently included in the panel of genes studied in patients with MB at our center, as an association between *CHEK2* and MB is reported in the literature, although not well established (Shah and Walter, 2018). The variant was inherited from the patient's father and has previously been reported as likely pathogenetic, associated with a hereditary cancer-predisposing syndrome (Manoukian et al., 2011).

NGS was also performed on genomic DNA extracted from the tumor sample. Sequencing analysis revealed a somatic variant (allele burden 40%) in *PTEN*, c.388C > G (p.Arg130Gly) in addition to the germline change p.Tyr27Asn (allele burden 48%). This variant has been reported in the literature in individuals with clinical features characteristic of a *PTEN*-related disorder and identified as somatic variant in multiple malignancies (Fusco et al., 2020). The p.Arg130Gly variant affects *PTEN* function abolishing the phosphatase activities (Han et al., 2000).The variant in *CHEK2* was also found in the tumor sample with an allele burden of 46%.

Post-surgical chemotherapy was performed according to the Italian Association of Pediatric Hematology and Oncology MB infant reccomandations. It consisted in three courses of induction chemotherapy (methotrexate 8 g/m2 plus vincristine 1.5 mg/m2 week 0; etoposide 2.4 g/m2 week 1; cyclophosphamide 4 g/m2 plus vincristine 1.5 mg/m2 week 4) and two courses of high-dose thiotepa (300 mg/m2 for 3 days, week 7 and 12) followed by autologous hematopoietic stem cell transplantation (Massimino et al., 2013). Four years after diagnosis, the child is currently in remission from MB.

At 3 years of age, the patient presented with blood and mucus in stools, inappetence, recurrent abdominal pain and weight loss. For these reasons a colonoscopy was performed, and colic polyposis was found (>50 sessile lesions, others pedunculated). Some skin lesions compatible with PHTS (Tan et al., 2011) were also found: punctate

keratosis of the palm-plantar region, hyperkeratotic papular lesions on the back of the hands and feet (trichilemmomas), numerous papular lesions on the back of the feet, periungual and axillary (acrochordon), papillomatous lesions of the oral cavity, and a melanocytic lesions in the abdominal region (compound melanocytic nevus). The child underwent the removal of 30 polyps (diameter 2 cm), during two endoscopic sessions; all polyps were hamartomatous (Figure 2).

At 4 years of age, an additional brain lesion (a right frontal with dural implant amartoma) was diagnosed and removed. The following year (at 5 years of age) severe hypoglycaemia was found and the patient needed to positionate a sensor and initially started feeding with the nasogastric tube. Severe hypoglycemia, although not always present in PTHS, is described in the literature as part of the clinical picture [always linked to *PTEN* regulation of the PI3K-AKT/mTOR pathway (Maines et al., 2021)].

4. Discussion

We present the first known case of a child carrying a germline and somatic pathogenic variant of *PTEN* associated with a germline and somatic variant of *CHEK2*, with a phenotype characterized by macrocephaly, DD, skin lesions, and very early onset of MB SHH and intestinal polyps.

There are only a few other cases with this type of MB-associated mutation that have been described: two pediatric cases, both with MB SHH (Table 1), and two cases of young adults (19 and 23 years) with MB SHH (Gröbner et al., 2018). It should be noted that the latter two patients are part of the series of Gröbner et al. (2018), which also includes a 4 year-old MB G3 patient with a *PTEN* low allele frequencies, in whom genetic analysis was performed only at the somatic level. Several studies have shown that *PTEN* variants are associated in 5% of cases with the development of CNS tumors (Liaw et al., 1997; Lynch et al., 1997; Staal et al., 2002; Sturm et al., 2014; Yakubov et al., 2016; Gröbner et al., 2018; Waszak et al., 2018; Kim et al., 2020), in particular glioblastoma, meningioma, dysplastic gangliocytoma, pineal tumor, and oligodendroglioma. The data currently available in the literature, although scarce, would suggest a possible association with MB as well. In contrast, it is not surprising that all patients with PHTS-associated MB belonged to the SHH subgroup, as more than 40% of pediatric SHH MBs have damaging germline mutations (Garcia-Lopez et al., 2021). The peculiarity of our case lies in the early onset of the brain tumor [presented before the first peak incidence of 3–4 years according to Millard and De Braganca (2016)], and the very early onset of the gastrointestinal manifestations, which usually occur in adulthood. Even cases of juvenile intestinal polyps in patients younger than 12 years with PHTS are rarely reported (Table 2). In fact, to our knowledge, this is the sixth described case of intestinal polyps in PHTS before the age of 12 years, and our patient is the youngest case reported so far. Due to the rarity of pediatric age intestinal manifestations, considering that even the National Comprehensive Cancer Network (NCCN) guidelines recommend "starting at 35 years old, unless symptomatic or close relative with colon cancer under age 40 years" a follow up with colonoscopy was not initially set (Daly et al., 2020).

Nearly 90% of patients with PHTS develop clinical manifestations before 20 years of age, although they may not be diagnosed until 30 years (Kim et al., 2020). There is an increased risk of developing breast or endometrial cancer for women, and thyroid cancer for both men and women. Colorectal cancer is also seen in 9–13% of cases, while polyps are found in 40–60% of cases (Heald et al., 2010). Other cancers were detected in the kidney and skin (Tan et al., 2011; Ngeow and Eng, 2015).

The *PTEN* protein acts as a potent suppressor of oncogenesis by inhibiting the PI3K-AKT/mTOR pathway and regulating cell proliferation and survival (Mester and Eng, 2013). Reinforcing the hypothesis that inhibition of this trail plays a crucial role in tumor pathogenesis. A study recently reported a reduction in hamartomas in patients with PHTS after rapamycin treatment, suggesting that patients with disorders in the *PTEN* hamartoma tumor syndrome spectrum might respond to therapies designed to inhibit the PI3-K/mTOR pathway (Marsh D. J. et al., 2008).

The SHH and PI3K pathways converge to promote the proliferation of granule cell progenitors in the outer granular layer of the cerebellum *in vitro* (Kenney et al., 2004). It has been observed in a mouse model that inactivation of the *PTEN* gene creates an abnormal perivascular proliferative niche in the cerebellum,

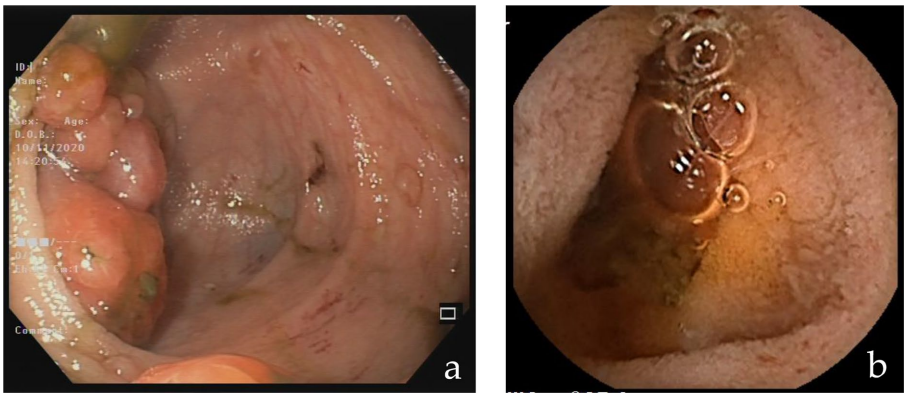

FIGURE 2
Endoscopic picture **(A)**: colon macro-polyp. Capsular picture **(B)**: middle ileum micro-polyp.

TABLE 1 Known pediatric cases of MB with germline variants of *PTEN*.

Age at diagnosis	Sex	Medulloblastoma subtype	Gene variant	Other PHTS features
[a]15 months	M	SHH	*p*.Tyr27Asn	Macrocephaly, DD, bowel polyps, papillomas, trichilemmomas, acrochordon
[b]12 months	F	SHH	*p*.(Thr286ProfsTer5)	Unknown
[c]14 months	F	SHH	*p*.(Glu7Argfs*4)	Macrocephaly

[a]Our case; [b]Waszak et al. (2018); [c]Tolonen et al. (2020); DD, developmental delay; F, female; M, male; PHTS, *PTEN* hamartoma tumor syndrome.

TABLE 2 Known cases of affected by PHTS with bowel polyps' onset before age of 12 years.

Age at diagnosis	Sex	PNET variant	Other PHTS features
[a]3 years	M	*p*.Tyr27Asn	Macrocephaly, DD, papillomas, trichilemmomas, acrochordon
[b]9 years	M	*p*.Phe337Ser	Macrocephaly, lipoma, tongue lesions, penile macules
[b]11 years	M	c.634 + 5G > A	Macrocephaly, lipoma, penile macules
[c]6 years	M	del(10)(q23.2q23.33)	Macrocephaly, DD, penile macules
[d]12 years	M	unknown	Lips polypoid excrescences, tonsillar papillomatosis
[e]4 years	M	del(10)(q23)	Macrocephaly, DD

[a]Our case; [b]Lachlan et al. (2007); [c]Tsuchiya et al. (1998); [d]Gorensek et al. (1984); [e]Hiljadnikova Bajro et al. (2013); DD, developmental delay; M, male; PHTS, *PTEN* hamartoma tumor syndrome.

persistent in adult animals, characterized by undifferentiated cells but without the tendency for malignancy, and in the absence of *TP53* or *PTCH1* codeletion (Zhu et al., 2017). Alterations in *PTEN* could therefore create a predisposing substrate for the development of MB, especially the SHH subgroup. A genomic analysis of medulloblastoma tumors showed that of 13 SHH subgroup patients, 2 had loss-of-function somatic mutations in *PTEN* (Robinson et al., 2012). Of 66 patients profiled from the other subgroups, none had loss of *PTEN*. Another study found a number of *PTEN* mutations in medulloblastoma tumors, one of which co-occurred with a homozygous PTCH mutation (Parsons et al., 2011). In addition, epigenetic inactivation of *PTEN* has been reported to occur at a high frequency in medulloblastoma samples (Hartmann et al., 2006). In our patient, sequencing analysis on tumor revealed the well-characterized loss of function somatic variant of *PTEN* p.Arg130Gly, that together with the germline missense change p.Tyr27Asn likely determines the complete loss of phosphatase activities of the protein, providing a strong evidence that the MB in our patient is associated with PHTS. On the contrary, we did not observe a loss of heterozygosity or the presence of a second deleterious somatic variant in *CHEK2*, suggesting this gene could have a marginal role in the tumorigenesis in our patient.

The increased susceptibility to develop hamartomatous polyps in the gastrointestinal tract is also related to uncontrolled cell growth in patients with *PTEN* mutation, especially subjects with heterozygous *PTEN* deletions developing intestinal epithelial dysplasia with subsequent invasion of the lamina propria, as described in adenoma-carcinoma progression (Marsh V. et al., 2008). The occurrence of bowel polyps has been described especially in patients with overlapping phenotypes between JPS and PTHS. *BMPR1A*, the gene associated with JPS, shares the same chromosomal region as *PTEN* (10q23.2): if large deletions encompass these genes the phenotypic expression can include features of both PHTS and JPS, most typically with juvenile polyposis of infancy (JPI), an aggressive subtype of JPS characterized by severe gastrointestinal symptoms, including diarrhea, intestinal bleeding, rectal prolapse, protein-losing enteropathy with a high risk of intussusception and consequently high infant mortality (Jelsig et al., 2014). The severity of this condition was hypothesized to be due to the loss of these two tumor suppressors, which function in two different but cooperative pathways (Delnatte et al., 2006; Salviati et al., 2006; Menko et al., 2008; Hiljadnikova Bajro et al., 2013). Our case did not present overlapping mutations between these genes, but rather a variant in the *CHEK2* gene.

The frameshift variant in the *CHEK2* gene, related to the *TP53* pathway, has been previously described in an Italian family with hereditary breast/ovarian cancer (HBOC) and is considered to be likely pathogenetic for cancer predisposition syndromes. However, the role of this variant is not yet fully understood, and it might be speculated that it elicits its effect in a context of polygenic inheritance, contributing to cancer risk in association with other susceptibility alleles and increasing the oncological recurrence risk in the family (Manoukian et al., 2011; Teodorczyk et al., 2013).

5. Conclusion

Although the association is rare, the panel of genes to be tested in the presence of an MB SHH could be extended to *PTEN*. The role of *CHEK2*, instead, remains uncertain at this time. The discovery of a *PTEN* germline mutation, even if in childhood, should induce the clinician to promptly provide genetic counseling in order to assess and monitor the occurrence of other PHTS clinical features and set up careful surveillance.

Author contributions

AMC, AM, EA, and LB: conceptualization. AMC and SR: investigation and writing—original draft preparation. AMC, SR, LB, EA, MM, and AntC: data curation. AM, LB, EA, MM, FT, GSC, and AT: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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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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Bridging the age gap: a review of molecularly informed treatments for glioma in adolescents and young adults

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Gliomas are the most common primary central nervous system (CNS) tumors and a major cause of cancer-related mortality in children (age <15 years), adolescents and young adults (AYA, ages 15–39 years), and adults (age >39 years). Molecular pathology has helped enhance the characterization of these tumors, revealing a heterogeneous and ever more complex group of malignancies. Recent molecular analyses have led to an increased appreciation of common genomic alterations prevalent across all ages. The 2021 World Health Organization (WHO) CNS tumor classification, 5th edition (WHO CNS5) brings forward a nomenclature distinguishing "pediatric-type" and "adult-type" gliomas. The spectrum of gliomas in AYA comprises both "pediatric-like" and "adult-like" tumor entities but remains ill-defined. With fragmentation of clinical management between pediatric and adult centers, AYAs face challenges related to gaps in medical care, lower rates of enrollment in clinical trials and additional psychosocial and economic challenges. This calls for a rethinking of diagnostic and therapeutic approaches, to improve access to appropriate testing and potentially beneficial treatments to patients of all ages.

KEYWORDS

gliomas, AYA (adolescents and young adults), WHO CNS5, targeted therapy, BRAF, histone mutations, PI3K-AKT pathway, IDH mutation

Introduction

Gliomas are the most common primary central nervous system (CNS) tumors across all ages (1, 2). The overall incidence rate of gliomas is estimated at 5.81 per 100,000 and is approximately three times higher in older adults compared to young children. In the adolescent and young adult (AYA, ages 15-39 years) group, gliomas constitute 29–35% of all CNS tumors with an incidence of 3.41 per 100,000 (3–5). Gliomas remain a global challenge and improving treatment strategies to reduce mortality and morbidity is a top priority in neuro-oncology. AYA patients are especially vulnerable, and gliomas represent a major cause of cancer-related mortality in this age group. Gains in overall survival rates of AYA patients after cancer diagnosis have been marginal over the last decades, especially for AYAs with CNS tumors compared with other tumor types (6), with some reports suggesting that mortality might in fact be rising for AYAs with gliomas (5, 7).

Clinical management, therapy response and outcome differ significantly between childhood and adult glioma patients. Prognosis of children diagnosed with high-grade gliomas (HGGs) is generally poor, with often limited long-term survival - months to a few years after diagnosis (8). However, prognosis for pediatric patients with low-grade gliomas (LGGs) is excellent in terms of overall survival (9), albeit being associated with high tumor- and treatment-associated morbidity (8, 10). In adults with LGG, the higher rate of malignant transformation [exceedingly rare in children (11)] leads to a poorer prognosis.

Recent advances in molecular profiling have uncovered key oncogenic drivers and distinct glioma entities. Identification of these drivers can improve diagnostic accuracy and facilitate implementation of molecularly tailored treatments. Targeting oncogenic drivers is already a cornerstone of treatment for a subset of glioma patients, most notably those with Neurofibromatosis 1 (NF1) mutations, BRAF fusions and BRAFV600E mutated LGG and HGG (12–15).

The fifth edition of the World Health Organization (WHO) CNS tumor classification (WHO CNS5), published in 2021, introduced several molecular markers to the nomenclature to improve the diagnostic accuracy of CNS tumors (16, 17). Concerning glioma classification, one of the main additions was the distinction between “pediatric-” and “adult-type” gliomas, highlighting the clinical and biological differences across age groups. Gliomas in AYA possess "pediatric-type" and "adult-type" features, but the degree of overlap and the prognostic implications of genetic alterations in AYAs remain poorly characterized (18).

Despite the significant incidence of gliomas in AYAs, they remain an understudied population with specific needs - often unmet due to gaps in clinical care and lack of research focus on this population. Even though the biological features of gliomas in pediatric and adult patients have been described, gliomas in AYA patients have not been characterized extensively yet. Further, barriers to treatment access, lower rates of enrollment in clinical trials, financial insecurities, and paucity of AYA-focused healthcare services also negatively affect the quality of care in AYAs (19, 20).

Here we review the main molecular alterations and their implications for diagnosis, prognosis, and treatment of gliomas across age groups (Figure 1). Highlighting current gaps in knowledge on the AYA population, we discuss targeted approaches currently under clinical investigation for patients with glioma, and potential strategies to improve access to diagnostic testing and biologically-informed treatments for AYAs.

Molecular features of pediatric and adult gliomas

Tumor profiling has revealed a complex glioma molecular landscape (Figure 2). The spectrum of genetic alterations and tumor subtypes is heterogeneous across the age continuum, with some typically diagnosed in children and others in adults (18, 21–25).

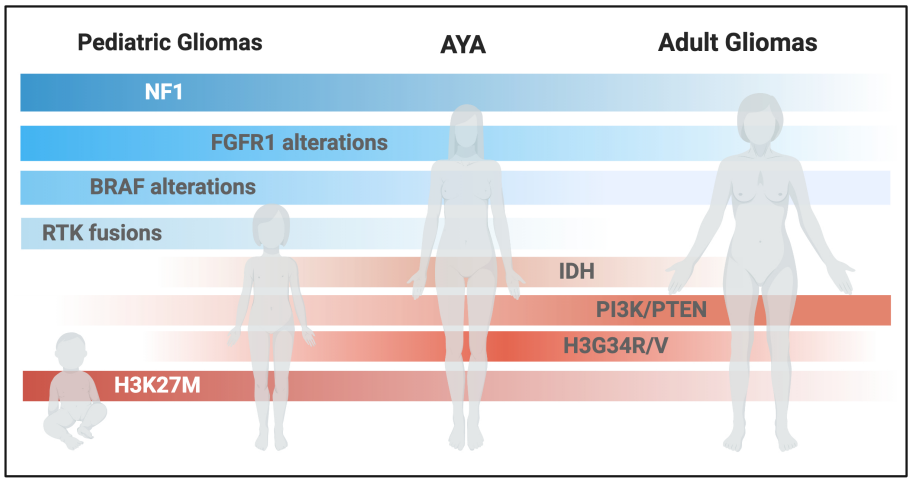

FIGURE 1
Schematic representation of glioma-associated molecular alterations across different ages. (Created with BioRender.com).

Reflecting this disparity, WHO CNS5 groups gliomas into six main entities, including adult-type diffuse gliomas, pediatric-type diffuse LGG, and pediatric-type diffuse HGG. Despite this updated terminology, the patient's age at diagnosis is not a diagnostic criterion. As such, older patients may be diagnosed with pediatric-type tumors and similarly, children may be diagnosed with adult-type tumors. While further research is needed to establish age-specific prognostic implications, we summarize how the WHO CNS5 classification highlights the biological distinctions between pediatric and adult gliomas, and potential implications for AYAs (find some details on prevalence of different types of glioma in AYAs, survival data and prognostic factors listed in Supplemental Table S1).

Pediatric-type gliomas

Pediatric LGGs (PLGG) comprise a variety of histopathologic and molecular entities. Most genetic alterations underlying PLGG development are typically confined within the Ras/mitogen-activated protein kinase (MAPK) pathway, most commonly at the level of the *BRAF* oncogene (25, 26). Several molecular markers were incorporated into the WHO CNS5 classification alongside previously established histological features and immunohistochemistry information.

The glioma family of "pediatric-type diffuse low-grade glioma" includes: "diffuse astrocytoma, MYB- or MYBL1-altered", "angiocentric glioma" (MYB::QKI fusions), "polymorphous low-grade neuroepithelial tumor of the young" (PLNTY, typically harboring FGFR fusions or BRAF alterations) (27), and "diffuse low-grade glioma MAPK pathway-altered" (BRAF alterations, including BRAF::KIAA1549 and BRAFV600E; and *FGFR1* alterations, including point mutations, *FGFR1* fusions and tyrosine kinase domain duplications) (16). This classification expedites diagnosis, highlighting the most common and informative molecular alterations, which should be screened for in the diagnostic workup of low-grade gliomas.

Meanwhile, HGGs are significantly less common in children compared to the adult population, where they represent the largest proportion of primary CNS tumors. HGGs are devastating diseases, associated with poor prognosis and a five-year survival below 20%, accounting for a disproportionate number of cancer-related deaths in children (28). Pediatric HGGs (PHGG) arising in midline structures of the CNS are usually driven by the somatic mutation in histones H3.1 and H3.3 encoding genes resulting in aberrant oncohistone H3K27M protein. Almost 80% of all midline PHGGs harbor H3K27M mutations while the rest exhibit overexpression of EZHIP which mimics mutant histone protein resulting in PRC2 sequestration and thus global hypomethylation (29–31). A subpopulation of PHGGs are also associated with frequent *EGFR* alterations (32, 33) which can be potential treatment targets. Tumors carrying the H3.1K27M mutation usually grow in the pons, as is the case in diffuse intrinsic pontine gliomas (DIPG); whereas H3.3K27M mutations are often identified in tumors growing in the brainstem and also other midline structures, such as the thalamus, representing diffuse midline gliomas (DMG) more generally. Interestingly, tumors with the H3.3K27M mutation are most commonly associated with brainstem location in children, whereas in AYA patients these tumors are often thalamic (18). Another common histone mutation is the H3.3G34R/V, which has been observed mostly in PHGGs of the cerebral hemispheres (23, 34), also prevalent in the AYA population (18). These primary molecularly defined entities are reflected in the WHO CNS5 "pediatric-type diffuse high-grade glioma" family, which includes "diffuse midline glioma, H3 K27-altered", "diffuse hemispheric glioma, H3 G34-mutant," and "diffuse pediatric-type high-grade gliomas, H3-, and *IDH*-wildtype" (16).

A rare subset of pediatric gliomas - infant-type hemispheric gliomas (IHG) - are driven by oncogenic fusions involving the receptor tyrosine kinase (RTK)-encoding genes *ALK, ROS1, MET*, and the *NTRK*-family (16, 35, 36). These fusions are common in gliomas diagnosed in very young children but have also been detected in adolescents and adults (37, 38). Though rare, these are highly targetable alterations and, in the absence of other more common alterations, should also be screened for in older patients (Figure 2).

Adult-type gliomas

In contrast to pediatric-type diffuse gliomas, which are separated into LGG and HGG, this distinction is not made for adult-type gliomas (16). In adult-type diffuse gliomas - the most common malignant primary CNS tumor in adults - one main molecular feature with prognostic implications is the isocitrate dehydrogenase (*IDH)1* or *IDH2* mutation status (Figure 2). Adult-type diffuse gliomas are thus subdivided into "astrocytoma, *IDH*-mutant", "oligodendroglioma, *IDH*-mutant, and 1p/19q-co-deleted", and "glioblastoma, *IDH*-wildtype". One important change, compared to the previous WHO CNS4 classification, is that glioblastoma is a more restricted diagnosis, encompassing diffuse and astrocytic *IDH*-wildtype tumors, typically harboring *TERT* promoter mutation and/or *EGFR* gene amplification and/or +7/−10 chromosome copy number changes. Further important molecular features implemented in the WHO CNS5 classification of gliomas include co-deletion of 1p/19q (oligodendroglioma, WHO grade 2-3), homozygous *CDKN2A/B* deletion (astrocytoma, *IDH*-mutant, WHO grade 4), as well as the presence of alterations in *MYB*, *MYBL1*, *MN1*, *YAP1*, *MYCN*, *FOXR2*, *BCOR*, *SMARCB1*, *FET-CREB*, and *DICER1* (39). In addition to the molecular classification, the presence/extent of necrosis and microvascular proliferation are still used for WHO grading (WHO grade 1-4).

Other gliomas

Alongside "pediatric-" and "adult-type gliomas", the WHO CNS5 includes further glioma tumor families: "circumscribed astrocytic gliomas" (including pilocytic astrocytoma, high-grade astrocytoma with piloid features, pleomorphic xanthoastrocytoma, subependymal giant cell astrocytoma, and astroblastoma, *MN1*-altered), as well as a heterogenous group of "glioneuronal and neuronal tumors".

Cancer predisposition syndromes and germline testing in AYA

Cancer predisposition is another important factor to consider when evaluating AYA patients with glioma. In the pediatric

population, there is a higher incidence of germline events associated with cancer predisposition. These are detected in approximately 10% of children and adolescents with cancer overall (40, 41), often with profound implications for patients and families. Though population-based data on prevalence of pathogenic germline mutations in AYAs with glioma are lacking, screening and genetic counselling should be considered, especially when family history or the presence of a somatic mutation potentially associated with cancer disposition raise suspicion for an inheritable alteration. A broad spectrum of cancer predisposition syndromes can be associated with gliomas, especially HGGs, including Li-Fraumeni syndrome (*TP53* mutation) and the germline DNA replication deficiency syndromes constitutional mismatch repair deficiency (cMMRD) syndrome and Lynch syndrome. Accurate diagnosis of constitutional mismatch repair deficiency (CMMRD)- and Lynch-associated hypermutant HGGs is critical, not only due to implications for family and tumor surveillance, but for treatment (see immunotherapy section below). For LGGs the most important cancer predisposition syndrome is NF1 leading to mainly optic pathway gliomas in 15-20% of the affected children (42).

Biologically informed therapies

Beyond the implications for accurate tumor classification, the detection of molecular markers can facilitate access to targeted therapies. As such, appropriate molecular profiling as part of routine diagnostic testing in AYAs is the first key step, to improve the implementation of the biologically informed therapies. Several strategies targeting molecular vulnerabilities are undergoing development and optimization for glioma therapy, though typically not with a focus on AYA population. As such, in this section we review new therapeutic approaches which may be of benefit to AYA patients, despite current extensive gaps in knowledge in this population.

