PEDIATRIC CENTRAL NERVOUS SYSTEM TUMORS: STATE-OF-THE-ART AND DEBATED ASPECTS

EDITED BY: Angela Mastronuzzi, Andrea Carai, Elisabetta Ferretti and Evelina Miele

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PEDIATRIC CENTRAL NERVOUS SYSTEM TUMORS: STATE-OF-THE-ART AND DEBATED ASPECTS

Topic Editors:
Angela Mastronuzzi, Bambino Gesù Children Hospital (IRCCS), Italy
Andrea Carai, Bambino Gesù Children Hospital (IRCCS), Italy
Elisabetta Ferretti, Dipartimento di Medicina Sperimentale, Facoltà di Medicina e Odontoiatria, Sapienza Università di Roma, Italy
Evelina Miele, Bambino Gesù Children Hospital (IRCCS), Italy

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EDITORIAL
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Editorial: Pediatric Central Nervous System Tumors: State-of-the-Art and Debated Aspects

Andrea Carai[1] *and Angela Mastronuzzi*[2]*

[1] Neurosurgery Unit, Department of Neuroscience and Neurorehabilitation, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [2] Neuro-Oncology Unit, Department of Onco-Hematology, Cell and Gene Therapy, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

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Editorial on the Research Topic

Pediatric Central Nervous System Tumors: State-of-the-Art and Debated Aspects

Central nervous system tumors are the second most frequent malignancy in children and young adults. Despite this, they remain rare conditions and management standardization continues to be challenging despite international networking efforts.

The Research Topic on "pediatric central nervous system tumors: state-of-the-art and debated aspects" we included innovative and original contributions on multiple aspects of pediatric neuro-oncology.

In reference to the neuroimaging, the work of Colafati et al. presents preliminary data suggesting direct involvement of the V cranial nerve at diagnosis as a negative prognostic marker in DIPG.

In their analysis, 13.8% of the children presented cranial nerve V involvement at diagnosis. This finding was associated with a poor prognosis (median overall survival: 7 vs. 13 months), concluding that cranial nerve V should be routinely evaluated with diagnostic scans.

Even if these findings need to be confirmed with a larger series, it is important to notice that, in the era of radiomics, accurate interpretation of traditional MR sequences can still contribute in advancing clinical knowledge.

In the paper by Yalon et al. an elevated neutrophil to lymphocyte ratio, which is a relatively simple blood-derived biomarker, is suggested to be a hallmark of malignant brain tumors. The biological justification for this observation would be both a reduction in lymphocytes, considered to be protective from cancer, and an increase in neutrophils, usually associated to tumor progression. This paper highlights two promising fields of pediatric neuro-oncology: the potential role of immunity modulation for treatment and the opportunity offered by the development of biomarkers to assist in the treatment of patients.

The contribution by Foster et al. offers a wide overview on the advancement of neuro-oncology surgery. Sophisticated techniques allow more accurate surgical planning, better visualization and orientation during surgery, and an increase of intraoperative safety. Advancing the possibilities in tumor resection while preserving neurological functions, will certainly overall contribute to better treatment results, specifically for patients with low-grade lesions that still suffer significant surgical morbidity.

In the last decades, significant progress in oncology has been achieved through molecular characterization of tumors and targeted therapies. Unfortunately, not all tumor types are eligible for these treatment options. into It is critical for the treatment to detect if the BRAF V600E mutation is found in a subset of pediatric low-grade gliomas, thus specific inhibitors of the mutated protein, such as Vemurafenib, can be used. In the paper by Del Bufalo et al., the authors investigated

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Paul A. Northcott,
St. Jude Children's Research Hospital, United States

***Correspondence:**
Angela Mastronuzzi
angela.mastronuzzi@opbg.net

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Vemurafenib's safety and efficacy as a single agent in pediatric patients with BRAFv600E positive LGG, which showed very encouraging results.

Petruzzellis et al. further demonstrated the efficacy and safety of this target therapy, in the setting of Pleomorphic Xanthoastrocytoma (PXA) associated with Down syndrome. PXA is a rare WHO grade II tumor that can harbor the BRAF mutation p.V600E. This case report describes the first occurrence of a PXA reported in a child with Down syndrome (DS) as well as the first use of Vemurafenib in DS. The treatment was well-tolerated, and the efficacy was seen by a partial response and a stabilization of the disease. In conclusion, despite the use of Vemurafenib, not yet standardized for pediatric patients affected by brain tumors and DS, we have shown the feasibility of this therapeutic approach.

In addition to the molecular characterization of tumors, several significant discoveries have contributed to shedding light on the role of epigenetic modification and cellular microenvironment in tumor growth and progression. Proteins of the Polycomb group (PcG), which is one of the major epigenetic modification, can be differentiated in polycomb repressive complexes (PRCs): PRC1 and PRC2. The trimethylation of lysine on Histone H3 is an epigenetic modification induced by enhancer of zeste homolog 2 (EZH2), the catalytic core subunit of PRC2, leading to the silencing of many tumor suppressor genes. Overexpression of EZH2, evidenced by a growing number in data, is associated with a poor outcome and progression in a large number of cancer cases.

Hypoxia inducible factor (HIF), a crucial transcription factor involved in promoting and regulating tumor development, promotes inflammation, angiogenesis, metabolic reprogramming, invasion, and metastatic fate.

In their review, Papale et al. analyzed the activity and influence of EZH2 and HIF in pediatric cancer progression, the correlation between them and the possible future role of specific inhibitors.

Medulloblastoma is among the most common malignant childhood brain tumors (WHO grade IV). Genomic studies have defined four consensus molecular subgroups (WNT, SHH, Group 3, and Group 4), each are characterized by distinct clinical outcomes, copy-number variation, transcriptional profiles, and somatic mutations.

Aberrant expression of long non-coding RNAs, which are normally expressed in the human brain, have been linked to neuro-oncological disorders. In their paper, Laneve et al. tried to explain the function of long non-coding RNAs in the medulloblastoma biology and development.

From another point of view, several studies have investigated the role of epigenetic modulators in various types of cancers. Recently, the molecular epigenetic deregulation in Medulloblastoma has been reviewed, highlighting the pathways implicated in the disease, their different biological behaviors and possible future target therapies. In this setting, Zwergel et al. have summarized and highlighted epigenetic modulators as promising drug targets in MB.

Moreover, Abballe et al. investigated the role of Numb in medulloblastoma's cancer cells. Their study showed that Numb p66, which is expressed in medulloblastoma stem-like cells and cerebellar neuronal stem cells (NSCs), modulates cancer staminality. In particular, the medulloblastoma samples analyzed in this study, showed low levels of Numb p66 and overexpression of Numb p72 compared to normal tissue. These results show different roles for the two major Numb isoforms evaluated in medulloblastoma, which highlighted a central role for Numb p66 in regulating stem-like cells and NCS maintenance.

Pediatric neuro-oncology remains a challenging arena for researchers with different expertise. We believe that the coordinated work on the study of different types of tumor, as found in this Research Topic, from various points of view, will be vital in the contribution to advancing the knowledge of these tumors, will be key to the improvement clinical results.

AUTHOR CONTRIBUTIONS

AC and AM have jointly contributed intellectually and materially to the work, and approved for publication.

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ORIGINAL RESEARCH
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Numb Isoforms Deregulation in Medulloblastoma and Role of p66 Isoform in Cancer and Neural Stem Cells

Luana Abballe[1†], Angela Mastronuzzi[2†], Evelina Miele[2], Andrea Carai[3], Zein Mersini Besharat[1], Marta Moretti[4], Enrico De Smaele[1], Felice Giangaspero[5,6], Franco Locatelli[2,7], Elisabetta Ferretti[1,6‡] and Agnese Po[4‡]*

[1] Department of Experimental Medicine, Sapienza University, Rome, Italy, [2] Department of Pediatric Hematology/Oncology and Cellular and Gene Therapy, Bambino Gesù Children's Hospital, Rome, Italy, [3] Neurosurgery Unit, Department of Neuroscience and Neurorehabilitation, Bambino Gesù Children's Hospital, Rome, Italy, [4] Department of Molecular Medicine, Sapienza University, Rome, Italy, [5] Department of Radiological, Oncological and Pathological Science, Sapienza University, Rome, Italy, [6] IRCCS Neuromed, Pozzilli, Italy, [7] Department of Paediatrics, University of Pavia, Pavia, Italy

Numb is an intracellular protein with multiple functions. The two prevalent isoforms, Numb p66 and Numb p72, are regulators of differentiation and proliferation in neuronal development. Additionally, Numb functions as cell fate determinant of stem cells and cancer stem cells and its abnormal expression has been described in several types of cancer. Involvement of deregulated Numb expression has been described in the malignant childhood brain tumor medulloblastoma, while Numb isoforms in these tumors and in cancer stem-like cells derived from them, have not been studied to date. Here we show that medulloblastoma stem-like cells and cerebellar neuronal stem cells (NSCs) express Numb p66 where its expression tampers stemness features. Furthermore, medulloblastoma samples evaluated in this study express decreased levels of Numb p66 while overexpressed Numb p72 compared with normal tissues. Our results uncover different roles for the two major Numb isoforms examined in medulloblastoma and a critical role for Numb p66 in regulating stem-like cells and NSCs maintenance.

Keywords: medulloblastoma, numb, NUMB isoforms, cancer stem cells, neural stem cells, sonic hedgehog signaling

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Rimas J. Orentas, Seattle Children's Research Institute, United States

Reviewed by:
Mingfu Wu, Albany Medical College, United States
Anshu Malhotra, Emory University, United States

****Correspondence:***
Agnese Po
agnese.po@uniroma1.it

[†]These authors share first authorship

[‡]These authors share last authorship

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INTRODUCTION

Medulloblastoma (MB) arises in the cerebellum and is the most common malignant pediatric brain tumor (1, 2), originating from both granule cell progenitor (GCPs) and neural stem cells (3). Human medulloblastoma is divided in at least 4 molecular subgroups (WNT, SHH, Group 3, and Group 4) which are characterized by different patterns of gene expression, genetic aberrations, and clinical outcomes (4). SHH MBs account for roughly 27% of tumors and are characterized by aberrant activation of the Sonic hedgehog (Shh) pathway. This aberrant activation is mostly achieved by genetic loss of negative regulators (i.e., Ptch) or amplification of positive regulators (i.e., Gli2) (4–6).

Cancer subpopulations with stemness features (stem-like cells, SLCs) are considered the ultimate reservoir of cancer cells and have been isolated and characterized in several solid tumors, including brain tumors (7). In our previous studies we isolated and characterized cerebellar NSCs and SLCs of human and murine origin and we showed that Shh is a major driver of stemness in this context, through the transcriptional activity of the transcription factor Gli1 and the post-transcriptional regulation of Gli1 activity (8–12).

Numb is an adaptor protein, evolutionary conserved from flies to mammals, (13), which is involved in many cellular processes (14), and is able to behave as a cell fate determinant, being responsible for daughter cell polarization in asymmetric division (14–16). Numb has been described in cortical ventricular zone cells (17) and neural crest lineages (18), where it segregates preferentially in neural daughter cell during asymmetric division (16). Mammalian Numb is transcribed in four isoforms, namely p65, p66, p71, and p72, produced by alternative splicing (19), of which the p66 and p72 have been the main focus of research. Several studies described the role of Numb in stem cells compartment's maintenance, acting as intrinsic determinant through the interaction with signaling pathways such as Notch, p53 and Shh (20–23). To this regard, Numb was shown to control Gli1 function by inducing Gli1 ubiquitination and degradation (23, 24). Interestingly, Numb is abnormally expressed in many cancer types (25–29), and has been demonstrated to play a role in cancer stem cell subpopulation of colorectal cancer (30) and gliomas (31).

The role of Numb as fate determinant in different types of stem cells, the important role of Shh in the maintenance of NSCs and MB SLCs and the molecular relationship between Numb and Gli prompted us to investigate the role of Numb in the mouse cerebellar neural stem cells (NSCs) as well as in medulloblastoma stem-like cells (MB-SLCs). Moreover, we aimed to investigate the expression of Numb isoforms in MB subgroups, since previous studies were conducted before the definition of the molecular subgroups and also the differential expression of Numb isoforms was not explored (23).

MATERIALS AND METHODS

Cell Cultures

Stem Cells. Cerebellar NSCs were obtained from cerebella of postnatal 4-day-old wild-type black 6 /C56 (C57BL/6) mice (Charles River). NSCs were derived as previously described (12).

Mouse Medulloblastoma stem-like cells (mMB-SLCs) were derived from spontaneous tumors arisen in Ptc+/– mice, as previously described (9, 32).

Human Medulloblastoma stem-like cells (hMB-SLCs) were derived from primary human MB during surgical resection as previously described (9). hMB-SLCs were immunostained with APC-conjugated anti-CD133 (Miltenyi Biotec) according to manufacturer's protocol and sorted using a FACSAriaIII (BD Biosciences) prior to experiments (32).

NSCs and MB-SLCs were cultured in Selective medium (SM) for stem cells enrichment, containing DMEM/F12 (Gibco) supplemented with 0.6% glucose, 25 mg/ml insulin, 60 mg/ml *N*-acetyl-L-cysteine, 2 mg/ml heparin, 20 ng/ml EGF, 20 ng/ml bFGF (Peprotech, Rocky Hill, NJ), 1X penicillin-streptomycin, and B27 supplement without vitamin A (Gibco).

For neurosphere/oncosphere forming assay NSCs and MB-SLCs were disaggregated to single cell and plated at clonal density (1–2 cells/mm^2) into 96-well plates, in selective medium. After 10–14 days, the number of neurospheres or oncospheres was divided by the number of cells plated to determine the percentage of neurosphere forming cells and oncosphere forming cells, respectively.

To induce differentiation, NSCs were disaggregated and plated on poly-lysine coated dishes in differentiation medium containing platelet-derived growth factor (PDGF; 10 ng/ml) (Sigma, P3076), for 48 h (9).

Animal experiments were approved by local ethic authorities and conducted in accordance with Italian Governing Law (D.lgs 26/2014; Prot. no. 03/2013).

P19 were purchased from ATCC and maintained in Alpha Minimum Essential Medium with ribonucleosides and deoxyribonucleosides supplemented with 7.5% bovine calf serum, 2.5% fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo scientific).

Treatments

Transduced NSCs were treated with a Smoothened antagonist cyclopamine-KAAD, (Calbiochem), at the 1 μM, and with Smo-agonist SAG (200 nM, Alexis), for 48 h. For differentiation experiment, cells were treated with platelet derived growth factor (PDGF, Sigma-Aldrich) for 48 h.

Immunofluorescence

To detect Gli1 and Numb, neurospheres were blandly disaggregated and plated on poly-lysine-coated Lab-Tek chamber slides (cover slips) for 2 h. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, incubated in blocking solution (5% normal goat serum, 1% BSA, 0.1% Triton X-100) and stained overnight with primary antibodies diluted in blocking solution and for 2 h with secondary antibodies. Primary antibodies were mouse anti-Gli1 and rabbit anti-Numb (Cell Signaling Technology Inc). 594- or 488-conjugated anti-mouse and anti- rabbit secondary antibodies were purchased from Molecular Probes (Invitrogen, Eugene, OR). Nuclei were counterstained with Hoechst reagent. Cover slips were mounted with fluorescence mounting medium (Dako, Carpinteria, CA). Images were acquired with Carl Zeiss microscope (Axio Observer Z1) and AxioVision Digital Image Processing Software.

Lentiviral Transduction

Neurospheres were transduced with pGreenZeo Lentiviral Reporter Vectors containing specific promoters for NANOG

Abbreviations: MB, Medulloblastoma; Shh, Sonic Hedgehog; NSC, Neural Stem Cells; SLC, Stem-like cells; LvNumb, Lentivirus Numb; hMB-SLC, Human Medulloblastoma stem-like cells; MB-SLC, Murine Medulloblastoma stem-like cells.

(Nanog-GFP) or CMV (Zeo-GFP) (9) and infected cells were selected with Zeocin (Thermo Fisher) treatment.

Numb p66 lentiviral infection was performed using a lentiviral vector pRRL–CPPT–CMV–PGK–GFP–WPRE (TWEEN) containing Numb p66 coding sequence (23). NSCs and MB-SLCs were infected for 48 h prior to analyses.

Knockdown Experiments

Silencing of endogenous Numb was performed using ON-TARGET plus Human NUMB siRNA (L-015902-00-0005) for hMB-SLCs and ON-TARGET plus Mouse Numb siRNA (L-046935-01-0005) for NSCs and mMB-SLCs. Dharmacon. Hiperfect reagent (Qiagen) was used for siRNA transfections according to manual instructions. After 72 h, cells were harvested and subjected to mRNA expression analysis.

RNA Extraction and Gene Expression Analysis

Total RNA was isolated from cells and human tissue using Trireagent (Ambion) and reverse transcribed in cDNA as previously described (12). cDNA was used for quantitative RT-PCR (qRT-PCR) analysis using ViiA ^{TM} 7 Real-Time PCR System and SensiFAST^{TM} Probe Lo-ROX (Bioline).

For each mRNA analysis 10 ng of cDNA were used. We selected best coverage TaqMan gene expression assay from Applied Biosystems and used according to the manufacturer's instructions. To analyze Numb isoforms, the following assay IDs were used: murine Numb p66 Mm01302754_m1; murine Numb p72 Mm01304901_m1, human Numb p66 Hs01105435_m1; human Numb p72 Hs01105426_m1.

mRNA quantification was expressed in arbitrary units and each amplification reaction was performed in triplicate. All Results were evaluated using the 2-ΔΔCT method and values were normalized to three endogenous controls: ß-actin, ß2-microglobulin, and Gapdh.

Western Blot Assay

Western blot was performed as previously described (33). Cellular pellets were lysed using lysis buffer: Tris-HCl pH 7.6 50 mM, deoxycholic acid sodium salt 0.5%, NaCl 140 mM, NP40 1%, EDTA 5 mM, NaF 100 mM, sodium pyrophosphate 2 mM, and protease inhibitors. Cellular lysates were separated on 8% acrylamide gel and western blot analysis was performed using standard procedures. Membranes were incubated overnight with the following antibodies: anti-Numb (ab4147; Abcam), anti-mouse Nanog (Cosmo Bio Co, Japan), anti-GAPDH (ab8245; Abcam), anti-Actin I-19 (sc-1616; Santa Cruz Biotechnology), anti-mouse Gli1 (#2643; Cell signaling), anti-NeuN (MAB377 Millipore), anti-βIII-tubulin (MAB 1637 Millipore). HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) were applied on membranes and signals were visualized by enhanced chemiluminescence (ECL Advansta). Densitometry was performed using ImageJ software and protein levels were normalized to the respective loading control. Error bars represent mean ± standard deviation of at least three experiments.

Human MB Samples and Controls

Surgical specimens of primary MBs were originated from a cohort of patients included in the present study, enrolled with Institutional Review Board approval, as previously described (34). Molecular subgroup classification was performed as described in (34). Number of MBs analyzed for each molecular subgroup: WNT n: 10; SHH n: 25; G3 n: 25; G4 n: 19.

Correlation analysis between the isoforms was measured using GraphPad Prism 6 software (La Jolla, CA, USA).

Commercial non-neoplastic cerebellum was purchased from Bio-chain Institute ($n = 4$: R1234039-50, Total RNA-Human Brain cerebellum Adult; $n = 4$: R1244041-50 and R1244040-50, Total RNA-Human Brain cerebellum Fetal).

RESULTS

Numb has a Pro-Differentiation Role in Cerebellar Neural Stem Cell (NSCs)

Involvement of Numb in cell determination and differentiation and in cortical neurogenesis has already been described (35), while the role of Numb in cerebellar neural stem cell (NSCs) differentiation has not been studied to date. First of all, we evaluated Numb protein expression in NSCs with respect to starting population (**Figure 1A**). NSCs were identified as the neurosphere forming cells after at least 30 days in selective medium (SM), and were compared to both the bulk cell population and to cerebellar cells after 5 days in SM. Notably, Numb protein level was lower at day 5 in SM with respect to both bulk population and NSCs, probably due to a selection of stem cells in medium, and its expression increased at day 30, when NSC culture was established (**Figure 1A**). Since only one band was revealed by western blot analysis, we compared Numb protein expression pattern of NSCs with the protein expression in murine embryonal carcinoma P19 cells after differentiation stimuli. P19 cells represent a model of neuronal differentiation which express both Numb p66 and Numb p72 isoforms (19). Interestingly, NSCs expressed high levels of the Numb p66 isoform while Numb p72 was not detectable (**Supplementary Figure 1**). To investigate the distribution of Numb positive cells in the heterogeneous population of neurosphere culture, we performed immunofluorescence staining of Numb and Gli1 (**Figure 1B**), a stemness marker in the context of cerebellar NSCs (9). Interestingly, Numb is expressed in both Gli1 positive and Gli1 negative cells.

To further investigate whether Numb was associated with stemness features in the neurosphere population, we sorted cells according to their expression of the stemness factor Nanog (9), and we observed that Nanog positive cells expressed significantly lower levels of Numb p66, with respect to control (**Figure 1C**).

In order to explore the role of Numb in influencing the balance between stemness and neural differentiation, we evaluated Numb p66 protein level with western blot analysis, in NSCs before and after *in vitro* differentiation (**Figure 1D**). Numb p66 protein level was increased in NSCs after differentiation stimuli such as platelet-derived growth factor (PDGF), together with an

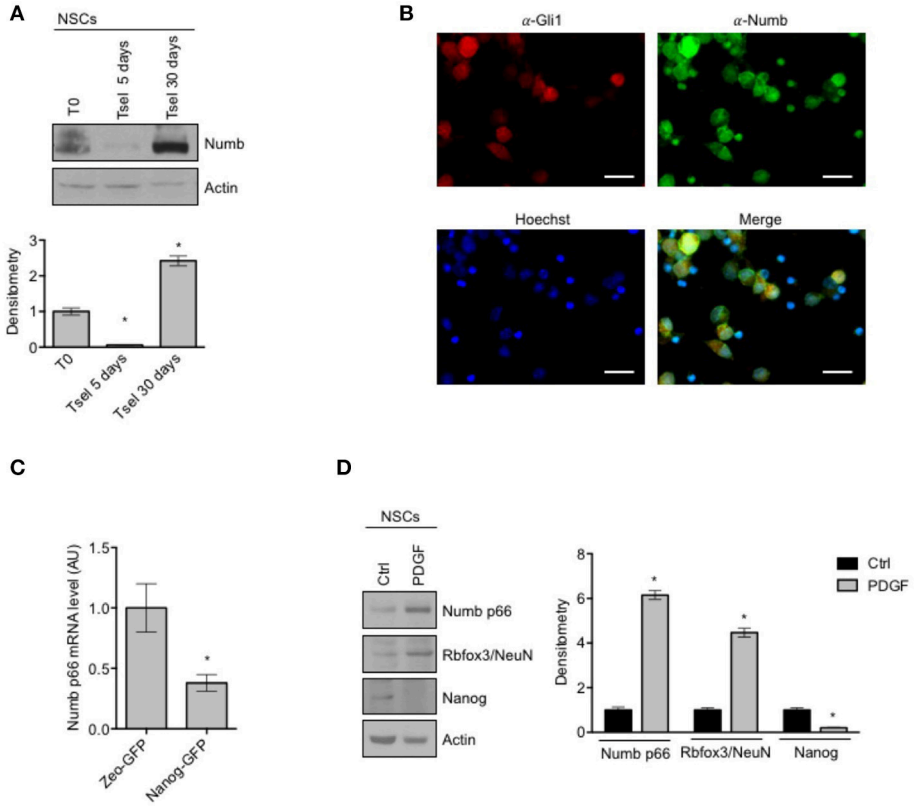

FIGURE 1 | Numb expression in NSCs. **(A)** Representative Western Blot (WB) (up) and densitometric analysis (bottom) of endogenous Numb isoforms (p66 and p72) in NSCs grown in selective medium for 5 or 30 days, compared to bulk cells (T0). Actin was used as loading control. *P*-values $^*p < 0.05$. **(B)** Representative images of immunofluorescence staining of NSCs for Numb (green) and Gli1 (red); nuclei are counterstained with Hoechst (blue). Scale bar: 10 μm for all panels. **(C)** qRT-PCR analysis showed mRNA expression of Numb p66, evaluated in NSCs infected with lentivirus carrying Nanog-GFP or Zeo-GFP as control and sorted for GFP. Data represent means ± SD from three independent experiments. *P*-values $^*p < 0.05$. **(D)** Representative Western Blot (WB) (left) and densitometric analysis (right) of NSCs before and after *in vitro* differentiation for 48 h (PDGF); p66, Rbfox3/NeuN, Nanog were evaluated. Actin was used as loading control. *P*-values $^*p < 0.05$. **(A–D)** Data are means ± SD from three independent experiments. Full-length images are presented in **Supplementary Figures**.

enhanced expression of differentiation markers (Rbfox3/NeuN) and a reduced expression of Nanog stemness marker.

We next proceeded to investigate the role of Numb p66 in NSCs by modulating its expression. We performed lentiviral infection of NSCs with a virus encoding the ORF of Numb p66 (LvNumb) and evaluated the effects after 48 h. LvNumb-transduced NSC cells showed a differentiated phenotype with adherent morphology (**Figure 2A,** left), up-regulation of differentiation neural markers (Rbfox3/NeuN and βIII-tubulin) and down-regulation of the stemness markers Nanog and Gli1 (**Figure 2A,** right). Thus, our data support the role for Numb in promoting the "differentiated phenotype" of NSCs.

To deepen our understanding of the relationship between Numb p66 expression, stemness features and Shh signaling, we evaluated the expression of relevant markers in LvNumb-transduced NSCs. In detail, we observed a strong reduction, at transcription level, of both key components in the Shh pathway (Gli1, Gli2) and the stemness markers Prom1 and Nanog compared with control cells (**Figure 2B**). Consistently with mRNA level data of stemness markers, Numb overexpression also impaired the ability of NSCs to form secondary neurospheres, i.e., their clonogenicity (**Figure 2C**) and Numb transduced NSCs formed smaller neurospheres. We previously showed that the modulation of the Shh signaling in NSCs is able to significantly enhance or impair clonogenicity (9). Interestingly, after modulation of the Shh signaling in LvNumb-transduced NSCs, we did not observe any significant variation of self-renewal, suggesting that Numb overexpression could counteract this pathway (**Figure 2C,** right).

In order to further investigate the role of Numb p66 in NSCs, we performed silencing of Numb (siNumb) and observed an upregulation of Nanog and Gli1 (**Figure 2D**). Altogether, these data support a role for Numb p66 in NSCs where it induces neural differentiation and controls stemness by negatively regulating the Shh signaling.

NUMB p66 Controls Self-Renewal of SHH MB Stem-Like Cells

To investigate the role of NUMB in cancer stem-like cells (SLCs) from SHH MB, we performed the following set of experiments in SLCs derived from a human SHH MB, referred to as hMB-SLCs, as previously described (32). First of all, we sorted hMB-SLCs for

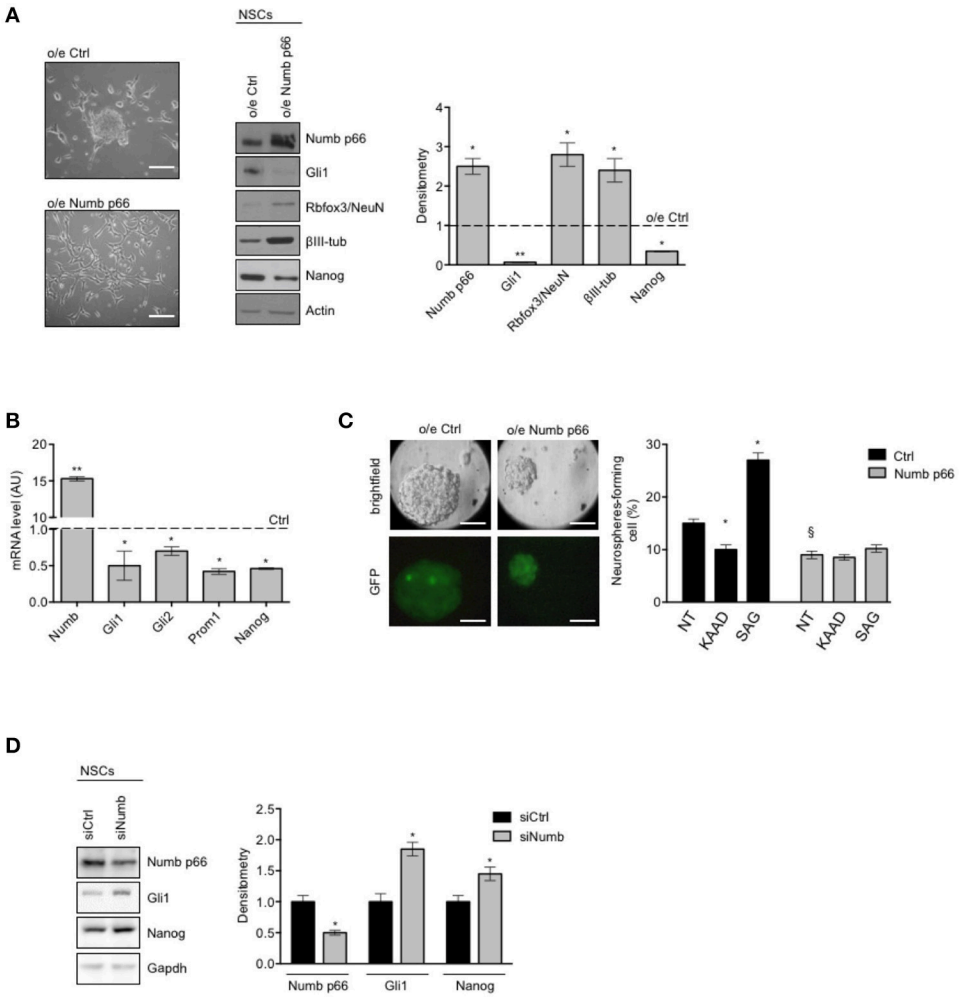

FIGURE 2 | Numb antagonizes stemness and promotes differentiation of cerebellar NSCs. **(A)** (Left) Representative bright-field images of neurospheres after overexpression of p66 (or Ctrl). Scale bar: 100 μm. Representative western blot images (center) and densitometric analysis (right) of analysis of p66, stemness, and/or Hedgehog markers Nanog and Gli1 and differentiation markers Rbfox3/NeuN and βIII-tubulin (βIII-tub), in NSCs after overexpression of p66 vs. Ctrl. Actin was used as loading control. *P*-values $*p < 0.05$. **(B)** mRNA expression levels of Numb p66 and stemness and/or Hedgehog markers (Gli1, Gli2, Prom1, and Nanog), in NSCs after overexpression of p66. Dashed bars represent control cells (Ctrl). Data are means ± SD from three independent experiments. *P*-values $*p < 0.05$; $**p < 0.01$. **(C)** (Left) LvNumb-transduced NSCs were plated for clonogenic assay as described in Material and Methods. Upper panels show brightfield images of secondary neurospheres, lower panels show GFP of transduced cells. Scale bar: 100 μm. (Right) Neurosphere-formation capacity of LvNumb-transduced NSCs and treated with cyclopamine-KAAD (KAAD) to suppress endogenous Hedgehog signaling or with Smo-agonist (SAG). Data are means ± SD from three independent experiments. *P-values (vs. NT), $*p < 0.05$. §P-value (vs. LvCtrl), $§p < 0.05$. **(D)** Representative Western Blot (WB) (left) and densitometric analysis (right) of NSCs before and after *in vitro* differentiation for 48 h (PDGF); p66, Rbfox3/NeuN, Nanog were evaluated. Actin was used as loading control. *P*-values $*p < 0.05$. GAPDH was used as loading control. **(A–D)** Data are means ± SD from three independent experiments. Full-length images are presented in **Supplementary Figures**.

the stemness marker CD133 (32), and analyzed the expression profile of CD133 positive (CD133+) and negative (CD133-) cells.

As shown in **Figure 3A**, expression level of NUMB p66 was significantly lower in CD133+ cells, with respect to CD133– ones, while NUMB p72 showed a positive trend in CD133 positive cells, without reaching statistical significance. Interestingly, mRNA levels of NUMB p66 resulted higher than NUMB p72 in CD133+ hMB-SLCs (**Supplementary Figure 1B**), in accordance with protein data. We also investigated markers of the Shh pathway (GLI1, CYCLIN D1, HIP1), that we previously showed to drive stemness in cerebellar NSCs and SHH MB SLCs (9). Shh pathway resulted more active in CD133+ (**Figure 3A**). These data suggest that NUMB p66 was associated with a reduced state of stemness hMB-SLCs.

We then proceeded to evaluate the role of NUMB in hMB-SLCs *in vitro*. As shown in **Supplementary Figure 1A**, hMB-SLCs express high protein levels of NUMB p66 isoform. To explore the role of NUMB p66 in hMB-SLCs, we performed lentivirus-mediated overexpression of NUMB p66 (**Figure 3B,** left). We then evaluated the clonogenicity of LvNumb-transduced hMB-SLCs cells, a significant decrease of

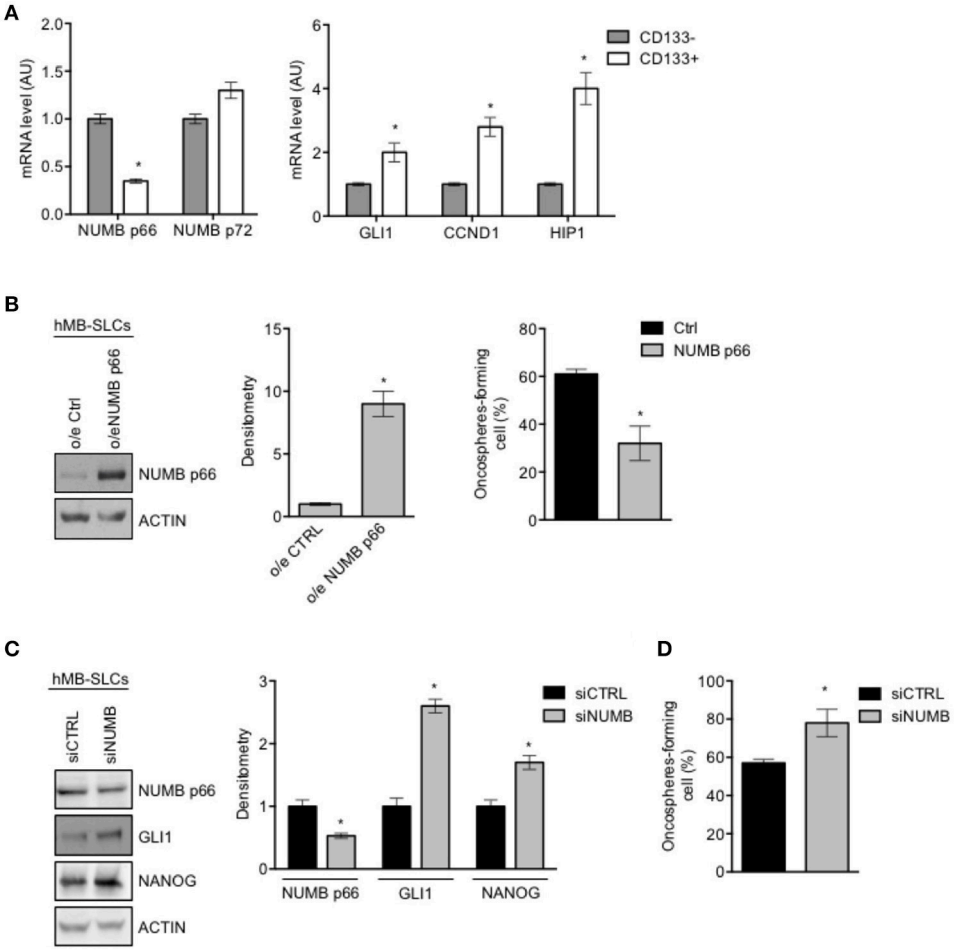

FIGURE 3 | Modulation of NUMB in hMB-SLCs. **(A)** qRT-PCR analysis showed mRNA expression of NUMB isoforms, p66, and p72, and of Hedgehog markers (GLI1, CCND1, HIP1) evaluated in hMB-SLCs CD133^{+} vs. CD133^{-}. Data represent means ± SD from 3 independent experiments. *P*-values $^{*}p < 0.05$. **(B)** Representative western blot images (left) and densitometric analysis (center) of p66 in hMB-SLCs, after overexpression of p66 vs. Ctrl. ACTIN was used as loading control. (Right) Oncospheres-formation capacity of LvNumb-transduced hMB-SLCs vs. LvCtrl. *P*-values $^{*}p < 0.05$. **(C)** Representative images of western blot (left) and densitometric analysis (right) analysis of p66, NANOG, and GLI1, in hMB-SLCs transfected with siRNA against NUMB (siNUMB) or non-targeting siRNA controls (siCTRL). ACTIN was used as loading control. *P*-values $^{*}p < 0.05$. **(D)** Oncospheres-formation capacity of siNUMB hMB-SLCs vs. siCTRL. *P*-values vs. siCTRL. Data represent means ± SD from three independent experiments. *P*-values $^{*}p < 0.05$. **(B–C)** Data are means ± SD from three independent experiments. Full-length images are presented in **Supplementary Figures**.

the oncospheres' forming capability was observed in hMB-SLCs overexpressing NUMB vs. control cells (**Figure 3B,** right).

To further understand the role of p66 in regulating stemness in MB-SLCs, we depleted the expression of NUMB using small interfering RNA (siNUMB) in hMB-SLCs. As shown in **Figure 2C**, following NUMB silencing, hMB-SLCs showed an increase of GLI1 protein level respect to control siRNA-transfected cells. Moreover, siNUMB hMB-SLCs showed an increase of NANOG stemness marker (**Figure 3C**) and oncosphere-forming ability (**Figure 3D**). These results suggest that NUMB may have a role in negatively regulating stemness features in hMB-SLCs. Additionally, we performed silencing of Numb in stem like cells isolated from murine SHH MB spontaneously arisen in Ptch +/− mice (9, 32). Numb silencing caused an increase in Gli1 and Nanog protein levels (**Supplementary Figure 2A**) and enhanced clonogenicity (**Supplementary Figure 2B**).

Our evidence show that NUMB plays a critical role in influencing MB-SLCs behavior, blocking the Sonic Hedgehog signaling and stemness features.