BRAF/MEK inhibitors

BRAFV600E mutation and *BRAF* fusions are key drivers of pediatric LGGs (25, 43–46) and the BRAFV600E mutation is detected in a subset of pediatric and adult HGGs. With recent implementation in clinical use, BRAF and MEK inhibitors are increasingly used in treatment of pediatric and adult patients with glioma (13, 47). Vemurafenib and dabrafenib are BRAF inhibitors proven to be safe and successful in the treatment of BRAFV600E-mutated LGG in children and adults, as monotherapy, or in combination with MEK inhibitors (14, 48–50). Patients with BRAFV600E-mutated HGGs also show response to treatment with BRAF inhibitors but it is insufficient as monotherapy for cure in these patients. A randomized trial assessing the overall response rate (ORR) and tolerability of treatment with dabrafenib and trametinib versus carboplatin and vincristine in a pediatric population with BRAFV600E-mutant LGGs revealed a higher ORR, longer progression-free survival (PFS), and fewer adverse events (51). Combining MEK and BRAF inhibition also showed meaningful responses in adult BRAFV600E-mutant LGG and HGG (14).

The MEK inhibitor selumetinib has shown significant anti-tumor activity in progressive NF1-mutated and BRAF-altered PLGGs (52–54). Another MEK inhibitor, trametinib, has also been studied and proven active in patients with progressive PLGG (15, 55). Questions remain regarding optimal duration of treatment, outcomes compared with conventional chemotherapy and potential combination with other established treatment regimens. Ongoing studies are expected to answer some of these questions, including trials comparing the upfront use of selumetinib vs carboplatin/vincristine (NCT03871257), as well as a comparison of selumetinib monotherapy vs selumetinib in combination with vinblastine in patients with progressive LGGs (NCT04576117). Though designed with the pediatric population in mind, both trials allow the inclusion of young adult patients.

The pan-RAF inhibitor tovorafenib (DAY101) is being investigated in an open-label, multi-center, international phase II study (FIREFLY-1) in patients between the ages of 6 months and 25 years with BRAF-altered recurrent or progressive LGGs. The promising results from the registrational arm show an ORR of 64% with a clinical benefit rate (CBR) of 91% (56). Another ongoing trial LOGGIC/FIREFLY-2 is comparing tovorafenib monotherapy to standard of care chemotherapy in patients with PLGG harboring a RAF alteration requiring front-line systemic therapy (NCT05566795). As for FIREFLY-1, this trial also allows for inclusion of young adult patients, up to 25 years of age.

FGFR inhibitors

Genetic alterations in *FGFR* such as point mutations or chromosomal rearrangements can occur in PLGG, whereas in adults they are more commonly detected in high-grade tumors. Emerging reports suggest that they are frequently encountered in AYA, in up to 16% of IDH-WT AYA gliomas (57). Data from this large cohort of FGFR-altered gliomas, encompassing patients aged 6 months - 87 years, fusions were more common in pediatric patients, while point mutations were more common in AYA patients. Most (87%) pediatric tumors had low-grade histology, whereas in AYA this percentage was lower (67%) and in older adult patients FGFR-altered tumors were typically high-grade. While the clinical and prognostic implications of these findings are still under investigation, this study highlights the importance of cross-age studies to uncover the landscape of molecular alterations in AYAs.

Several FGFR inhibitors have been tested in pediatric and adult patients with glioma, including erdafitinib (58) and the FGFR1–3 inhibitor infigratinib (59), which was investigated in a multicenter phase II study in patients with recurrent gliomas and FGFR alterations. Despite a low ORR of 3.8%, 4 patients had prolonged disease control (59). A pediatric study testing the oral FGFR inhibitor Debio1347 on a small cohort of 3 PLGG patients and 2 PHGG patients detected some responses (60), whereas none were observed in adult patients with HGG. Despite relatively low response rates, these early findings suggest that some patients might have durable responses to FGFR inhibition. It is likely that

specific FGFR alterations and/or the presence of other concomitant alterations dictate response to FGFR inhibitors. Further studies are needed to explore these and other open questions but, given the high prevalence in AYA and positive responses in some pediatric patients, FGFR alterations should be screened for and targeted approaches considered in this patient population.

HDAC inhibitors

Histone deacetylase (HDAC) inhibitors are increasingly used to treat H3K27M-altered DMGs and DIPGs. At a molecular level, mutated H3K27M induces an inhibition of the H3K27me3 methyltransferase complex, Polycomb repressive complex 2 (PRC2), leading to increased histone acetylation and decreased histone methylation. This global alteration of epigenetic marks results in increased expression on oncogenic programs. HDAC inhibitors have been developed with the goal to enzymatically remove histone acetyl groups from the genome under tumorigenic circumstances. One of the HDAC inhibitors under clinical evaluation for DIPGs/DMGs is panobinostat, which has also been used to treat many other cancer types. Treatment with panobinostat lead to an increase in histone acetylation, demonstrating biological activity. The therapy is generally well tolerated, despite up to 30% pediatric patients showing thrombocytopenia and anemia (61, 62). Seven children and adolescents (5-21 years) with newly diagnosed DIPG received repeat doses of convection enhanced delivery (CED) with MTX-110 (aqueous panobinostat) in the PNOC015 trial which was tolerated well. Most toxicities patients experienced were of neurological etiology. Compared with historical controls, the OS with a median of 26.1 months was encouraging but due to the limited number of participants must be interpreted with caution (63). New HDAC inhibitors are under clinical investigation to overcome the drawbacks from panobinostat, among them, quisinostat and romidepsin. Recent studies have demonstrated the efficacy of quisinostat and romidepsin in preclinical DMG models, with good BBB penetration and inhibition of tumor growth (62).

Imipridones

Imipridones are small inhibitor molecules that have shown anti-tumor effects with promising results for several cancer treatments (64). ONC201 is a type of imipridone for which the anti-tumor effects are still being investigated and which has shown efficacy in hematological malignancies (65), as well as in H3K27M DMGs in combination with radiation (66). ONC201 was first discovered during its involvement in activating the TNF-Related Apoptosis Inducing Ligand (TRAIL)-pathway and the integrated stress response (ISR)-pathway, which are important modulators in balancing both cell survival and cell death (66, 67). ONC201 works as an antagonist for the dopamine receptors DRD2 and DRD3, both belonging to the G-protein coupled receptor family. ONC201 crosses the BBB and blocks DRD2, resulting in the activation of the ISR-pathway, TRAIL-induced apoptosis and inhibition of the AKT/ERK pathway (67). Another trial with ONC201 is ongoing for adult patients with recurrent, mainly thalamic (location in the pons or spinal cord excluded) H3K27M glioma (NCT03295396). The results of this trial will contribute to our knowledge on treating these tumors in AYA patients as H3K27M mutated gliomas are mainly located thalamic in AYAs. A new derivate of the ONC201 imipridone, ONC206, has been shown in preclinical studies (68) to be more potent than ONC201 and is currently under clinical investigation in children and young adults (up to 21 years of age) with DMG or other recurrent high-grade CNS tumors (NCT04732065). Both drugs bind to and activate the mitochondrial serine protease ClpP (caseinolytic protease proteolytic subunit), leading to mitochondrial damage, release of reactive oxygen species, activation of ISR-pathway, and apoptosis (68–70).

H3K27M DMGs are universally associated with dismal prognosis and, though affecting mostly pediatric patients, they are also prevalent in the AYA and adult population. Given the lack of curative and treatment options, there is a strong rationale for the design of age-inclusive clinical trials for DMGs.

PI3K/mTOR inhibitors

Overactivation of the PI3K/mTOR pathway - through the presence of activating mutations (e.g. in *PIK3CA*), loss of the negative regulator *PTEN*, and/or activation of upstream receptor tyrosine kinase receptor signaling - underlies tumor growth and is a key oncogenic driver in most human cancers, including gliomas. As such, targeting the PI3K/mTOR pathway, either using a monotherapy or combinatorial approach, is a strategy that has been amply explored. The mTOR inhibitor everolimus is used to treat several CNS tumor entities. A well-known indication for therapy with everolimus is the presence of relevant, unresectable subependymal giant cell astrocytomas (SEGAs) in patients with tuberous sclerosis complex (TSC) (71, 72). Patients with TSC and associated SEGA treated with everolimus typically show a significant reduction in tumor size and a significant reduction of seizure frequency (73). Also, children with recurrent/progressive NF1-associated LGGs showed good responses to everolimus (74). Due to the known common activation of the PI3K-pathway in DIPG, everolimus was included as one of the drugs tested in the biomarker-driven platform trial BIOMEDE (NCT02233049) for children and young adults (up to 25 years of age) (75). Everolimus showed a trend towards better efficacy (not statistically significant) when compared to erlotinib and dasatinib, with a good toxicity profile.

Paxalisib is a PI3K-inhibitor under clinical investigation, which has shown encouraging responses in adult patients with recurrent HGGs (76, 77). Paxalisib is also being evaluated for safety and efficacy in HGGs, including DIPG/DMG in combination with ONC201 (NCT05009992) (78, 79).

NTRK/ALK inhibitors

Several inhibitors have been developed targeting neurotrophic tropomyosin kinase receptors (NTRK) and/or anaplastic

lymphoma kinase (ALK)-fusion proteins (80). Second-generation ALK inhibitors, such as alectinib and brigatinib have been designed with an enhanced BBB penetration to treat ALK-driven non-small cell lung cancer (NSCLC) with CNS metastasis (81). Lorlatinib, a third-generation ALK inhibitor with enhanced BBB penetration, has shown efficacy in several pediatric and adult malignancies, including in a child with ALK-fused infant-type hemispheric glioma (IHG) (82).

The first-generation TRK inhibitor larotrectinib has been approved for treatment in adult and pediatric patients with NTRK-fused CNS tumors (83). Entrectinib has also shown activity against NTRK-, ROS1-, and ALK-fused malignancies, especially in adults with NSCLC with CNS metastases. Entrectinib was approved in 2019 by the FDA to treat children >12 years old and shown to have positive anti-tumor activity both in adult and pediatric patients with NTRK- and ALK-driven CNS tumors (84, 85).

IDH inhibitors

Tumor-driving isocitrate dehydrogenase (*IDH)* mutations have been identified in different types of cancer, leading to the development and implementation of several *IDH* inhibitors in clinical practice. As adult-type gliomas commonly harbor *IDH1* (and less commonly, *IDH2*) mutations, testing the efficacy of IDH inhibitors in these tumors has become a research focus in recent years.

Ivosidenib (AG-120), an *IDH1* inhibitor, was tested in *IDH*-mutant solid cancers and is being evaluated for efficacy in *IDH1*-mutant LGGs in adults. The BBB-penetrant *IDH1* inhibitor DS-1001b was evaluated in a phase I clinical trial in adult patients with *IDH1*-mutant recurrent/progressive glioma with promising results (86). Vorasidenib, an inhibitor of mutant *IDH1* and *IDH2*, was investigated in adult patients with IDH-mutant WHO grade 2 gliomas in a randomized phase III trial. Treatment with vorasidenib prolonged PFS compared to placebo-treated patients. Furthermore, the time to next therapeutic intervention was significantly longer in patients receiving vorasidenib compared to the placebo group (87).

IDH mutations are rare in the pediatric population but detected in up to 35% of glioma adolescent patients aged 14 years or older (88). This calls for a lower age of inclusion and/or AYA-focused trials (such as NCT03749187) evaluating the role of IDH inhibition in gliomas also in adolescent patients.

EGFR inhibitors

Epidermal growth factor receptor (EGFR) gain of function, due to amplification or the presence of its active mutant EGFRvIII, is common in adult patients with HGGs, exceedingly rare in pediatric and rare in adult patients under 35 years of age (89). As such, most clinical trials developed over the last decades focused on the adult/older adult patient population. Multiple biological agents targeting EGFR, including tyrosine kinase inhibitors (e.g. gefitinib, erlotinib), monoclonal antibodies (e.g. cetuximab), antibody-drug conjugates (e.g. depatuxizumab mafodotin), as well as immunotherapeutic approaches, such as anti-tumor vaccines and EGFRvIII-specific chimeric antigen receptor (CAR) T cells, have been tested in adult glioma patients, with generally underwhelming results (90–93). The reasons for treatment failure are multifactorial and include mechanisms leading to target independence (through alteration of the structure or loss of target expression), activation of alternative signaling pathways, and limited agent distribution due to BBB's properties (94–96).

Combination treatment of osimertinib and bevacizumab was explored in patients with tumors harboring EGFR amplification and EGFR variant III mutations but as the study cohort was small (15 patients), further evaluation is needed (97). Tesevatinib is a second-generation tyrosine kinase inhibitor that crosses the BBB and targets EGFR, human epidermal growth factor 2 (HER2)/neu, and Src, currently in phase II clinical trials (NCT02844439) (98).

Immunotherapies

Another growing field with new treatment options for (high-grade) glioma is immunotherapy. Based on success in hematological malignancies and other solid tumors, expectations to identify immunotherapies which are effective for gliomas were built up in the past few years (34, 99, 100). Immunotherapeutic approaches include checkpoint inhibitors, cellular immunotherapy, anti-tumor vaccines and oncolytic viruses.

Drugs targeting the immunoregulatory checkpoint proteins programmed cell death protein 1 (PD1) and its ligands PD-L1 and PD-L2 and cytotoxic T-lymphocyte-associated-protein 4 (CTLA-4), which inhibit T-cell-mediated response of the patients' immune system have been tested in clinical trials. Several of these clinical trials evaluating checkpoint inhibitors so far did not show significantly prolonged OS or PFS in pediatric and adult HGG and glioblastoma patients (101–105).

In a phase II clinical trial (Ipi-Glio trial) comparing the efficacy of ipilimumab and temozolomide versus temozolomide alone in adults with newly diagnosed glioblastoma, no difference in PFS or OS was observed (106).

The exception to this is patients with cMMRD or Lynch syndrome associated HGGs (107). These patients are unlikely to respond to temozolomide, which requires an intact MMR system for activity. After early reports suggested a benefit for patients with cMMRD-associated hypermutant HGGs treated with immune checkpoint inhibitors (108), further studies confirmed objective responses and a three-year survival of 41.4% (107). AYA patients are more likely to be diagnosed with Lynch syndrome (monoallelic germline pathogenic variants in mismatch repair genes), given that patients with cMMRD (biallelic germline pathogenic variants in MMR genes) are typically diagnosed with tumors at young age. Though Lynch syndrome-associated hypermutant tumors have a lower mutational burden compared to cMMRD-associated tumors, especially those with concomitant polymerase proofreading deficiency (genomic predictor of response to PD-1 inhibition), there are objective responses to immune checkpoint inhibitors in these patients.

CAR T cells have revolutionized treatment of refractory hematologic malignancies, but are not yet established for solid and CNS tumors (109, 110). Several targetable antigens have been identified in adult and pediatric HGG, including Ephrin-A2 (EphA2)-receptor, human epidermal growth factor receptor 2 (HER-2), B7-H3 (CD276), interleukin-13 receptor subunit α-2 (IL13Rα2), and glycolipid tumor antigen 2 (GD2) (111, 112). For both adult and pediatric HGG patients several clinical trials with different treatment strategies have been carried out and are still ongoing. Out of 16 evaluable patients (adults and children/adolescents), eight showed a clinical benefit (partial response or stable disease) to treatment with intravenous HER-2- (and pp65)-targeted CAR T cells and treatment was considered to be safe (113). Clinical trials testing HER-2-directed CAR T therapy in children with CNS tumors, EGFR-directed CARs for children and AYAs with CNS tumors and B7-H3-specific CAR Ts in patients with DIPG/DMG or refractory pediatric CNS tumors are ongoing (NCT03500991, NCT03638167 and NCT04185038). For H3K27M-altered DIPG/DMG, GD2-CAR T cells (114) and B7-H3 CAR T cells are currently under clinical investigation with promising preliminary results (115).

Vaccination has been a focus of immunotherapy research for three decades. In a randomized trial, rindopepimut, a peptide vaccine targeting EGFRvIII-positive glioblastoma in adults did not prolong survival (92). More recent developments include vaccines targeting histone H3 mutations. In a trial with patients aged 3-21 years, patients with H3.3K27M-specific CD8+ immunological responses had longer OS compared to non-responders.

Oncolytic viruses are (re-)emerging as important immunotherapeutic options, especially for pediatric and young adult patients with DIPG/DMG. Of 9/12 children with DIPG treated with the oncolytic adenovirus DNX-2401 a reduction in tumor size was documented, making this treatment another interesting development for these very high-risk tumor entities (116). On the other hand, 49 patients with recurrent glioblastoma treated with intratumoral delivery of the oncolytic DNX-2401 virus followed by intravenous pembrolizumab did not develop any dose-limiting toxicities but treatment also did not result in a statistically relevant increase of the overall response rate (117).

Discussion

The WHO CNS5 introduced the distinction between pediatric-type and adult-type gliomas, highlighting the biological differences between tumors in these age groups. This sets the stage for further research and therapy developments, tailored to the specific needs of the pediatric and adult populations. While this will certainly be beneficial and support a focus on age-relevant research questions for those patient groups, there is a concern that AYAs will remain poorly defined, "unseen" and medically underserved.

Understanding the longitudinal overlap and glioma evolution from childhood to adulthood is an important research gap. The prevalence and prognostic impact of molecular alterations in AYA gliomas is largely unknown. While medicine in general, and oncology in particular, evolve towards biologically-informed treatment, this lack of knowledge on AYA gliomas has critical consequences. Gliomas represent a significant cause of cancer-related morbidity and mortality in AYAs and survival gains for these patients have been minimal to non-existent, with some studies suggesting that mortality might in fact be rising (5, 7).

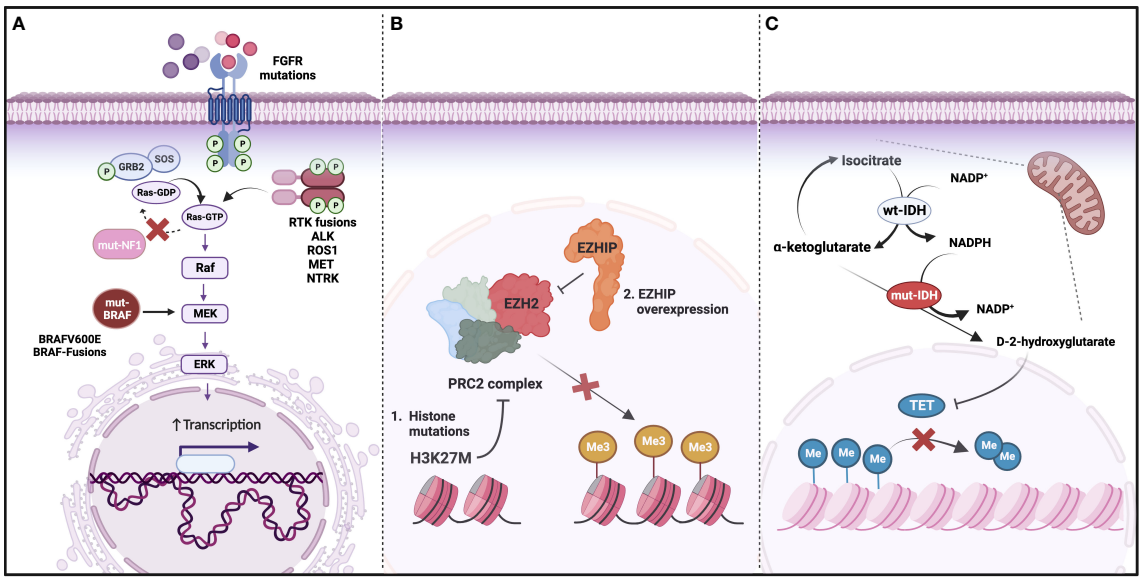

FIGURE 2
Main molecular drivers of glioma. **(A)** Genetic alterations activating the Ras/MAPK pathway, including loss of function mutations in *NF1* and gain of function mutations or fusions in *BRAF* and Receptor Tyrosine Kinases (RTKs); **(B)** DNA hypomethylation as a result of Polycomb repressive complex 2 (PRC2) inhibition by H3K27M or *EZHIP* overexpression (mutually exclusive); **(C)** IDH mutations leading to an accumulation of D-2 hydroxyglutarate and decrease in TET-mediated DNA demethylation. (Created with BioRender.com).

Treatment optimization, including implementation of targeted therapies, starts with the adoption of appropriate molecular testing as part of the diagnostic work-up, for biomarker identification. Given the pediatric versus adult focus of WHO CNS5, recent consensus statements and recommendations from experts in the field are key in ensuring appropriate and timely diagnostic testing for AYA patients (118, 119).

Even though the molecular features vary between pediatric, adult, and - most likely - AYA gliomas, these tumors also share common tumorigenic pathways, including overexpression of oncogenes, activation of RTKs, epigenetic dysregulations, and increased metabolic pathways, which should be explored for introducing new therapies in age-inclusive clinical trials. As discussed above, several pediatric studies and study consortia are starting to increase the upper limit of age of inclusion, to allow enrollment of young adults with "pediatric-type" diseases, a much-needed step to increase access to innovative therapies for AYAs.

Currently, clinical management of AYA patients is highly fragmented between pediatric and adult centers, which can further limit access to therapy due to lack/disconnected information exchange between health care practitioners. To bridge this gap and offer this vulnerable group of patients better treatment options, exchange of expertise and close collaboration between pediatric and adult neuro-oncologists - and broader multidisciplinary clinical teams - is indispensable. Several centers are implementing regular joint case discussions within dedicated tumor boards, to improve the quality of care for AYA patients and increase inclusion of AYA patients in clinical trials.

Furthermore, it is important to also consider the socioeconomic and mental health burden that AYA patients experience. Due to prognostic uncertainty and treatment limitations, AYA patients report being under long-term stress due to lack of control over their future, feeling burdened, and social isolation (120). Support from specialized social workers, physical therapists and psychologists, ideally in AYA-focused treatment facilities, would contribute to advise, guide, and support AYAs during and after tumor therapy. Specialized departments also offer the possibility to connect with other patients in similar age groups, and tailored activities, such as physical activities/sports for AYA patients.

Conclusion

There is still much to learn about gliomas in AYAs and much to do to improve clinical care and treatment. The growing awareness and identification of specific gaps in knowledge is a step in the right direction and hopefully broader changes will follow. Ensuring access to appropriate molecular testing to detect key biomarkers, designing age-inclusive clinical trials for gliomas and creating multidisciplinary teams, bridging the pediatric/adult divide, are some of the many actions needed and being implemented in several centers across the world. Further, research focusing on AYAs should be encouraged and supported, to bring new insights into tumor biology in this population.

Author contributions

AW: Writing – original draft, Writing – review & editing. ASB: Writing – original draft, Writing – review & editing. CM: Writing – original draft, Writing – review & editing. JB: Writing – review & editing. PR: Writing – original draft, Writing – review & editing. RR: Writing – review & editing. JN: Writing – original draft, Writing – review & editing. AGS: Writing – original draft, Writing – review & editing.

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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.2023.1254645/full#supplementary-material

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DNA methylation-based diagnosis confirmation in a pediatric patient with low-grade glioma: a case report

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Central nervous system (CNS) tumors in children comprise a highly heterogenous and complex group of diseases. Historically, diagnosis and confirmation of these tumors were routinely based on histological examination. However, recently obtained data demonstrate that such a diagnostic approach is not completely accurate and could lead to misdiagnosis. Also, in recent times, the quantity and quality of molecular diagnostic methods have greatly improved, which influences the current classification methods and treatment approach for pediatric CNS tumors. Nowadays, molecular methods, such as DNA methylation profiling, are an integral part of diagnosing brain and spinal tumors in children. In this paper, we present the case of an infant with a posterior fossa tumor who demonstrated a non-specific morphology and whose diagnosis was verified only after DNA methylation.

KEYWORDS
children, pediatric oncology, CNS tumors, pilocytic astrocytoma, DNA methylation, Sanger sequencing, molecular diagnostics

Introduction

Central nervous system (CNS) tumors are a highly heterogenous group of diseases, and their accurate pathological and molecular diagnosis is crucial for providing optimal treatment. However, the standardization of the diagnostic process remains challenging. Methylome profiling is a novel molecular approach that may have a substantial impact on tumor identification and may also be used as a surrogate marker for tracing genetic events (1). Data collected from the literature confirm that the availability of this method may lead to a change in diagnosis in up to 12% of prospective cases (2). Incorrect diagnosis will lead to the wrong therapeutic strategies and could deteriorate patient outcomes.

In this study, we present a challenging clinical case of a patient with a posterior fossa tumor and a complicated diagnostic pathway. The right diagnosis in this case was established only after DNA methylation profiling of the tumor, which allowed us to choose the right therapeutic strategies and treat the patient appropriately.

Case description

A 5-month-old Caucasian girl presented to our pediatric department with a history of frequent regurgitation, loss of appetite, and macrocephaly. A brain MRI with contrast

enhancement revealed a posterior fossa tumor with invasion into the lateral and third ventricles, called obstructive hydrocephalus (Figure 1). For relief from hypertension, the patient underwent ventriculoperitoneal shunting.

For subsequent treatment, the patient was admitted to the Department of Pediatric Neurosurgery of the Almazov National Medical Research Centre, where surgical removal of the cerebellar and fourth ventricle tumors was performed. A postoperative brain and spinal MRI with contrast enhancement was performed 48 h after surgery. A brain MRI showed hydrocephalus, residual tumor in the vermis, patterns of restricted diffusion, and pathological contrast accumulation in the walls of the resection cavity up to 1 mm, in the pia mater on the back of the brain stem, and the area of the right and left lateral apertures (Figures 2, 3). Spinal MRI with contrast enhancement revealed a thickening of the shell-like dura mater up to 2-4 mm, intensive contrast accumulation along the whole spine, and irregular contrast accumulation of the pia mater (Figure 4).

A morphological examination of the tumor sample showed fragments of a polymorphic tumor. Areas of small cells having hyperchromatic nuclei with Homer–Wright rosettes and fields of larger cells with optically scant cytoplasm (neurocyte-like cells) were detected, and an increased number of mitoses and blood vessels were also seen. An immunohistochemistry analysis (IHC) showed positive staining of beta-catenin, filamin, glial fibrillary acidic protein (GFAP), synaptophysin, positive nuclear staining of INI1 and p53 (5%), and GAB1-negative staining. The rate of the Ki-67 proliferation index was 20% (Figures 5, 6). From the collected morphological and IHC data, a pathological diagnosis of medulloblastoma was made. Following local clinical practice, the material was sent for reference diagnostics, which normally take 10–14 days.

The data collected from pathological diagnosis and MRI were in conformance with the medulloblastoma of the cerebellar vermis and the fourth ventricle, R + M3 stage, according to the Chang Staging System. To prevent deterioration of the patient's condition, adjuvant chemotherapy was started, and one cycle of intensified induction was performed in accordance with HIT-MED 2014 (version 5.1, 2020).

Tumor fragments with medium and high cellularity and proliferating vessels were described in accordance with the reference histological examination. Cells were polymorphic with an oligodendro-like morphology with a round nucleus and an optically scant cytoplasm, predominantly with high mitotic activity (Figure 7). IHC analysis revealed positive cytoplasmatic beta-catenin, S100, GFAP, weak-positive p53, and negative CD34, OTX2, NSE, and chromogranin A staining (Figures 8, 9). The Ki-67 index rate ranged between 10% and 15%. The final pathological diagnosis was diffuse high-grade glioma.

Since the diagnosis was changed, a third reference histological examination was performed. Intermediate-intensity chemotherapy (vincristine and cyclophosphamide) was continued until the results were obtained. A microscopic examination revealed a tumor composed of cells with an optically scant cytoplasm and thin proliferating vessels. Dense-packed cells, mitosis, and structures resembled perivascular rosettes, and areas of small cells with

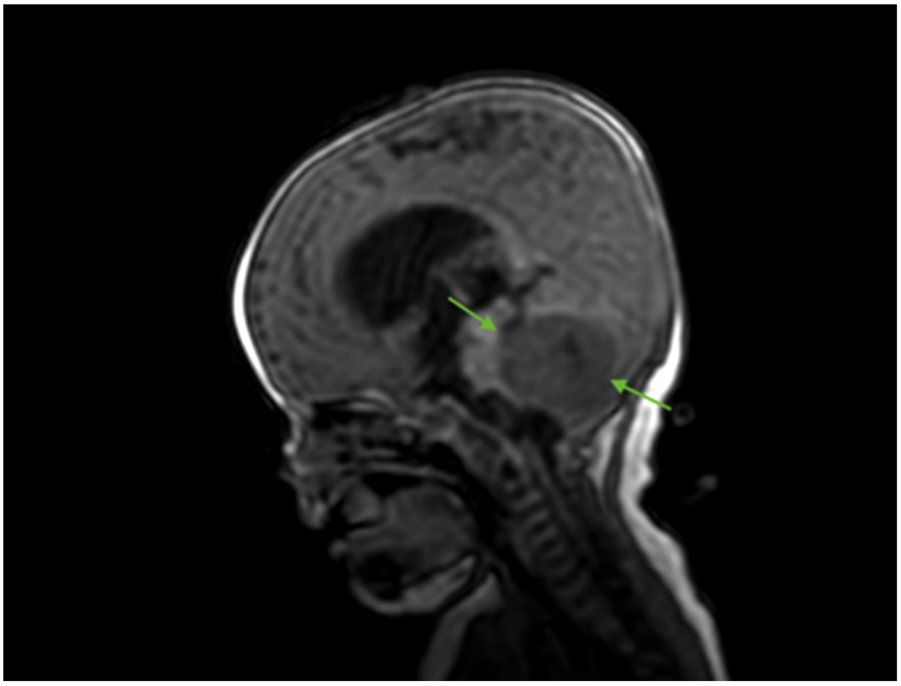

FIGURE 1
Brain MRI (sagittal T2 + C). Preoperative MRI shows a posterior fossa tumor (green arrows).

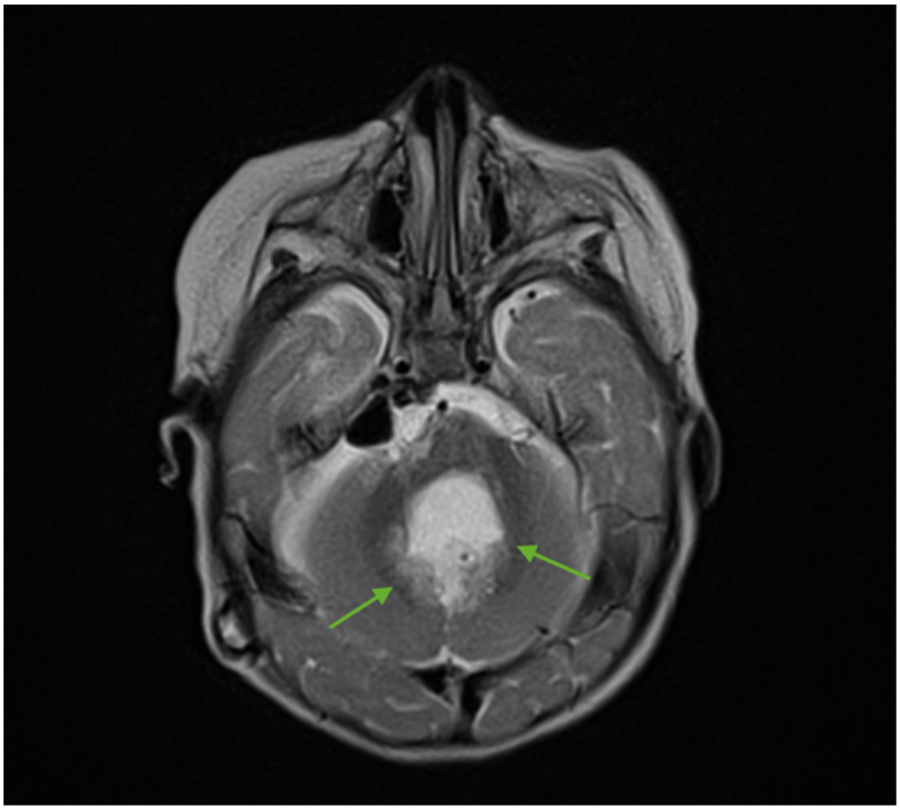

FIGURE 2
Brain MRI (axial T2 + C). Postoperative changes, pathologic contrast accumulation in the walls of the resection cavity, and residual tumor in the vermis (green arrows).

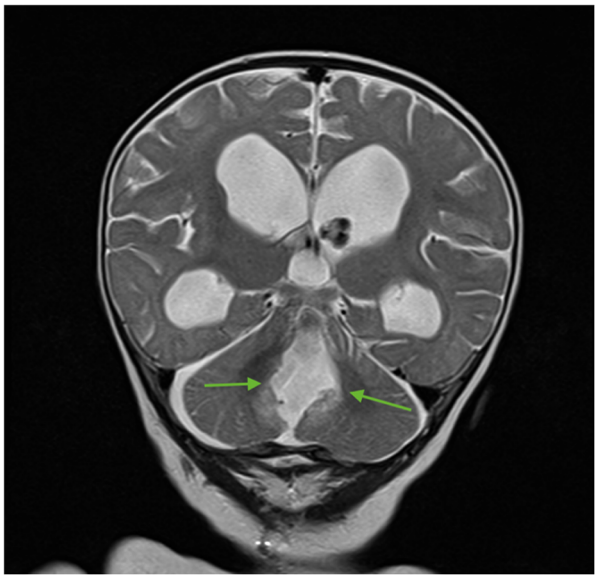

FIGURE 3
Brain MRI (coronal T2 + C). Postoperative changes, pathologic contrast accumulation in the walls of the resection cavity, and residual tumor in the vermis (green arrows).

astrocytic differentiation were described (Figure 10). IHC analysis revealed positive staining for GFAP, focal EMA expression, and a high Ki-67 index, which could be attributed to the young age of the patient (Figures 11–13). Olig2 immunostaining, which could be helpful in making a differential diagnosis between glioma and ependymoma, was not performed because of the absence of this antibody in the laboratory at that time. With Olig2 immunostaining, a third possible diagnosis —anaplastic ependymoma or, less likely, pilocytic astrocytoma, was considered. For reference diagnostics, it is necessary to underline that the same tumor sample was used.

Considering the discrepancies in the diagnosis in three reference centers and the limited number of tumor samples, a decision was made to perform a molecular investigation of the tumor sample with DNA methylation profiling using the Illumina NextSeq 550 (Illumina Inc, USA) using Illumina Infinium MethylationEPIC BeadChip kit. An analysis of the results was conducted on the platform MolecularNeuropathology.org using version 11b4/version 12.5 of the brain classifier. The analytical results using v11b4 were interpreted as methylation class low-grade glioma and subclass posterior fossa pilocytic astrocytoma, whereas the analytical

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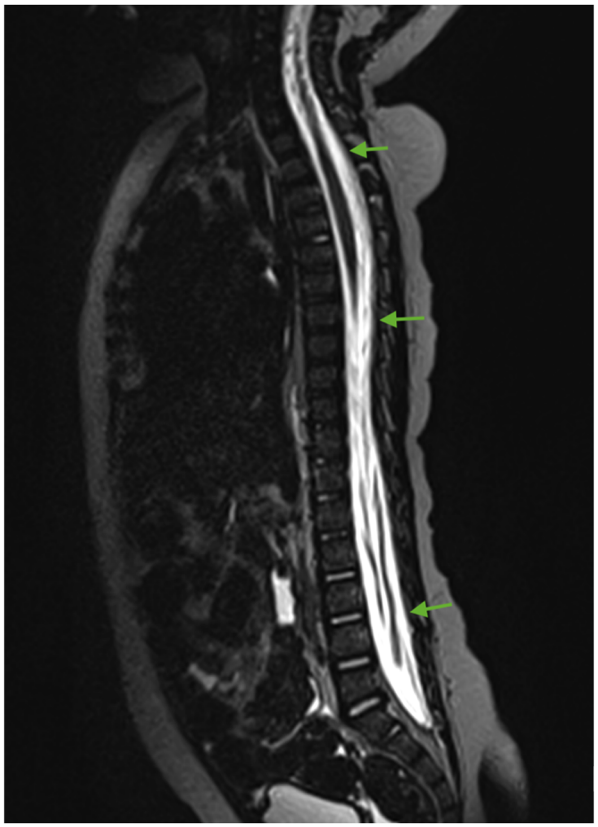

FIGURE 4
Spinal cord MRI (sagittal T2 + C). Thickening of the dura mater and intense contrast accumulation along the whole spine (green arrows).

results using v12.5 were interpreted as infratentorial pilocytic astrocytoma (**Figures 14**, **15**). The calibration score for pilocytic astrocytoma by using version 11b4 was 0.39, whereas it was 0.99 by using version 12.5. In addition, at the time of writing this paper, the analysis of the results was repeated using the latest version 12.8 of the brain classifier, and the calibration score was 0.95.

Since the diagnosis of pilocytic astrocytoma was verified, an additional molecular analysis for *BRAF* alterations was performed. Direct Sanger sequencing was conducted on an Applied Biosystems 3500 SeqStudio™ Flex - Genetic Analyzer (Thermo Fisher Scientific, Waltham, USA), and the results were analyzed using the Sequencing Analysis Software 6 program (for electropherogram visualization). MegAlign Pro was also used to align the investigated fragment to the reference genome. Moreover, real-time polymerase chain reaction (PCR) was performed on the QuantStudio 5 Applied Biosystems (Thermo Fisher Scientific), and the results were analyzed using the QuantStudio™ Design and Analysis Software v1.4.3/v1.5.1 program. Direct Sanger sequencing revealed the absence of *BRAF* mutations. However, according to the PCR analysis, a *BRAF-KIAA 15-9* fusion was found (**Figure 16**).

The final integrated diagnosis was infratentorial pilocytic astrocytoma, *MGMT* unmethylated, *BRAF* wild type, and *BRAF-KIAA 15-9* fusion.

When this diagnosis was made, the patient had already completed two cycles of chemotherapy, and a control examination was performed. A brain MRI with contrast enhancement detected a residual tumor in the vermis and a less

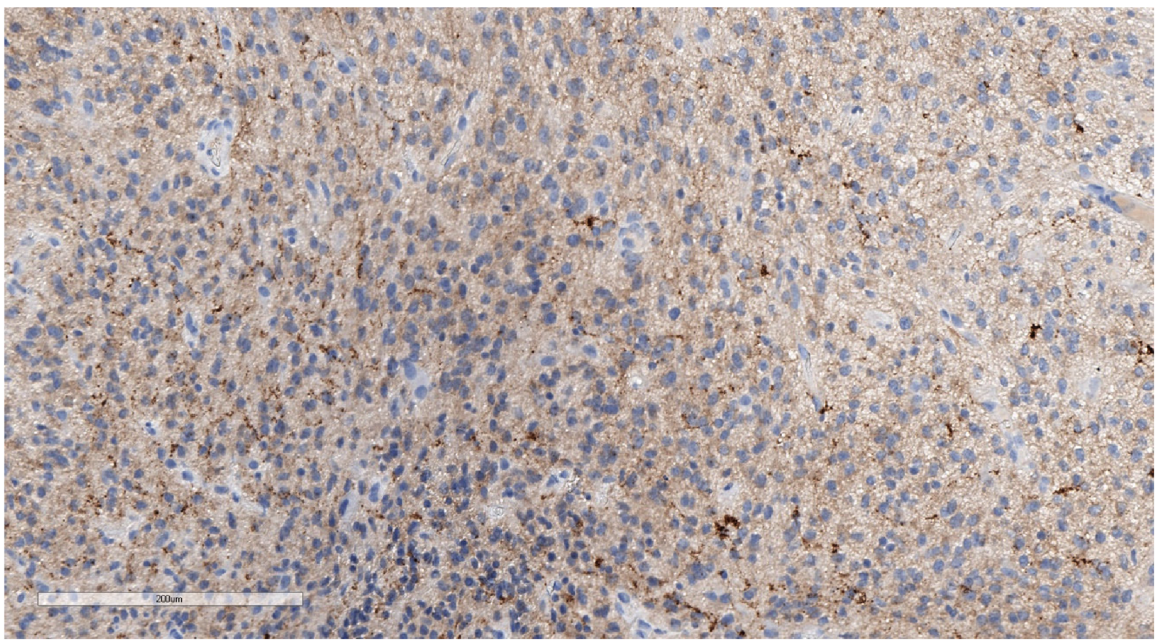

FIGURE 5
Positive synaptophysin staining confirms the neuronal origin of the tumor. IHC with synaptophysin, magnification ×200.

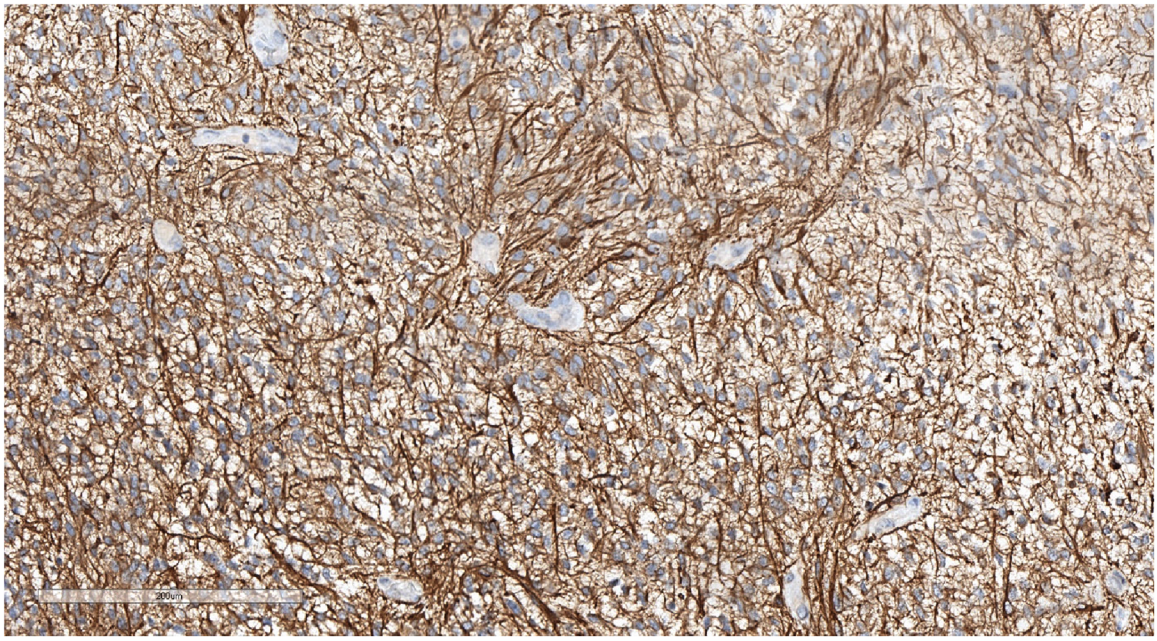

FIGURE 6
Positive GFAP staining underlining perivascular pseudorosettes. IHC with GFAP, magnification ×200.

intense contrast accumulation in the walls of the resection cavity, in the pia mater at the back of the brain stem, and the area of the right and left lateral apertures. A spinal MRI with contrast enhancement revealed a distinct regression of dura mater thickening and contrast accumulation along the spinal cord.

Considering the final diagnosis of pilocytic astrocytoma, the treatment scheme was changed, and the patient was given 21 weeks of induction chemotherapy in accordance with the SIOP-LGG 2004 (version 3.0, 2010) protocol. The age of the patient (8 months) was a limiting factor for the application of

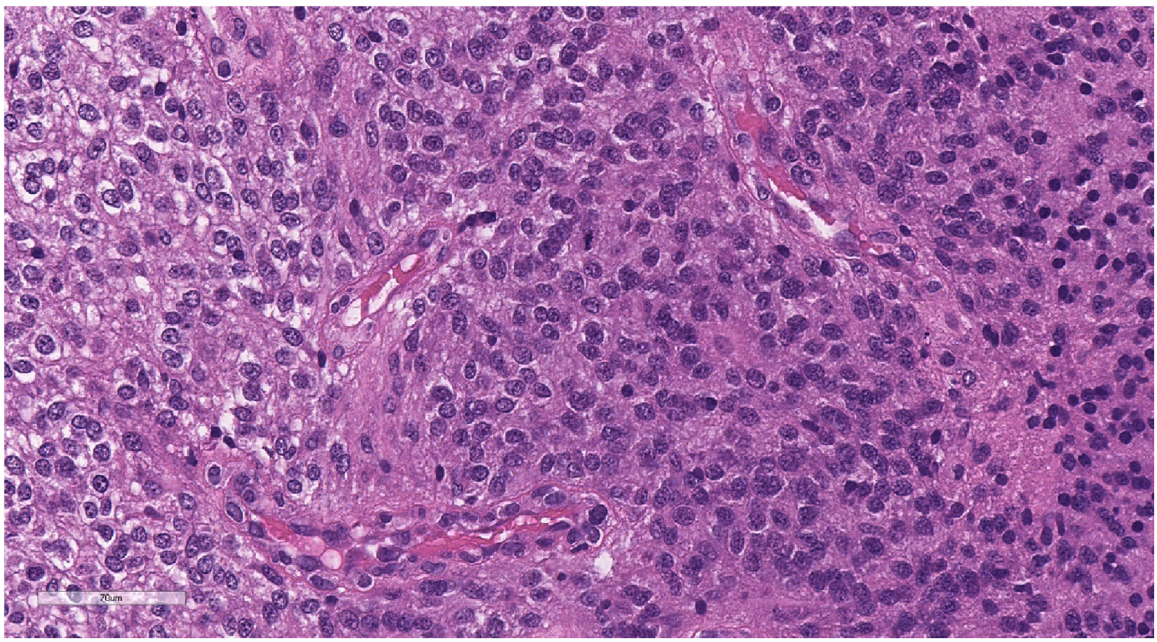

FIGURE 7
Morphologic picture of high-grade glioma. Hematoxylin and eosin staining, magnification ×300.

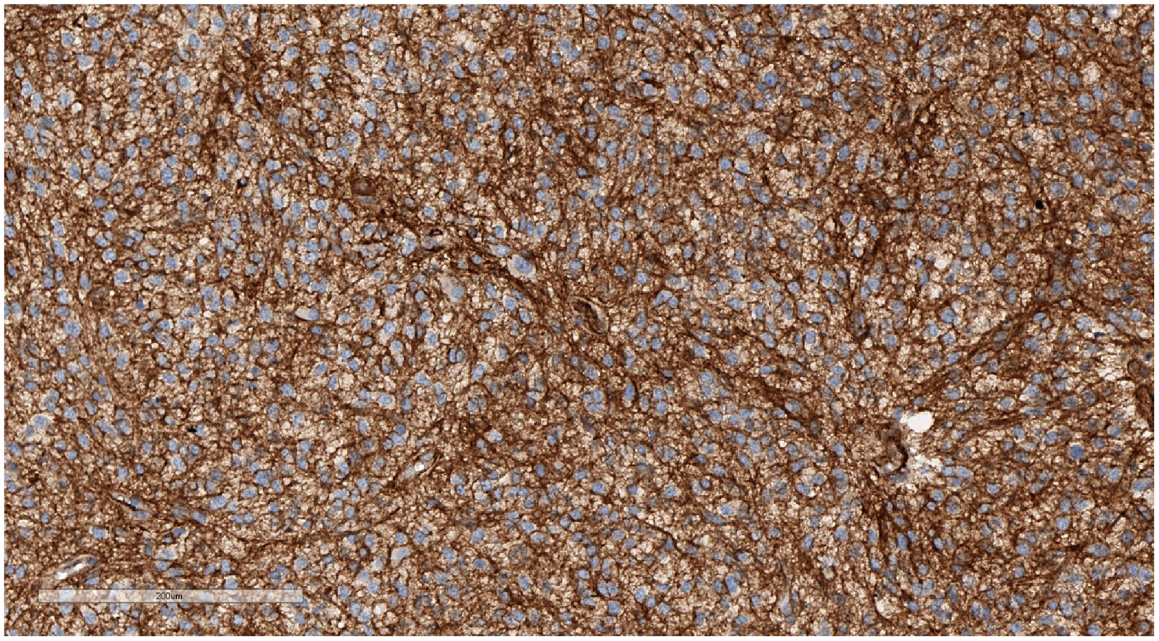

FIGURE 8
Positive cytoplasmic, but not nuclear staining for beta-catenin, confirming that the sample is not WNT-activated medulloblastoma. IHC with beta-catenin, magnification ×200.

targeted therapy with MEK inhibitors. A subsequent brain MRI with contrast enhancement revealed regression of the internal hydrocephalus, size reduction, and contrast accumulation of the residual tumor in the vermis, in the walls of the resection cavity, and the pia mater at the back of the brain stem. A spinal MRI showed a regression of the previously visualized area of minimal contrast accumulation by the pia mater at the level of C7-Th1.

The patient was confirmed to have stable disease and continued chemotherapy in accordance with the SIOP-LGG 2004 (version 3.0, 2010) protocol. In the event of disease progression, second-line therapy with BRAF inhibitors will be considered.

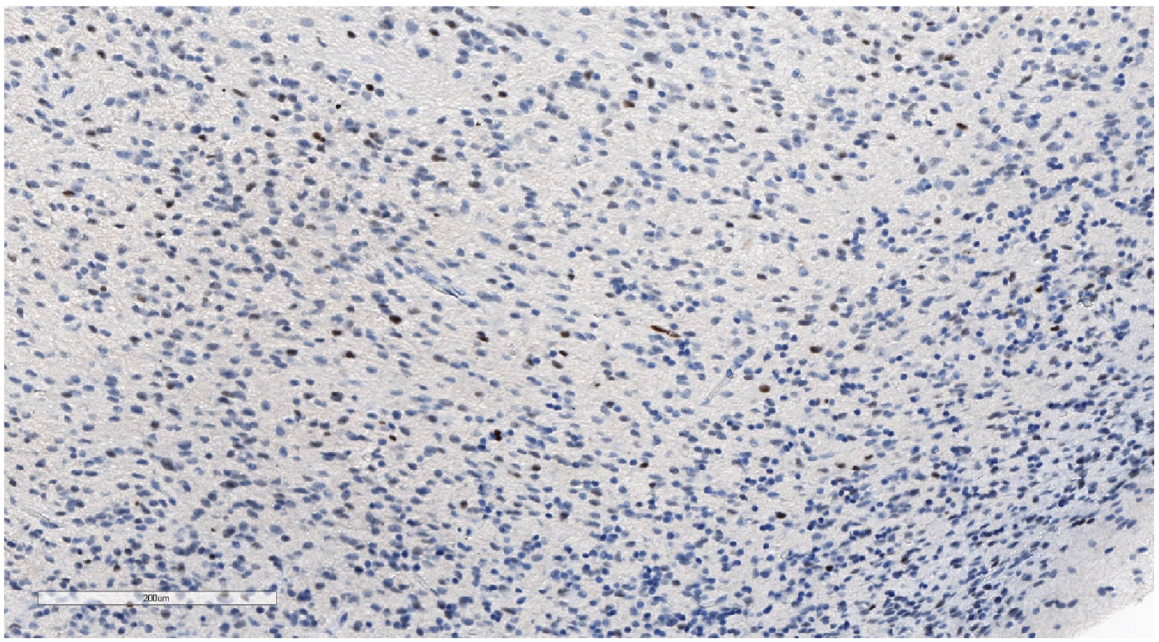

FIGURE 9
Positive nuclear staining for p53 and what might be observed in high-grade gliomas. IHC with p53, magnification ×180.