Numb Alternative Splicing in Medulloblastoma

We then investigated NUMB expression in a cohort of MB using the MB dataset provided by Cavalli (Tumor Medulloblastoma–Cavalli—763 -rma_sketch—hugene11t) in the R2 platform (36). MB samples were divided into molecular subgroups and according to total NUMB expression level (low or high). As shown in **Supplementary Figure 3**, low expression of NUMB was

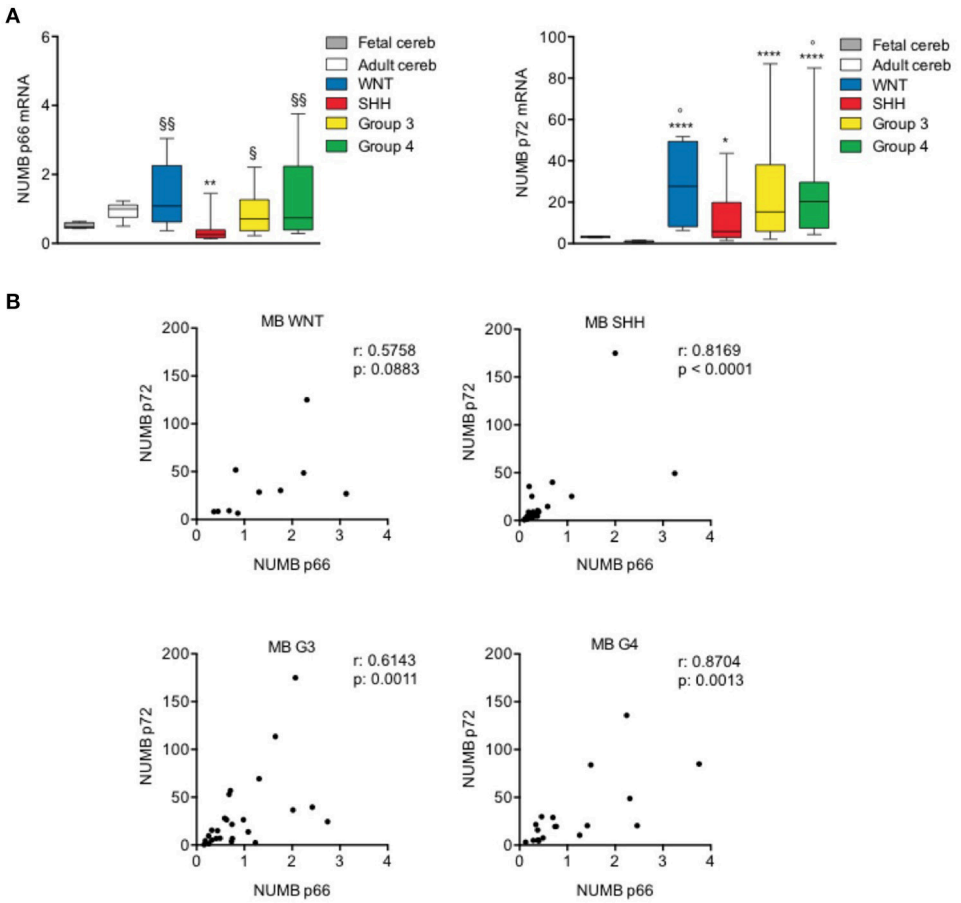

FIGURE 4 | NUMB isoforms expression in human MB samples. **(A)** qRT-PCR analysis showed NUMB isoforms [p66 (left) and p72 (right)] mRNA expression for molecular subgroups of primary MBs (WNT *n*: 10; SHH *n*: 25; G3 *n*: 25; G4 *n*: 19), adult normal cerebellum (Adult cereb) and fetal normal cerebellum (Fetal cereb). **P*-value vs. Adult cereb, **p* < 0.05; ***p* < 0.01; *****p* < 0.0001. ° = *P* vs. Fetal cereb, °*p* < 0.05. §*P*-value vs. SHH MBs, §*p* < 0.05; §§*p* < 0.01. **(B)** Pearson's correlation test of p66 vs. p72, in human MB subgroups, to evaluate the link between the expression of NUMB isoforms (r = correlation coefficient; *p* < 0.05 significance).

associated with shorter overall survival (OS) in SHH and G3 subgroups.

Unfortunately, in the publicly available datasets the present probes do not discriminate the different isoforms of NUMB.

The expression level of NUMB 66 (p66) [Isoform 2, identifier: P49757-2] and NUMB 72 (p72) [Isoform 1, identifier: P49757-1] was evaluated in a cohort of human MB patients' samples characterized and divided in molecular subgroups.

The transcript level of p66 resulted significantly reduced only in SHH subgroup, whereas the p72 mRNA level was significantly up-regulated in all tumor molecular subgroups, as shown in **Figure 4A**, compared with normal adult cerebellar tissue (control). Interestingly, the up-regulation of p72 was stronger and more significant in G3, G4, and WNT with respect to SHH, although not reaching statistical significance in the comparison among subgroups. Furthermore, the expression of Numb p66 transcript was significantly higher in non-SHH tumors with respect to SHH. We also investigated the mRNA expression of NUMB isoforms in normal fetal cerebellum. With respect to adult tissues, fetal cerebellum showed a trend toward down-regulation of p66 and a trend of up-regulation of p72, even though it didn't reach statistical significance (**Figure 4A**). NUMB p66 expression in MBs failed to show statistical significance in the comparison with fetal cerebellum, and NUMB p72 resulted statistically up-regulated only in G4 MBs and WNT MBs. These data support previous findings that showed that SHH MB derive from of proliferating granule cell progenitors in the external granular layer (EGL) (37), that physiologically dissipates during the first year of postnatal life (38).

Next, we performed a correlation analysis of the expression level of the two isoforms analyzed that indicated significant positive correlation ($p < 0.05$) in SHH, G3, and G4 subgroups. WNT subgroup showed a trend toward positive correlation but didn't reach the statistical significance, possibly due to the small number of samples included in the study, with respect to other subgroups (**Figure 4B**).

Together, these data suggest a different role of NUMB p66 and p72 isoforms in human MB samples. Interestingly, we demonstrated a decreased expression of p66 only

in the SHH subset, suggesting that different isoforms may exert divergent functions in the different MB subgroups.

DISCUSSION

In this study we investigated for the first time, the expression of the two main NUMB isoforms p66 and p72 in a wide MB cohort, finding that different NUMB isoforms were differentially expressed among subgroups.

Indeed, NUMB p66 and p72 have been shown to have different/opposite roles regulating cellular functions. In murine embryonic carcinoma cells, it is described that p66 isoform is involved in differentiation but not in proliferation, whereas p72 has a role in proliferation but not in differentiation (39). This difference in the role of each isoform could explain the different functions described for NUMB in different types of cancer.

Specifically, NUMB has been described as an oncosuppressor in breast cancer (29), esophageal squamous cell carcinoma (27) and mesothelioma (40), but evidence showed also a role for NUMB as an oncogene in hepatocellular carcinoma (41), in astrocytomas (42) in cervical squamous carcinoma cells (43), and in endometrial cancer (44).

These different roles could reflect the different isoforms' expression in our cohort of human MB samples. Indeed, regardless of the molecular subgroup, NUMB p72 is up-regulated. The expression of this isoform could have a role in accelerating cell proliferation and promoting EMT features in MB as suggested by studies in other context (41, 45).

Interestingly, we found that NUMB p66 was significantly down-regulated in SHH MB only. Numb p66 is expressed during development and in adult brain (39, 46), and in murine P19 embryonic carcinoma cell line, p66 is reported to promote neural differentiation (39). Numb p66 was also shown to be down-regulated in murine models of SHH MB and to control Shh pathway activation through the regulation of Gli1 function, via its ubiquitination and proteasome dependent degradation (23, 24). Thus, our results strongly suggest that low levels of NUMB p66 in SHH MB contribute to Shh pathway deregulation keeping cancer cells in an undifferentiated state and enhancing their cancer stemness features. Altogether these results point out to different roles for NUMB isoforms in the MB subgroups possibly reflecting the diverse cell signaling pathways governing them. We believe that the dual role of NUMB as oncosuppressor or oncogene might be ascribed to the different isoform expressed, a topic that has not been fully investigated in the cited literature.

NUMB isoforms differential expression is controlled by alternative splicing. Of note, RBFOX3 a member of RNA-binding Fox (Rbfox) family, is one of the most important regulators of NUMB alternative splicing in neuronal lineages (47). RBFOX3/NEUN is expressed in neurons (47, 48) and during spinal neuronal development, RBFOX3/NEUN regulates neuronal differentiation promoting the alternative splicing of NUMB mRNA, from the isoform p72 to p66 (47). Interestingly, we found up-regulated RBFOX3/NEUN both after *in vitro* differentiation of NSCs (**Figure 1D**) and after Numb p66 over-expression (**Figure 2A**), suggesting that there could be a regulatory feedback between Numb p66 and RBFOX3/NEUN. As shown in **Figure 1D**, RBFOX3/NEUN is up-regulated during differentiation of NSCs along with Numb. Moreover, SHH MB shows the highest expression of RBFOX3/NEUN compared to the other molecular subgroups (**Supplementary Figure 4**). These evidence suggest that RBFOX3/NEUN could be responsible for NUMB alternative splicing in SHH MB context, leading to isoforms shift from p72 to p66.

Notably, we identified expression of Numb p66 also in NSCs and in MB-SLCs, as a common feature of "stem cell phenotype." We have previously demonstrated that these cellular models are characterized by Hedgehog pathway activation (9, 49). Numb p66 expression inversely correlates with stem cells features: indeed, NSCs and SHH MB-SLCs show high clonogenic potential when Numb is silenced (**Figures 2C**, **3C**), where it cannot antagonize survival pathways, such as Hedgehog signaling (23). We demonstrated the role of the Numb p66 isoform in promoting neural differentiation, antagonizing the expression of stemness/Shh markers and inhibiting self-renewal of stem cells.

In conclusion, in this study we demonstrated for the first time the expression of Numb isoforms in Medulloblastoma, highlighting possible different roles for each isoform in MB subgroups, that we believe are worthy of further investigation in follow up studies. Moreover, we described the suppressive role of Numb p66 on stemness features of cerebellar NSC and of SHH MB-SLCs.

AUTHOR CONTRIBUTIONS

LA and AP designed experiments, analyzed the data, and wrote the manuscript. AM, EM, AC, ZB, and MM performed the experiments and analyzed the data. EDS, FG, FL, and EF assisted the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2018.00315/full#supplementary-material

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REVIEW
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Pediatric Central Nervous System Tumors: State-of-the-Art and Debated Aspects

Mitchell T. Foster, Lalgudi Srinivasan Harishchandra and Conor Mallucci*

Department of Neurosurgery, Alder Hey NHS Foundation Trust, Liverpool, United Kingdom

Pediatric neuro-oncology surgery continues to progress in sophistication, largely driven by advances in technology used to aid the following aspects of surgery: operative planning (advanced MRI techniques including fMRI and DTI), intraoperative navigation [preoperative MRI, intra-operative MRI (ioMRI) and intra-operative ultrasound (ioUS)], tumor visualization (microscopy, endoscopy, fluorescence), tumor resection techniques (ultrasonic aspirator, micro-instruments, micro-endoscopic instruments), delineation of the resection extent (ioMRI, ioUS, and fluorescence), and intraoperative safety (neurophysiological monitoring, ioMRI). This article discusses the aforementioned technological advances, and their multimodal use to optimize safe pediatric neuro-oncology surgery.

Keywords: intraoperative magnetic resonance imaging, pediatric neuroimaging, technology in surgery, neurooncology, pediatric brain tumors

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Alessandro De Benedictis, Bambino Gesù Ospedale Pediatrico (IRCCS), Italy

****Correspondence:***
Mitchell T. Foster
mitchell.foster@doctors.org.uk

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INTRODUCTION

Following clinical and radiological diagnosis, the surgical management of pediatric brain tumors involves tumor biopsy, tumor excision, and the management of perioperative surgical complications including CSF diversion. There is a balance between maximizing surgical resection whilst minimizing surgical morbidity. This has to be balanced with post-operative plans for further oncological treatment, and the natural history of the tumor in question. Advances in preoperative and operative tools and techniques help to optimize this process of perioperative decision making and the operative intervention itself. A full discussion of pre-operative workup is beyond the scope of this article. In short, MRI techniques & radiologist expertise have evolved such that preoperative prediction of not only the tumor histology but also molecular subgroup is increasingly accurate (1, 2).

OPERATIVE INTERVENTION

Tumor Biopsy

Biopsy may be performed with an open technique (through craniotomy), through a small burr hole, or endoscopically.

The precision of biopsy has evolved over time with progression of technology: first open biopsy, then stereotactic frame biopsy, then frameless biopsy which continues to evolve in sophistication, including the use of robotic assisted biopsy which is now becoming mainstream (3).

Neuro-navigation is used to plan biopsy entry sites, trajectory and the intracranial target. This can be done with precision to avoid eloquent gray or white matter and vessels within the trajectory.

Volume MRI is used alongside stereotactic frame, optical neuro-navigation, or electromagnetic neuro-navigation (4). Stereotactic frame and optical techniques require pin fixation to the skull; this

makes them more accurate, but contraindicated in infants. Electromagnetic navigation allows head movement, is not dependant on skull pin fixation, but as a consequence has less precision. Optical and electromagnetic techniques allow real-time tracking of the biopsy tip during the procedure (5).

In cases where the surgical target is small, intraoperative MRI can be used to confirm the biopsy site's accuracy to the planned target prior to waking the patient, should further biopsy be required.

Tumor Excision

Planning

The surgical plan may be for tumor debulking or complete excision. Within this range, there is complex decision making to maximize safe resection whilst minimizing morbidity which depends on two main factors: tumor type (influencing propensity for recurrence, metastasis, overall survival and the need for adjuvant therapies), and tumor location (proximity to eloquent areas of brain and vital neurovascular, or neuroendocrine structures).

Software can be used to mark out the tumor extent and a resection plan which can be used intraoperatively to navigate to the tumor. Functional MRI can be used to identify eloquent areas involved in motor generation, speech and language (6). The applicability of fMRI in pediatric neurosurgery is limited because it needs patient cooperation and some form of sedation, especially in children under 6 years (7). Diffusion weighted MRI can be processed using diffusion tensor imaging based tractography to map out white matter tracts in the vicinity of the resection cavity (8). Tracts may be for example within tumor, abutting the tumor, or split by the tumor, which may impact the decision of both resection extent and direction of approach (**Figure 1**). DTI has the disadvantage that it does not offer any functional information, and based on the *post-hoc* analysis it can be represented in several ways (9).

In complex intrinsic tumors, multi-voxel MR spectroscopy is used to identify the most aggressive components and aid resection planning (**Figure 1**).

Preoperative volume MRI, once registered for neuro-navigation can be used to plan an optimal skin incision and craniotomy on the patient on the table (10).

Operative Microscope

The history and vital role of the microscope in neuro-oncology surgery is well described elsewhere (11). It provides magnification and illumination, while allowing ergonomic movement to resect tumors with increased precision. Each successive iteration of microscope has improved their functionality and utility. The most recent advances include 3D stereoscopic visualization to an external screen for the operating surgeon if desired, assistant(s) and theater staff (12). This is beneficial for safety, as the non-operating surgical team can visualize the current surgical activity in real-time, and respond promptly to problems such as hemorrhage. This also benefits teaching within the department. Another development is angled micro-endoscopy (12). This system permits visualization around corners, and has been used with success in brainstem tumor resection in our department.

Navigation

Neuro-navigation can be used for tumor biopsy as discussed above, and for operative planning for resection as described above. Intraoperatively, it is used to confirm the location of tumor alongside normal anatomy. Plans made preoperatively which include anatomical regions of interest, fMRI, or tractography can be used in real-time to ensure maximal safety.

A navigated "pointer" is the most commonly used tool. In addition, the operative microscope can be integrated with the neuro-navigation system such that the point of maximal focus (indicated by the convergence of two laser pointers in the operative field) becomes visible on the navigation screens. It is also possible to overlay navigation onto the microscope view to the surgeon (**Figure 2**). Recently, a navigable suction catheter has been developed to allow synchronous tumor resection with navigation, reducing the need to continually re-site a navigation pointer.

Navigation has become a standard technique for pediatric neuro-oncological surgery, and indeed much of all cranial neurosurgery. However it is not without limitations: It is subject to error during the fusion of different image sequences, and at the point of registration to the patient. Once craniotomy and durotomy have been performed, the brain will be permitted to shift. The variable degree to which this occurs can unpredictably diminish navigation's accuracy to the intracranial anatomy. This inaccuracy increases with progressive tumor resection as the brain shifts more. Operating theaters can be crowded with staff and equipment; it can be challenging to ensure the navigation camera's field of view can see the optical reference points at all times.

Furthermore, two recent Cochrane reviews have highlighted the lack of evidence for intraoperative neuro-navigation in terms of tumor resection and quality of life (13, 14). While there is a lack of evidence in support of these techniques, there is no evidence against, and most surgeons would agree, this is an invaluable resource that is a mainstay of neuro-oncology surgery (11).

There is anticipated to be a greater role for "augmented reality" techniques in neuro-navigation as technology progresses (15).

Intraoperative MRI

Tejada et al provide a comprehensive summary of intraoperative MRI at our center, with the largest published series of ioMRI tumor resections (16).

The patient's head is placed in a non-magnetic frame with (or without) pin fixation. The patient is registered to their preoperative volume MRI and the operation is performed. After tumor resection, at a point of safety with adequate hemostasis, the skin is loosely approximated, and the wound is draped to ensure sterility. The MRI coil head is placed over the drapes and secured. The patient is then transferred to the neighboring ioMRI room, scanned, and returned to the operative theater. Optical markers on the MRI coil allows the patient to be automatically re-registered to the new intraoperative scan.

The choice of MR sequence is determined by the pre-operative findings. For example, a low grade tumor that does not enhance on T1 MRI with gadolinium, but is visible on FLAIR

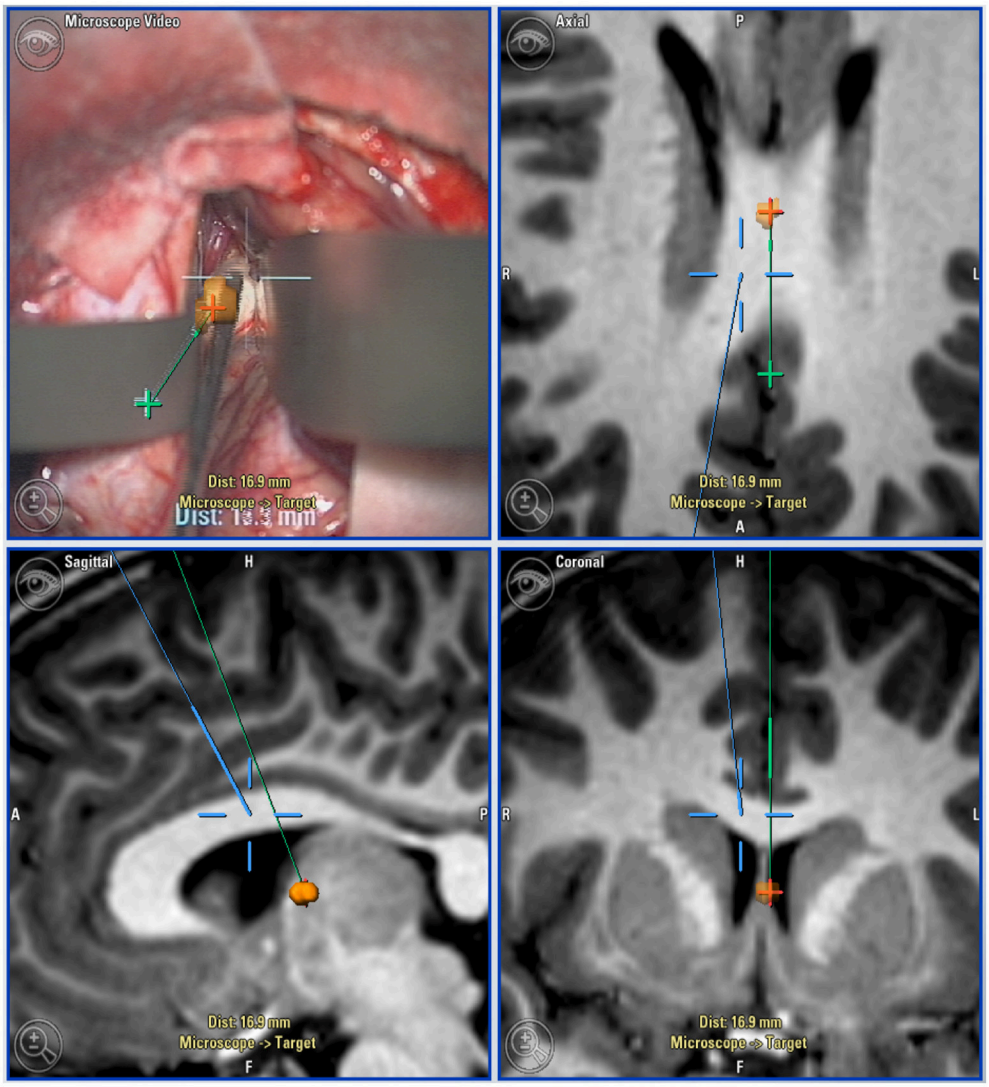

FIGURE 1 | Neuro-navigation guided surgical approach: Top left: microscope view with navigation overlaid. Top right (axial), Bottom left (sagittal), and bottom right (coronal): Views of target lesion with planned trajectory (green) and microscope line of sight (blue).

sequences, would have this sequence performed intraoperatively, and then mapped onto an intraoperative volume T1 sequence for intraoperative navigation. Images are reviewed with a consultant neuro-radiologist familiar with the case and intraoperative MRI alongside the operating surgeon.