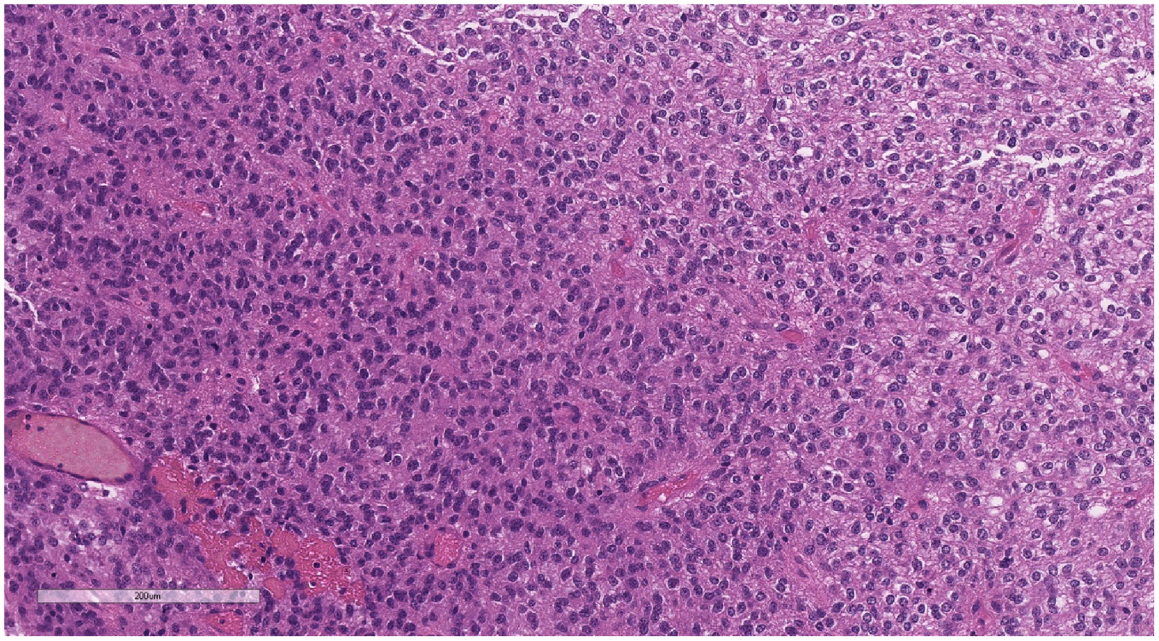

FIGURE 10
Perivascular pseudorosettes (typical of ependymoma). Hematoxylin and eosin staining, magnification ×160.

Discussion

Pediatric CNS tumors demonstrate clinical and biological diversity and variability in the morphological picture, which can lead to misdiagnosis and wrong therapeutic strategies. These diagnostic challenges can be overcome by using novel technological diagnostic approaches such as DNA and RNA sequencing, RNA expression profiling, fluorescence *in situ* hybridization, and DNA methylation (1). DNA methylation latter has been shown to be a powerful tool in terms of classification and diagnosis verification of CNS tumors and has been used in many investigations (2–6). The principle of DNA methylation

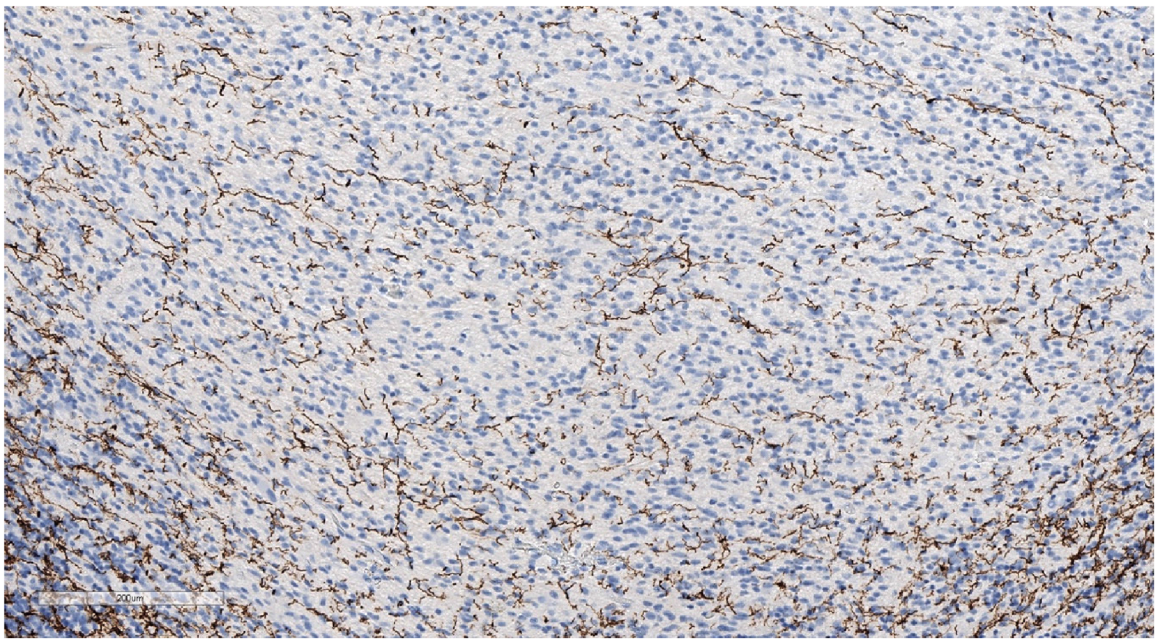

FIGURE 11
Focal weak-positive staining of neurofilaments may be seen in a well-defined tumor. IHC with NF, magnification ×140.

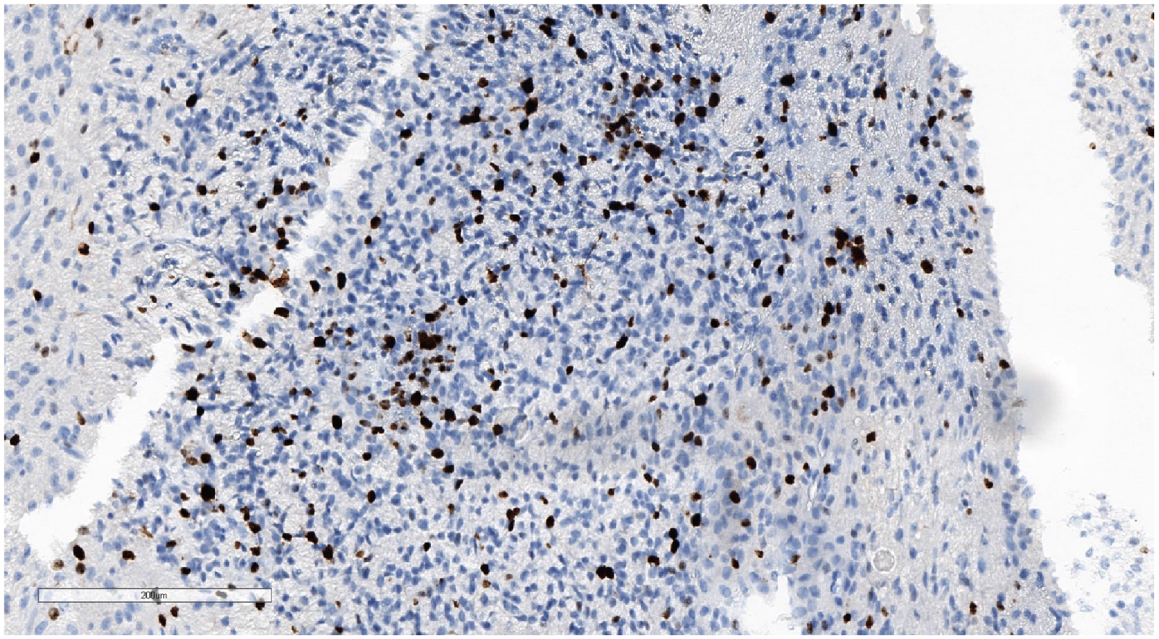

FIGURE 12
High Ki-67 proliferation index. IHC with Ki-67, magnification ×170.

analysis is based on the detection of specific methylation patterns that contain the methylation profile of the tumor, which is subsequently analyzed by using a special brain tumor classifier, and an entity-specific methylation class is also defined (6). It should be underlined that the brain tumor methylation classifier is constantly being improved, thus becoming more refined and complete. It allows the possibility of diagnosing older cases that may have not been previously classified.

Our case demonstrates the complexity of diagnosing a CNS tumor in a pediatric patient, which was caused by a non-specific clinical and morphologic picture of the tumor itself, which twice led to misdiagnosis and a wrong therapeutic

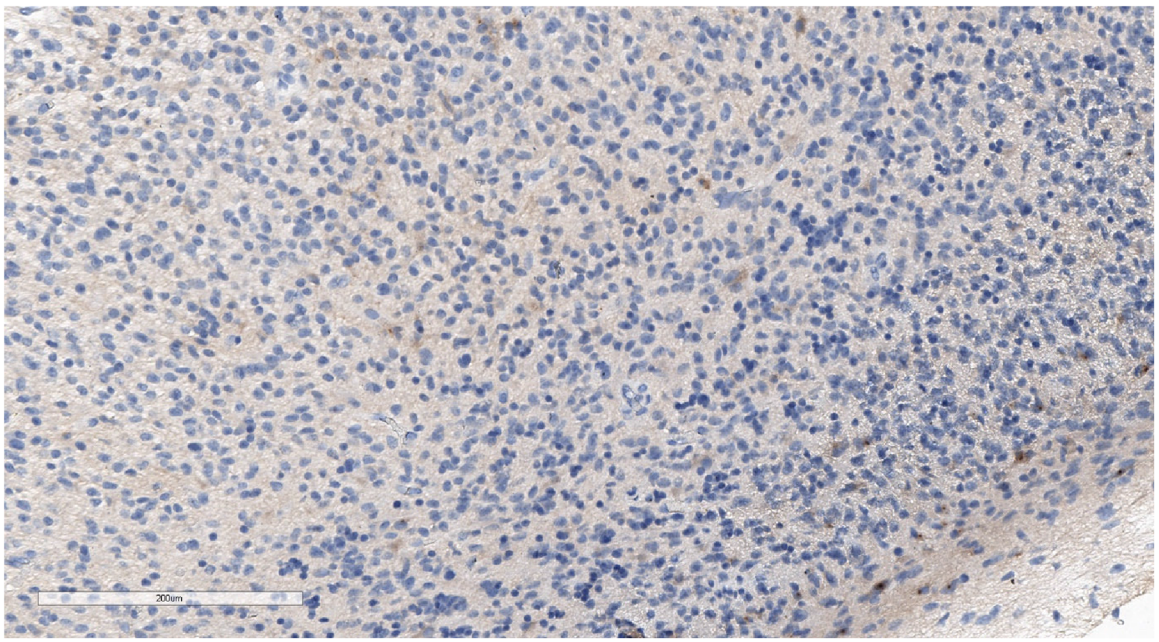

FIGURE 13
Focal EMA staining in tumor cell cytoplasm. IHC with EMA, magnification ×400.

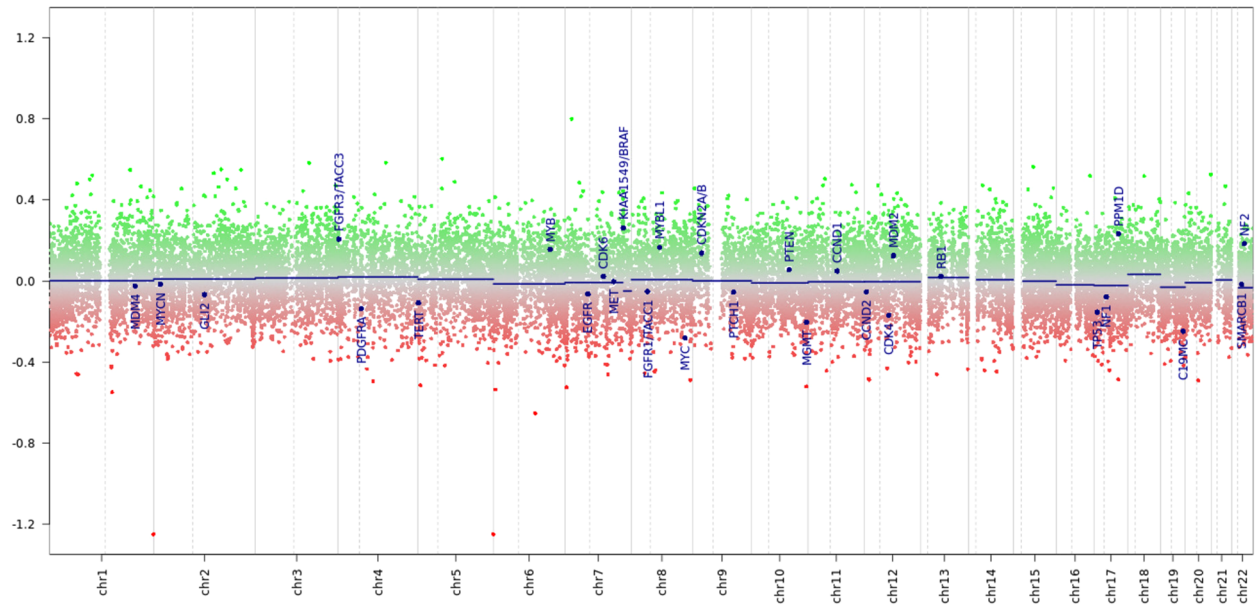

FIGURE 14
Chromosome profile derived from tumor DNA methylation. Amid a signal scatter, a *KIAA::BRAF* fusion is suspected (suspected peak at the 7q34 locus).

approach. In the event of a misdiagnosis, it is important to ensure that our patient is treated with more intensive chemotherapy and radiation therapy, which, however, could have serious consequences in terms of short- and long-term toxicity. Moreover, an additional molecular analysis allowed us to find a potential target for precision therapy, which may be useful in the event of disease progression. Also, it is important to note that the implementation of DNA methylation in low- and middle-income countries could be challenging due to the technical complexity and the high cost involved. Nevertheless, in complex diagnostic cases, at least a complete IHC and simple molecular methods [PCR, fluorescence in situ hybridization (FISH)] should be used. Specifically, in our case, diagnostics could be simplified by using a complete IHC panel. In particular, we could use Olig2 immunostaining, which has been shown to be a useful marker in the differential diagnosis of astrocytic and ependymal pediatric neoplasms (7).

In conclusion, our case highlights the strong need for the implementation of molecular methods, especially tumor DNA methylation, in the diagnosis of CNS neoplasms in children.

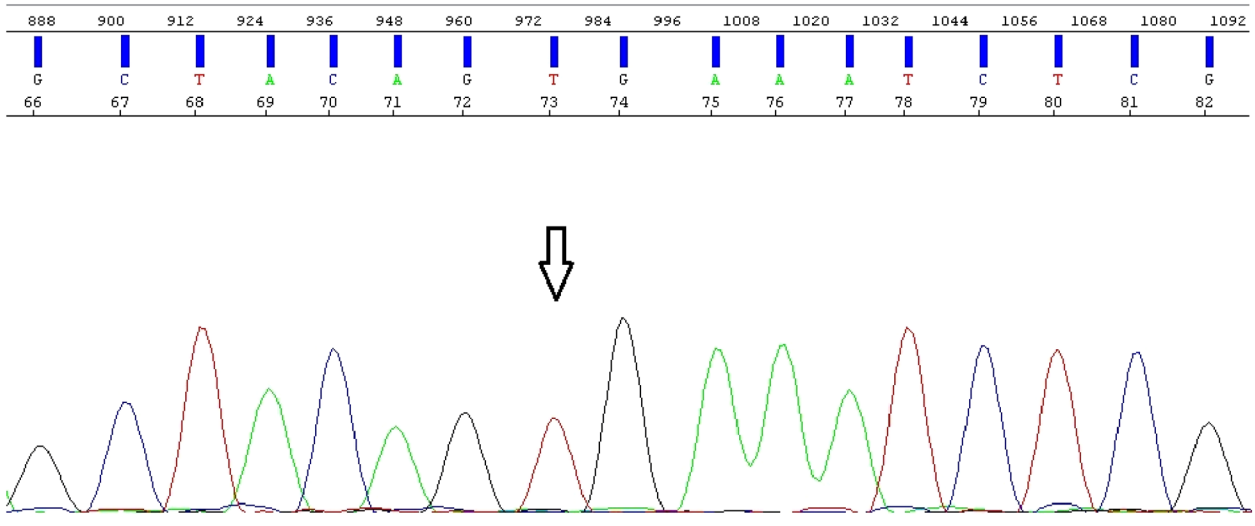

FIGURE 15
Electropherogram obtained from direct Sanger sequencing shows *BRAF* wild-type and absence of mutations.

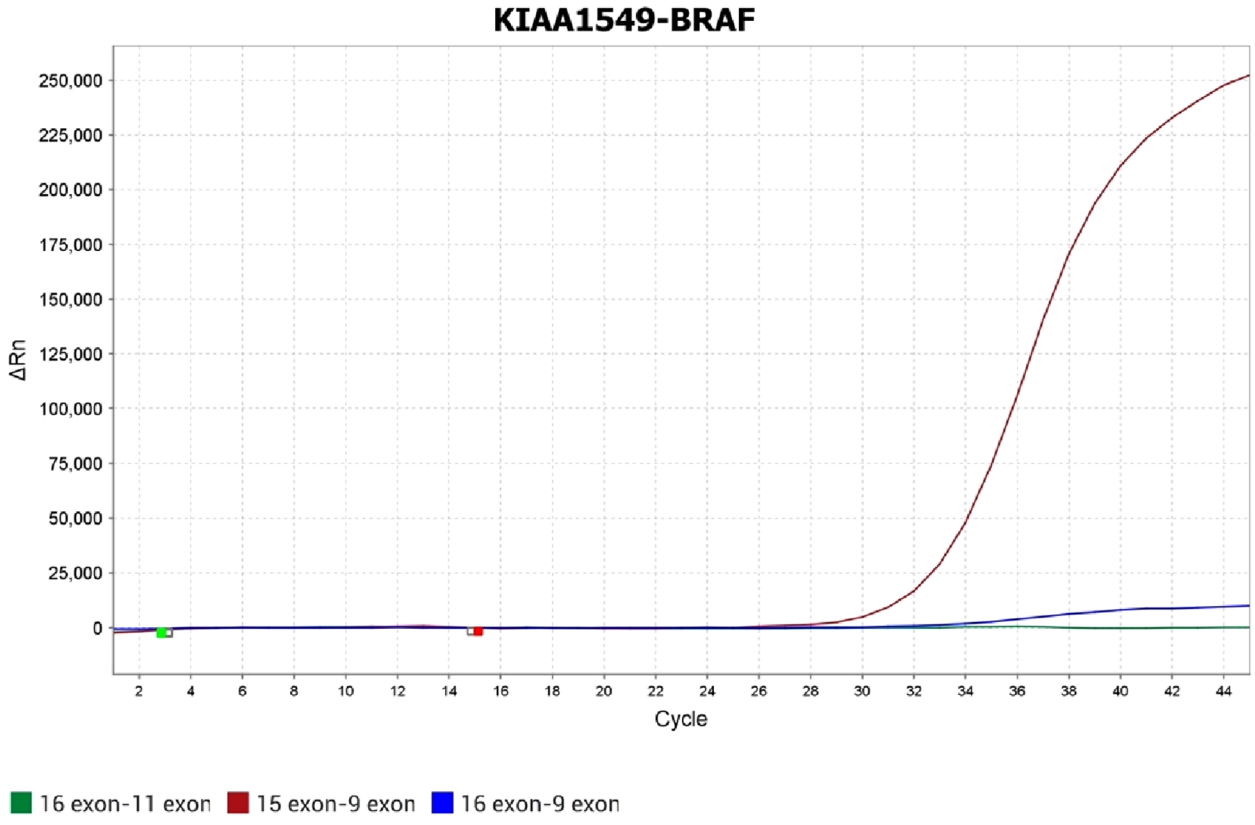

FIGURE 16
Fusion between exon 15 *KIAA* and exon 9 *BRAF*, which is confirmed by real-time PCR.

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

Written informed consent was obtained from the legal guardian/next of kin of the minor(s)' for publication of any potentially identifiable images or data included in this article.

Author contributions

DM: Writing – Original draft. MR: Writing – Original draft. OZ: Writing – Review and editing. MB: Writing – Review and editing. YD: Writing – Review and editing.

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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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Targeted therapy for pediatric central nervous system tumors harboring mutagenic tropomyosin receptor kinases

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The family of the neurotrophic tyrosine kinase receptor (*NTRK*) gene encodes for members of the tropomyosin receptor kinase (TRK) family. Rearrangements involving *NTRK1/2/3* are rare oncogenic factors reported with variable frequencies in an extensive range of cancers in pediatrics and adult populations, although they are more common in the former than in the latter. The alterations in these genes are causative of the constitutive activation of TRKs that drive carcinogenesis. In 2017, first-generation TRK inhibitor (TRKi) larotrectinib was granted accelerated approval from the FDA, having demonstrated histologic-agnostic activity against *NTRK*s fusions tumors. Since this new era has begun, resistance to first-generation TRKi has been described and has opened the development of second-generation molecules, such as selitrectinib and repotrectinib. In this review, we provide a brief overview of the studies on *NTRK* alterations found in pediatric central nervous system tumors and first and second-generation TRKi useful in clinical practice.

KEYWORDS

pediatric tumors, central nervous system tumors, tropomyosin receptor kinases, NTRK, gene fusions, point mutations, acquired resistance, targeted therapy

1 Introduction

Central nervous system (CNS) tumors are the commonest solid neoplasm in children aged 0-14 (1).

In CNS tumors, which commonly have no effective therapies, significant frequencies of neurotrophic tyrosine receptor kinase (*NTRK*) fusions have been revealed and their detection has become a cornerstone in the diagnostic evaluation of these cancers and

treatment through specific therapies (2, 3). NTRKs are a family of tyrosine kinases receptors of neurotrophins implicated in neuronal development, among them the development of memory and the growth and function of neuronal synapses (4). The *NTRK1/2/3* genes produce three members of the tropomyosin receptor kinases (TRKs) called tropomyosin receptor kinases TRKA, TRKB and TRKC, respectively, and are characterized by an extracellular binding domain, a transmembrane region and an intracellular kinase domain (4, 5).

TRK is usually activated in tumors via fusions involving *NTRK1/2/3*, caused by rearrangements of chromosomes between *NTRK* genes, which include the kinase domain, with several partner genes. The fusion products are chimeras with a constitutively activated TRK, regardless of the ligand they bound (6, 7).

The rearrangement between tropomyosin 3 (*TPM3*) and *NTRK1* in colorectal cancer was the first detected *NTRK* fusion (8). Afterward, *NTRK* fusions were found with several partners in a wide diversity of cancer typologies: among the fusions involving *NTRK1* are known the fusions with ROS Proto-Oncogene 1, Receptor Tyrosine Kinase (*ROS1*) and Lamin A/C (*LMNA*), involved in spitzoid neoplasms and in soft tissue sarcomas (STS), respectively (9). The *LMNA-NTRK1* is involved also carcinoma of lung and colorectal (10). Translocated promoter region (*TPR*) with *NTRK1* was found in thyroid cancer, and sequestosome 1 (*SQSTM1*)-*NTRK1* fusion in STS and non-small cell lung cancer (NSCLC) (11–15). The fusion that involved ETS variant of transcription factor 6 (*ETV6*) and *NTRK3* was found, for example, in congenital fibrosarcoma, congenital mesoblastic nephroma, PTCs and colorectal cancer (9, 16–18). Regardless of this review, *NTRK* gene fusions occur in more than 2.5% of low-grade gliomas (LGGs) and 5.3% of high-grade gliomas (HGGs) in children (19), and contribute to defining infant-type hemispheric gliomas, a new type of HGG, in the 2021 WHO classification of CNS tumors (20).

In this review, we explain a brief overview of the studies on *NTRK* alterations found in pediatric CNS tumors and first- and second-generation TRKi targeted therapy.

2 NTRK fusions: from detection to treatment

2.1 Tropomyosin receptor kinase and cell cycle

Briefly, neurotrophin growth factors bind and activate TRKs in a specific manner: nerve growth factor neurotrophin (NGF) to TRKA; brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT-4) that bins to TRKB; and neurotrophin 3 (NT-3) to all three TRK proteins, although it has a higher kinship for TRKC (21–26).

The RAS/MAPK, PI3K/AKT, and PLC/PKC signaling pathway is triggered by the bond between ligand to the extracellular domain that causes the homodimerization and transactivation of TRK receptors via autophosphorylation of tyrosine residues (Figure 1). Activation of the above pathways promotes cell proliferation, differentiation, and survival (5, 6, 27, 28).

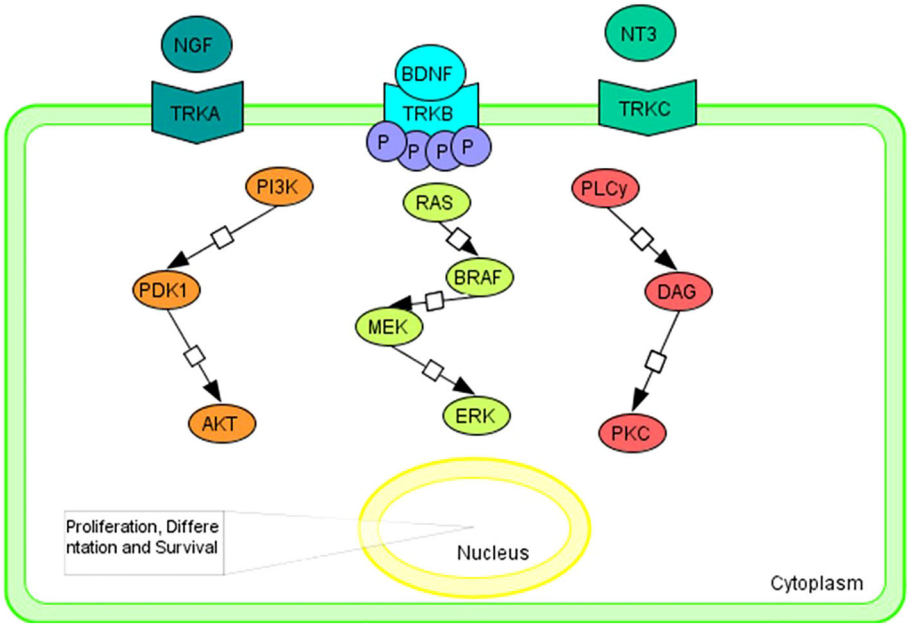

FIGURE 1
Graphical representation of the main intracellular signaling pathways associated with TRK family members. Tropomyosin receptor kinase A (TRKA); tropomyosin receptor kinase B (TRKB); tropomyosin receptor kinase C (TRKC); nerve growth factor neurotrophin (NGF); brain-derived neurotrophic factor (BDNF); neurotrophin 4 (NT-4); neurotrophin 3 (NT-3); PhosphatidylInositol 3-Kinase (PI3K); Pyruvate Dehydrogenase Kinase 1 (PDK1); AKT Serine/Threonine Kinase (AKT); B-Raf Proto-Oncogene, Serine/Threonine Kinase (BRAF); Mitogen-activated protein kinase kinase (MEK); extracellular signal-regulated kinase (ERK); Phospholipase C y (PLCy); diacylglycerol (DAG); Protein Kinase C (PKC).

2.2 *NTRK* fusions in pediatric central nervous system tumors

In CNS tumors, significant frequencies of *NTRK* fusions have been identified and their detection has become a cornerstone in the diagnostic evaluation of these cancers (3).

Several studies including large cohorts of pediatric CNS tumors found that *NRTK1-3* alterations occur mostly in very young children and tumors localized to the hemispheric lobs (29, 30). These results converged in the 2021 WHO Classification of CNS Tumors, in which *NTRK* alterations contribute to defining novel entities among both HGGs and LGGs in children, namely infant-type hemispheric glioma and diffuse LGG, MAPK pathway-altered, respectively (20). Despite the high-grade histology, the first subgroup benefits from a better outcome compared to its counterpart without tyrosine kinase fusions (29, 30).

NTRK fusions found in several studies are depicted in Figure 2.

2.3 NTRK inhibitors

There have been only limited *in vitro* or preclinical studies of signaling performed to illuminate the effect of TRKi on downstream cascade signaling or the time span of inhibition, but meaningful clinical responsiveness to these drugs has been shown in several types of tumors such as soft tissue sarcomas, childhood fibrosarcoma, lung cancer, colon cancer, melanoma (40–45).

First-generation TRKi were developed in 2015 that included larotrectinib and entrectinib. The recruiting clinical trials of either larotrectinib or entrectinib are listed in Supplementary Table 1.

Larotrectinib, developed simultaneously for pediatric and adult cancer, is the first oral treatment with a "tumor-agnostic" indication: discovered in 2015, it obtained accelerated approval from FDA in 2017. It is a small-sized competitive inhibitor of ATP and selective pan-TRK, with a 50% inhibitory concentration (IC50) of 5-11 nm *in vitro* and a specificity >100 times for TRK (46, 47). Inhibition of RAF-MEK-ERK or PI3K-AKT pathways, caused by larotrectinib, inhibits the growth of some cell lines that contained targeted *NTRK* fusions, as well *TPM3-NTRK1*, *TRIM24-NTRK2*, and *ETV6-NTRK3* (48, 49). An awesome overall response rate (ORR - the percentage of patients who experienced a complete or partial response) of 79% and a well-tolerated profile of toxicity were found in phase I/II clinical trials enrolling both adult and pediatric patients (50). In brain tumors, ORR was 30% and in particular, a retrospective study showed the results on the efficacy and safety for patients (n=33) with progressive or refractory CNS tumors enrolled in the SCOUT (NCT02637687) and NAVIGATE (NCT02576431) trials; among the 26 pediatric patients (79%), 13 pediatric HGGs and 7 pediatric LGGs were included. The observed ORR was 38% (38% in HGGs and 43% in LGGs, respectively), with three complete responses and seven partial responses. Importantly, the disease control rate at 24 weeks was 77% for pediatric HGGs and 100% for pediatric LGGs (43). In Supplementary Table 2 are reported results on patents with CNS tumor and treated with Larotectinb.

The Food and Drug Administration approved entrectinib in August 2019 to treat adult and pediatric populations with *NTRK* fusion tumors (51).

Robinson and colleagues published the first interim results based on 29 enrolled patients, aged 5 months to 20 years. The ORR was 100% in 11 pts [(high-grade CNS tumors (n=5) and extracranial solid tumors (n=6)] (52). In 2020, an expanded cohort of 39 patients confirmed an ORR of 77%. CNS tumors were in 14 patients, of which 11 displayed *NTRK* fusions. Notably, the ORR in this subgroup reached 64% (53). Desai et al. demonstrated that entrectinib had a rapid and durable responses in pediatric patients with solid tumors harboring *NTRK1/2/3* or *ROS1* fusions (54). In Supplementary Table 2 are reported results on patents with CNS tumor and treated with entrectinib. In addition, Liu et al. reported weight gain, dizziness and withdrawal pain in a several patients who were treated with TRKi (55).

Usually, both Larotrectinib and Entrectinib are administered until disease progression or unacceptable toxicity occurs (42, 43, 54). Treatment discontinuation is reported in extracranial tumors in which tumor size reduction has made complete resection

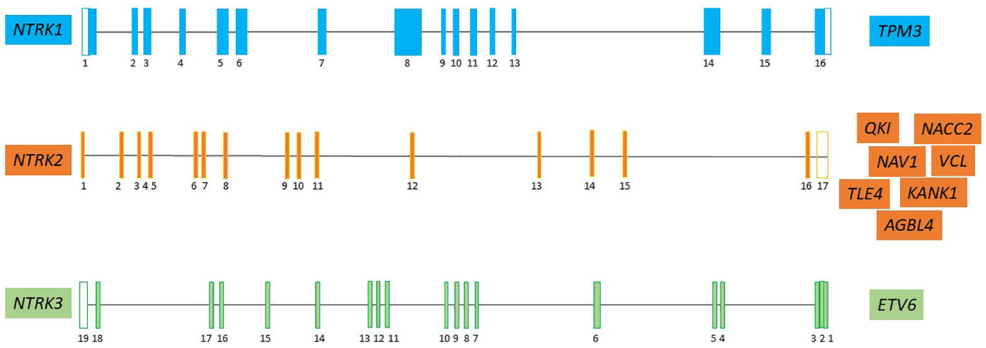

FIGURE 2
The major NTRK partner fusion genes in pediatric CNS tumor (7, 19, 28, 31–39). Neurotrophic tyrosine kinase receptor 1 (*NTRK1*), neurotrophic tyrosine kinase receptor 2 (*NTRK2*), neurotrophic tyrosine kinase receptor 3 (*NTRK3*), Tropomyosin 3 (*TPM3*); QKI, KH Domain Containing RNA Binding (*QKI*); NACC Family Member 2 (*NACC2*); Neuron Navigator 1 (*NAV1*); KN Motif And Ankyrin Repeat Domains 1 *(KANK1)*; actin-binding protein vinculin (VCL); ATP/GTP-binding protein (*AGBL4);* TLE Family Member 4, Transcriptional Corepressor (*TLE4*); ETS variant transcription factor 6 (*ETV6*).

possible; interestingly, patients who discontinued treatment following an initial response and subsequently experienced disease progression may still benefit from restart of therapy (41).

Mutations called on target and off target, respectively, on the *NTRK* gene or in genes associated with the MAPK pathway, are responsible for resistance to those drugs in several type of cancers (5, 56–59). In the *NTRK3* gene, acquired variants p.G623E and p.G623R have been identified to confer resistance to either larotrectinib or entrectinib (48, 57, 59, 60). Additionally, acquired variants p.F617L and p.G696A specifically confer resistance to larotrectinib (50, 57, 61). In the *NTRK1* gene, acquired variants p.V573M and p.G667S have been found to induce resistance to both larotrectinib and entrectinib, whereas the acquired variant p.F589L in the same gene only confers resistance to larotrectinib (50, 57, 62–64).

As a result, the need for second-generation TRKi, such as selitrectinib (loxo-195), taletrectinib (DS-6051b, AB-106), and repotrectinib (tpx-0005), has arisen (5, 30, 56). Taletrectinib works as a multi-kinase inhibitor that can overcome resistance from solvent-front replacements involving TRKA, TRKB and TRKC such as others involving ROS1 (65). Selitrectinib is a selective TRKi studied in a phase I trial involving both children and adults with tumors that have developed resistance mediated by TRK kinase mutations, in which a preliminary efficacy was found (66). Repotrectinib functions as a kinase inhibitor encoded by the *NTRK*, *ROS1*, and *ALK* genes. It effectively binds to the ATP-binding pocket of the kinase, preventing steric hindrance caused by various clinically resistant mutations (57). A clinical trial investigating its use in pediatric patients with solid tumors that include CNS neoplasms is currently ongoing (NCT04094610).

On the other hand, mutations that involved other RTKs or downstream pathway mediators can result in off-target resistance to TRKi. Specifically, MET amplification, $BRAF^{V600E}$ mutation, or KRAS alterations have been found in patients with TRK fusion and who show a progression of the tumor during the treatment of TRKi (56). Of note, the TRKi monotherapy was not effective for resistance mediated to overcome the mutational pathway, while a dual blockade of TRK and other pathways involved in the resistance mechanism could effectively control tumor growth (67). For instance, the combination of the inhibitors of TRK and MET has been found to be effective in a patient carried a *TRK* fusion and MET amplification that drives the resistance to the TRKi alone (56).

3 Conclusions

Tropomyosin receptor kinase inhibitors, such as larotrectinib and entrectinib, have showed high efficacy in pediatric patients, also in CNS tumors carrying alterations in *NTRK* genes. To date, additional research is necessary to help us to understand better the mechanism of action of these drugs and to identify biomarkers that can help identify patients who will benefit most from therapy.

Author contributions

AM, AC, and LB conceptualized the work. SC, FF, GB, and GM wrote the manuscript. AM, AC, and LB contributed to the finishing of the work and revised it critically for important intellectual content. All authors finally approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. 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

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Pediatric CNS tumors and 2021 WHO classification: what do oncologists need from pathologists?

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The fifth edition of the WHO Classification of Tumors of the Central Nervous System (CNS), published in 2021, established new approaches to both CNS tumor nomenclature and grading, emphasizing the importance of integrated diagnoses and layered reports. This edition increased the role of molecular diagnostics in CNS tumor classification while still relying on other established approaches such as histology and immunohistochemistry. Moreover, it introduced new tumor types and subtypes based on novel diagnostic technologies such as DNA methylome profiling. Over the past decade, molecular techniques identified numerous key genetic alterations in CSN tumors, with important implications regarding the understanding of pathogenesis but also for prognosis and the development and application of effective molecularly targeted therapies. This review summarizes the major changes in the 2021 fifth edition classification of pediatric CNS tumors, highlighting for each entity the molecular alterations and other information that are relevant for diagnostic, prognostic, or therapeutic purposes and that patients' and oncologists' need from a pathology report.

KEYWORDS

pediatric CNS tumors, brain tumors, molecular biology, WHO classification, neuro-oncology, neuropathology

1 Introduction

The 2021 WHO Classification of Central Nervous System (WHO CNS5) is the fifth edition of the international standard for the classification of brain and spinal cord tumors. The WHO CNS5 combined the previous 2016 WHO classification with novel molecular pathogenic alterations that are fundamental for the most accurate classification of CNS neoplasms (Louis et al., 2021). The fifth edition changes relate mainly to pediatric CNS tumor classification, requiring an entire chapter for CNS pediatric tumors within the WHO Classification (Pfister et al., 2022). These changes included the integration of histologic diagnosis with molecular profile to formulate an integrated diagnosis; the introduction of novel molecular diagnostic techniques such as DNA methylation analysis for tumor classification and crucial diagnostic criterion, particularly for difficult-to-diagnose cases; the differentiation between "pediatric-type" and "adult-type" tumor categories, considering the different behaviors; the association with cancer-predisposition syndromes; and the identification of novel tumor entities (Torp et al., 2022). In the 2021 WHO classification, contrary to previously, CNS tumor grades are written using Arabic numerals, according to the classification of cancer in other organ systems and decreasing the mistakes of pathology report. In previous classification, CNS tumors received a grade assigned to each entity, and grades were used across different entities predicted to have similar survival. However, in WHO CNS5, the switch to within-tumor-type grading has been used to many tumor types (Tran and Bielle, 2022). Moreover, based on the recommendations of the 2019 cIMPACT-NOW Utrecht meeting, WHO CNS5 has simplified tumor nomenclature for better clinical utility, for example, "anaplastic astrocytoma" and "anaplastic oligodendroglioma" are no longer used; instead, such tumors are simply referred to as grade 3. Several new tumor types and subtypes are introduced in the 2021 classification because of novel diagnostic technologies, including NGS or DNA methylome profiling (Morganti et al., 2019; Wong et al., 2020). This updated WHO classification has important implications for diagnosis, management, and development of novel treatments, with the application of targeted therapies and use of combined immunological and molecular approaches (Horbinski et al., 2022). Although WHO CNS5 classification is a major advance for clinicians to choose the most tailored therapies and identify more homogeneous patient populations with the same clinical outcomes, its implementation on a routine clinical basis presents some challenges that will require real-world interaction in multidisciplinary molecular tumor board (MTB). These meetings comprise different physician figures with specialties in oncology, radiology, surgery, pathology, molecular biology, informatics, etc., which are held to discuss the multidisciplinary management of SNC patients. The roles of MTB is to try to indicate appropriate therapy based on the identified histopathological features and genetic alteration, understanding clinical and radiological treatment responses to achieve long-term survival with a good quality of patient life (Tamborero et al., 2022).

2 Pediatric low-grade gliomas and glioneuronal tumors (pLGG/GNTs)

2.1 Overview

Although relatively rare, low-grade gliomas and glioneuronal tumors account for approximately 30% of pediatric CNS tumors (Ostrom et al., 2022). Many tumor types and subtypes are included in the pLGG/GNTs group (pediatric low-grade gliomas/glioneuronal tumors), showing histological diversities that were recognized and described over years of microscopical and immunohistochemical studies. However, these tumors frequently show overlapping morphological features, and in some cases, the salient aspects may also be absent due to limited tumor sampling (Bale and Rosenblum, 2022). Over the past decades, the development of novel molecular techniques has led to revolutionary insights into the genetic drivers of these tumors (Ryall et al., 2020a). Among novel molecular diagnostics, methylome profiling is of particular interest in pLGG/GNTs (Qaddoumi et al., 2016). MAP kinase pathway alterations are almost universally present across pLGG/GNTs, even though they may occur in different forms. In fact, specific genetic alterations may have different meanings in the diagnostic algorithm of pLGG/GNTs. In this regard, fifth edition of the 2021 WHO Classification of Tumors of the Central Nervous System put together the current knowledge regarding the clinical, histopathological, immunohistochemical, and molecular features of these tumors, opening the doors for further precision in classification and treatment of these tumors (WHO Classification of Tumours Editorial Board, 2021). In the 2021 WHO, the pLGG/GNTs group has been subclassified into three different families: pediatric-type diffuse low-grade gliomas, circumscribed astrocytic gliomas, and glioneuronal and neuronal tumors (Table 1).

2.2 Pediatric-type diffuse low-grade gliomas

2.2.1 Diffuse astrocytoma, *MYB*- or *MYBL1*-altered

Diffuse astrocytoma, *MYB*- or *MYBL1*-altered is a diffusely infiltrative astroglial neoplasm composed of monomorphic cells and characterized by genetic alterations regarding *MYB* or *MYBL1* genes. This new entity has been assigned to CNS WHO grade 1 (WHO Classification of Tumours Editorial Board, 2021). *MYB*-altered neoplasms are characterized by MYB overexpression, deriving from different mechanisms. *MYBL1* belongs to the same *MYB* gene family of transcriptional transactivators and, though less studied, it shows similar structure and functions. Evidence suggests that, regardless of age, *MYB*-/*MYBL1*-altered diffuse gliomas typically behave as WHO grade 1 neoplasms and are generally indolent (Wefers et al., 2020). Diffuse astrocytoma, *MYB*- or *MYBL1*-altered is rare, accounting for only 2% of pediatric low-grade gliomas (Ryall et al., 2020a). To date, the largest series reported a median age of 29 years, with a wide range from 4 to 50 years and a male preponderance (Wefers et al., 2020), even though other series showed no clear sex predilection (Tatevossian et al., 2010; Zhang et al., 2013). Most commonly, the tumor is located in the cerebral hemispheres, preferentially in the temporal lobe (42.5% of the cases) (Slegers and Blumcke, 2020). Rarely, it may also occur in the brainstem (Ryall et al., 2020a). This tumor is part of the wide group of LEATS (long-term epilepsy-associated tumors) (Slegers and Blumcke, 2020; Wefers et al., 2020). At the MRI, the tumor appears mostly in a well-defined way but may also have, at least focally, a diffuse growth pattern (Chiang et al., 2019; Wefers et al., 2020). Histologically, diffuse astrocytoma, *MYB*- or *MYBL1*-altered typically shows low-to-moderate cellularity and is composed of well-differentiated neoplastic astrocytes with small, round-to-ovoid nuclei, diffusely permeating neuropil (Chiang et al., 2019; Wefers et al., 2020). Immunohistochemically, tumor cells reveal positivity for GFAP only,

TABLE 1 2021 WHO Classification of pLGG/GNTs group, subdivided into three families: pediatric-type diffuse low-grade gliomas, circumscribed astrocytic gliomas, and glioneuronal and neuronal tumors.

Pediatric-type diffuse low-grade gliomas	Circumscribed astrocytic gliomas	Glioneuronal and neuronal tumors
Diffuse astrocytoma, MYB- or MYBL1-altered	Pilocytic astrocytoma	Ganglioglioma
Angiocentric glioma	**High-grade astrocytoma with piloid features (HGAP)**	Gangliocytoma
Polymorphous low-grade neuroepithelial tumor of the young (PLNTY)	Pleomorphic xanthoastrocytoma (PXA)	Desmoplastic infantile ganglioglioma (DIG)/desmoplastic infantile astrocytoma (DIA)
Diffuse low-grade glioma, MAPK pathway-altered	Subependymal giant cell astrocytoma (SEGA)	Dysembryoplastic neuroepithelial tumor (DNT)
	Chordoid glioma	**Diffuse glioneuronal tumor with oligodendroglioma-like features and nuclear clusters (DGONC)**
	Astroblastoma, MN1-altered	Papillary glioneuronal tumor (PGNT)
		Rosette-forming glioneuronal tumor (RGNT)
		Myxoid glioneuronal tumor (MGNT)
		Diffuse leptomeningeal glioneuronal tumor (DLGNT)
		Multinodular and vacuolating neuronal tumor (MVNT)
		Dysplastic cerebellar gangliocytoma (Lhermitte-Duclos disease)
		Central neurocytoma
		Extraventricular neurocytoma
		Cerebellar liponeurocytoma

In bold, there are highlighted the new entities that have been included in the fifth WHO edition of CNS Tumors.

while they are negative for MAP2, OLIG2, and CD34 (Wefers et al., 2020). Molecular analysis is mandatory to define tumors with compatible features as "diffuse astrocytoma, *MYB*- or *MYBL1*-altered." FISH may be useful to demonstrate rearrangements of *MYB* or *MYBL1* genes, but sequencing allows to determine the nature of the fusion between *MYB* or *MYBL1* and a partner gene (most frequently *PCDHGA1*, *MMP16*, and *MAML2*). *QKI* has been rarely observed as partner of *MYB* in this entity, while *MYB*::*QKI* fusion is typical of angiocentric glioma (Zhang et al., 2013; Qaddoumi et al., 2016; Chiang et al., 2019; Wefers et al., 2020), which represents the most difficult differential diagnosis. However, almost all angiocentric glioma show *MYB* rearrangements and, most frequently, a *MYB*::*QKI* fusion. Nevertheless, it is of greater importance to distinguish these diffuse low-grade gliomas from other IDH-mutant or IDH-wild-type diffuse astrocytic gliomas, considering the different biological behavior and therapeutic approach. Diffuse astrocytoma, *MYB*- or *MYBL1*-altered shows a benign clinical behavior, even though the available outcome data are limited due to its rarity. The majority of cases had no evidence of disease or stable disease after long-term follow-up. In some cases, recurrence may occur, which seems to be more likely in patients who did not receive an initial gross resection (Chiang et al., 2019; Ryall et al., 2020a). Moreover, approximately 90% of patients, presenting with epilepsy, became seizure-free after resection. However, the remainder showed a reduction in seizure frequency after surgery (Wefers et al., 2020; Alzoubi et al., 2023).

2.2.2 Angiocentric glioma

Angiocentric glioma is a diffuse glioma characterized by thin, cytologically bland, bipolar cells that aggregate in perivascular spaces. Almost all angiocentric gliomas have MYB alterations, with the most frequent rearrangement being represented by *MYB*::*QKI* fusion. This tumor has an indolent behavior and is assigned to CNS WHO grade 1. Angiocentric glioma was first described by Wang et al. (2005). In 2021, WHO has been reclassified as a form of pediatric-type low-grade diffuse gliomas (Fabbri et al., 2022; Kurokawa et al., 2022a). The epidemiological data regarding this entity are limited by its exceptional rarity (Ampie et al., 2016). Typically, angiocentric glioma presents as a supratentorial tumor, though in some cases, the brainstem has been reported. The most common clinical presentation is represented by long-term and drug-resistant epilepsy and is included in the LEAT group (Ampie et al., 2016; Kurokawa et al., 2022a). Histologically, angiocentric gliomas are composed of monomorphic, bipolar, spindle cells, with an infiltrative appearance, and they tend to show a perivascular arrangement (Wang et al., 2005). Rare cases with high mitotic activity (Miyata et al., 2012) or anaplastic transformation have been reported, but the clinical significance is unclear (McCracken et al., 2016). Tumor cells are GFAP-positive but negative for OLIG2 and neuronal markers. EMA usually reveals a dot-like or ring-like (i.e., microlumina) positivity, which is similar to ependymomas (Wang et al., 2005; Ni et al., 2015). The diagnosis of angiocentric glioma does not mandatorily require the demonstration of *MYB* rearrangements. Rare cases with co-occurring *BRAF* p.V600E mutation have been reported (Qaddoumi et al., 2016). *MYB* alterations are in common with diffuse astrocytoma, *MYB*- or *MYBL1*-altered, which represents the closer differential diagnosis and, in rare cases, may also harbor the hallmark fusion of angiocentric glioma. Angiocentric gliomas are biologically indolent, and gross total resection is usually curative (Ampie et al., 2016).

2.2.3 Polymorphous low-grade neuroepithelial tumor of the young

Polymorphous low-grade neuroepithelial tumor of the young (PLNTY) comprise a group of neoplasms with variable morphology

but characterized by indolent behavior, diffuse growth pattern, oligodendroglioma-like elements, calcifications, CD34 expression, and genetic alterations activating MAPK signaling. This tumor has been first described in 2017 by Huse et al. and included as a new entity in the 2021 classification with a CNS WHO grade 1 (Huse et al., 2017; WHO Classification of Tumours Editorial Board, 2021). Most commonly, PLNTY regards children, adolescents, and young adults, but it has been reported in a wide range of age (Huse et al., 2017; Riva et al., 2018). PLNTY is a supratentorial tumor, and it is typically characterized by solid and cystic components with dense calcification (Johnson et al., 2019; Chen Y. et al., 2020). At microscopic examination, PLNTY may be characterized by intratumoral heterogeneous morphology. An oligodendroglioma-like component is usually present, but cells may vary from uniformly rounded with perinuclear halos to spindled elements and may exhibit also nuclear pleomorphism and intranuclear pseudoinclusions. Calcifications are typically observable and are usually confluent (Huse et al., 2017). Immunohistochemically, tumor cells express glial markers (i.e., GFAP and OLIG2) and CD34, which may be patchy or diffuse and may also display non-neoplastic ramified neural elements in the associated cerebral cortex. In some cases, immunohistochemistry may show positivity for BRAFV600E (Huse et al., 2017; Johnson et al., 2019; Chen Y. et al., 2020). In fact, PLNTYs are characterized by the MAPK pathway activating alterations, whose demonstration is mandatory. *BRAF* p.V600E represents the most common genetic mutation, but fusions regarding *FGFR2* and *FGFR3* genes are also encountered. Based on the current literature, the *FGFR2*::*CTNNA3* fusion seems to be exclusive of this tumor even though present only in some cases (Bale, 2020). *FGFR3*::*TACC3* fusion, usually associated with a rare subtype of adult-type diffuse glioma, IDH-wildtype, has also been reported in a single case of PLNTY (Chen Y. et al., 2020). However, some features of this case may suggest some doubts in classifying it as a PLNTY: adult age, histological, and biological malignant transformation, co-occurring alterations in TP53, ATRX, PTEN *TEK,* and *RB1* genes. Additionally, the DNA methylation profile was not assessed for this case; therefore, we do not know if it would have shown the characteristic methylation signature of PLNTYs. For these reasons, pathologists and oncologists must always remember that *BRAF* and *FGFR3* alterations are not specific of PLNTYs but may also be encountered in high-grade gliomas, showing PLNTY-like morphological features (Bielle et al., 2018). Hence, further studies are needed for a complete understanding of the clinico-pathological significance of *FGFR3*::*TACC3* fusion and a better characterization of diffuse gliomas, harboring this peculiar rearrangement. For rare cases of PLNTY with recurrence or less favorable prognosis, the presence of specific MAPK-signaling alterations may suggest possible future applications of target treatments, paving the way for personalized therapy in pLGGs (Cipri et al., 2023).