IoMRI may occasionally be performed after the wound has been closed: this occurs in cases when the surgeon is confident of the surgical resection, or if further resection is thought to be too high risk for post-operative morbidity. This is logistically advantageous for theater efficiency, but still preserves sterility of the field, in case the MRI identifies anything requiring further surgery (for example unexpected resectable residual tumor or haematoma). If as predicted, no further surgery is required, the patient can then simply be undraped and woken. This technique can also be done for biopsy cases, to confirm the target site. In most cases, this will serve as the immediate post-operative scan, and the patient will proceed to extubation and recovery. However, the patient remains within the ioMRI frame, to permit automatic re-registration to the ioMRI, for re-opening of the wound if deemed necessary (for example for further tumor resection, repeat biopsy or hemorrhage control).

There are multiple advantages to ioMRI: It allows diagnosis of residual tumor and surgical damage (ischemia, or hemorrhage). It allows automatic reregistration to an accurate scan to correct for brain shift, and the anatomic distortion after tumor resection. This means the surgeon can then navigate to the area of residual tumor quickly and efficiently. This is particularly helpful in tumors where there is no overt tumor plane, or where the appearances are similar to that of normal tissue (for example low grade glioma).

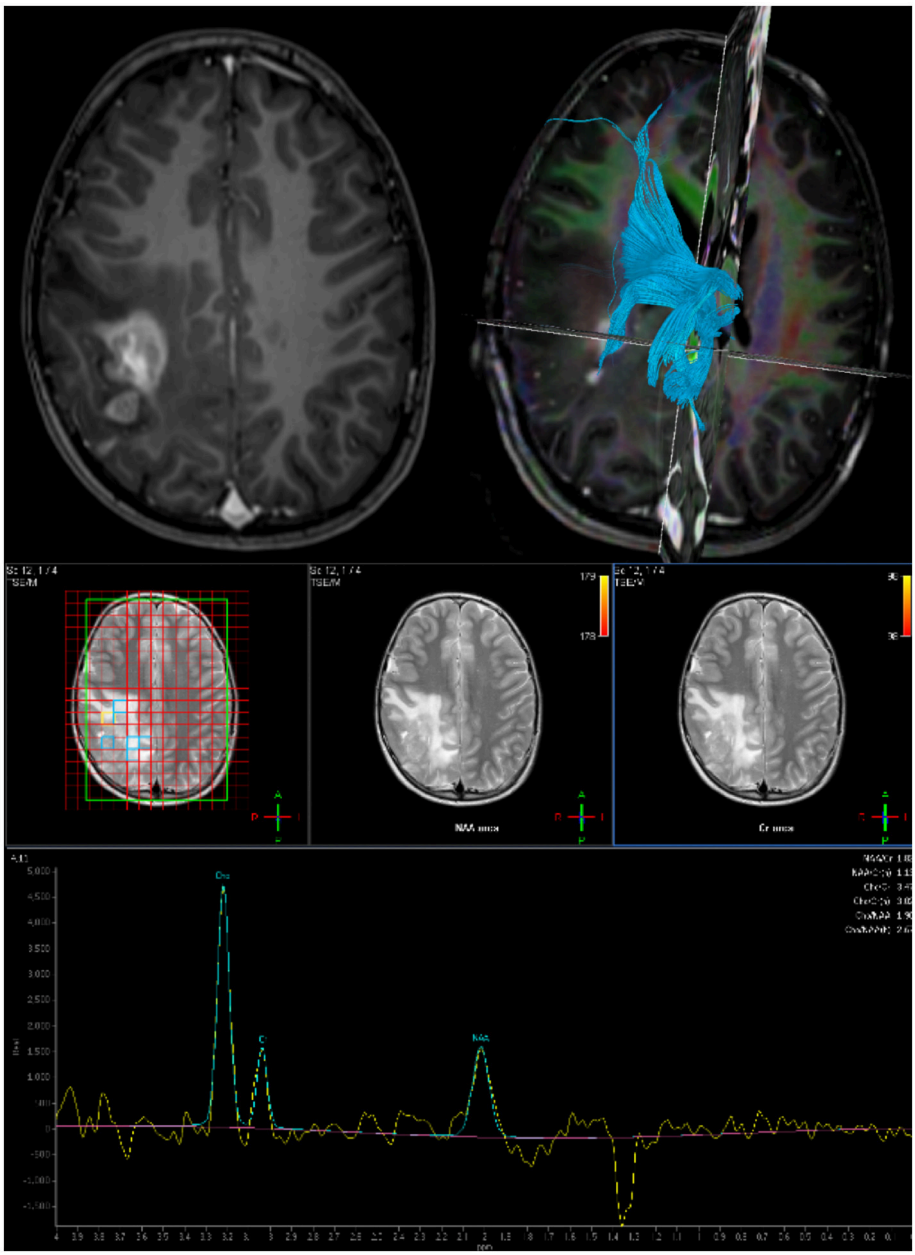

FIGURE 2 | Preoperative imaging for planning surgical approach and resection extent: **Top Left:** Axial post gadolinium T1 MRI. **Top Right:** DTI derived tractography to map white matter bundles in vicinity of tumor. **Bottom:** Multi-voxel MR Spectroscopy to define resection target.

IoMRI can also identify hemorrhage not immediately visible in the operative field; since the introduction of ioMRI, there have been no returns to theater for post-operative hemorrhage in our unit.

Nevertheless, ioMRI also has limitations: setup and running cost is a major prohibitory factor. A two room setup (as in our unit) can offset this, as the scanner can be used as a routine diagnostic tool when not in use intraoperatively (11). Another ioMRI suite option involves a ceiling mounted, moveable ioMRI scanner between two operating theaters (17).

While there is limited evidence in support of ioMRI in the pediatric neuro-oncology practice, an RCT in adults receiving craniotomy for glioma resection has been performed: the ioMRI group had 96% complete resection vs. 68 in the non ioMRI group ($p = 0.02$) (18).

Endoscopy

Technological advances in neuro-endoscopy have improved illumination, image resolution, and field of view (19). Recent technological developments include

the use of smartphone integrated endoscopes (20), and the exoscope (21). The role and applicability of these emerging techniques in pediatric neuro-oncology surgery will become clearer as technology becomes more widely available.

The range of neuro-endoscopes offer different qualities making each one more appropriate for certain procedures. In our center, rigid endoscopy is performed exclusively. However, flexible neuro-endoscopy has been described for use in tumor excision (22).

Endoscopic (or endoscopic assisted) surgery may be used during the following: intraventricular surgery [tumor biopsy (23) tumor resection (24), perioperative hydrocephalus management including endoscopic third ventriculostomy (25)], endoscopic endo-nasal-trans-sphenoidal surgery (for resection of sella lesions) (26, 27), supra-cerebellar infra-tentorial endoscopic approaches to pineal region tumors, (28) and as an adjunct to the microscope during open craniotomy.

Pure endoscopic tumor resection is described for select cases, but is seldom performed in our practice, and is better described elsewhere (29, 30). The dexterity of endoscopic instruments and techniques is improving; the advent of endoscopic lasers, and ultrasonic aspirators has expanded the capability of endoscopic tumor resection (31). Endoscopy can be combined in a multimodal approach for example alongside neuronavigation and ioMRI (**Figure 3**).

Ultrasound

2D or 3D ultrasound can be used as a standalone tool (32), or in tandem with MRI for neuro-navigation (33). It allows real-time visualization of the parenchymal and ventricular anatomy. Doppler USS can be performed to assess vessels if required. US is also helpful in identifying normal ventricular anatomy which may be dynamic. It has limitations, being user dependent with a learning curve, susceptibility to artifact and non-uniform resolution (33). Echogenicity of tumors can vary depending on their type, meaning ioUS may sometimes be of little use. Furthermore, the walls of the resection cavity may be hyperechoic which can lead to overestimation of tumor residual (11).

Intraoperative Fluorescence

Intra-operative fluorescence techniques can occasionally be used in pediatric neuro-oncology surgery. Orally administered 5-aminolevulinic acid (5-ALA) induces fluorescent porphyrin accumulation within certain tumors, which can be visualized with a modified microscope (34). The use of 5-ALA in pediatric patients is off label, but has been described (11, 35). An RCT in adults found 5-ALA improved the extent of tumor resection and benefitted progression free survival (36), however evidence in pediatric surgery is limited. Roth et al. noted that fluorescence is only seen in a small proportion of pediatric brain tumors (outside of glioblastoma multiforme), and therefore advise against the routine use of 5-ALA (35).

Indocyanine green is used in vascular neurosurgery to provide fluorescence to vessels directly visible in the operative microscope (11). This can be useful in pediatric neuro-oncology surgery if the tumor is especially vascular, or in close proximity to vital vascular structures (11).

The use of other fluorescence agents has also been described, including fluorescein, hypericin, 5-aminofluorescein-human serum albumin, and endogenous fluorophores, albeit with less evidence, especially in pediatrics (37).

Intraoperative Raman spectroscopy is an emerging technique with promise in distinguishing normal from pathological tissue (38). It has recently been described *in vivo* for core needle "biopsy" and delineation of pathological vs. normal tissue within resection cavity margins in a pig model (39).

Ultrasonic Aspirator

There are various ultrasonic aspirators available that utilize ultrasound to emulsify and aspirate tissues (11). The weak intracellular bonds and high liquid content of tumor tissue make it susceptible to ultrasonic aspiration. Conversely, vessels and nerves with higher elastin and collagen content are less likely to be damaged (11).

Intraoperative Neuromonitoring (ION)

Neuromonitoring intraoperatively is considered to be the gold standard in localizing brain function in brain tumor surgery (40). Mapping is the process of identifying the proximity to eloquent areas in the brain and avoiding damage to these regions (41). Both cortical and subcortical mapping can be performed (42). In pediatric neurosurgery where most of the supratentorial tumors are low grade lesions, the use of neuromonitoring helps in maximizing the extent of safe resection with least possible morbidity. Continuous, dynamic subcortical mapping with a suction monopolar device has recently been described (43).

Neurophysiological monitoring can also be used during resection of intramedullary spinal tumors by acquisition of data to confirm the integrity of neural pathways in the form of Motor evoked potentials (MEPs), D-waves and Somatosensory evoked potentials (SSEPs) (41, 44).

When using these techniques, total intravenous anesthesia is preferred, with avoidance of neuromuscular paralysis (11). Intraoperative neurophysiology is different in children to adults (especially infants), and requires and age adjustment to the stimulation techniques and interpretation of results because of the immaturity of the developing brain (41).

Awake craniotomy can be performed to allow real-time monitoring of neurological deficit alongside with cortical and subcortical mapping (45). However, this is seldom performed in children, although it has been described (46, 47).

Though there is sufficient evidence to support the use of ION in *predicting* neurological injury, there isn't much evidence for injury *prevention* (40).

IMPROVED UNDERSTANDING OF NEUROSCIENCE AND NEUROANATOMY

Neuroanatomy education has benefitted from the capability of both 3D digital, and 3D printed models (48). In general,

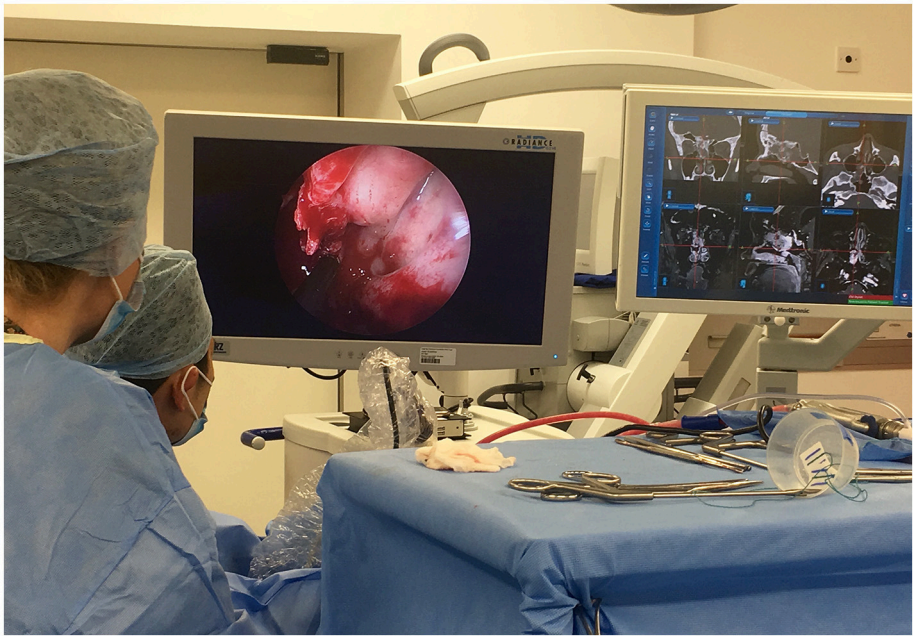

FIGURE 3 | Multimodal use of technology: The use of intraoperative endoscopy for an extended endo-nasal approach, in tandem with neuro-navigation and intraoperative MRI was vital in maximizing resection of this complex recurrent atypical meningioma.

the interest in, and sophistication of general neurosurgical simulation is continually increasing (49), however there remains a need for more sophisticated tools to accurately replicate the challenges of pediatric neuro-oncology surgery.

Recent deeper understanding of the mechanisms behind cerebellar mutism following posterior fossa surgery have led to refinements in surgical technique to avoid damage to the proximal efferent cerebellar pathway (50).

DISCUSSION

Surgery has progressed through advances in the following areas: visualization (microscopes, endoscopes), Navigation (ioUS, MRI), and delineation of tumor resection (ioMRI, ioUS, 5ALA), all of which, in tandem can improve the ability to carry out maximal safe resection. However, many more major advances in pediatric neuro-oncology are non-surgical: the molecular classification of tumors, and the advances in chemotherapy and radiotherapy. The impact of these on surgical decision making is complex, and a full discussion of is beyond the scope of this article. However, one notable paradigm shift is seen in with molecular subcategorization of Medulloblastoma: gross total resection conferred no survival advantage in comparison with near total resection when taking into account molecular subgroups (51). Therefore, re-look surgery for residual disease is now not necessarily recommended if the risk of neurological morbidity from complete excision is high (51, 52). In contrast, the survival benefit from complete resection of Ependymoma is established (53, 54), and current consensus opinion is that molecular subcategorization should not change this surgical decision making (52, 55) As a result, re-look surgery for residual disease is recommended, and may involve referral to a quaternary center such as ourselves for second opinion.

In our unit, we use a combination of the following imaging modalities to delineate tumor resection: pre-operative MRI, intraoperative macroscopic, and microscopic assessment of tissues, intraoperative ultrasound, and intraoperative MRI. Since the introduction of the ioMRI in our unit, there have been no returns to theater for post-operative haematoma following craniotomy for tumor.

The best methodology for ensuring maximal safe tumor resection continues to be debated; however it is clear that no operative tool is flawless in isolation. MRI (preoperative or intraoperative) has superior anatomical resolution to ultrasound, but is a static, historic image. Conversely, ultrasound is in real-time, but is very user dependant, with variable tumor echogenicity. Advanced pediatric neuro-oncology surgery therefore utilizes a multimodal approach with navigation, microscopy, endoscopy if appropriate, and ioUS. The combination of tools with varying strengths can offset the limitations of tools also in use.

While the use of state of the art equipment discussed here has advanced pediatric neuro-oncology surgery, the importance of the perioperative and intraoperative MDT cannot be understated: without a complete, competent team (neuro-anesthetists, theater staff, nursing staff, neuro-radiologists, physiotherapy, occupational therapy, speech and language therapy, oncologists, endocrinologists, neurologists), outcomes would be compromised.

AUTHOR CONTRIBUTIONS

MF drafted article. LH critically appraised and edited. CM is a lead surgeon in unit, critically appraised and edited.

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ORIGINAL RESEARCH
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BRAF V600E Inhibitor (Vemurafenib) for *BRAF* V600E Mutated Low Grade Gliomas

Francesca Del Bufalo[1†], Giulia Ceglie[1†], Antonella Cacchione[1], Iside Alessi[1], Giovanna Stefania Colafati[2], Andrea Carai[3], Francesca Diomedi-Camassei[4], Emmanuel De Billy[1], Emanuele Agolini[5], Angela Mastronuzzi[1] and Franco Locatelli[1,6]*

[1] Department of Paediatric Haematology/Oncology, IRCCS Bambino Gesù Children's Hospital, Rome, Italy, [2] Department of Imaging, Neuroradiology Unit, IRCCS Bambino Gesù Children's Hospital, Rome, Italy, [3] Department of Neuroscience and Neurorehabilitation, Neurosurgery Unit, IRCCS Bambino Gesù Children's Hospital, Rome, Italy, [4] Department of Laboratories, Pathology Unit, IRCCS Bambino Gesù Children's Hospital, Rome, Italy, [5] Laboratory of Medical Genetics, IRCCS Bambino Gesù Children's Hospital, Rome, Italy, [6] Department of Paediatric Sciences, University of Pavia, Pavia, Italy

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Suzie Chen,
Rutgers University, The State University of New Jersey, United States

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Koch Institute for Integrative Cancer Research at MIT, United States
Min Hee Kang,
Texas Tech University Health Sciences Center, United States

***Correspondence:**
Angela Mastronuzzi
angela.mastronuzzi@opbg.net

[†]These authors have contributed equally to this work

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Low-grade gliomas (LGG) are the most common central nervous system tumors in children. Prognosis depends on complete surgical resection. For patients not amenable of gross total resection (GTR) new approaches are needed. The *BRAF* mutation V600E is critical for the pathogenesis of pediatric gliomas and specific inhibitors of the mutated protein, such as Vemurafenib, are available. We investigated the safety and efficacy of Vemurafenib as single agent in pediatric patients with V600E^{+} LGG. From November 2013 to May 2018, 7 patients have been treated in our Institution; treatment was well-tolerated, the main concern being dermatological toxicity. The best responses to treatment were: 1 complete response, 3 partial responses, 1 stable disease, only one patient progressed; in one patient, the follow-up is too short to establish the clinical response. Two patients discontinued treatment, and, in both cases, immediate progression of the disease was observed. In one case the treatment was discontinued due to toxicity, in the other one the previously assessed *BRAF* V600E mutation was not confirmed by further investigation. Two patients, after obtaining a response, progressed during treatment, suggesting the occurrence of resistance mechanisms. Clinical response, with improvement of the neurologic function, was observed in all patients a few weeks after the therapy was started. Despite the limitations inherent to a small and heterogeneous cohort, this experience, suggests that Vemurafenib represents a treatment option in pediatric patients affected by LGG and carrying *BRAF* mutation V600E.

Keywords: pediatric central nervous system tumors, low-grade gliomas, vemurafenib, targeted therapies, pediatric neuro-oncology

INTRODUCTION

Low-grade gliomas (LGG) are common tumors in children. The prognosis varies widely among the different tumor subgroups and is determined by several factors, including grading, location, age at diagnosis, and extent of surgery, the gross total resection (GTR) being one of the main factors affecting the chance of cure. Surgery, along with radio- and chemotherapy, is currently the

standard of care in the treatment of these neoplasms. However, there is a subgroup of patients that is judged not amenable of GTR, mainly because of the localization and extent of the mass. Despite the low biological malignity of these tumors, patients with unresectable masses and with clinical progression of the disease undergo chemotherapy or radiotherapy, suffering for the short- and long-term toxicities associated with these regimens. The main approaches of conventional chemotherapy for LGG includes carboplatin and vincristine, TPCV (thioguanine, procarbazine, lomustine, and vincristine) and weekly vinblastine monotherapy (1). Bevacizumab is another promising approach as it has shown improvements in the treatment of optic pathway gliomas (2). All this considered, new approaches, tailored on the biological characteristics of the disease, are needed for these patients.

The main molecular alterations shown by LGG relate to the activation of the MAP Kinase (MAPK) pathway and can be caused by either duplication or mutation of the *BRAF* gene (3). Therefore, inhibitors of the MAPK pathway have been considered as a potential target of therapy for tumors harboring these types of alterations (4, 5). Vemurafenib is a competitive small molecule that selectively recognizes the ATP-binding domain of the $BRAF^{V600E}$ mutant. It has proved effective in the treatment of metastatic melanoma, a neoplasm frequently mutated for *BRAF*. More recently, an activity of this drug was proved also in pediatric $BRAF^{V600E}$ mutated malignant astrocytomas (6–8), while less data are available on the use of the drug in patients with LGG (9, 10). We herein present a retrospective, monocentric analysis of the safety and efficacy of Vemurafenib as single agent for the treatment of 7 patients affected by unresectable LGG.