2.2.4 Diffuse low-grade glioma, MAPK pathway-altered

Diffuse low-grade glioma, MAPK pathway-altered is a generic category that includes a group of gliomas showing infiltrative growth pattern and being composed by bland cells with astrocytic, oligodendroglial, or mixed morphology. These tumors are characterized by pathogenic MAPK pathway alterations, such as *BRAF* p.V600E mutations or *FGFR1* alterations, in the form of *FGFR1* internal tandem duplication (ITD), tyrosine kinase domain (TKD) mutation, or fusion gene (WHO Classification of Tumours Editorial Board, 2021). This entity typically occurs in children, but epidemiological data are limited by its rarity (Ryall et al., 2020a). The localization is variable through the craniospinal axis, although it tends to privilege the cerebral hemispheres and, interestingly, seems to show site-specific genetic alterations (Ryall et al., 2020a). Histologically, MAPK pathway-altered diffuse low-grade gliomas are composed of mildly atypical glial cells infiltrating normal brain parenchyma, which may only show a moderately higher cell density. Morphological aspects may vary on the basis of the pathogenic MAPK-pathway genetic alteration. Instead, *FGFR1*-altered tumors classically show oligodendroglial-like morphology. These tumors may occasionally have a vaguely nodular architecture. Interestingly, these two entities, belonging to two different families (pediatric-type diffuse low-grade gliomas and glioneuronal tumors, respectively), share the same genetic alterations involving the *FGFR1* gene (Qaddoumi et al., 2016; Ryall et al., 2020a). *FGFR1* alterations may also be shared with other glioneuronal tumors, such as rosette-forming glioneuronal tumor (RGNT) and extraventricular neurocytoma, or with pilocytic astrocytoma (i.e., a circumscribed glioma). Comprehensively, we may refer to these as "*FGFR1*-altered low-grade neuroepithelial tumors," having a common pathogenic genetic alteration, but leading to clinico-pathological entities that differ on the basis of tumor location, histologic features (even though with some overlap), accompanying genetic alterations, and epigenetic signature (Lucas et al., 2020). Because of the limited data regarding prognosis, a CNS WHO grade has not been assigned yet. However, it seems to have a better outcome when compared with CNS WHO grade 2 diffuse gliomas, but prognosis may depend on location, histology, and molecular alterations. In fact, the identification of specific alterations may provide important prognostic information and be predictive of therapeutic response to novel MAPK pathway-targeted therapies. Specifically, *FGFR1*-altered tumors may have beneficial effects from MEK inhibitor therapy (Cipri et al., 2023). Conversely, *BRAF* p.V600E-mutant tumors may respond to the administration of BRAF inhibitors (Hargrave et al., 2019). Furthermore, MEK inhibitors may also be useful for rare cases of diffuse low-grade glioma, MAPK pathway-altered, showing non-canonical *BRAF* mutations or other MAPK pathway-related gene alterations (Cipri et al., 2023).

2.3 Circumscribed astrocytic gliomas

The term circumscribed refers to the growth pattern, which is opposed to the "diffuse" tumors. They include pilocytic astrocytoma, high-grade astrocytoma with piloid features (HGAP), pleomorphic xanthoastrocytoma (PXA), subependymal giant cell astrocytoma (SEGA), chordoid glioma, astroblastoma, and MN1-altered (Table 1).

2.3.1 Pilocytic astrocytoma (PA)

PA is a low-grade astrocytic tumor (CNS WHO grade 1) characterized by MAPK pathway alterations (typically, *KIAA1549*::*BRAF* gene fusion). It represents the 5% of pediatric brain tumors, arising during the first two decades of life. In children, it is located most commonly in the cerebellum, but the whole neuraxis and the midline structures could be involved (Bartek et al., 2020). Clinical manifestations are due to mass effect or increased intracranial pressure; in infant, primary dissemination is common (Perilongo

et al., 1997). On imaging, it appears as a well-circumscribed lesion with a cystic and a solid component; the latter is hyperintense on T2, and the cystic wall has a variable contrast enhancement (Kornreich et al., 2001). On histology, they present as a low-to-moderately cellular tumor, composed of cells with a wide range of aspects as piloid features, oligodendrocyte-like cells, and multinucleated cells with nuclear clusters. Hyperchromatic and pleomorphic nuclei are sometimes present, but mitotic figures are uncommon. In a few cases, there is a high mitotic rate, which could indicate aggressive behavior (Rodriguez et al., 2010). Eosinophilic granular bodies and rosenthal fibers are frequent. Calcifications, hyalinized arteries, hemorrhages, and myxoid background with microcystic changes are common (Collins et al., 2015). The oligodendrocyte-like pattern may be linked to *FGFR1* mutations (Ryall et al., 2020b). PA express GFAP, S100, and OLIG2; synaptophysin is also frequently positive. IDH1 p.R132H expression and the H3 p.K28M (K27M) stain are negative. The Ki-67 index is usually low, and only focal increase may occur.

2.3.1.1 Subtypes

- Pilomyxoid astrocytoma: It is an infantile tumor that develops in the hypothalamic/chiasmatic area, has a worse prognosis than a standard pilocytic astrocytoma, and has a tendency to spread throughout the CSF fluid (Jeon et al., 2008). A diffusely myxoid background is the hallmark of this subtype, while Rosenthal fibers and eosinophilic granular bodies are often absent (Alkonyi et al., 2015).
- Pilocytic astrocytoma with histological features of anaplasia: It presents the same morphological features of pilocytic astrocytoma, but with vigorous mitotic activity and sometimes necrosis and/or anaplasia. Anaplastic alterations could be observed at either the initial diagnosis or recurrence. Necrosis, subtotal resection, alternative telomere lengthening, and ATRX deletion are linked to poorer overall survival (Rodriguez et al., 2019). The molecular alterations in this subtype are similar to PA, but this tumor may sometimes show a specific methylome signature known as "DNA methylation class anaplastic astrocytoma with piloid features" (Reinhardt et al., 2018). Although this methylation class is more prevalent in neoplasms identified as pilocytic astrocytomas with histological anaplasia, there are still some controversial issues.

Pilocytic astrocytomas have a favorable overall survival in the majority of cases, even after numerous progressions. Radiation therapy is frequently adopted with a positive overall outcome (Nelson et al., 2019). Additionally, the altered MAPK pathway genes might give a target therapy through MEK inhibitors. However, the long-term results are still unknown. Pilomyxoid astrocytoma are known to behave more aggressively, while it is important to better classify and further determine the prognostic significance of pilocytic astrocytomas with histological anaplasia (Tihan et al., 1999).

2.3.2 High-grade astrocytoma with piloid features (HGAP)

High-grade astrocytoma with piloid features (HGAP) is a high-grade astrocytic tumor histologically characterized by cells with thin fibrillary cytoplasmic process, which is suggested by the name itself (piloid). MAPK pathway gene alterations along with homozygous deletion of *CDKN2A/B* and/or ATRX mutation are distinctive of this tumor that clusters into a specific DNA methylation class. It is a rare tumor with a higher incidence in adults (Priesterbach-Ackley et al., 2020), with a median age of 40 years. Posterior fossa is the typical location of this tumor, but spinal and supratentorial regions can be also involved (Reinhardt et al., 2018). Histologically, HGAPs are mildly cellular, composed of moderately pleomorphic astrocytic cells with piloid features; glomeruloid proliferation of vessels is frequently observed. Necrosis and solid areas can be present. Rosenthal fibers and eosinophilic granular bodies are often observed. Although clinical, histologic, and molecular features may suggest the diagnosis of HGAP, DNA methylation analysis now represents one of the essential criteria for this tumor (Reinhardt et al., 2018). In fact, HGAP is a novel entity that is only defined by its methylome at present. Genes of the MAPK pathway are the most common reported, involving NF1 alteration most frequently, followed by *KIAA1549*::*BRAF* fusions and *FGFR1* mutations. *BRAF* p. V600E mutation can occur with a very low percentage. Furthermore, 80% of tumors harbor *CDKN2A/B* homozygous deletion, rarely *CDK4* amplification. Further less frequent chromosomal aberrations are partial gain of 12q and 17q, losses of 1p and 8p, and partial losses of chromosomes 14 and 19q. In 45% of cases, *ATRX* mutations have been described, leading to a loss of ATRX expression in neoplastic cells. In addition, in a small percentage of tumors, *TERT* promoter mutations have been found. Outcome data from a retrospective study showed an overall 5-year survival of approximately 50% (Reinhardt et al., 2018). No prognostic association with histological features or methylated *MGMT* promoter has been identified. A definitive CNS WHO grade has not yet been assigned.

2.3.3 Pleomorphic xanthoastrocytoma (PXA)

Pleomorphic xanthoastrocytoma (PXA) is an astrocytic tumor (CNS WHO grade 2 or 3), typically harboring *BRAF* p. V600E point mutation associated with homozygous deletion of *CDKN2A* and/or *CDKN2B*. Its incidence is <1% (Ostrom et al., 2022); it arises in children and young adults with a median age of 20 years, but it may also occur in older patients (Perkins et al., 2012). The supratentorial compartment is interested in 98% of cases, and the temporal lobe is most frequently involved. Tumors located in the infratentorial compartment and spinal cord have been observed. Leptomeninges are frequently infiltrated by the tumor (Ida et al., 2015). On MRI, these tumors exhibit a cystic component and a solid component, with heterogeneous contrast enhancement. Histologically, they show a wide morphological spectrum, being composed of spindle, epithelioid, and/or multinucleated astrocytes, sometimes with a xanthomatous appearance. Intratumoral lymphocytes and eosinophilic granular bodies are frequently present. CNS WHO grade 2 tumors have a low mitotic activity (<5 mitoses/10 HPF) and a circumscribed growth pattern, while grade 3 PXAs show a brisk mitotic activity (≥ 5 mitoses/10 HPF) and may reveal, at least focally, an infiltrative pattern and anaplastic features (Vaubel et al., 2018). Classically, PXAs show a diffuse S100 positivity and focal GFAP expression, despite their astrocytic nature. Neoplastic cells can also show CD34 (Reifenberger et al., 2003) and focal neuronal markers positivity (Powell et al., 1996). Immunostain for BRAF V600E protein is observed in approximately 70% of tumors (Phillips et al., 2019). Reticulin staining is usually diffused within PXAs. The most common molecular alteration in these tumors involves MAPK pathway genes, leading to an aberrant activation of this pathway, and *BRAF* p.V600E accounts for

approximately 80% (Vaubel et al., 2021). Less frequently, alterations in *NTRK1*, *NTRK2*, *NTRK3*, *RAF1*, and *NF1* genes can be detected (Vaubel et al., 2018). The contemporaneous presence of *CDKN2A* and/or *CDKN2B* homozygous deletion, detected in 90% of PXAs (Vaubel et al., 2021), and *BRAF* p.V600E mutation is highly suggestive (but not exclusive) of a PXA diagnosis (Nakajima et al., 2018). Further molecular alterations can be identified such as *TERT* promoter mutation or amplification, mainly in anaplastic tumors (Vaubel et al., 2021). The only essential criterion for the diagnosis of PXA is "an astrocytoma with pleomorphic tumour cells, including large multinucleated cells, spindle cells, xanthomatous (lipidized) cells, and eosinophilic granular bodies" (WHO Classification of Tumours Editorial Board, 2021). However, because of the wide morphological spectrum (sometimes resembling epithelioid glioblastomas, astroblastomas, gangliogliomas, or even atypical teratoid/rhabdoid tumors) and the possible absence/overlap of typical molecular alterations, DNA methylation profiling may be helpful in very challenging cases. Extent of resection is the major prognostic factor associated with recurrence. Even though PXAs mostly show a circumscribed growth pattern, they may disseminate at progression, and the recurrence is frequent (Kepes et al., 1979). Recurrent tumors and a high mitotic activity (CNS WHO grade 3) relate with survival (Ida et al., 2015); some studies showed that *TERT* promoter mutation can be associated with a more aggressive behavior (Vaubel et al., 2021). The presence of *BRAF* p.V600E mutation gives this tumor an important option for targeted therapy, predominantly in cases of incomplete resection.

2.3.4 Subependymal giant cell astrocytoma (SEGA)

Subependymal giant cell astrocytoma (SEGA) is a low-grade astrocytic tumor (CNS WHO grade 1), composed of cells with ganglion-like appearance, usually located in the periventricular area. It is the most frequent brain tumor associated with tuberous sclerosis (TS), with an incidence rate of 5–15%, among these patients (Ahlsén et al., 1994). It arises during the first two decades of life, rarely after the age of 20–25 years or among infants. Lateral ventricular involvement is the typical site, followed by the third ventricle and retina. On imaging, it appears as a solid and partially calcified lesion; MRI appears heterogeneously hyperintense on the T2-weighted images with homogeneous and evident enhancement after contrast administration (Inoue et al., 1998). On histology, this tumor is composed of three elements: spindle cells with fibrillary cytoplasm, intermixed with gemistocytic-like cells, and large ganglion-like elements with vesicular nuclei (Sharma et al., 2004). The immunophenotype is quite characteristic as this tumor exhibits a diffuse positivity for S100, a variable expression of GFAP, and neuronal markers. Moreover, TTF1 nuclear expression is common (Hewer and Vajtai, 2015). The typical genetic alteration of SEGA in patients with TS is the biallelic inactivation of *TSC1* or *TSC2* genes, either by loss of heterozygosity or germline mutation (Henske et al., 1997). Other infrequent alterations comprise *BRAF* p.V600E mutation (Bongaarts et al., 2017) and mTOR pathway activation (Franz et al., 2016). Prognosis of this tumor is good when a macroscopically total resection is made, though big lesions tend to have a superior morbidity (de Ribaupierre et al., 2007). Because of the mTOR pathway activation in SEGAs, treatment with inhibitors may lead to substantial reduction in tumor size (Franz et al., 2016).

2.3.5 Chordoid glioma

Chordoid glioma is a slow growing and well-circumscribed tumor (CNS WHO grade 2) characterized by *PRKCA* mutation. The incidence is <1%, occurring during the fourth or fifth decade of life with a median age of 45 years and a female predominance (Ampie et al., 2015). This tumor is typically localized to the third ventricle (Leeds et al., 2006), leading to obstructive hydrocephalus or visual field disorders as a consequence of the optic chiasm compression. On MRI, it appears as an isointense multilobulated mass on T1-weighted images, with homogeneous enhancement (Pomper et al., 2001). Microscopically, these tumors are composed of cords of epithelioid cells, which are embedded in mucoid/myxoid matrix. Lymphoplasmacytic infiltrate with Russell bodies are frequently observed. Mitotic figures are absent or very rare. The immunophenotype reflects the astrocytic nature of chordoid gliomas with a diffuse GFAP expression. Nuclear staining for TTF-1 (SPT24 clone) is frequent; the expression of S100 and EMA is variably present. Point mutation of *PRKCA* p.D463H is the hallmark alteration (Rosenberg et al., 2018); this alteration enhances proliferation of astrocytes and can represent a targetable mutation for therapy. Gross total resection (GTR) is the goal treatment for chordoid gliomas; when GTR is not applicable because of proximity to neurovascular structures, adjuvant radiotherapy can be considered (Ampie et al., 2015).

2.3.6 Astroblastoma, *MN1*-altered

Astroblastoma, *MN1*-altered, is a rare circumscribed glial tumor harboring *MN1* alterations. The incidence rate is between 0.45 and 2.8%, and it occurs most frequently in women and the median age is 15 years. The supratentorial compartment is more commonly involved, although the cases of the brainstem and spinal cord have been recorded (Chen W. et al., 2020). On histology, they show the characteristic astroblastic pseudorosettes, which is composed of tumor cells anchored to a central blood vessel by eosinophilic cytoplasmic processes (Mhatre et al., 2019). Vascular and stromal sclerosis is another typical feature of this tumor. Neoplastic cells are organized in papillary or pseudopapillary structures. MN1-altered astroblastoma has no CNS WHO grade assigned. Neoplastic cells exhibit a varying expression of GFAP protein and Olig2; EMA and L1CAM expression is frequent (Mhatre et al., 2019). These tumors display a distinct DNA methylation pattern, and the characteristic molecular aberration is the MN1 rearrangement at chromosome band 22q12.1 with fusion partner genes, such as *BEND2,* and, less frequently, *CXXC5* at chromosome band Xp22.13 (Hirose et al., 2018). In the spinal cord, tumors with astroblastoma-like morphology have been described, harboring different gene fusions involving chromosome X and 22q12 breakpoint regions as EWSR1::BEND2 fusion (Lehman, 2023). Further pathogenic alterations have been identified in a subset of tumors, such as *CDKN2A* homozygous deletion (Lehman et al., 2019). The copy number profiles of MN1-altered astroblastomas variably demonstrated loss of chromosomes 22q, 14, and broad regions of X, reflecting the rearrangement processes (Lehman et al., 2019). MN1-altered astroblastomas have limited outcome data, and specific clinical, histological, or molecular characteristics do not appear to be associated with outcomes (Lehman et al., 2019). Tumors with high-grade histology are associated with recurrence, tumor progression, and poor prognosis (Bonnin and Rubinstein, 1989). Patients with

MN1-altered astroblastomas have a high rate of local recurrence but good overall survival when associated with safe surgeries. Other than surgical resection, no additional prognostic factors have been identified (Tauziède-Espariat et al., 2019b). Because of the high survival rate, conservative treatment might be justified (Chen W. et al., 2020). A combination of radiotherapy and chemotherapy seems beneficial in cases where surgery is not feasible (Mhatre et al., 2019).

2.4 Neuronal and glioneuronal tumors

Glioneuronal tumors are rare tumors composed of both neural and glial components in different proportions. All neuronal and glioneuronal tumors are immunoreactive for neuronal cell markers, such as synaptophysin or neuron-specific enolase (NSE). However, in addition to neuronal marker positivity, only the glioneuronal subgroup of tumors are immunoreactive for markers of glial differentiation, such as glial fibrillary acidic protein (GFAP) or oligodendrocyte transcription factor 2 (Olig2). The fifth CNS WHO improves the role of molecular diagnostics in CNS tumor classification, combining them with traditional histology and immunohistochemistry (Krauze, 2021). The fifth CNS WHO comprises 14 different subtypes, including 3 new entities: multinodular and vacuolating neuronal tumor (MNVNT) which was only mentioned in the 2016 classification, diffuse glioneuronal tumor with oligodendroglioma-like features and nuclear clusters (DGONC), which is a provisional type, and myxoid glioneuronal tumor (MGT) (WHO Classification of Tumours Editorial Board, 2021). Multinodular and vacuolating neuronal tumor (MVNT) is a CSN WHO grade 1 neoplasm, arising in the temporal/frontal lobe of adult patients, with few pediatric examples. Most patients present with seizures, headache, episodic confusion, and dizziness. Histologically, MVNT shows clear hypomyelinated nodules with a fibrillary matrix, prominent vacuolar alteration, and monomorphic neuronal cells, which is haphazardly distributed or aligned along capillary vessels. Neoplastic cells are positive for OLIG2, doublecortin, and non-phosphorylated NFP and may express synaptophysin, and MAP2. Ki-67 is frequently low (<1%). CD34 expression may be observed in ramified neural elements and GFAP-positive reactive astrocytes of the adjacent cortex (Barresi et al., 2022). Molecular analyses may reveal MAPK pathway-activating abnormalities. Less commonly, they are associated with *BRAF* mutations or *FGFR2* fusions. Generally, they do not recur after gross total resection and remains stable also in the case of subtotal resection (Bale and Rosenblum, 2022). Diffuse glioneuronal tumor with oligodendroglioma-like features and nuclear clusters (DGONC) represents a new entity, which is included as a provisional type to the group of glioneuronal tumors. Its incidence is unknown but is presumed to be exceptionally rare. DGONC mainly occurs in pediatric age, with no sex predilection, and is localized in the cerebral hemispheres, mainly in the cortical or subcortical area of the temporal lobe. Histopathological features represent the hallmark of this tumor type, which are characterized by a diffuse growth of oligo-like and multinucleated cells, with nuclear clusters disposed as "pennies on a plate." Tumor cells display diffuse positivity for OLIG-2 and synaptophysin, focal positivity for Neu-N and MAP2, and negativity for GFAP. The mitotic index is variable, and the Ki-67 labeling index can be up to 30%. The molecular hallmark of DGONC is monosomy of chromosome 14, which has been found in all the cases reported (Bale and Rosenblum, 2022). These tumors present a specific DNA methylation profile; however, if DNA methylation profiling is unavailable, morphological and immunohistochemical features may suggest the diagnosis. Due to the low number of cases with an available follow-up, DGONC was not assigned to a CNS WHO grade. To date, outcome data are only available for 26 patients, indicating a 5-year progression-free survival rate equal to 81% and 5-year overall survival rate equal to 89% (Barresi et al., 2022). Myxoid glioneuronal tumour (MGT) represents a newly introduced entity, which is located in septum pellucidum and deep periventricular white matter and classified as CNS WHO grade 1 due to its favorable outcome (Bale and Rosenblum, 2022). These are uncommon primary brain tumors with a peak incidence in the second and third decades of life. Histologically, MGT is circumscribed tumor composed of oligo-like cells immersed in a myxoid stroma. Some cases may show floating neurons and perivascular neuropil, which are similar to DNTs. Mitoses are very rare or absent, and the proliferative index is low. The oligo-like cells are immunoreactive for OLIG2, SOX10, GFAP, and MAP2 and negative for synaptophysin. Floating neurons, perivascular neuropil, and neurocytic rosettes are synaptophysin-positive. MGT shows a recurrent *PDGFRA* p.K385L/I dinucleotide somatic mutation, typically occurring in the absence of accompanying *PDGFRA* gene amplification. Outcome is good, even in the cases showing local recurrence or dissemination throughout the ventricular system (Barresi et al., 2022).

3 Pediatric-type diffuse high-grade gliomas

3.1 Overview

The tumor family defined as "pediatric-type diffuse high-grade gliomas" represents one of the main changes in the fifth WHO Classification of CNS Tumors (WHO Classification of Tumours Editorial Board, 2021; Gianno et al., 2022a). The term "pediatric-type" has been introduced to distinguish these tumors from the adult-type counterpart. In fact, compared with adult-type diffuse high-grade gliomas, these tumors present different clinico-pathological characteristics, with diverse prognostic and therapeutic implications (Annese et al., 2022; Tamma et al., 2023). The term "diffuse" reflects the growth pattern of these tumors, even if some cases of H3-wildtype and IDH-wildtype glioma and infant-type glioma may show a circumscribed growth pattern (Guerreiro Stucklin et al., 2019; Clarke et al., 2020). The term high-grade reflects both morphology and biologic behavior of these tumors, even though occasionally, especially in some cases of H3 K27-altered diffuse midline glioma, they may show a misleading low-grade morphology, not corresponding to their undoubtful aggressive behavior (Buczkowicz et al., 2014). Finally, even though defined as "gliomas" and surely belonging to this category, these tumors may sometimes show neuronal or embryonal differentiation (Andreiuolo et al., 2019a; Tauziède-Espariat et al., 2019a). Pediatric-type diffuse high-grade gliomas are subdivided into four different clinico-pathological entities: diffuse midline glioma, H3 K27-altered; diffuse hemispheric glioma, H3 G34-mutant; diffuse pediatric-type high-grade glioma, H3-wildtype and IDH-wildtype; and infant-type hemispheric glioma. All of these tumors represent novel entities, which is first included in the 2021 CNS WHO. The only

exception is diffuse midline glioma, H3 K27-altered, which has been revised and renamed with respect to 2016 CNS WHO (WHO Classification of Tumours Editorial Board, 2021).

3.2 Diffuse midline glioma, H3 K27-altered (DMG)

Diffuse midline glioma (DMG) is an aggressive tumor occurring in the midline structures of the CNS. It is recognized as CNS WHO grade 4, due to its dismal prognosis, independently from its microscopical appearance. It is characterized by the loss of H3 K27me3 and is subdivided into three molecular subtypes: (1) DMG, H3 K27-mutant (encompassing K27M or K27I mutation in H3.3, H3.1, or H3.2); (2) DMG, with EZHIP overexpression (H3-wildtype); and 3) DMG, EGFR-mutant (including either EGFR mutation or amplification) (2021 CNS WHO). The H3 K27-mutant subtype is characterized by somatic heterozygous mutation in one of the genes encoding histone *H3* variants (in order of frequency: *H3.3, H3.1*, and *H3.2*) (Castel et al., 2015, 2018). The DMG subtype with EZHIP overexpression represents the rarest subtype. The increased expression of EZHIP protein may be assessed both by immunohistochemistry or molecular analyses (Castel et al., 2020). In both subtypes, the final result is a loss of H3 K27 trimethylation (H3 K27me3) due to the inhibition of the methyltransferase activity of EZH2, which is the catalytic subunit of PRC2. In the first subtype, this inhibitory effect is a consequence of the *H3* mutation (Bender et al., 2013; Lewis et al., 2013), whereas in the second subtype, this inhibitory effect is probably mediated by EZHIP overexpression, acting as an endogenous mimic of mutated *H3* genes (Jain et al., 2019). EGFR-mutant subtype commonly shows small in-frame insertions/duplications within exon 20, which encodes the intracellular tyrosine kinase domain (TKD). Alternatively, it may also present missense mutations in other exons. In some cases, also *EGFR* gene amplification has been reported, which may co-occur with *EGFR* mutation or in absence of it, hence it might be more precisely defined as EGFR-altered subtype. Furthermore, in this specific subtype, *EGFR* abnormalities seem to always co-occur with *H3* mutations or alternatively with EZHIP overexpression and are more frequently observable in DMG with a bithalamic or monothalamic presentation (Mondal et al., 2020; Sievers et al., 2021b). DMG is considered as a rare tumor, preferentially occurring in children. When located in the pons, DMG is also defined as "diffuse intrinsic pontine glioma" (DIPG) (Ostrom et al., 2022). Another common location is represented by the thalamus. In children, it usually presents with a bilateral thalamic involvement, while in adolescents and young adults, DMG tends to prefer a monothalamic or spinal location (Hoffman et al., 2016; Chai et al., 2020, 2023). On MRI, DMG appears as a solid mass, with diffuse infiltration of the surrounding structures. It is hypointense on T1, hyperintense on T2, and may show variable or no contrast enhancement on FLAIR (Poussaint et al., 2011; Giagnacovo et al., 2020). Histologically, DMG usually presents as a hypercellular diffusely infiltrative tumor, composed of neoplastic glial cells, showing a variegate spectrum of morphologies (Solomon et al., 2016). Mitosis, necrosis, and microvascular proliferation are frequently observable but are not essential for diagnosis. Immunohistochemically, they are usually positive for GFAP, OLIG2, and MAP2, with a variable expression. IDH1 is negative, while 50% of the cases show overexpression of p53, and 15% of cases present a loss of ATRX nuclear expression. In most of the cases, immunohistochemistry is sufficient to reach the diagnosis because it may assess the loss of H3 K27me3 expression (the cutoff is at least 80%) and H3 K27M or EZHIP positivity (Huang et al., 2018; Castel et al., 2020). In cases with loss of H3 K27me3, but not showing positivity for neither H3 K27M nor EZHIP, molecular analyses are mandatory in order to obtain a diagnosis. In rare cases, molecular analyses may reveal infrequent co-occurring alterations, such as *IDH1/2* mutations, *CDKN2A/B* homozygous deletions, *TERT* promoter mutations, and *MGMT* promoter methylation (Mackay et al., 2017, 2018). It is noteworthy to mention the possible co-occurrence of *H3* K27M and *BRAF* V600E mutations, which has been described not only in DMG (Gestrich et al., 2022) but also in glioneuronal tumors with a midline location (Nguyen et al., 2015; Pagès et al., 2017). Moreover, even though its significance still needs to be fully understood, the eventual presence of *BRAF* mutations or other MAPK pathway-related genetic alterations, such as *FGFR1* mutations, may be related to a better outcome and might also be predictive of response to *BRAF* or *MEK* inhibitors (Schüller et al., 2021). Considering the wide morphological spectrum and the possible presence of co-occurring mutations, DMG presents numerous differential diagnoses. The appropriate application of 2021 CNS WHO criteria is a fundamental aid to correctly diagnose this tumor. In difficult cases, molecular analyses may be a useful tool to correctly diagnose DMGs and identify the specific subtype. Moreover, molecular results may also be useful for predicting prognostic differences and, hopefully in the very next future, might suggest possible target therapies. Unfortunately, DMG prognosis currently remains invariably poor, with a 2-year survival rate of <10% (Mackay et al., 2017). Up-to-date, the therapeutic approach is based on surgery, frequently limited by location, radiotherapy, and chemotherapy (Vallero et al., 2023). CAR-T cells have yielded very promising preclinical and clinical results but are not yet part of the standard of care (de Billy et al., 2022; Majzner et al., 2022; Vitanza et al., 2023). Different clinical trials obtained promising results for the treatment of DMG with CAR T cells using different targetable antigens, such as GD2 (Majzner et al., 2022; Mackall, 2023), which are recently found to be highly expressed in DMG/DIPG. For these reasons, pathologists and oncologists should always try to obtain extensive information on each and every DMG case, not only for a precise diagnosis of specific tumor subtype but also for possible prognostic and therapeutic implications, widening the understanding of this rare and still lethal neoplasm.