MATERIALS AND METHODS

We retrospectively evaluated the medical reports of patients diagnosed with LGG and treated with Vemurafenib at Bambino Gesù Children's Hospital in Rome. The histological diagnosis was obtained in all patients either from biopsy or resected part in those children undergoing partial resection. $BRAF^{V600E}$ mutation was assessed by immunohistochemistry in all patients and in one case, due to a progression of the disease under treatment, also through Sanger sequencing of the *BRAF* gene.

Exon 15 of BRAF, spanning the V600 locus, was amplified by PCR using the KAPA2G Fast HotStart PCR Kit (Kapa Biosystems) according to the manufacturer's protocol with the following primers: BRAF_Ex15_Fw: CTTCATAATGCTTGCTCTGATAG and BRAF_Ex15_Rv: CTAGTAACTCAGCAGCATCTCAG. The amplification product was purified and sequenced by using the BigDye Terminator Version 3.1 Cycle Sequencing Kit according to the manufacturer's protocol on a 3130XL automatic sequencer (Applied Biosystems).

As for immunohistochemistry, 3-micron paraffin embedded sections were dehydrated, pretreated with avidin block for 15' and biotin block for 15', then incubated with BSA for 30' and incubated with mouse anti-human BRAF V600E monoclonal antibody (clone VE1, Spring Bioscience) at room temperature for 60' (dilution 1:50, PT-link antigen retrival at high pH). Incubation with biotinylated secondary antibodies for 15' at room temperature and incubation with alkaline phosphatase or 3,3′-diaminobenzidine-tetrahydrochloride-dihydrate (DAB) conjugated streptavidin for 15' at room temperature were performed. The BRAF staining for all patients is shown in **Figure 1** and a quantitative measure of the intensity of the staining can be found in **Table 1**. Normal cerebellar parenchyma served as negative control (shown in the same Figure).

In the absence of pharmacokinetic and pharmacodynamic data of Vemurafenib in a pediatric population, we decided to start the treatment with the minimal dose proved to be active in adults (240 mg/day *per os* in 2 administrations). Subsequent dose adjustment was evaluated case by case, depending on drug tolerance. Radiologic response to treatment was evaluated and classified according to RECIST criteria; major responses included complete response (CR), partial response (PR) and stable disease (SD). Toxicity was graded according to the Common Terminology Criteria for Adverse Events (CTCAE), v 4.0. The mean time for the radiologic evaluation of response was 6 months for all the patients.

All the parents/legal guardians of patients provided formal, informed consent to the treatment and the study was approved by Institutional Review Board (Ospedale Pediatrico Bambino Gesù).

RESULTS

From November 2013 to May 2018, 7 patients have been treated; the main characteristics of the patients are reported in **Table 1**. The median age at diagnosis was 75.2 months (range 1–125); F:M ratio was 1:6. Histological diagnosis were: Gangliogliomas (GG) (4 patients), Pleomorphic Xantoastrocytoma (PXA, 1 patient), Ganglio-neurocytoma (1 patient) and Pylocitic Astrocytoma (PA, 1 patient). Only one of the seven patients had undergone previous treatment consisting of surgery and chemotherapy according to the treatment protocol SIOP LGG 2004 (including mainly Vincristine, Carboplatin, Etoposide).

Vemurafenib was started at a median time from diagnosis of 17 months (range 1–52) and was administered orally for a median of 22 months (range 3–52). Dose adjustments were carried out depending on observed toxicity and efficacy; in one case the dose remained 240 mg/day, in two cases was increased up to 480 mg/day and in four cases up to 960 mg/day. The median follow-up time from initiation with Vemurafenib therapy is currently 24 months (range 2–54).

The treatment was overall well-tolerated. Skin toxicity developed in 5/7 pts, reaching grade 3 (CTC v4.0) in one of them (at the dose of 960 mg/day) and leading to a 2-month discontinuation of the treatment that was later restarted without safety issues at the same dose. One patient developed grade 2 toxicity and the remaining 5 cases experienced only grade 1 toxicity, not requiring discontinuation of the treatment. The skin changes were mainly in the photo-exposed areas and consisted of a maculo-papular rash and diffused xerosis. These adverse effects occurred around 1 month after the treatment was started.

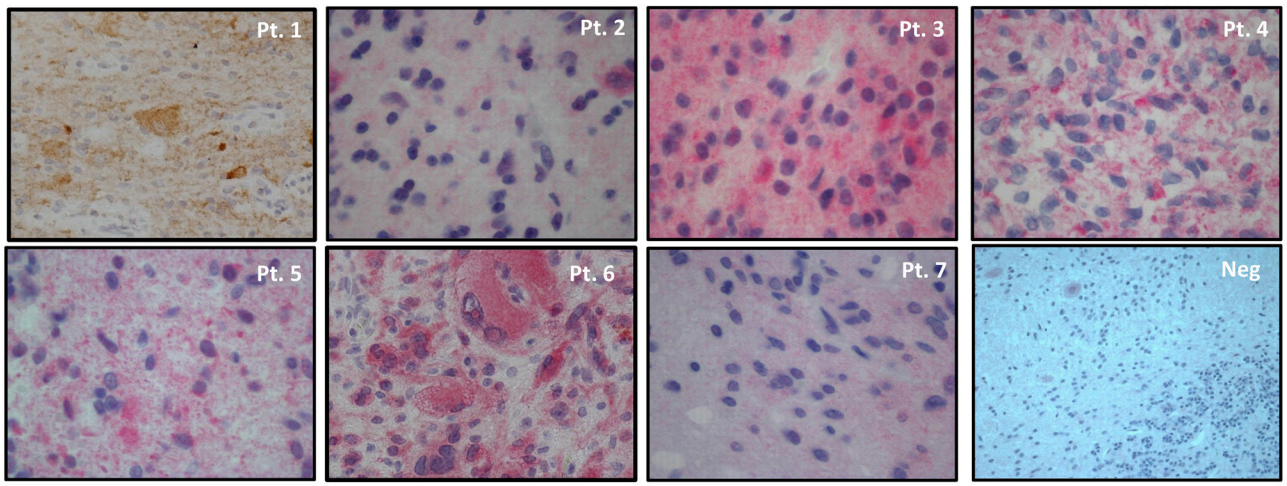

FIGURE 1 | $BRAF^{V600E}$ immunohistochemistry in tumor samples of patients' series and in normal cerebellar parenchyma (small neurons of cortical molecular layer, Purkinje cells and underlining white mater resulted negative). Magnifications: Pt. 1 20x, Pts. 2-7 40x, Neg. 20x. 3,3′-diaminobenzidine-tetrahydrochloride-dihydrate (DAB) substrate for Pt.1 and alkaline phosphatase substrate for Pts. 2-7 and negative control. Hematoxylin counterstain in all samples.

TABLE 1 | Clinical-pathological characteristics of patients and $BRAF^{V600E}$ immunostain intensity.

	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5	Pt 6	Pt 7
Age (months)	28 m	54 m	1 m	108 m	89 m	122 m	125 m
Site	Cervico-medullary	Vermis + cerebellar hemisphere	Medulla oblongata	Midbrain	Medulla oblongata	Midbrain	Optic chiasm
Histology	GG	GG	Ganglioneurocytoma	PXA	GG	GG	Pylocitic Astrocytoma
$BRAF^{V600E}$ IHC	++	++	+++	+++	+++	+++	+
Surgery	Biopsy	Partial resection	Partial resection	Partial resection	Partial resection	Partial resection	Partial resection
Previous CT	SIOP LGG 2004	No	No	No	No	No	No
Max dose	960 mg/day	480 mg/day	240 mg/day	960 mg/day	480 mg/day	480 mg/day	960 mg/day
Toxicity	Skin (grade 3)	No	No	Skin (grade 1)	Skin (grade 1)	Skin (grade 1)	Skin (grade 2)
Best response	PR	CR	PD	PR	SD	Insufficient follow-up	Treatment stopped for $BRAF^{V600E}$ negativity at Sanger Sequencing
Follow-up/(months)	54 m	40 m	4 m	30 m	13 m	2 m	24 m
Duration of treatment (months)	54 m	40 m	3 m	30 m	13 m	2 m	9 m

Pt 4 is also affected by constitutional chromosome 21 trisomy.
SIOP-LGG 2004: (Vincristine, Carboplatin, Etoposide). Acronyms: GG, ganglioglioma; PXA, pleomorphic xantoastrocytomas; IHC, immunohistochemistry; CT, Chemotherapy; PR, Partial Response; CR, Complete Response; PD, Progressive Disease; SD, Stable Disease.

In one case (the most severe one), the maximum grade of the lesions appeared after 15 months from the initiation of treatment, but shortly after (i.e., 1 month) the increase of the dose to 960 mg/day. The patients experiencing the highest toxicity (grade 2 and 4) were both receiving the maximum dose (960 mg/day). No skin tumor developed in any patient.

Although the cohort is too small and heterogeneous in terms of tumor histology, we observed 57% (4/7) major responses in our cohort. One child affected by GG obtained CR after 6 months and 2 patients (1 GG and 1 PXA) reached PR. Amongst these 2 latter patients, the one affected by PXA (who also had constitutional trisomy 21) had the best response to treatment after 3 months, while the other one showed the best response 12 months after the treatment was started, 2 months after initiating with the highest tolerated dose level of 960 mg/day. For the remaining 2 patients affected by GG, one remains in stable disease after 18 months of treatment and the other has a follow-up of 2 months only, therefore too short to evaluate the radiological response; he shows, however, a clinical response with improvement of the main neurological deficits (ataxia and visual impairment).The best radiological response was obtained 3 months after Vemurafenib was started and a mean sustained response of 2 years was observed. No correlation between $BRAF^{V600E}$ positivity and treatment outcome was found.

Remarkably, a clinical response with improvement of the neurological function was observed early in all the responding patients, after 2 weeks of treatment.

The patient with the ganglio-neurocytoma progressed under treatment. This child had a bulky lesion involving the brainstem and cerebellum and presented with severe clinical conditions at diagnosis.

Lastly, in one patient affected by PA we observed disease progression under treatment with Vemurafenib. To understand the possible cause of this lack of response, we performed sequencing of the *BRAF* gene, which showed a negativity for the V600E mutation. The therapy was then interrupted, and the patient underwent partial resection with stabilization of the disease.

Moreover, two of the patients that showed a response (after obtaining PR in one case and CR in the other case), experienced tumor regrowth under treatment, 24 and 15 months after the start of the treatment respectively, suggesting the development of resistance (RM images in **Figure 2**). One case was treated with radiotherapy, obtaining a reduction of the mass and Vemurafenib was later reintroduced with a complete control of the disease. In the other case, the treatment was continued at the same dose, obtaining a new stabilization of the disease until the latest follow-up (40 months).

Not surprisingly, an immediate tumor re-growth was seen after suspension of the treatment for toxicity in one responder; the therapeutic benefit was, however, re-obtained with the resumption of the drug.

DISCUSSION

In the past few years, dramatic progresses have been made in the understanding of the biology of LGG, thanks to the advent of new methodologies in genomics. The identification of specific molecular signatures of these tumors is changing the treatment strategies and targeted therapies are currently being explored in clinical trials. In fact, the conventional treatment strategies for unresectable LGG include chemotherapy and radiotherapy, with the long term disabilities that these treatments determine. In view of the long term survival associated with these tumors, and especially when considering the pediatric age, reducing long-term toxicities is mandatory. Targeted agents have, by definition, less systemic and long term adverse effects, even if their relatively recent use does not enable a full characterization of these aspects yet.

One of the most notable findings in this ever-changing landscape includes abnormalities in the RAS/MAPK pathway, such as *BRAF* activation. The availability of specific inhibitors of the $BRAF^{V600E}$ alteration such as Vemurafenib is an example of the possible therapeutic translation of these findings, paving the way for an innovative treatment option for a selected population of pediatric patients affected by LGG and harboring $BRAF^{V600E}$.

We have presented the case of 7 patients with *BRAF* mutated LGG treated with Vemurafenib. This is, to the best of our knowledge, the first study to investigate safety and efficacy of this molecule in a pediatric population affected by LGG.

In all patients, the *BRAF* mutation was assessed through immunohistochemistry, and a further investigation through molecular assay was performed only in one case because of the poor response to the treatment observed, revealing a false positivity of the staining. Even though the gold standard method to assess BRAF status in patients with metastatic melanoma is based on molecular assays, the high costs and expertise required for the molecular assay is responsible for the limited use of the test. On the other side, the development of a mutation-specific monoclonal antibody (VE1), which enables the detection

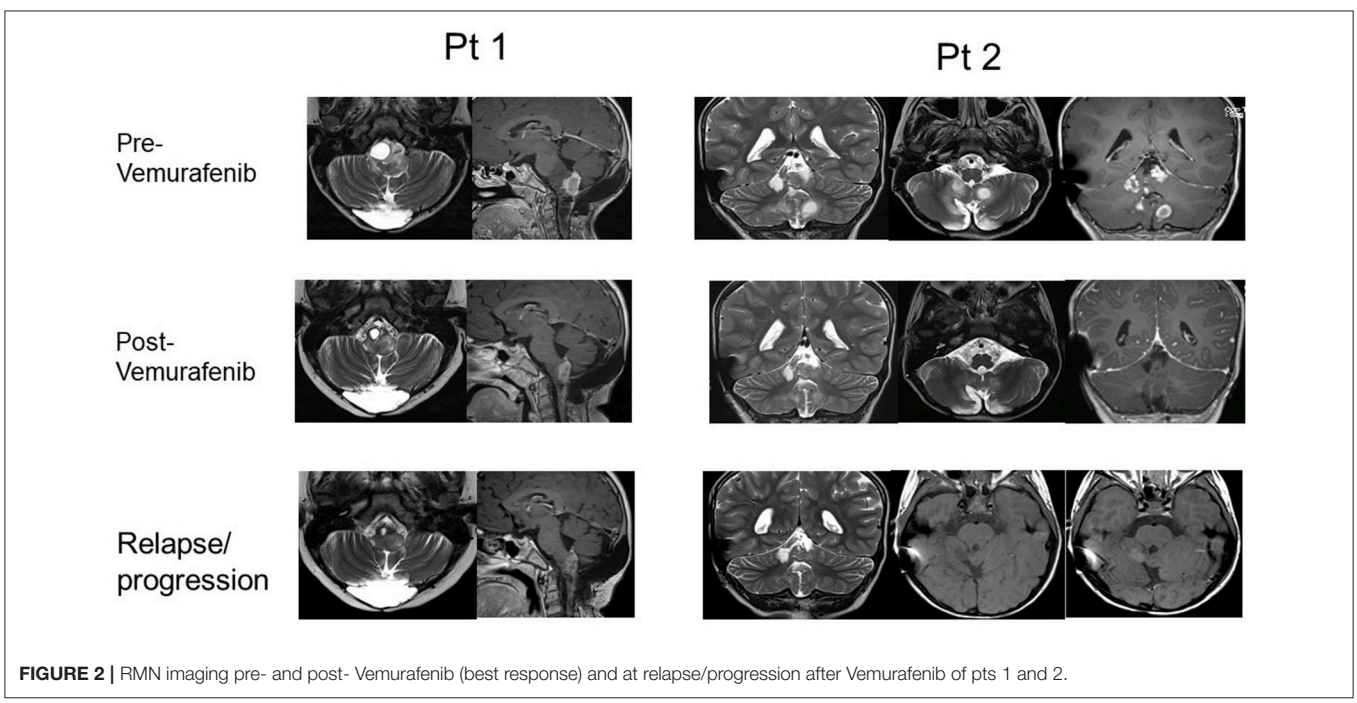

FIGURE 2 | RMN imaging pre- and post- Vemurafenib (best response) and at relapse/progression after Vemurafenib of pts 1 and 2.

of the $BRAF^{V600E}$ mutated protein by immunohistochemistry, made the analysis more widely available and less expensive, although less accurate as shown by our child with PA. In this regard, the possible inconsistency between these two methods (immunohistochemistry vs. molecular testing) is an issue that must be addressed. Immunohistochemistry may represent the best screening method in LGG patients, thanks to the wider availability and lower cost, but its lower specificity and sensibility when compared to molecular analysis must be always taken into account, particularly in those patients where the specific therapy does not seem to control the disease. In these cases, a molecular confirmation of the immunohistochemistry results is recommended.

The treatment appears to be well-tolerated, with only mild toxicity; the safety was confirmed also in a patient with constitutional trisomy of the chromosome 21. We observed exclusively skin toxicity, and only in one case the severity of the adverse effect led to the discontinuation of the drug. Dermatological toxicity is a known adverse effect of the drug and includes photosensitivity, keratinocyte proliferation and differentiation dysfunctions; the development of malignant lesions of the skin has also been reported (11).

The immediate progression of the disease noted after discontinuation of the drug and the prompt response after Vemurafenib reintroduction prove the strong dependence of the disease on the continuous inhibition of the $BRAF^{V600E}$ activity. This evidence underlines the need for further combinations/options to possibly achieve complete tumor eradication.

The efficacy of the treatment in our small cohort is promising, with approximately 60% of response, although the small number of patients, given by the rarity of the condition, does not allow to draw firm conclusions on a statistically significant base. Only one patient with a bulky neurocytoma of the brainstem did not respond to the treatment (12). Taking into consideration the tolerability of the treatment, it represents a valuable option for these patients, sparing the long-term neurocognitive and endocrinological sequelae associated with chemotherapy and radiotherapy in a group of patients that have an excellent overall survival. Therefore, it is extremely important to reduce the possible impact of the treatment for these children and to preserve their quality of life during adulthood. It must be, however, underlined that evidence of the long-term safety of the treatment with Vemurafenib is lacking and should be prospectively confirmed. Moreover, as mentioned, although it can represent an excellent option to control the disease and could serve as a bridge to a more definitive treatment, neither it can be considered as a definitively curative approach itself nor a lifelong treatment can be envisioned.

Moreover, the regrowth of the disease observed under treatment, suggests the development of resistance, a well-documented finding in melanoma. The biological mechanism underlying the development of resistance has not been unraveled yet, but several studies are currently ongoing to find novel therapeutic strategies for these patients (13–15). Further molecular characterizations of the non-responders is necessary to evaluate the alternative pathways of the MAPK signaling.

Confirmation of our findings in larger and prospective studies is required. However, our results suggest that Vemurafenib could be a well-tolerated and effective option in pediatric patients affected by *BRAF* V600E-mutated LGG.

AUTHOR CONTRIBUTIONS

FD, AM, and FL designed the study. FD, GC, AC, and IA cured the collection of the data. FD, GC, IA, AC, AM, GSC, ED FD, and F-DC interpreted and analyzed the data, FD and GC drafted the manuscript, AM and FL critically revised the manuscript for intellectual content. EA took care of the molecular characterization of the BRAF gene.

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REVIEW
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EZH2, HIF-1, and Their Inhibitors: An Overview on Pediatric Cancers

Marco Papale[1], *Elisabetta Ferretti*[1,2], *Giuseppe Battaglia*[2], *Diana Bellavia*[3], *Antonello Mai*[4]* *and Marco Tafani*[1]*

[1] Department of Experimental Medicine, Sapienza University, Rome, Italy, [2] IRCCS Neuromed, Isernia, Italy, [3] Department of Molecular Medicine, Sapienza University, Rome, Italy, [4] Department of Drug Chemistry and Technologies, Sapienza University, Rome, Italy

During the past decades, several discoveries have established the role of epigenetic modifications and cellular microenvironment in tumor growth and progression. One of the main representatives concerning epigenetic modification is the polycomb group (PcG). It is composed of different highly conserved epigenetic effector proteins preserving, through several post-translational modifications of histones, the silenced state of the genes implicated in a wide range of central biological events such as development, stem cell formation, and tumor progression. Proteins of the PcG can be divided in polycomb repressive complexes (PRCs): PRC1 and PRC2. In particular, enhancer of zeste homolog 2 (EZH2), the catalytic core subunit of PRC2, acts as an epigenetic silencer of many tumor suppressor genes through the trimethylation of lysine 27 on histone H3, an essential binding site for DNA methyl transferases and histone deacetylases. A growing number of data suggests that overexpression of EZH2 associates with progression and poor outcome in a large number of cancer cases. Hypoxia inducible factor (HIF) is an important transcription factor involved in modulating cellular response to the microenvironment by promoting and regulating tumor development such as angiogenesis, inflammation, metabolic reprogramming, invasion, and metastatic fate. The HIF complex is represented by different subunits (α and β) acting together and promoting the expression of vascular endothelial growth factor (VEGF), hexokinase II (HKII), receptor for advanced glycation end products (RAGE), carbonic anhydrase (CA), etc., after binding to the hypoxia-response element (HRE) binding site on the DNA. In this review, we will try to connect these two players by detailing the following: (i) the activity and influence of these two important regulators of cancer progression in particular for what concerns pediatric tumors, (ii) the possible correlation between them, and (iii) the feasibility and efficiency to contrast them using several inhibitors.

Keywords: HIF-1, EZH2, tumors, cancers, oncology, hypoxia, epigenetics

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Rimas J. Orentas, Seattle Children's Research Institute, United States

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Chunliang Li, St. Jude Children's Research Hospital, United States
Johan Holmberg, Karolinska Institutet (KI), Sweden

***Correspondence:**
Marco Tafani
marco.tafani@uniroma1.it
Antonello Mai
antonello.mai@uniroma1.it

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INTRODUCTION

During the past decades, a growing number of scientific data has led to the discovery of epigenetics, defined as the changes during the development of an organism that are not related to DNA sequences. Changes in the chromatin configuration at the gene promoter influenced by chemical modification such as acetylation, methylation, ubiquitylation, and phosphorylation are part of an exact mechanism that regulates the accessibility to DNA and the consequent transcription

and expression of coding genes (1). Some of these modifications are considered inheritable in plants, invertebrates, and also vertebrates both cell division and cell generation (germline). In fact, environmental pressures as well as lifestyle can produce epigenetic changes that can be maintained through two or three generations even in the absence of the specific stimulus (2). However, epigenetic modifications, unlike genetic mutations, are reversible because they fail to alter the nucleotide sequence.

One of the most important epigenetic regulators is represented by the polycomb group (PcG) of proteins influencing the expression of many genes implicated in the development of the organism from childhood to adulthood. Moreover, the PcG represents evolutionarily conserved multiprotein repressive complexes such as polycomb repressive complex 1 (PRC1) and PRC2. In particular, histone methyltransferase enhancer of zeste homolog 2 (EZH2), a specific component of PRC2, regulates the trimethylation of Lys 27 on histone 3 (H3K27) implicated in the expression of some essential genes in the early steps of X-chromosome inactivation in women (3). Moreover, deregulation of this particular complex has been associated with a large number of tumors, and the expression of PcG proteins is closely associated with carcinogenesis (4).

The development of a tumor is a complicated process in which a large number of *influencers* play their own role. The correlation between tumors and microenvironment was hypothesized in the last century, and it was part of Stephen Paget's (1889) "seed and soil" theory Paget's 1889 "seed and soil" theory postulating that metastatic cells of a specific cancer ("the seed") usually metastasize to some sites ("the soil") depending on the affinity between initial and secondary tumor sites (5).

Nowadays, we know that particular conditions of the hypoxic tumor microenvironment could promote the expression of specific oncogenes resulting in the progression of cancer. One of the most important factors regulating the response of tumor cells to hypoxia is represented by hypoxia-inducible factor 1 (HIF-1) (6, 7).

Tumor environment is characterized by the lack of oxygen, and the cells are exposed to hypoxia. In this specific hypoxic "habitat," HIF-1 is activated. Specifically, HIF-1 is a heterodimeric protein composed of two subunits: HIF-1β (constitutively expressed) and HIF-1α (O_2-regulated). Under normoxic conditions, the newly synthesized HIF-1α is hydroxylated at proline residues 402 and/or 564 by specific prolyl hydroxylase domain (PHD) proteins (i.e., PHD2) using O_2 and α-ketoglutarate as substrates to catalyze a dioxygenase reaction. During such a reaction, one oxygen is inserted into the proline residue while the other oxygen is inserted into α-ketoglutarate leading to the formation of succinate and CO_2. Next, the protein OS-9 interacts with both PHD2 and HIF-1α, promoting hydroxylation. This step is essential to bind the von Hippel-Lindau protein (VHL) that, in turn, interacts with elongin C forming a ubiquitin ligase complex. This process leads to the proteasomal degradation of HIF-1α. Contrastingly, under hypoxia conditions, oxygen (O_2) deprivation inhibits the hydroxylation reactions and/or increases the mitochondrial production of reactive oxygen species (ROS), which oxidize the ferrous ion in the catalytic site of hydroxylases, thereby inhibiting these enzymes. In this way, the stability and the transactivation function of HIF-1α are increased, promoting dimerization with HIF-1β. This determines the activation of the complex and the consecutive binding to the hypoxia responsive element (HRE) sequence 5′-(A/G)CGTG-3′ in target genes, increasing the transcription into mRNA (6, 7).

More than 40 different HIF-1 target genes are regulated in this way. Importantly, these genes regulate crucial cellular processes such as tumor progression, remodeling of the microenvironment, cell proliferation, survival, angiogenesis, glucose metabolism, and iron homeostasis. Proteins encoded by these genes, such as vascular endothelial growth factor (VEGF), mediate increased O_2 delivery via the formation of new blood vessels. The expression of other HIF-1-regulated proteins, such as hexokinase II (HKII), which catalyzes the first step of glycolysis (8), glucose transporters (GLUT), and glycolytic enzymes, allow cells to adapt their metabolism to a specific environment distinguished by a reduction or lack of oxygen. Finally, the expression of a third group of gene products may influence the balance between apoptotic and anti-apoptotic signals that determine cell survival (9).

EZH2 and Its Inhibitors

Enhancer of zeste homolog 2 is the catalytic subunit of the PRC2, a highly conserved histone methyltransferase, involved in the methylation of lysine 27 of histone 3.The overexpression of EZH2 has been correlated with many cancers, such as prostate, breast, bladder, whereas other kinds of cancer, such as B-cell lymphomas or other forms of breast cancer, can be promoted by specific mutations (10). For this reason, EZH2 can be considered as an interesting target for the development of targeted anticancer strategies (10).

The PRC2 complex operates as a chromatin and it is expressed in several organisms from plants to flies modifier, and humans. It interferes with the transcription of target genes (i.e., silencing genes involved in differentiation) by trimethylating lysine 27 on histone 3 (H3K27me3), considered as its major function "*in vivo*." The human PRC2 complex is composed of five different subunits: EZH2, SUZ12, EED, AEBP2, and RbAp46/48 (10–12). In addition, EZH2, which represents the main subunit of PRC2 sequentially catalyzes three methylation reactions at H3K27, generating monomethylated, dimethylated, and trimethylated H3K27 (H3K27me1, H3K27me2, and H3K27me3). In the PRC2 complex, EZH2 represents the most important subunit, SUZ12 and EED are important for maintaining the integrity of the entire structure, while AEBP2 is necessary to stabilize the structure of the complex. Mutations of these subunits could determine a lack of PRC2 function. In contrast, the RbAp48 subunit does not seem to be necessary during the enzymatic activity, and therefore it may be not essential for the histone methyltransferase action of EZH2 (12).

Given its specific activities, EZH2 can be considered as an interesting target in several types of cancer. For this reason, during the past years, a large number of EZH2 inhibitors have been discovered, such as 3-deazaneplanocin A (DZNep), EPZ005687, EI1, GSK126, or UNC1999 (**Figure 1**). Obviously, the development of these inhibitors is limited to those cancers

EZH2 INHIBITORS	CANCER TYPE	ACTIVITY
DZNep (3-DEAZANEPLANOCIN A) *ABCAM* *MERCK*	BREAST, COLON	REDUCTION OF EZH2, INTERFERING PRC2
EPZ005687 *CAYMAN CHEMICAL* *BPS BIOSCIENCE*	LYMPHOMAS	INHIBITS EZH2 MUTANTS Y641 AND A677
EI1 *NOVARTIS* *CAYMAN CHEMICAL*	B-CELL LYMPHOMAS	LOSS OF H3K27 METHYLATION FUNCTION (INHIBITS EZH2 MUTANT Y641)
GSK126 *CAYMAN CHEMICAL* *CHEMIETEK*	DIFFUSE LARGE B-CELL LYMPHOMAS	INHIBTS PROLIFERATION OF EZH2 MUTANTS
UNC1999 *CAYMAN CHEMICAL* *MERCK*	DIFFUSE LARGE B-CELL LYMPHOMAS	INHIBITS EZH2 MUTANT Y641N
REFERENCE	10	

FIGURE 1 | EZH2 inhibitors 3-deazaneplanocin A (DZNep), EPZ005687, EI1, GSK126, and UNC1999. DZNep is a S-adenosylhomocysteine hydrolase inhibitor, depletes EZH2 and the associated H3K27me3, and induces apoptosis in breast and colon cancer cells. EPZ005687 inhibits H3K27 methylation by the EZH2 mutants Y641 and A677, and it has been shown to selectively kill lymphoma cells that are heterozygous for one of these EZH2 mutations. EI1 is active against different forms of EZH2, while GSK126 effectively inhibits the proliferation of the protein mutants in DLBCL cell lines. UNC1999 represents the first orally bioavailable EZH2 inhibitor.

in which there is a gain of function of EZH2. Therefore, in this section, we describe different types or tumors indicating, when possible, the state of EZH2 (wild type, overexpressed, mutated but still targetable by inhibitors or mutated with a loss of function). The reader must keep in mind that EZH2 inhibitors are useless or even harmful in those cancers characterized by a loss of function of EZH2. Nonetheless, this knowledge is important for both scientists as well as patients.

The drug DZNep is an S-adenosylhomocysteine hydrolase inhibitor that reduces EZH2 and H3K27me3 levels, thereby inducing apoptosis in breast and colon cancer cells. This compound alters the PRC2 pathway even if the exact mechanism is yet to be elucidated, but it is considered as a promising drug for future cancer therapies. The drug EPZ005687 was discovered as an inhibitor with a 50 fold higher selectivity for EZH2 than for EZH1 (10). Moreover, EPZ005687 can also inhibit H3K27 methylation induced by two EZH2 mutants (Y641 and A677), thereby killing lymphoma cells carrying the Y641 or A677 EZH2 mutant (10, 13). In addition, EI1 is active against different forms of EZH2, while the most potent inhibitor of EZH2, GSK126, is effective in inhibiting proliferation of the protein mutants in DLBCL cells (10). The drug UNC1999 an analog of GSK126, represents the first orally bioavailable EZH2 inhibitor. *In vitro* UNC1999 has shown high efficiency against wild type and mutant

EZH2 against many epigenetic and non-epigenetic targets. In cells, UNC1999 seems to reduce H3K27me3 levels ($IC_{50}<50$ nmol/L) reducing the vitality of DLBCL cell lines with the Y641N mutation (14). Recent genetic studies in a wide range of organisms have shown an evolutionarily conserved antagonistic relationship between polycomb proteins and switch/sucrose non-fermentable (SWI/SNF) complexes, which utilize the energy of ATP hydrolysis for chromatin remodeling. These complexes are composed of 12 to 15 subunits that are mutated in 20% of all human cancers. Moreover, in several cases, it has been demonstrated that EZH2 gain-of-function mutations such as in A687 and A677 can lead to cellular transformation as reported in non-Hodgkin lymphomas(15, 16). Unopposed EZH2 activity is also a driver of cancers determined by the loss of the core subunit SNF5/SMARCB1 in the SWI/SNF complex as demonstrated in rhabdoid tumor, a highly malignant aggressive type of pediatric cancer (17, 18). Additionally, EZH2 has been shown to also possess non-enzymatic functions, leading to a situation that raises the possibility that the enzymatic inhibitors currently employed in clinical trials may not fully suppress EZH2 activity as well as its tumor promotion. However, this effect may be reduced by blocking the interaction between EZH2 and other PRC2 subunits using a peptide known as *stabilized alpha-helix of EZH2* (SAH-EZH2) obtained from the EZH2 domain interacting with the EED subunit. The SAH-EZH2 interaction interferes with the EZH2–EED complex, reducing EZH2 levels, and stopping H3K27 trimethylation. This peptide is effective against EZH2-dependent MLL–AF9 leukemia and EZH2-mutant lymphoma cells, whereas it does not have any effect on non-transformed and EZH2 controls. Remarkably, the anti-proliferative effect of SAH-EZH2 has been linked more to the reduction of EZH2 expression than to H3K27me3 reduction. These results are consistent with the observations stressing the important role of the non-enzymatic function of EZH2 in SWI/SNF-mutant cancers (11).

HYPOXIA MASTER REGULATOR HIF-1 AND ITS INHIBITORS

During recent years, a growing number of evidence has laid the groundwork for a better comprehension of all the complicated processes implicated in the development of cancers. Transforming cells are characterized by an uncontrolled growth forming the tumor mass, and in the internal core, the reduction of oxygen induces a great number of cellular modifications leading the cells to adapt to this particular microenvironment. All these hypoxia-adaptive changes include the switch from oxidative phosphorylation to glycolysis in cell metabolism, an augmented synthesis of glycogen, and a switch from glucose to glutamine as a substrate for the synthesis of fatty acids. In this particular condition, HIF-1 acts as a primary controller of a great number of proteins implicated in a wide variety of cellular activities. The presence of hypoxic regions inside the tumor results in (I) necrosis of cells distant from vessels of host tissue and (II) activation of the HIF-1 complex in an attempt to increase the survival of sublethally damaged tumor cells. In this situation, HIF-1 expression increases not only the survival of tumor cells but also their commitment to malignancy (19). In cancer cells, HIF-1 can also be activated by loss of function of the tumor suppressor VHL and by gain of function of oncogenes leading to the activation of the PI3K/AKT/mTOR pathway. Moreover, HIF-1 activation regulates the metabolism to increase cancer progression and resistance to therapy. For this reason, HIF-1 or metabolic enzyme inhibitors may be used to target the metabolic flexibility of cancer cells increasing their sensitivity to anticancer therapies (20).

During the past years, a growing number of molecules have been demonstrated to inhibit HIF-1 activity. A large number of these inhibitors work by reducing HIF-1α mRNA or protein levels, HIF-1 DNA-binding activity, or the trans-activation of some HIF-1 related genes. These drugs reduce the expression of HIF-1α operating on the synthesis or degradation of proteins. In many cancers, the synthesis of HIF-1α is strictly related to mTOR activity. In fact, in cancer cells, the continuous activation of tyrosine kinase receptors (such as HER2neu, BCR-ABL, and EGFR) and/or of the downstream phosphatidylinositol 3-kinase/AKT and RAS/MAP kinase signal transduction pathways determines the growth of mTOR activity and the consecutive activation of HIF-1. Thus, inhibitors of these pathways are able to determine the loss of HIF-1 activity and other biological activities such as a reduction of tumor vascularization promoting the therapeutic effect (21).

Moreover, HIF-1 expression can be also controlled by redox (reduction-oxidation)-dependent processes. In fact, it has been demonstrated that by treating purified HIF-1 with diamide, hydrogen peroxide, or N-ethyl-maleimide, a specific alkylator of cysteine sulfhydryl groups, it is possible to cause a complete loss of DNA binding activity (22).

On the basis of these data, it is obvious to consider HIF-1 as an important target for anticancer therapy, and during the past years, several natural products (generally, the term "natural product" is used to indicate low molecular weight secondary metabolites produced by animals, plants, and microbes for chemical defense and growth advantage) or synthetic compounds have been shown to possess an anti HIF-1 activity.

NATURAL INHIBITORS

Several emerging scientific data show the efficacy of many natural compounds to inhibit the activity of HIF-1 operating on protein synthesis or protein degradation. Actinomycin D (dactinomycin) is a metabolite produced by *Streptomyces parvullus* (formerly *S. antibioticus*) that acts as an inhibitor of transcription, and it is able to block hypoxia-induced HIF-1 activity in the human hepatoma cell line Hep3B. Moreover, other data showed the efficacy of actinomycin D to block HIF-1α induction by angiotensin II (Ang II) in the vascular smooth muscle cells of rats without altering hypoxia induction. Inhibition of HIF-1 depended on the cell type, stimulus, and drug concentration. Another cytotoxic compound, GL331, is a semisynthetic podophyllotoxin-derived topoisomerase II inhibitor with IC_{50} values in the range of 0.5 μm to 2 μm that

has been shown to work against a large number of tumor cell lines. In the human lung adenocarcinoma cell line CL1-5, using a concentration of 10 μm of GL331, it has been possible to decrease HIF-1α mRNA levels, probably through transcriptional inhibition. However, such an inhibitory effect is not specific for HIF-1. At the same concentrations, the compound shows cytotoxicity and inhibits the expression of cyclin D1 in CL1-5 cells. In addition, GL331 demonstrated no effects against gastric cancer in a clinical study (23).

Emerging data have discovered a growing number of natural products capable of reducing HIF-1α mRNA levels despite the fact that the mechanism is not completely clear for all of them. The Indian traditional medicine glycoside known as picroliv (in which the major compound found is a purified iridoid glycoside fraction picroside-I and kutkoside from the roots of *Picrorhiza kurrooa*) reduced HIF-1α and VEGF mRNA levels *in vitro* (24, 25).

Other compounds have the ability to promote HIF-1 degradation through different ways. For example, geldanamycin (GA), a metabolite of *Streptomyces hygroscopicus*, interferes with hsp90 through the amino-terminal ATP/ADP binding pocket. In addition, GA blocks the activation of the HIF-1 complex promoting the degradation of the α-subunit through a VHL-independent proteasomal mechanism both in normoxic and hypoxic conditions (26, 27).

Other emerging scientific data have shown that by using the antifungal antibiotic cycloheximide isolated from *Streptomyces griseus*, it is possible to inhibit general eukaryotic protein synthesis and to interfere with the accumulation and activation of the HIF-1α protein. Another important group of compounds with inhibitory effects on HIF-1α protein synthesis is represented by microtubule disrupting agents (MDA) (23). Additionally, other hsp90 inhibitors such as 17-N-allylamino-17demethoxygeldanamycin, resveratrol, the antibiotic novobiocin, and many others have been shown to have an activity against the accumulation of HIF-1 (23).

Finally, another natural compound with an anti HIF-1 activity is the red Korean ginseng, even if the mechanisms of action and the antitumor effect are yet to be clarified (19, 28).

Therefore, as reported in this review, there are several natural drugs and compounds capable of interfering with HIF-1 activity, accumulation, and function, and this can possibly lead to the development of new anticancer strategies in the future (**Figure 2**). However, even if a growing number of evidence is collected day by day, still several sets of data are necessarily required to go on in this way.

HIF–EZH2: TWO SIDES OF THE SAME COIN?

During the past years, great progresses have been made in many areas of pediatric oncology even if tumors of the central nervous system (CNS) remain a significant challenge because of their complicated nature. Basing on recent scientific data, it has been possible to achieve a better comprehension of cancer molecular mechanisms and to develop new therapeutic approaches focused on different molecules and pathways (**Figure 3**).

In this review, we paid particular attention on some specific pediatric tumors such as high grade glioma and pediatric low grade glioma.