3.3 Diffuse hemispheric glioma, H3 G34-mutant

Diffuse hemispheric glioma, H3 G34-mutant is a diffusely infiltrative tumor, arising in the cerebral hemispheres and assigned to CNS WHO grade 4 (WHO Classification of Tumours Editorial Board, 2021). Although classified as a glioma, it may show morphological and immunohistochemical aspects of neuronal differentiation, which is also confirmed by transcriptomic and epigenomic studies, suggesting a possible neuronal origin (Chen C. C. L. et al., 2020). The characteristic pathogenic alteration is a missense mutation of the *H3-3A* gene, resulting in a substitution of glycin 34 with an arginine (G34R) or less frequently with a valine (G34V) in the H3.3 protein

(Wu et al., 2012; Korshunov et al., 2016), and to a consequent inhibition of SETD2 methyltransferase (Jain et al., 2020) and KDM2A lysine demethylase activity (Cheng et al., 2014). Studies on H3 G34-mutant cells demonstrate that differential binding of H3 K36me3 induces a transcriptional reprogramming, recapitulating that of the developing forebrain, and causes prominent upregulation of the proto-oncogene *MYCN* (Bjerke et al., 2013). Co-occurring alterations are *TP53* and *ATRX* mutations (present in 90–95% of cases), *MGMT* promoter methylation (Korshunov et al., 2016), and *PDGFRA* mutations (present in 50–70% of cases) (Chen C. C. L. et al., 2020). This tumor occurs at a median age of 15 years and is mainly located in the temporal or parietal lobe (Mackay et al., 2017). On MRI, it exhibits features comparable to those of other high-grade gliomas (Kurokawa et al., 2022b). Histologically, H3 G34-mutant diffuse hemispheric glioma typically presents as a highly cellular, infiltrative astrocytic tumor with brisk mitotic activity. Some cases may show an alternative pattern, which is morphologically similar to CNS embryonal tumors (Andreiuolo et al., 2019a). Immunohistochemically, GFAP expression may be variable. The embryonal-like variant usually expresses synaptophysin in a diffuse and strong manner. The negativity for OLIG2, together with ATRX loss of expression and p53 overexpression, is highly suggestive for this entity, though not specific. Hence, the demonstration of H3 G34 mutations is mandatory to diagnose this tumor, as specified in the 2021 CNS WHO diagnostic criteria. In the majority of the case, *H3* G34R and *H3* G34V mutations may be detected immunohistochemically by the two respective antibodies. However, false negative cases have been described (Gianno et al., 2021). Therefore, in cases with negative immunohistochemistry, but presenting the appropriate clinico-pathological context, molecular analyses are needed to demonstrate the presence of *H3* G34 mutation or address possible differential diagnoses. Molecular investigations may be also useful to stratify the prognosis in a more precise manner. In fact, better prognosis is associated with the presence of *MGMT* promoter methylation and *MUC* gene mutations, while a worse prognosis is associated with *PDGFRA* mutations and the amplification of oncogenes, such as *PDGFRA*, *EGFR*, *CDK4,* and *MDM2* (Korshunov et al., 2016; Hu et al., 2022; Vuong et al., 2022). Furthermore, the demonstration of alterations in *PDGFRA* and *MUC* genes might be potentially useful to open up new therapeutic options for these patients (Lucas et al., 2021; Hu et al., 2022).

3.4 Diffuse pediatric-type high-grade glioma, H3-wildtype, and IDH-wildtype

Diffuse pediatric-type high-grade glioma, H3-wildtype and IDH-wildtype (pHGG H3/IDH WT) represents a heterogeneous group of tumors, which is characterized by histological high-grade features, absence of histone H3 and IDH mutations, and aggressive biological behavior (CNS WHO grade 4) (WHO Classification of Tumours Editorial Board, 2021). The pHGG RTK1 subtype is characterized by *PDGFRA* amplifications. The pHGG RTK2 presents *EGFR* amplifications and *TERT* promoter mutations. The pHGG MYCN subtype, as its name suggests, is enriched for *MYCN* amplifications (Korshunov et al., 2017). Hence, in order to obtain a diagnosis of pHGG H3/IDH WT, the identification of *PDGFRA*, *EGFR,* or *MYCN* alterations is essential. Alternatively, this diagnosis may be obtained by demonstrating the alignment of the tumor methylation profile with the pHGG RTK1, pHGG RTK2, or pHGG MYCN subtypes. Some cases of pHGG H3/IDH WT may develop the following therapeutic radiation, or in the context of Li Fraumeni syndrome or germline mismatch repair deficiency (i.e., CMMRD or Lynch syndrome), and usually belong to the pHGG RTK1 subtype (López et al., 2019). On MRI, pHGG H3/IDH WT is similar to other high-grade gliomas, which usually appears as contrast-enhancing tumors with mass effect. Differently from other subtypes, pHGG MYCN tumors may show more specific characteristics, being better circumscribed, with slight perilesional edema and homogeneous contrast enhancement (Tauziède-Espariat et al., 2019a, 2020). At microscopical examination, pHGG H3/IDH WT may show either a glioblastoma-like or a primitive, undifferentiated morphology that may also co-exist in the same tumor. Giant cells may be variably present and might raise the suspicion of mismatch repair deficiency, which may be also assessed by immunohistochemistry. Therefore, in cases of pediatric high-grade gliomas presenting severe pleomorphism and/or giant cells, pathologists should always ask for immunohistochemical evaluation of mismatch repair (MMR) proteins (MLH1, PMS2, MSH2, and MSH6), in order to exclude a constitutional mismatch repair deficiency (CMMRD) (Guerrini-Rousseau et al., 2019). Immunohistochemically, they show at least focal positivity for glial markers, such as GFAP and/or OLIG2, even though MYCN subtype may also be completely negative for glial markers, expressing neuronal markers. As a defining feature, these tumors are always negative for IDH1 (R132H) and H3K27M antibodies and show a preserved nuclear expression of H3K27me3. The number of possible differential diagnoses is high. The application of 2021 CNS WHO diagnostic criteria is greatly helpful for managing differential diagnoses, although some critical issues may be raised (Gianno et al., 2022a). First, the characteristic genetic alterations of the three pHGG H3/IDH WT subtypes are not exclusive of this entity and may be frequently found in other CNS tumors. Moreover, in a considerable percentage of pHGG H3/IDH WT tumors, the subtype-specific genetic alterations (*PDGFRA*, *EGFR*, *TERT*, and *MYC*) are absent, and the diagnosis may be reached, only demonstrating an aligned methylation profile (Korshunov et al., 2017; Tauziède-Espariat et al., 2020). Hence, neither the absence nor the presence of these alterations alone should never suggest to certainly exclude or confirm this diagnosis, without an appropriate clinico-pathological and molecular context. At the state of the art, prognosis for these tumors remains unfavorable, with some differences as regard the three subtypes: worst for MYCN (median OS of 14 months), intermediate for RTK1 (21 months), and better for RTK2 (44 months). *PDGFRA* and *EGFR* alterations may represent potential therapeutic targets in these tumors but yet to be demonstrated and validated. Another therapeutic chance may be represented by immunotherapy, especially in the context of mismatch repair deficiency-related cases (Korshunov et al., 2017; Mackay et al., 2018).

3.5 Infant-type hemispheric glioma

Infant-type hemispheric glioma is a high-grade diffuse glioma arising in cerebral hemispheres during early childhood. CNS WHO grade has not been assigned for this new entity due to the lack of prospective outcome data. This tumor is characterized by receptor tyrosine kinase (RTK) fusions regarding NTRK family, *ROS1, ALK,* or

MET (WHO Classification of Tumours Editorial Board, 2021). These fusions lead to an aberrant expression of a kinase domain, which drives tumorigenesis via the activation of PI3K and/or MAPK pathways. Generally, co-occurring genetic alterations are missing, even though *NSD1* mutations, with still uncertain significance, have been reported in rare cases (d'Amati et al., 2023b). Most of the cases occur very early in childhood, in particular during the first year of life. At histological examination, they appear highly cellular and composed of astrocytic cells, with mild-to-moderate nuclear pleomorphism. Rarely, gemistocytic elements, ganglion cells, or ependymal differentiation may be present (Olsen et al., 2015; Guerreiro Stucklin et al., 2019; Clarke et al., 2020). High mitotic activity, necrosis, and microvascular proliferation are frequently observable, even though biphasic tumors with low-grade and high-grade components have been reported (Ng et al., 2019; Valera et al., 2020). Immunostaining is scarcely useful for the dentification of gene fusions, as ALK positivity can be found only in some ALK-fused tumors, and NTRK shows high expression also in the normal brain tissue. Molecular analyses are fundamental in order to demonstrate the presence of a specific *RTK* fusion or an aligned DNA methylation profile. However, methylation profiling only recognizes a common subgroup, regardless of *RTK* fusion type, whereas the identification of these fusions is potentially useful for targeted therapeutic options (Olsen et al., 2015; Guerreiro Stucklin et al., 2019; Clarke et al., 2020). Finally, it is important to note that the identification of an *RTK* fusion in a morphologically high-grade glioma does not correspond to the diagnosis of infant-type hemispheric glioma. In fact, *RTK* rearrangements are also occasionally found in adult-type glioblastoma, IDH-wildtype, probably representing additional molecular events as a consequence of clonal evolution (Ferguson et al., 2018; Woo et al., 2020). Regarding prognosis, although data are currently limited for this new entity, the onset in early childhood is historically related to better outcomes as compared with pediatric-type diffuse high-grade gliomas, occurring in older children. Furthermore, each specific fusion seems to be associated with distinct prognosis. On the basis of data reported in a single study, ALK-rearranged tumors show the best prognosis (53.8% 5-year OS) as compared with the intermediate prognosis of NTRK-fused tumors (42.9% 5-year OS) and the poorer prognosis of tumors harboring *ROS1* alterations (25% 5-year OS) (Guerreiro Stucklin et al., 2019). However, to support these preliminary findings, prospective studies with bigger cohorts are required. The same consideration may be true regarding the effectiveness of small-molecule inhibitors that are showing promising responses in these tumors harboring RTK-activating fusions, although further studies are required for a complete validation (Drilon et al., 2017; Ziegler et al., 2018).

4 Ependymal tumors

4.1 Overview

Ependymal tumors are a heterogeneous group of neuroepithelial neoplasms arising from the progenitors of the ependymal cells, which line the inner cavities of the CNS. These are rare tumors, accounting for only 2–3% of all primary CNS neoplasms (Ostrom et al., 2022). They may arise along the whole neuroaxis, but most of the pediatric cases usually occur intracranially, whereas the spinal cord represents the preferential location among adults (McGuire et al., 2009). During the last years, significant novel molecular data allowed to identify distinct tumor subtypes, which are characterized by specific DNA methylation profile and genetic alterations. These molecular developments led to a substantially revised classification of the ependymal tumors, which are included in the 2021 WHO Classification of the CNS Tumors. The current classification, which is based on a combination of clinical, histological, immunohistochemical, and molecular features, subdivides ependymal tumors into three main groups, according to their location: supratentorial, infratentorial, and spinal.

4.2 Supratentorial ependymomas

Supratentorial ependymomas account for approximately 30% of all intracranial ependymomas, arising more frequently in pediatric age (Vera-Bolanos et al., 2015; Elsamadicy et al., 2020). Histologically, they show characteristic morphologic features of ependymomas, which appear similar across different anatomic sites. Perivascular pseudorosettes represent the hallmark feature and are composed of tumor cells organized around a central blood vessel. True ependymal rosettes, composed of tumor cells around an ependymal channel, are rarer to be observed. Immunohistochemically, ependymomas arising in other locations, are characterized by positivity for GFAP, negativity for OLIG2, and dot-like or ring-like cytoplasmic positivity for EMA. Regarding grading, ependymomas are classified as CNS WHO grade 2 or 3, principally on the basis of mitotic activity and independently from their location. However, there is no established cutoff, and the association between histological grading and outcome is not consistent. Supratentorial ependymomas are subclassified into *ZFTA*- and *YAP1*-fusion positive; therefore, molecular evaluation is necessary to demonstrate the presence of these entity-defining gene fusions (Andreiuolo et al., 2019b; Pagès et al., 2019). A significant part of supratentorial ependymomas do not show fusions involving *ZFTA* or *YAP1* genes and is currently classified as "Supratentorial Ependymomas, NEC" (Louis et al., 2018). In this context, a recent study reported that some supratentorial tumors preferentially occurred in pediatric age, showed ependymoma-like morphological and immunohistochemical characteristics, and characterized by recurrent fusions in *PLAGL1* genes (Sievers et al., 2021a).

4.2.1 Supratentorial ependymoma, ZFTA fusion-positive

Supratentorial ependymoma, ZFTA fusion-positive, is a circumscribed ependymal tumor, which is characterized by a fusion involving *ZFTA* (formerly C11orf95) gene (WHO Classification of Tumours Editorial Board, 2021). *ZFTA* rearrangements are believed to be the principal oncogenic driver of the disease and originate from chromothriptic events on chromosome 11 (Parker et al., 2014). In most of the cases, *ZFTA* is fused with *RELA*, which encodes the p65 subunit of NF-κB transcription factor complex, leading to pathological activation of NF-κB signaling (Pietsch et al., 2014). In other cases, supratentorial ependymomas may present *ZFTA* fusions with gene partners that differ from *RELA*, such as *NCOA1/2, MAML2,* and *MN1*. These cases also show significant histopathological heterogeneity and lack pathological activation of NF-κB signaling (Tauziède-Espariat et al., 2021). ZFTA-fused supratentorial ependymomas are

more common in children but may occur also in adults, and the most frequent location is represented by the frontal or parietal lobe (Lillard et al., 2019). Rare cases have also been reported, presenting an intracranial extra-axial location (Nowak et al., 2019) or a midline transtentorial involvement (Cardoni et al., 2023). Histologically, they usually show the typical features of other ependymomas, although unusual morphologies have been described, particularly for non-RELA tumors. Ependymomas with *ZFTA*::*RELA* fusion reveal cytoplasmic positivity for L1CAM and diffuse nuclear staining for p65 protein (encoded by *RELA* gene). The positivity for both or one of these two antibodies is reliable in predicting the presence of *ZFTA*::*RELA* fusions, although it always requires molecular confirmation. Instead, the negativity for both L1CAM and p65 consistently predicts the absence of *RELA* fusions. Conversely, non-RELA, ZFTA-fused tumors, usually show positivity only for L1CAM and negativity for p65. However, the molecular demonstration of *ZFTA* fusions is always mandatory for diagnosis, as reported in the 2021 CNS WHO criteria (2021 CNS WHO). Regarding prognosis, available data indicate that ZFTA fusion-positive tumors have the worst outcome. However, these studies exhibit significant variation, necessitating the use of prospective therapeutic trials to validate those findings (Pajtler et al., 2015; Figarella-Branger et al., 2016). In a series of ZFTA::RELA-fused ependymomas, homozygous deletion of *CDKN2A/2B* has been found to be an independent predictor of poorer survival (Jünger et al., 2020).

4.2.2 Supratentorial ependymoma, YAP1 fusion-positive

Supratentorial ependymoma, YAP1 fusion-positive, is a circumscribed ependymal tumor, which is characterized by fusions involving *YAP1* gene (WHO Classification of Tumours Editorial Board, 2021). Most frequently, *MAMLD1* represents the fusion gene partner, although other genes may rarely be involved (Andreiuolo et al., 2019b). *YAP1*::*MAMLD1* fusion exerts oncogenic activity through the recruitment of nuclear factor I (NFI) and TEAD family members (Pajtler et al., 2019). As for ZFTA-fused ependymomas, these tumors preferentially occur in young children but are rarer. Differently from ZFTA-fused ependymomas, they are negative for both L1CAM and p65. The molecular demonstration of *YAP1* fusions is essential for diagnosis. Compared with ZFTA fusion-positive ependymomas, this entity appears to have a more favorable prognosis (Upadhyaya et al., 2019).

4.3 Posterior fossa ependymomas

Posterior fossa (PF) ependymomas, as suggested by the name, arise intra-axially in structures located in the posterior cranial fossa, mainly in the fourth ventricle or in the cerebellopontine angle (Witt et al., 2011). Numerous subgroups of posterior fossa ependymomas were identified through analysis of DNA methylation profiling and DNA/RNA NGS data, but two main subgroups, PFA and PFB, were reliably confirmed in independent investigations and have been included in the 2021 WHO classification (Mack et al., 2014, 2018). In most cases, immunostaining for H3 K27me3 serves as a reliable surrogate of DNA methylation profiling, being a crucial marker for the diagnostic categorization of PF ependymomas into two PFA and PFB subgroups (Panwalkar et al., 2017). As for other CNS tumors, the inability to perform appropriate immunohistochemical and/or molecular analyses prompts the addition of "NOS" (Louis et al., 2018). Posterior fossa ependymomas can be assigned CNS WHO grade 2 or 3. As for ependymomas arising in other sites, brisk mitotic activity and microvascular proliferation seem to be more reliable in defining histological grading, compared with necrosis and pleomorphism, but inconsistent results have been reported in the literature (Godfraind et al., 2012). Regarding prognosis, PFA show a poorer prognosis compared with PFB (Pajtler et al., 2015). Independently from the molecular group, the identification of a chromosome 1q gain is a reproducible indicator of adverse outcome (Kilday et al., 2012).

4.3.1 Posterior fossa ependymoma, group A (PFA)

PFA is a circumscribed ependymal tumor, arising in the posterior fossa and aligning with the PFA molecular group of ependymomas. An ependymoma can be classified as PFA by immunohistochemical demonstration of H3 K27me3 loss of nuclear expression or DNA methylation profiling (WHO Classification of Tumours Editorial Board, 2021). Most commonly, PFA occur in younger children and show poorer prognosis compared with PFB (Pajtler et al., 2015; Witt et al., 2018). The oncogenesis of PFA is driven by epigenetic alterations, consisting in CpG islands hypermethylation and global DNA hypomethylation, associated with a reduction in the repressive histone mark H3 K27me3 (Mack et al., 2014). This reduction is the consequence of EZHIP overexpression, which mimics the oncohistone H3 K27M by binding to the EZH2 subunit of PRC2 complex and then inhibiting its methyltransferase activity (Hübner et al., 2019; Jain et al., 2019; Ragazzini et al., 2019). Immunohistochemistry is useful to demonstrate the loss of H3 K27me3 and also the presence of EZHIP overexpression (Antin et al., 2020; Nambirajan et al., 2021). Furthermore, since they are mutually exclusive, the demonstration of EZHIP overexpression allows to exclude the presence of *H3* K27 mutations, which are typical of DMG, H3 K27-altered, but has been rarely reported also in some PFA (Castel et al., 2020). However, the significance of *H3* K27 mutations reported in some PFA has yet to be determined (Gessi et al., 2016; Ryall et al., 2017). Remarkably, the fact that DMG and PFA share molecular features and often location, arising in neighboring regions of the brainstem and posterior fossa, suggests that certain cell populations in the developing hindbrain/posterior fossa are particularly sensitive to H3K27me3 states, and that, deregulated mechanisms of hindbrain/posteriors fossa development are fundamental to the biology of these tumors (Pun et al., 2023). To note, it has been reported that also germinomas arising in posterior fossa may show strong nuclear EZHIP positivity, associated with a loss of H3 K27me3, suggesting that the spectrum of neoplasms sharing these molecular features may be wider but strictly related to deregulated development of hindbrain/posterior fossa. In addition, isolated cases of MYC methylation class AT/RT and WNT-activated medulloblastoma have been shown to present EZHIP positivity, but this was only focal (<1% of positive tumor cells), and thus, this was not to considered as true EZHIP overexpression (>90% of positive tumor cells) (Antin et al., 2020).

4.3.2 Posterior fossa ependymoma, group B (PFB)

Posterior fossa group B (PFB) ependymoma is an ependymal tumor aligned with the PFB molecular group of ependymomas. Nuclear expression of H3 K27me3 is typically retained, but it is not specific of PFB. Therefore, according to 2021 CNS WHO criteria, an

ependymoma can be classified as PFB only by DNA methylation profiling (WHO Classification of Tumours Editorial Board, 2021). Different from PFA, they are more common in adults and are associated with a better prognosis (Witt et al., 2011). Preliminary data identified five molecular subgroups of PFB, with different epidemiological features: PFB-1, PFB-2, and PFB-3 are common in patients aged 25–30 years; PFB-4 occurs in younger people (median age: 15 years); and PFB-5 occurs in older people (median age: 40 years). Several cytogenetic alterations have been described in PFB, especially chromosomal aberrations, such as chromosome 6 monosomy, chromosome 18 trisomy, and loss of chromosome 22q, but the pathogenesis still remains currently unclear. However, even though biomarkers of worse prognosis are still fully elucidated, incomplete surgical removal and loss of chromosome 13q have been associated with poorer outcome in a single study (Cavalli et al., 2018).

4.4 Spinal ependymomas

According to 2021 WHO Classification of CNS Tumors, the spinal location has been recognized three types of ependymomas: (1) spinal ependymoma (morphologically similar to other ependymomas); (2) MYCN-amplified spinal ependymoma (characterized by MYCN-amplification and poorer prognosis); (3) myxopapillary ependymoma (identifying morphological features and usually localized in conus medullaris/filum terminale) (WHO Classification of Tumours Editorial Board, 2021).

4.4.1 Spinal ependymoma

Spinal ependymoma is a circumscribed ependymal tumor, demonstrating classic histological features of ependymoma and lacking features of myxopapillary ependymoma or subependymoma. When testing is feasible, *MYCN* amplification is absent (WHO Classification of Tumours Editorial Board, 2021). The experimental inactivation of *NF2* in mice led to enhanced proliferation and decreased apoptosis of embryonal spinal cord neural progenitor cells, supporting the idea that *NF2* plays a significant role in the pathogenesis of spinal ependymomas (Garcia and Gutmann, 2014). With a median age at diagnosis ranging from 25 to 45 years, these neoplasms account for approximately 20% of primary spinal tumors (Koeller et al., 2000). As regards histopathological features, the rare tanycytic aspect, characterized by spindle cells with bipolar processes, is more frequently observable in spinal location. This morphology may mimic the histological appearance of schwannoma or pilocytic astrocytoma, representing a possible diagnostic pitfall. Immunohistochemistry may be helpful in differential diagnosis, showing typical ependymoma immunophenotype, along with SOX10 negativity (Vege et al., 2000). Frequent loss of chromosome 22q and mutations of *NF2* are characteristic alterations of spinal ependymomas, but molecular analyses are not essential for the diagnosis. According to the previously described morphological characteristics, CNS WHO grade 2 or 3 is assigned; nonetheless, CNS WHO grade 3 is uncommon in this anatomic compartment. Overall, 5–10 year survival rates of 90–100% indicate a favorable outcome (Panwalkar et al., 2017).

4.4.2 Spinal ependymoma, MYCN-amplified

Spinal ependymoma, MYCN-amplified is a rare spinal ependymal tumor that has been recently characterized and included as a new entity in the 2021 WHO Classification of CNS Tumors (WHO Classification of Tumours Editorial Board, 2021). It shows a median age of 31 years, with a higher incidence in women, and is usually localized to the cervico-thoracic levels (Ghasemi et al., 2019; Swanson et al., 2019). Leptomeningeal dissemination is frequently observed at diagnosis or later during the course of the disease (Ghasemi et al., 2019; Swanson et al., 2019; Raffeld et al., 2020). This tumor shows the same morphological aspects of other ependymomas but almost always displays CNS WHO grade 3 histological features: microvascular proliferation, necrosis, and high mitotic count. *MYCN*, an oncogene belonging to the *MYC* family, encodes a transcription factor that controls neuronal development (Beltran, 2014). It plays a role in the pathogenesis of several tumors, such as medulloblastoma and neuroblastoma, but the mechanisms involving *MYCN* in ependymomas development are still unknown. *MYCN* amplification may be demonstrated as a surrogate by immunohistochemistry, showing strong and diffuse nuclear expression in these tumors. In some cases, a partial loss of H3 K27me3 has been observed (Swanson et al., 2019), but this is not constant (Ghasemi et al., 2019). Anyway, immunohistochemistry for *MYCN* may be a useful screening method in spinal ependymomas showing suspicious clinico-radiological and histopathological features, but the amplification should always be demonstrated by molecular analyses, such as FISH. Spinal ependymoma, MYCN-amplified also has a DNA methylation profile which is different from other ependymal tumors and CNS tumors with *MYCN* amplification (Raffeld et al., 2020). Compared with other spinal ependymomas, MYCN-amplified spinal ependymoma is an aggressive tumor with low progression-free and overall survival rates. Despite intensive treatments, all patients with reported follow-up data had recurrences (Ghasemi et al., 2019; Swanson et al., 2019; Raffeld et al., 2020).