Pediatric high grade gliomas (pHGGs) have the same histological, but not molecular, features of adult HGGs (aHGGs) and have been classified, in accordance with the World Health Organization (WHO), as grade III and IV CNS tumors. Grade III glioma (anaplastic astrocytoma) is characterized, histologically, by atypical nuclei, an increased cellularity, and an increased mitotic activity. Grade IV glioma, also known as glioblastoma multiforme (GBM), is the most pathologically advanced and clinically aggressive tumor. A wide number of genomic analyses on several pediatric tumors such as glioblastomas, anaplastic astrocytomas, and diffuse intrinsic pontine gliomas (DIPG) have revealed frequent mutations of the *H3F3A* gene (29) (encoding H3.3) in which the lysine residue at position 27 was substituted with a methionine (K27M) or the glycine residue at position 34 with an arginine or valine (G34R/V) (30–33). Conversely, just a limited number of DIPGs revealed a replacement of the lysine residue at position 27 with a methionine (K27M) in the *HIST1H3B* gene encoding for the histone H3.1 (33). In fact, pediatric GBM with *H3F3A* K27M mutations shows a reduction in H3K27me3 when compared with wild type tumors. Moreover, anaplastic astrocytomas also revealed the presence of a significant fraction of hypoxic tissue areas. In fact, hypoxia has been shown to play an important role in the malignant progression of various tumor entities, and hypoxic areas have been found in malignancies such as breast, prostate, pancreas, and lung cancer, soft tissue sarcomas, non-Hodgkin's lymphomas, melanomas, liver tumors, etc (34). Hypoxia leads to the activation of HIF-1 via the stabilization of its α-subunit. Importantly, a number of studies have indicated a widespread expression of HIF-1α (35–39) and its target genes glucose transporter (GLUT)-1 (40) and carbonic anhydrase (CA) IX (40, 41), in malignant astrocytomas. Hypoxia has been suggested as an adverse prognostic factor for patient outcome. In fact, several studies of tumor hypoxia involving the direct assessment of the oxygenation status have suggested worse disease-free survival for patients with hypoxic cervical cancers or soft tissue sarcomas. In head & neck cancers, studies suggest that hypoxia is prognostic for survival (34). In fact, the identification and quantification of hypoxic regions is extremely important for the management of the disease. A large number of predictive assays for tumor oxygenation status have been developed in the past years showing differences in the degrees of success. To date, functional imaging techniques based on positron emission tomography (PET) have been demonstrated to be fundamental for both pretreatment and tumor response evaluation during therapy. Several hypoxia-specific PET markers have been developed in several clinics to quantify hypoxic tumor sub-volumes for personalized treatment planning. Moreover, a large number of new radiotracers are actually under investigation. In addition, PET-derived functional parameters and tracer pharmacokinetics can be used to obtain very useful input data to create computational models aimed to simulate or interpret PET acquired data to develop new treatment

HIF-1 NATURAL INHIBITORS	ACTIVITY	REFERENCE
DACTINOMYCIN (ACTINOMYCIN D)	INHIBITION OF TRANSCRIPTION, BLOCKS INDUCTION OF HIF-1α	23
GL331	INHIBITION OF HIF-1 BY DECRASING mRNA HIF1-α	23
PICROLIV	REDUCTION OF HIF-1α, VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) mRNA LEVELS IN VITRO	24,25
GELDANAMYCIN	HSP90 INTERFERING, HIF-1α DEGRADATION	26,27
CICLOHEXIMIDE	INHIBITION OF EUKARYOTIC PROTEINS, INTERFERING HIF1-α ACTIVATION	23
MDA (MICROTUBULE DISRUPTING AGENTS)	INHIBITION OF HIF-1α PROTEIN SYNTHESIS	23
17N-ALLYLAMIN-17DEMETHOXYGELDANAMYCIN	HSP90 INHIBITION	23
RESVERATROL	HSP90 INHIBITION	23
NOVOBIOCIN	HSP90 INHIBITION	23
RED KOREAN GINSENG	INHIBITION OF HIF-1α DIMERIZATION	19,28

FIGURE 2 | Natural inhibitors of HIF-1. During these years, a large number of compounds have been tested, and their natural inhibitory effect during hypoxia has been demonstrated. Here, we reported some of them presenting different mechanisms of action such as inhibition of transcription, inhibition of VEGF, inhibition of HIF-1 synthesis or dimerization, etc.

planning or radio/chemotherapy response prediction programs (42).

Primary GBM is characterized by increased vascular proliferation and necrosis in addition to the features of grade III glioma (43). In particular, pHGGs have a specific wide distribution in contrast to aHGGs that are more frequent in the cerebral cortex (44). Molecularly, pHGGs are distinct from aHGG and are characterized by a great number of activating or inactivating gene modifications, such as gene amplifications, deletions, and inactivation of p53 and histone 3.3 (H3F3A), causing them to be activated and inactivated (45).

The canonical treatment for pHGG occurring in the cerebrum typically includes initial surgery followed by radiation and chemotherapy. There is widespread agreement that total resection of tumor tissue improves patient outcome, but anyway, focal radiation therapy has also become standard in the treatment of patients greater than 3 years of age with pHGG (43). Chemotherapy is frequently used to treat patients with pHGG; although evidence of efficacy is modest, chemotherapeutic agents are often employed in the treatment of these patients in combination with temozolomide even if the ability of this drug is still debated (46–48).

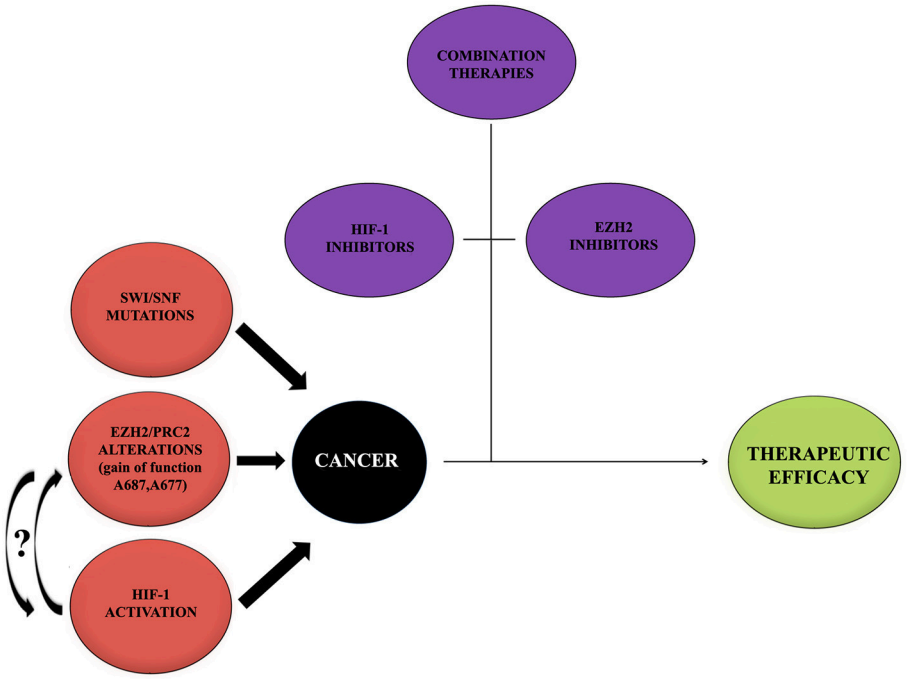

FIGURE 3 | Cancer onset influencing mediators. Cancer represents a multifactorial pathology in which a large number of alterations act to promote the onset and development. Alterations of EZH2, HIF-1, and mutations of SWI/SNF may provide a negative influence promoting tumors. Curved arrows with question marks refer to the fact that no data or very little data are available regarding the possible interaction between EZH2 and HIF-1 during cancer onset. On the contrary, thanks to recent studies, the importance of single treatments with either HIF-1 or EZH2 inhibitors, combined with other therapeutic approaches, to contrast the tumor and to obtain a therapeutic efficacy has been shown. It would be interesting to try a new approach with inhibitors for both EZH2 and HIF-1.

Medulloblastoma (MB) is another pediatric brain tumor, representing 15–20% of the tumors occurring at a young age. While it can occur at any age from infancy through adulthood, it is most typically seen in children with bimodal incidence peaks between 3 and 4 or 8 and 9 years of age (49). MB is characterized by four distinct molecular subgroups, such as those dependent on Wingless (WNT) or Sonic Hedgehog (SHH) signaling and those that are less well characterized (Group 3 (G3) and Group 4 (G4)), showing multiple responses to common therapeutic approaches (50). These tumors present, in contrast to most other cancers, some recurrent mutations. These four MB subgroups have different mutational landscape, gene expression, pathology, and prognosis (50–54). In fact, about 25% of MBs are G3 tumors with high MYC levels as a result of somatic MYC gene amplification in about 15–20% of G3 cases. Moreover, some G3 MBs have been associated with relatively high levels of EZH2 and to increased global H3K27me3 chromatin repressive marks (53). In contrast, the H3K27me3-specific demethylase, KDM6A/UTX, is mutated in a reciprocally exclusive manner for the most part in G4 MBs (53, 55–57). Basing on this knowledge, recently, it has been possible to investigate the relationship between G3 MB and EZH2 and to explore its functional relationship with other genes that are important for the development of MB, using gene editing systems. In fact, in this way, it has been possible to demonstrate that the deletion of EZH2 accelerates de novo MB development and the progression of established MBs and that EZH2 inactivation in G3 MB implicates Gfi1 (a proto-oncogene frequently activated in human G3 MBs whose disruption antagonizes the tumor promoting effects of Ezh2 loss) as an oncogenic target gene. Therefore, enforced expression of Gfi1 promotes secondary G3 MB formation mimicking the effects of the absence of Ezh2, whereas the lack of Gfi1contrasted the effects of Ezh2 inactivation, together providing at least a partial rationale for Ezh2-mediated tumor suppression (58). Importantly, these new results point the attention to the complexity of the pathways directly and indirectly influenced by EZH2 to the point that this protein can function as either a tumor suppressor or promoter depending on the cellular context.

Ependymomas occur in both children and adults and can arise throughout the entire neuraxis. They are divided, according to the WHO, into three types classified as grade I, II, and III. While spinal cord tumors are more common in adults, in pediatric patients, approximately 70% of ependymomas arise in the posterior fossa. The canonical treatment for these specific tumors is based mainly on surgical resection, and in the post-operative period, radiation can be used to improve the survival frequency (49).

During the pediatric age, other kinds of tumor such as low grade glioma (PLGG) can cause several problems to patients. This kind of tumor represents the most common type of pediatric astrocytoma and pediatric brain tumor in general.

According to the WHO classification, PLGGs are grade I or II and include pilocytic astrocytoma (PA), subependymal giant cell astrocytoma (SEGA), pilomyxoid astrocytoma (PMA), pleomorphic xanthoastrocytoma (PXA), and low-grade fibrillary astrocytoma or diffuse astrocytoma. Among these tumors, PA is considered the most common glioma in children.

Actually, these tumors are treated using different drugs including carboplatin, vincristine, temozolomide, etc. Anyway, surgical resection is commonly used to treat subependymal giant cell astrocytoma especially in the case of intracranial hypertension.

Apart from pediatric brain tumors, epigenetic alterations have an important role in several cancers; for example in leukemias, repressive proteins of the polycomb group, PRC1 and PRC2, are the main epigenetic regulators in developmental and transcriptional repression.

For example, early T cell precursor acute lymphoblastic leukemia (ETP-ALL) represents an aggressive subtype of ALL characterized by the presence of stem cells and transcriptional programs observed in myeloid cells (59). It has been demonstrated that inactivating alterations of the PRC2 components are recurrent in this kind of human tumor, but their functional role is yet to be entirely defined (60). Some data about the involvement of EZH2 have been collected using a murine model of *NRASQ61K*-driven leukemia that shows the phenotypic and transcriptional aspects of ETP-ALL (60). In particular, homozygous inactivation of EZH2 cooperates with oncogenic NRASQ61K to favor leukemia development. In addition, EZH2 acts as silencer of stem cell- and early progenitor cell-associated genes, and its loss is associated with an increased activation of signal transducer and activator of transcription 3 (STAT3) by tyrosine 705 phosphorylation (60). Moreover, the activity of STAT3 maintains the co-option of a pro-metastatic program in activated astrocytes in a metastatic lesion. These astrocytes support metastatic cells through their ability to modulate the innate and acquired immune systems, and it has been demonstrated that patients who have reactive astrocytes with active STAT3, show a reduced survival from the diagnosis of intracranial metastases. In fact, if in reactive astrocytes the signaling of STAT3 is prevented, it is possible to reduce the formation of metastasis in the brain from different primary tumor sources. Thus, by using an orally bioavailable treatment against STAT3, it is possible to obtain an antitumor effect in patients even with advanced systemic diseases such as brain metastasis (61).

Therefore, according to these data, the inactivation of EZH2 can be linked to the activation of transcriptional programs in stem cells and to increased growth/survival signaling, characteristics determining a poor prognosis in patients (60).

T-cell acute lymphoblastic leukemia (T-ALL) represents about 10–15 % of pediatric ALL cases and, when compared with B-progenitor ALL patients, shows some aggressive clinical features such as higher risk for primary resistant disease, and frequent early isolated central nervous system relapse. In recent years, thanks to different therapeutic strategies based on intensive chemotherapy, T-ALL prognosis in children and adolescents has improved. However, it is still worse when compared with B-lineage ALL in particular if associated to a lack of initial response to the therapy with prednisone (62). Another approach to treat this tumor is based on 3-deazaneplanocin-A (DZNep) blocking EZH2. This agent showed a good activity inducing a strong apoptosis of cancer cells; however, additional data have to be collected regarding its potential for treating T-cell ALL (63).

Other therapeutic approaches for the treatment of this cancer are based on daunoblastine, an anti-leukemic agent used for decades despite the fact that its success is frequently linked to the use of other drugs. For this reason, recently, the efficacy of this drug in combination with DZNep has been shown. A combination of these drugs in the ratio 25:50 showed a synergistic effect at the CalcuSyn elaboration (a software to explore the relative contribution of each agent to the synergism) and induced apoptosis in Jurkat cells. In particular, DZNep caused 63 % of apoptosis if used alone, whereas a stronger effect up to the 67% was observed if combined with daunoblastine after 72 h of treatment (64). Therefore, DZNep inhibits Jurkat cell proliferation through the activation of check-points that block cell cycle arrest at S phase, an effect that results in the induction of apoptosis. In fact, EZH2 was upregulated in natural killer/T-cell lymphoma, through Myc-mediated mRNA inhibition. Such EZH2 upregulation determines the activation of cyclin D transcription promoting cell proliferation, an effect that is independent of its methyltransferase activity (65).

Even if the principal activity of EZH2 as a gene silencer proceeds through the methylation of H3K27, a large number of studies have shown that the transcriptional activation of EZH2 in many types of cancer is achieved independently of H3K27me (66). This activity has been demonstrated especially in natural killer/T-cell lymphoma, in which cyclin D transcription is activated by EZH2 upregulation via Myc-mediated mRNA inhibition promoting cell proliferation without a methyltransferase activity (65). Similarly, in Jurkat cells, when EZH2 is upregulated, there is an activation of cyclin D transcription demonstrating the effect of DZNep. The data obtained showed that using both agents causes a change in the expression and activity of several central proteins such as caspase-3, 9, Bcl-2, Erk, and EZH2. Specifically, a reduction of Bcl-2 and an increase of cleaved caspase-3 and caspase-9 especially in cells treated with therapy combination has been observed. Moreover, the combination also inhibited Erk-mediated proliferation pathway. This observation has been considered extremely important since it gives the possibility to develop new therapeutic approaches based on the combination of DZNep and daunoblastine to treat T-ALL cells, in particular the subgroup with a much worse prognosis (63).

Along with PRC2, another main factor promoting the development and progression of cancer is represented by the microenvironment and its main responder HIF even for what concerns pediatric tumors. In fact, several studies reported a large number of brain and non-brain tumors in which HIF plays a primary role (39, 67–69). One example is that of metformin, a well-known insulin-sensitizer frequently used for type 2 diabetes therapy, which can be potentially considered as a very interesting drug in oncology also. In fact, the use of metformin has been hypothesized to contrast pediatric sarcomas

such as osteosarcoma, Ewing sarcoma, and rhabdomyosarcoma, representing common pediatric sarcomas, that depend on insulin-like growth factor (IGF) and insulin for pathogenesis and progression. *In vitro* results showed antiproliferative and chemosensitizing effects, but these results have not been confirmed in *in vivo* experiments related to Ewing sarcoma even if combined with vincristine. It is suggested that hypoxia, present in solid tumors, may be the cause of the discrepancy between *in vitro* and *in vivo* effects. In fact, some evidence confirmed that hypoxia can prevent the antitumoral mechanism of action of metformin, that is, activation of AMPK and inhibition of mTOR signaling. Thus, the use of metformin for conventional chemotherapy may be limited to low hypoxic tumors. For this reason, the influence of hypoxia should be always considered when using novel therapies to treat these sarcomas in particular (67).

As frequently demonstrated, the development and progression of a tumor is linked to a large number of molecules and/or to a long sequence of events and modifications leading to the proliferation of cells. The tumor suppressor gene p53 and its family members p63/p73 are the main regulators of the cell cycle and critical determinants of tumorigenesis. ΔNp63 is a splice variant of p63, which lacks the N-terminal transactivation domain and acts as an antagonist of p53-, p63-, and p73- dependent translation, contrasting their tumor suppressor activity. Recent studies of pediatric neuroblastoma and osteosarcoma, demonstrated an increased expression of 1Np63, but it has not been possible to correlate ΔNp63 expression with p53 mutation status (70). For this reason, a possible mechanism leading ΔNp63 itself to favor the cells' malignant transformation with a gain of function independently by any p53 antagonism has been supposed. The overexpression of ΔNp63, independent of p53, increases the secretion of interleukin-6 (IL-6) and interleukin-8 (IL-8), leading to the elevated phosphorylation of STAT3 (Tyr-705). This condition leads to the stabilization of the HIF-1α protein, resulting in VEGF secretion. These data suggest that high levels of ΔNp63α are expressed in pediatric neuroblastoma and osteosarcoma, ΔNp63α has oncogenic effects in neuroblastoma and osteosarcoma, ΔNp63α regulates VEGF activity and promotes migration, induces STAT3 phosphorylation, and increases the transcription of interleukin (IL-6 and IL-8), and that ΔNp63 expression is enhanced in osteosarcoma lung metastases (70).

As reported for many kinds of tumor, hypoxia plays a primary role in the development, and progression of the pathology, influencing the specific microenvironment in which tumor cells live. One of the most dangerous but also uncommon tumors with <50 cases reported in literature (71, 72), with a poor cure of approximately 2 months (72) in infancy even with intensive therapy, is congenital glioblastoma (cGBM). The prognosis of this tumor is absolutely poor, but surgery and/or chemotherapy can increase long-term survival (24–33 months) (72) if the infants survive birth and initial surgery or biopsy. Histologically, they are very similar to other GBMs and present themselves with some typical features such as hypercellularity, high ability to infiltrate in the glia with necrosis and/or pseudopalisading necrosis, vascular proliferation, and increased mitotic activity and MIB-1 rate (72, 73). Following these observations, recently, it has been carried in a study on five patients with congenital GBMs in the first 3 months of life. Glioblastomas were situated in the deep gray matter close to the ventricles, and three of the patients had an extension of the tumor parenchyma into the intraventricular space. Four patients survived surgery, but the tumor was completely removed only in one of them. Cytogenetic studies on one patient demonstrated the presence of a diploid population; meanwhile, in two of the three patients, a diploid clone was observed in combination with aneuploidy in a near tetraploid context, while in the other, tetraploidy was seen in combination with diploidy. Furthermore, another interesting observation of this study is the difference in ploidy between this tumor and the small cell astrocytoma/GBM. However, FISH analysis revealed that this tumor type possesses disseminated diploid populations (74). Hierarchical clustering analysis in the three cGBMs has shown a similar gene expression also comparable to other high-grade adult and pediatric gliomas. Comparing cGBMs with pediatric and adult GBMs revealed that only 31 genes were differentially expressed. This can be considered to be, despite the different scale, very similar to the differences observed between pediatric GBMs and adult GBMs. Furthermore, in all three cGBM samples measured, EGFR gene expression was very low, and additionally amplification in EGFR was not observed, a result very similar to pediatric GBMs. Moreover, functional analysis showed that more that 50% of the differing genes had a role in signal transduction, including four receptor tyrosine kinases, and 39% of the differing genes in cGBMS, were involved in glucose metabolism. Thanks to these evidence, it has been possible to differentiate cGBMs from pediatric and adult GBMs. Furthermore, in some cases, cGBMs may have a better prognosis than pediatric or adult GBMs, however, this is strongly dependent on the ability of the infants to survive birth, tolerate surgery, and undergo chemotherapy. Patients who were exposed to surgery with subtotal resection or biopsy also did well, suggesting that it is not necessary to base the therapeutic approach on aggressive surgery probably inducing more morbidity in this population. Since these tumors are characterized by high vascularization and bleeding, patients with cGBMs show a good response to moderately intense chemotherapy regimens without requiring stem cell rescue or dose-dense chemotherapy to obtain a response. In conclusion, this study provides important information on a rare tumor. Unfortunately, such information is limited due to the small sample size, and for this reason, additional evidence is necessary to solidify both the molecular and chemotherapeutic results (75).