4.4.3 Myxopapillary ependymoma

Myxopapillary ependymoma is a circumscribed ependymal tumor, which is histologically characterized by a radial arrangement of tumor cells around blood vessels and perivascular myxoid changes. It commonly arises in the cauda equina, filum terminale, or conus medullaris and in the 2021 WHO Classification of CNS Tumors have been assigned to CNS WHO grade 2 (previously grade I, WHO 2016) (WHO Classification of Tumours Editorial Board, 2021). It may occur at all ages but is more common in adults (Bates et al., 2016). The pathogenic mechanisms of myxopapillary ependymomas are still unknown, although some recurring copy-number variations (Rogers et al., 2018) and upregulations of enzymes promoting a Warburg metabolic phenotype have been described (Mack et al., 2015). Histologically, tumor cells are usually arranged around hyalinized fibrovascular cores, forming multiple papillary structures. Deposition of myxoid material around blood vessels and microcysts is frequent (Prayson, 1997). Very rare cases of "anaplastic myxopapillary ependymomas," showing high-grade morphological features, have been described (Lee et al., 2019). Immunohistochemistry shows positivity for GFAP, negativity for OLIG2, and absence of dot-like EMA positivity, which instead characterizes other ependymomas. Moreover, positivity for S100, CD99, CD56, and AE1/AE3 pancytokeratin may be found (Lamzabi et al., 2013). The prognosis is usually favorable and similar to conventional spinal ependymomas.

4.5 Subependymoma

Subependymoma is a rare ependymal tumor, with an excellent prognosis, assigned to CNS WHO grade 1 (WHO Classification of Tumours Editorial Board, 2021). It is more common in adults and sometimes discovered as incidental findings (Nguyen et al., 2017). Their typical location is fourth or lateral ventricles (Bi et al., 2015). At microscopic examination, subependymomas appear composed of small neoplastic glial cells, typically forming nuclear clusters within a fibrillary matrix and associated with microcysts and calcifications. In some cases, these tumors may be admixed with more classic ependymoma-like areas. These so called "mixed ependymoma-subependymoma" cases are considered to have a more aggressive behavior, comparable to conventional ependymomas (Rushing et al., 2007). Their immunophenotype is similar to ependymomas. Molecular analyses are usually not required for diagnosis, though they have shown interesting epigenetic results, presenting distinct site-specific DNA methylation profiles (Neumann et al., 2020). Outcome is usually excellent, with very rare recurrences, even after subtotal surgical resection. A single study reported some tumors with brainstem location, showing subependymoma morphology and H3 K27M mutations. Interestingly, these tumors seem not to be associated with the adverse outcome of DMG, although data are limited to the few cases of this study (Yao et al., 2019).

5 Embryonal tumors

5.1 Overview

Embryonal tumors of the CNS are characterized by genetic driving events, which are extremely aggressive, mainly affecting children (Sturm et al., 2016). DNA analysis and gene expression profiling allowed the identification of novel entities, leading to a reclassification of these tumors (Louis et al., 2014). One example is the diagnosis of medulloblastoma, which combines histopathological and molecular features (Taylor et al., 2012). Atypical teratoid/rhabdoid tumor (AT/RT), usually characterized by *SMARCB1* (or alternatively *SMARCA4*) inactivation, includes three genetically, epigenetically, and clinically different molecular subgroups: ATRT-TYR, ATRT-SHH, and ATRT-MYC (Ho et al., 2020). Embryonal tumor with multilayered rosettes (ETMR) typically harbors the amplification of a microRNA cluster on chromosome 19 (C19MC). In the new WHO classification, ETMR received an updated designation due to the newly discovered *DICER1* mutation in this tumor. Two newly introduced entities are FOXR2-activated CNS neuroblastoma and CNS tumor with *BCOR* internal tandem duplication (ITD) (WHO Classification of Tumours Editorial Board, 2021).

5.2 Medulloblastoma

Medulloblastoma is categorized in WHO CNS5 based on a combination of molecular and histological characteristics. Extensive transcriptome and DNA profile studies have led to the present molecular classification, which reflects the clinico-biological variability of this neoplasm (Ellison, 2020). Children are most commonly affected by medulloblastomas, which can occur at any age. This tumor accounts for approximately 20% of intracranial neoplasms in this age group, which is second only to high-grade gliomas (Ostrom et al., 2022). Several inherited cancer syndromes are associated with medulloblastomas (Waszak et al., 2018). A variety of germline mutations can be found in *ELP*, *SUFU, PTCH1* (naevoid basal cell carcinoma syndrome/Gorlin syndrome), *TP53*, *APC*, *PALB2*, and *BRCA2*. Medulloblastoma may grow in the fourth ventricle or be located in the cerebellar parenchyma (Blaser and Harwood-Nash, 1996), displaying symptoms and signs of elevated intracranial pressure caused by non-communicating hydrocephalus. Medulloblastomas have the ability to spread regionally, the leptomeninges, or occasionally outside the CNS. The majority of metastases are discovered adhering to the pia mater. The cerebrospinal fluid (CSF) or a hematogenous way is two possible mechanisms, mainly for SHH and non-WNT/non-SHH groups. Moreover, non-WNT/non-SHH medulloblastomas virtually always have distant CNS metastases at the time of the recurrence (Hill et al., 2020). Despite the fact that some molecular groupings and subgroups of medulloblastoma, such as WNT-activated tumors, exhibit a very good response to current therapy regimens and almost all of these individuals can be treated, all types of medulloblastomas are classified as embryonal tumors and CNS WHO grade 4. The histology is dominated by small, poorly differentiated cells with a high N:C ratio and high levels of mitotic activity and apoptosis. Architectural and cytological variation, on the other hand, classifies medulloblastomas into four histological subtypes: classic, desmoplastic/nodular, medulloblastoma with extensive nodularity, and large cell/anaplastic (Giangaspero et al., 1992; McManamy et al., 2007; Smolle et al., 2012). Such a wide range of morphological traits can be found in medulloblastoma molecular groupings. The new classification preserves the original four key molecular groups defined by the previous CNS WHO classification; wingless activated (WNT), sonic hedgehog (SHH) activated, and non-WNT/non-SHH. SHH tumors are classified based on *TP53* status in *TP53*-mutant and *TP53*-wildtype tumors (Taylor et al., 2012; Table 2). DNA methylation profiling, on the other hand, has resulted in the detection of 12 subgroups (four subgroups for SHH medulloblastoma and eight subgroups for groups 3 and 4) (Cavalli et al., 2017). This segmentation of molecular subgroups has important biological and clinical implications for prognosis and treatment options (Massimino et al., 2013; Goschzik et al., 2018). To distinguish between WNT, SHH, and non-WNT/non-SHH medulloblastomas, immunohistochemistry is even useful. The nuclear immunoreactivity for beta-catenin, which is found in the majority of malignant, helps to identify the WNT-activated group. The GAB1 and YAP1 protein immunostaining in the cytoplasm identifies the SHH-activated group. The medulloblastoma WNT and SHH groups both have cytoplasmic positivity for filamin A. Non-WNT/non-SHH tumors are immunonegative for GAB1 and YAP1 (Gianno et al., 2022b). Nevertheless, the gold standard for assessing the status of a medulloblastoma subgroup is DNA methylation profiling (Schwalbe et al., 2017). The best prognostic and predictive data come from combining morphological interpretation with molecular analysis. The level of diagnostic accuracy is further improved by incorporating information into genetic changes. To increase accuracy, additional genetic changes, such as *MYC* amplification, currently employed in the risk categorization, are incorporated into an integrated diagnosis. When a medulloblastoma develops in the context of a hereditary tumor syndrome, an integrated approach to diagnose with commentary provides a chance to focus on the clinical implications of germline. New potential treatment options derived from recent studies regarding metabolic changes during cancer progression. Indeed, MB subgroups

TABLE 2 Clinico-pathological and molecular aspects of Medulloblastoma subgroups.

	Subgroup	WNT		SHH				G3			G4		
Clinico-pathological aspects	Subtype	α	β	α	β	γ	δ	α	β	γ	α	β	γ
	Frequency	10–15%		28–30%				25–28%			40–45%		
	Anatomic location	Cerebellopontine angle/Cerebellar peduncle		Cerebellar hemisphere				Midline (filling fourth ventricle)			Midline (filling fourth ventricle)		
	Histology	Mostly classic, rarely LCA		Mostly ND, classic and LCA (less frequent)				Classic (most common), LCA			Classic and LCA (less frequent)		
	Age	6–12	>17	3–17	0–3	0–3	>17	0–10	3–17	0–10	3–17		
	Metastatic disease at diagnosis	8.6%	21.4%	20%	33%	8.9%	8.4%	43.4%	20%	39.4%	40%	40.7%	38.7%
	Prognosis (5-year survival)	97%	100%	69.8%	67.3%	88%	88.5%	66.2%	55.8%	41.8%	66.8%	75.4%	82.5%
Molecular aspects	Genetics	CTNNB1, DDX3X, KMT2D		PTCH1, TP53 KMT2D, DDX3X, MYCN ampl, BCOR, LDB1, GLI2 ampl				MYC ampl, OTX2 gain, SMARCA4, NOTCH, TGF-β			MYCN ampl, CDKN6 apml, SNCAIP duplications		
	Chromosomal abnormalities	Monosomy of chromosome 6		9q deletion; loss of 10q and 17p; gain of 3q and 9p				17q, 1q gain; loss of 5q and 10q			loss of 8, 10, 11; gain of 4, 7, 17, and 18		
	Genetic predisposition	APC (germline), most tumors lack CTNNB1 mutation		SUFU, PTCH1, TP53, PALB2, and BRCA2				PALB2 and BRCA2 (rare)			PALB2 and BRCA2 (rare)		

LCA, large cell/anaplastic; ND, nodular desmoplastic [data from Funakoshi et al. (2023)].

demonstrate different gene expression, leading to dysregulated metabolic pathways (lipid metabolism, nucleotide metabolism, and oxidative phosphorylation) (REF indicate) associated with different prognosis. These contributed to metabolic clustering and further risk stratification groups. In particular, high-risk metabolic clusters comprise G3/G4 methylation subgroup, MYC-amplified. MYC amplification displayed upregulation of genes related to nucleotide metabolism and oxidative phosphorylation, making them a potential therapeutic target (Gwynne et al., 2022; Funke et al., 2023).

5.3 Atypical teratoid/rhabdoid tumor (AT/RT)

AT/RT is a high-grade neoplasm characterized by the ability to differentiate along three germ layer lines, making this tumor unique. AT/RT is distinguished genetically by biallelic inactivation of *SMARCB1* (known as INI1 or BAF47), less frequently, *SMARCA4* (BRG1) (Hasselblatt et al., 2014). It is assigned to grade 4 CNS WHO classification. The incidence rate of AT/RTs is 1.6% of all pediatric CNS tumors, and they can occur as familial cases in the context of rhabdoid tumor predisposition syndromes 1 (Hasselblatt et al., 2014), although there have been reports of *de novo* germline mutations (Bourdeaut et al., 2011). The median age of the patients is 20 months (Ostrom et al., 2022). Adult occurrence is uncommon (Chan et al., 2018). These tumors are located mainly in the supratentorial compartment, but the whole neuraxis can be involved. Immunohistochemistry reveals positivity for synaptophysin, EMA, and AML. Loss of INI1 or BRG1 expression represents the surrogate of the underlying gene mutations. DNA methylation analysis combined with gene expression profiling has identified three molecular subgroups: AT/RT-SHH, AT/RT-TYR, and AT/RT-MYC. These subgroups stratify patients based on their age, place of origin, and gene alteration pattern (Frühwald et al., 2020; Table 3). AT/RT-SHH is characterized by the upregulation of proteins in the SHH and Notch signaling pathways. Heterozygous *SMARCB1* point mutations are frequently observed (Ho et al., 2020). AT/RT-TYR is distinguished by an increase in the expression of proteins involved in the melanosomal system (tyrosinase), the bone morphogenetic protein (BMP) pathway, and transcription factors related to the development. The deletion of the *SMARCB1* gene is caused mostly by a mutation in one allele, and the second hit leads to a total or partial loss in the second allele of chromosome 22 (Ho et al., 2020). AT/RT-MYC expresses the *MYC* oncogene and the *HOX* cluster genes. It arises more frequently in the supratentorial compartment, although they rarely happen in the spinal cord. This group also includes the uncommon AT/RTs, affecting adults restricted to the sella (Ho et al., 2020; Broggi et al., 2022). A recent study also found a high correlation between histological patterns and molecular grouping (Zin et al., 2021). The prognosis for patients with AT/RT is often dismal. Clinical studies have revealed, however, that AT/RTs do not always result in a bad outcome. High-dose chemotherapy combined with stem cell rescue and radiation was linked to a 4-year survival rate of 43% (Reddy et al., 2020). Epigenomic landscapes of AT/RT subtypes may be associated with varied treatment response so that it may be possible to stratify patients with AT/RT (Mittal and Roberts, 2020).

5.4 Embryonal tumor with multilayered rosettes (ETMR)

ETMR is an embryonal rare malignancy with characteristic morphological features, which is characterized by a *C19MC* alteration

TABLE 3 Clinico-pathological and genetics of AT/RT molecular subgroups [data from Federico et al. (2022)].

Molecular subgroups	Median age	Location	*SMARCB1* alterations	Involved pathway
AT/RT-SHH	2–5 years	Mainly supratentorial	Point mutations	SHH and NOTCH pathway
AT/RT-TYR	0–1 years	Mainly infratentorial	Point mutations	BMP and melanosomal pathway
AT/RT-MYC	>3 years	Mainly supratentorial	Extensive deletions	Overexpression of *MYC* gene and *HOX* cluster genes

or, less commonly, a *DICER1* mutation (CNS WHO grade 4). The median age of children affected by ETMR is less than 4 years. Three main histological arrangements can be observed: embryonal tumor with abundant neuropil and true rosettes (ETANTR), ependymoblastoma, and medulloepithelioma. On DNA methylation profile and gene expression, these three patterns cluster together. Different patterns of epithelial or mesenchymal development can be recognized (WHO Classification of Tumours Editorial Board, 2021). ETMRs exhibit widespread immunopositivity for LIN28A, which represents a very helpful marker for the identification of these tumors (Spence et al., 2014). The molecular detection of *C19MC* amplification or *DICER1* mutations is mandatory. Only ETMRs have the *C19MC* microRNA cluster mutation at 19q13.42, which is present in 90% of cases (Korshunov et al., 2010). Copy number profiling array FISH analysis is effective ways to identify *C19MC* changes. Only 5% of ETMRs do not show *C19MC* amplification, harboring *DICER1* mutations. Rare ETMRs that do not have a *DICER1* mutation or *C19MC* change should be categorized as NEC. The survival rates for ETMR are still extremely low, despite rigorous multimodal treatment.

5.5 CNS neuroblastoma, FOXR2-activated (CNS NB-FOXR2)

CNS neuroblastoma FOXR2-activated is a rare recently-described embryonal tumor displaying various degrees of neuroblastic/neuronal development and foci of ganglion elements and neuropil-rich stroma. It typically harbors rearrangements activating the transcription factor FOXR2 (CNS WHO grade 4). It arises in children classically within the supratentorial compartment, rarely with intraventricular location (Sturm et al., 2016). On MRI, this tumor typically manifests as a delineated mass with a cystic and a solid component, exhibiting a mild contrast enhancement (Holsten et al., 2021). Histologically, CNS NB-FOXR2 exhibits embryonal architecture-organized sheets. Homer-Wright rosettes and vascular pseudorosettes can also be observed. The immunoprofile shows a significant positivity for OLIG2. Synaptophysin is positive in regions with neurocytic/ganglionic differentiation. The majority of cases also have TTF1 overexpression (Holsten et al., 2021). CNS NB-FOXR2 is a recent addition to CNS 2021 WHO, discovered by using DNA methylation analysis, which revealed that several tumors may have belonged to distinct entities. This novel entity has chromosomal rearrangements with overexpression of *FOXR2* gene (Sturm et al., 2016; Louis et al., 2020). Next-generation sequencing is required for the discovery of *FOXR2* rearrangements, but copy-number analysis may be able to reveal changes to the *FOXR2* locus on chromosome Xp11.21. However, DNA methylation profiling greatly aids in the diagnosis of these cancers. Data on the prognosis of CNS NBFOXR2 are limited, but studies show that they have strong response to the current treatment (von Hoff et al., 2021).

5.6 CNS tumor with *BCOR* internal tandem duplication (ITD)

CNS tumor with *BCOR* internal tandem duplication (ITD) is a malignant CNS neoplasm that has an ITD in exon 15 of the BCOR gene. The reported patients' median age at presentation is 3.5 years (the range is 0 to 22 years). The cerebral or cerebellar hemispheres are most frequently involved (De Lima et al., 2020). On MRI, they show a central cystic area and inhomogeneous contrast enhancement (Bremer et al., 2020). Some regions may exhibit a glioma-like appearance, and compact fascicular patterns are commonly connected with a branching capillary network. Myxoid or microcystic region is quite distinctive. Mitosis and palisading necrosis can also commonly occur. They diffusely express vimentin and CD56, while absent or sparse expression of OLIG2, GFAP, or S100 supports the diagnosis (Louis et al., 2020). Although nuclear expression of *BCOR* is a sensitive marker, it is not specific (Kao et al., 2016; Ferris et al., 2020). The molecular detection of the specific ITD is required for a conclusive diagnosis. It is possible to distinguish CNS tumors with *BCOR* ITD from other CNS tumors by DNA methylation profiling and gene expression patterns. Patients with these malignancies have low survival rates (Wen and Packer, 2021).

6 Mesenchymal tumors

Mesenchymal tumors of the central nervous system (CNS) are a broad group of tumors, showing different clinical, pathological, and biological features. In the CNS, mesenchymal tumors usually originate from the meninges, more rarely in the CNS parenchyma or choroid plexus. Nomenclature and histology of these neoplasms are often similar to the extra-CNS counterparts, but there are also some entities showing peculiar site-specific characteristics, part of them arising exclusively in the CNS. Meningioma represents the most frequent tumor arising from the meninges (Ostrom et al., 2022) but, in rare cases, may arise in unusual locations, such as the lung (Kemnitz et al., 1982) or head and neck (Kershisnik et al., 1993). However, meningiomas are believed to develop from arachnoid cap cells (Perry et al., 2004), whose origin is still topic of discussion whether they are mesenchymal or not. Contrarily to meningiomas, mesenchymal non-meningothelial tumors are uncommon. In the 2021 World Health Organization (WHO) Classification of CNS Tumors (WHO Classification of Tumours Editorial Board, 2021), the mesenchymal non-meningothelial tumors include those neoplasms that exclusively occur in the CNS, presenting particular histological or molecular features, or that are relatively common in the CNS with respect to other sites. These tumors are subclassified on the basis of their differentiation: fibroblastic and myofibroblastic tumors (solitary fibrous tumor), vascular tumors (hemangiomas and vascular

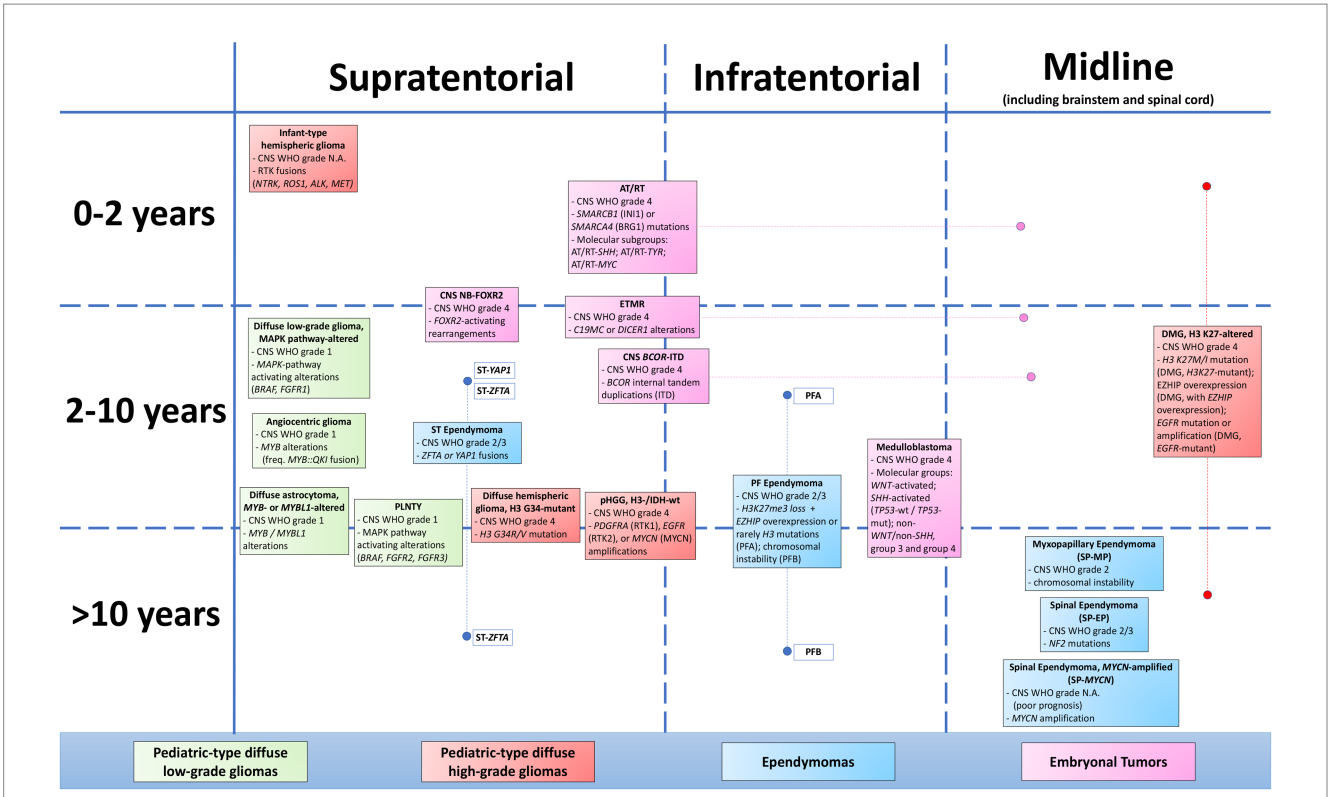

FIGURE 1
Map of pediatric CNS tumors, according to age of onset (left column) and location (first row). CNS WHO grade and relevant molecular features of each entity are presented in the figure, belonging to the groups of diffuse low-grade gliomas, diffuse high-grade gliomas, ependymomas, and embryonal tumors.

malformations, hemangioblastoma), skeletal muscle tumors (rhabdomyosarcoma), chondrogenic tumors (mesenchymal chondrosarcoma, chondrosarcoma), notochordal tumors (chordoma), and tumors of uncertain differentiation (Meredith and Alexandrescu, 2022). Among tumors of uncertain differentiation, there are Ewing sarcoma and three recently described tumors, which have been recognized as new entities and included in the fifth edition of the WHO Classification of CNS Tumors: primary intracranial sarcoma, DICER1-mutant; CIC-rearranged sarcoma; intracranial mesenchymal tumor, FET::CREB fusion-positive. Overall, mesenchymal non-meningothelial tumors of uncertain differentiation often show variable and not specific histology and immunophenotype, making their diagnosis challenging. The application of molecular techniques allowed a better understanding of these tumors and led to the inclusion of novel entities in the 2021 WHO Classification of CNS tumors, mandatory requiring the identification of specific molecular alteration for the diagnosis. However, as demonstrated by recently reported molecular alterations in CNS tumors that are still missing an appropriate classification (d'Amati et al., 2023a), we are currently far from having fully understood the wide spectrum of morphological and molecular aspects that characterize CNS mesenchymal tumors.

7 Discussion

Over the last decade, molecular studies have identified an increasing number of key genetic alterations in cancers, and this has improved our knowledge and understanding of the molecular basis underlying tumor biology. Identification of these cancer-specific alterations had changed clinical approach and improved diagnosis, classification, and prognosis of CNS tumors. In the past, histologic and immunohistochemical features alone were considered for classification of CNS tumors; nowadays, the molecular findings have led to disease stratification including molecular alterations as diagnostic criteria in the fifth edition of WHO classification of tumors of the CNS. According to WHO CNS5 classification, the relevant features of some types of CNS tumors, integrating grade and molecular alterations with age and location, are presented in Figure 1. The rationale of this "molecular classification" is also related to the effective and experimental molecular therapies, targeting some cancer-specific genetic events. Additionally, molecular classification is crucial because many patients are being considered for clinical trials of targeted treatments based on the genetics described on the underlying tumor. Then, this molecular stratification has identified specific classes of entities that appear homogeneous also in their response to treatment and clinical outcomes. Regardless of this progress, further modification is needed, particularly for rare and poorly characterized tumor. These important implications for clinical practice highlight the necessity to adopt the new classification when considering therapeutic options (clinical trials, targeted therapies) and discussing prognosis.

Author contributions

Ad'A: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. LBa: Validation, Writing – original draft. SR: Conceptualization, Data curation, Validation, Writing – review & editing. AC: Conceptualization, Data curation, Validation, Writing – review & editing. LBe: Data curation, Writing – review & editing. VBa: Data curation, Validation, Writing – review & editing. ME: Data

curation, Validation, Writing – review & editing. AB: Data curation, Writing – review & editing. SA: Data curation, Writing – review & editing. GM: Data curation, Writing – review & editing. GD: Data curation, Writing – review & editing. AM: Conceptualization, Data curation, Writing – review & editing. EM: Conceptualization, Data curation, Writing – review & editing. FD'A: Conceptualization, Data curation, Writing – review & editing. ES: Conceptualization, Data curation, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. VBi: Conceptualization, Data curation, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. MM: Conceptualization, Data curation, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. MG: Data curation, Writing – review & editing. MA: Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing. FG: Conceptualization, Data curation, Investigation, Supervision, Writing – original draft, Writing – review & editing.

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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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