HIF–EZH2 CONNECTION

The correlation between HIF-1 and EZH2 is an interesting but yet uncharted aspect of tumor biology. There are few scientific studies connecting hypoxia, HIF-1, and EZH2 during tumor formation or tumor progression. The majority of such studies concord with the idea that hypoxia in general and HIF-1 in

particular increase EZH2 expression. In fact, in pancreatic cancer cells, hypoxia stabilizes HIF-1α that, in turn, increases TWIST expression. TWIST accumulation increases EZH2 expression and tumorigenesis measured as epithelial-mesenchimal transition (EMT) and xenograft growth (76). Similarly, in hepatocellular carcinoma (HCC), hypoxia and HIF-1α increases NIPP and EZH2 levels, an effect that then results in augmented invasive and metastatic potential (77). Interestingly, abrogation of NIPP and EZH2 expression, prevented such a malignant phenotype (77).

Another example regarding the correlation between HIF-1 and EZH2 has been revealed in a study conducted some years ago in which the expression of EZH2 in breast tumor initiating cells (BTICs) is enhanced by hypoxia through HIF1α-mediated transactivation, which promotes the expansion of BTICs and cancer progression (78, 79). The enhanced expression of EZH2 in BTICs determines the downregulation of DNA damage repair proteins and the accumulation of genomic abnormalities that mediate deregulated signaling (RAF1-ERK-β-catenin) resulting in BTIC expansion and cancer progression. Moreover, it has been revealed that the cancer-predisposed hypoxic microenvironment may promote BTICs through the upregulation of EZH2 expression. Furthermore, a specific genomic aberration mediated by EZH2-impaired DNA damage response has been linked to the expansion of BTICs and, finally, it has been possible to show a previously unidentified therapeutic effect of the inhibitors of RAF1-ERK signaling (e.g., AZD6244, a specific MEK/ERK inhibitor, already tested in multiple clinical trials) to prevent breast cancer progression by eliminating BTICs with important clinical implications (79).

Interestingly, in colorectal cancer, HIF activation is achieved through the overexpression of the type II Na/Pi co-transporter SLC34A2 causing ROS accumulation. In fact, increased ROS stabilize the HIF-1α protein inducing EZH2 overexpression. The silencing of SLC34A2 completely prevented EZH2 overexpression, as well as colorectal cell proliferation and chemo-resistance (80). Another mechanism through which HIF-1α can increase EZH2 expression has been demonstrated in prostate cancer cells. In these studies, authors show that HIF-1α activation during hypoxia is accompanied by a decrease in the expression of miR-101 and the increase of EZH2 (81). In fact, normally, expression of miR-101 inhibits EZH2 production and the invasiveness of prostate cancer cells (81). Upon HIF activation, however, miR-101 levels drop, thereby increasing EZH2 expression and the invasion of prostate tumor cells (81). However, the study of the HIF-EZH2 interplay is complicated by the observation that, in glioblastoma, EZH2 activates HIF-1α expression by inhibiting the eleven-nineteen lysine-rich leukemia associated factor 2 (EAF2) that, in the prostate, regulates transcriptional elongation of RNA Poll II (82). In fact, EAF2 binds and stabilizes VHL, thereby reducing HIF-1α levels. Therefore, by decreasing EAF2 expression, EZH2 reduces VHL activity stabilizing HIF-1α expression, which is an effect that, in turn, increases tumor growth and metabolism favoring glycolysis (82).

In addition, in multiple myeloma, EZH2 inactivation upon phosphorylation is observed during the acquisition of multi drug resistance. Interestingly, in this tumor, EZH2 inactivation is accompanied by increased expression of HIF-1α and anti-apoptotic genes, an effect that then causes multidrug resistance (83).

Finally, it has been demonstrated that, under hypoxia, HIF-1α regulates the switch from the tumor suppressive to the oncogenic function of EZH2. This interesting study tries, for the first time, to reconcile these two apparently opposing effects of EZH2 by showing that EZH2 has a tumor suppressive function when part of the PRC2 and a tumor promoting function when bound to Forkhead box M1 (FoxM1) by increasing matrix metalloproteinase expression and tumor cell invasion. Importantly, HIF-1α regulates the formation of these complexes by shifting EZH2 from PRC2 to FoxM1. Such an effect is achieved through an HIF-1α-mediated decrease in the expression of the PRC2 complex (84). In conclusion, even if scientific evidence correlates that HIF-1 and EZH2 are accumulating, still there is a need to obtain more additional data before we can completely elucidate and connect the mechanisms involving both HIF-1 and EZH2 in a tumor specific context.

SOMETHING FOR THE FUTURE

During recent years, a large number of scientific studies revealed the importance of therapeutic targeting of PRC2. Such a consideration emerged when it was observed that EZH2 overexpression or mutations increasing EZH2 activity could initiate, promote, or maintain oncogenesis. Some of the molecules that have been already developed such as the small-molecule inhibitors of PRC2 or EZH2 are actually under clinical evaluation for the treatment of some kinds of cancers including germinal-center B-cell lymphomas characterized by EZH2 mutations, which represent about 15–20% of all cases. PRC2, or more precisely its subunit EZH2, has been implicated in the development of various hematopoietic drugs. Furthermore, some drugs that have already been developed could be used to develop more efficient therapeutic approaches and personalized therapies.

Basing on this knowledge, it is important to consider the wide potential of these new generation of drugs not only for the actual but also for future therapeutic strategies. However, caution must be taken when planning to use EZH2 inhibitors and in interpreting the results. In fact, as recently demonstrated, our knowledge of the role of EZH2 as a tumor suppressor or tumor promoter is still too limited, and new players and interlinked pathways must be detailed and studied before we can provide a valid antitumor strategy that uses inhibitors of EZH2. Moreover, the use of such inhibitors will be context-specific according to the role of EZH2 in that specific tumor.

As reported in this review, HIF-1 plays a central role in tumor growth/progression. In fact, numerous *in vitro/in vivo* experiments have demonstrated an inhibition of tumor growth elicited by many HIF-1 inhibitors at the point to activate clinical trials for some of them. In particular, 2ME2 and other

derived-molecules are undergoing phase I and II clinical trials in patients with breast, prostate, and ovarian cancer (83, 84). Other drugs such as analogs of GA are employed to treat patients with VHL disease, breast cancer, etc. in phase II clinical trials (85, 86). In addition, bortezomib has been approved by the FDA for multiple myeloma treatment, based on the results from the phase II trials. Finally, EZN-2968 is currently in phase I clinical trials (85, 86).

As reported in this review and depicted in **Figure 3**, both EZH2 and HIF-1 play major roles in the regulation of cellular activities, and interference in their normal functions may alter the cellular homeostasis resulting in the development of different pathologies such as cancer. Even if little is known, new experiments that aim to unravel the connection between EZH2 and HIF may lay the groundwork for a better comprehension of the pathologic molecular mechanisms interlinking these two molecular players and will provide the possibility to develop, day by day, an increasing number of specific drugs to use in personalized therapies against cancer.

AUTHOR CONTRIBUTIONS

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

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MINI REVIEW
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Application of Small Epigenetic Modulators in Pediatric Medulloblastoma

Clemens Zwergel[1†], Annalisa Romanelli[1†], Giulia Stazi[1], Zein Mersini Besharat[2], Giuseppina Catanzaro[2], Marco Tafani[2], Sergio Valente[1] and Antonello Mai[1,3]*

[1] Department of Drug Chemistry and Technologies, Sapienza University of Rome, Rome, Italy, [2] Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy, [3] Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza University of Rome, Rome, Italy

Medulloblastoma is one of the most frequent among pediatric brain tumors, and it has been classified in various subgroups. Some of them already benefit from quite good therapeutic options, whereas others urgently need novel therapeutic approaches. Epigenetic modulators have long been studied in various types of cancer. Within this review, we summarize the main preclinical studies regarding epigenetic targets (such as HDAC, SIRT, BET, EZH2, G9a, LSD1, and DNMT) inhibitors in medulloblastoma. Furthermore, we shed light on the increasing number of applications of drug combinations as well as hybrid compounds involving epigenetic mechanisms. Nevertheless, in the studies published so far, mainly un-specific or old modulators have been used, and the PKs (brain permeability) have not been well-evaluated. Thus, these findings should be considered as a starting point for further improvement and not as a final result.

Keywords: brain cancer, medulloblastoma, epigenetic modulators, targeted therapy, innovative therapy concepts

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***Correspondence:**
Antonello Mai
antonello.mai@uniroma1.it

[†]These authors have contributed equally to this work

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INTRODUCTION

Medulloblastoma (MB) is one of the most frequent and extensively studied pediatric brain tumors. According to the WHO-classification of central nervous system tumors, four main genetically defined subgroups have been described: WNT, SHH, group 3, and group 4. Each of these groups has its unique expression signature and clinical outcome (1–4). Guerreiro Stucklin et al. recently well-summarized the differences in biological and clinical behavior between subgroups (3). Because of the heterogeneity of the various groups of MB, a targeted and efficient therapy, specifically for young patients, is very challenging (2). Epigenetic modulators have long been studied in various types of cancers, and some of them have been approved mainly for the treatment of hematological malignancies (5). These compounds are a particularly appealing therapy approach because they do not alter irreversibly the genetic code but act on reversible epigenetic marks, with a lower risk of side effects. In MB, a malignant brain tumor, the main challenge relies on the fact that whatever small molecule used as a therapeutic agent has to be able to cross the blood-brain barrier (6). The molecular epigenetic deregulation in MB has been recently reviewed, shedding light onto the pathways involved in the disease, on their biological importance as well as on the possible targets to hit (7, 8).

In this review we would like to highlight the latest preclinical and clinical efforts regarding the application of epigenetic modulators in MB. An overview of the presented compounds, their targets and effects in MB can be found in **Table 1**.

SINGLE EPIGENETIC MODULATORS IN MEDULLOBLASTOMA

Histone (De)acetylation Modifiers/Readers

HDAC Inhibitors (HDACi)

HDACi are among the oldest and deeply studied class of epigenetic modulators. In time, the most widely studied HDACi have not been isoform selective, but they were targeting more than one HDAC, specially class I and/or IIa/b HDACs. Nevertheless, selective isoform-specific modulators are more and more developed (9). Vorinostat, romidepsin, and belinostat are FDA-approved drugs to treat rare T-cell lymphomas by re-expressing silenced tumor suppressor genes. Currently, many preclinical studies are evaluating the effects of these inhibitors on MB (8). Surprisingly, relatively few clinical studies have been conducted with these validated inhibitors in MB, and most of them are closed or finished (10).

Older studies described valproic acid (VPA) as a potential treatment in various MB cell lines (11, 12), nevertheless this compound seems no longer under evaluation as newer studies cannot be found. Parthenolide, an HDAC1i, has been shown to be active in DAOY and D283med MB cancer stem cells, which aberrantly overexpress HDAC1 (13). In the same study also other HDACi, such as vorinostat, entinostat, or romidepsin, were tested resulting less active. However, the authors of this study noticed that very low concentrations of the HDACi resulted in an increase, rather than a decrease, in proliferative activity (13).

Recently, another well-known pan-HDACi, panobinostat, was reported to suppress leptomeningeal seeding, a rare complication in MB spreading, causing brain and spinal cord inflammation in a mouse model (14).

Milde et al. developed a Group 3 MB HD-MB03 cell line and xenograft model with high HDAC expression levels and sensitivity to HDACi, such as vorinostat and panobinostat (15).

A meaningful example of the involvement of HDACi in the SHH signaling pathway has been given by Canettieri et al. They showed that the HDAC1/2 selective inhibitors HDiA and HDiB blocked GLI1 and GLI2 activity through their acetylation, and SHH MB cell growth in several SHH MB cell lines (16). Despite these interesting results, no follow up studies have been published so far.

The natural compound curcumin, through HDAC inhibition and HDAC4 level depletion, reduced tumor growth and significantly increased survival in the Smo/Smo transgenic MB mouse model displaying HDAC4 overexpression. However, due to the pleiotropism displayed by curcumin, these positive results might not only be ascribed to HDAC4 inhibition but also to other off-target effects (17).

In 2015, Ecker et al. used the class IIa-selective HDACi MAZ1863 and MAZ1866 in Group 3 MB cancer cells and compared them to vorinostat (pan-HDACi) and to the class I specific inhibitor MS-275 (entinostat). MAZ1863 and MAZ1866 had only very weak effects on MYC-MB cells, whereas vorinostat and entinostat efficiently reduced the metabolic activity in MYC-MB cells. These results give precious hints on the development of novel therapies with selective HDACi in MYC- dependent MB (10).

Interestingly, when tested in the MED8A MB cell line, the novel non-toxic HDAC6/8/10 inhibitor TH34 modestly impaired colony growth and specifically induced caspase-dependent programmed cell death in a dose-dependent manner (18). TH34 warrants deeper evaluation and could be an interesting candidate for *in vivo* studies.

To sum up, the well-established and approved HDACi have so far failed to demonstrate a significant antitumoral effect in solid malignancies in preclinical and clinical settings (19), in contrast to leukemias and lymphomas. The failure of translating preclinical results into clinical success has been extensively discussed (20). Most likely, insufficient pharmacological study design regarding the clinical situation such as compound concentrations and their pharmacokinetic as well as dynamic properties are the primarily suspected factors (21).

SIRT Inhibitors

Sirtuins (SIRTs), also known as class III HDACs, are NAD^+ dependent deacetylases considered as a separate family of enzymes including seven different isoforms (hSIRT1-7). So far, there is very little literature evidence about the use of SIRT modulators in MB (22). In 2013, Ma et al. demonstrated that SIRT1 was overexpressed in human MB cells. In their work, they showed that lowered SIRT1 expression levels by siRNA or SIRT1 pharmacological inhibition with nicotinamide resulted in growth arrest and apoptosis in MB cells (23).

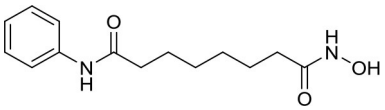
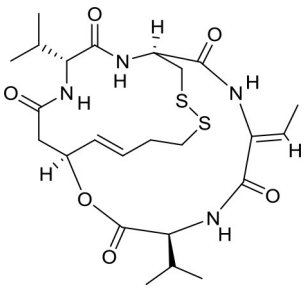
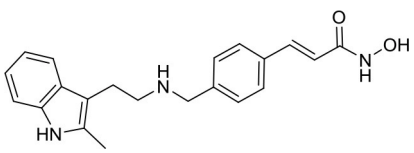
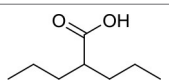
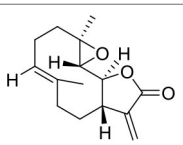
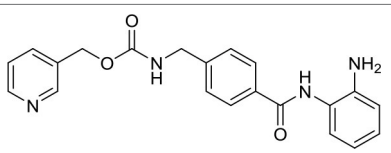
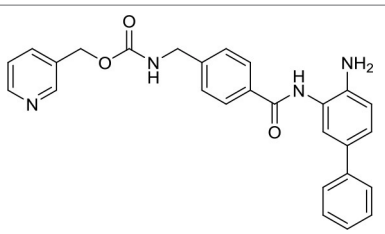
In contrast, Tiberi and coworkers found that the downregulation of the BLC6/BCOR/SIRT1 complex, a potent repressor of the SHH pathway, led to MB growth in human cells and in a mouse model. They demonstrated that SIRT1 is necessary for the BCL6 function (24), thus SIRT1 inhibition might be a double-edged sword in MB treatment. Therefore, researchers should proceed with caution for SIRT1 modulation in MB. The different results reported by these research groups well-summarize the problem of the context-dependent function of epigenetic targets (in this case SIRT1) in different experimental settings and MB subgroups.

BET Inhibitors (BETi)

The BET (Bromodomain and Extra-Terminal domain) proteins BRD2, BRD3, and BRD4, have been extensively studied in brain tumors including MB (25). These proteins are epigenetic readers as they recognize acetyl-lysine residues and acetylated chromatin, which usually mark active enhancers, thus they are important mediators of gene activation. High levels of H3K27Ac mark super-enhancers regulate key genes in cancer growth, and are sensitive to BET inhibition (26).

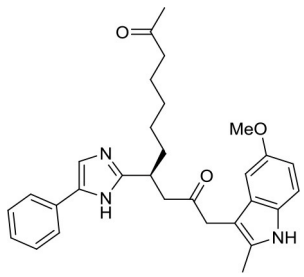
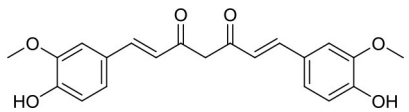
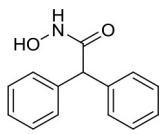
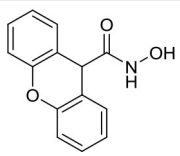
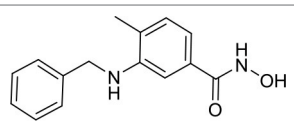
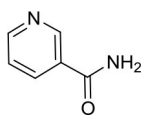
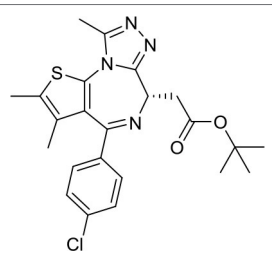
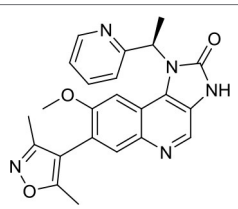
The BETi JQ-1 is one of the most studied in the literature. Tang et al. demonstrated that reduced expression of BRD4 via RNAi or its pharmacologic inhibition by JQ-1 resulted in decreased proliferation and tumor growth in SHH MB, reducing the expression of the glioma-associated oncogenes GLI1 and GLI2 (27). The same compound also led to positive results in Group 3 MB, as MYC-driven MBs are sensitive to BETi. Henssen et al. described JQ-1 to be active in a human Group 3 MB xenograft model via MYC downregulation, as it reduced tumor volume and

TABLE 1 | Summary of the epigenetic modulators and combinations active in MB.

Compound	Structure	Target	Results	Combination
Suberoylanilide-hydroxamic acid, vorinostat		HDACs	Active in DAOY and D283med MB cancer stem cells (13) Active in HD-MB03 cell line and xenograft model (15) Efficiently reduced the metabolic activity in MYC-MB cells (10).	Synergistic effect in D283med cells, but not in DAOY with decitabine (41). Newer study for both cell lines (13). Synergistic effects of VPA and vorinostat with irradiation in MB (42). The aurora kinase inhibitor MLN8237 had additive inhibition effects on MB group 3 cell lines (43)
Romidepsin		HDAC1/2	Active in DAOY and D283med MB cancer stem cells (13)	–
Panobinostat		HDACs	Suppressed leptomenigeal seeing in a MB mouse model (14) Active in HD-MB03 cell line and xenograft model (15)	–
Valproic acid, VPA		Class I/IIa HDACs	Potential treatment in various MB cell lines (11, 12)	Synergistic effects of VPA and vorinostat with irradiation in MB (42)
Parthenolide		HDAC1	Active in DAOY and D283med MB cancer stem cells (13)	–
MS-275, entinostat		Class I HDACs	Active in DAOY and D283med MB cancer stem cells (13) Efficiently reduced the metabolic activity in MYC-MB cells. (10)	–
HDiA		HDAC1/2	Block GLI1/2 activities and SHH MB growth (16)	–

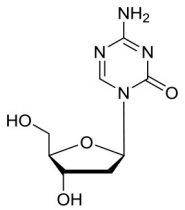
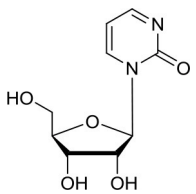
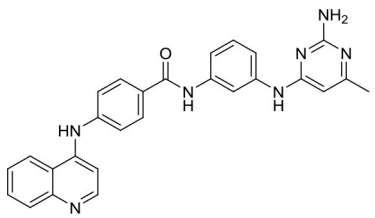
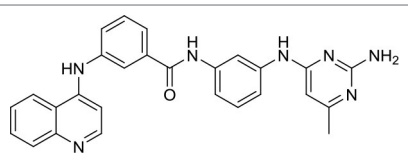
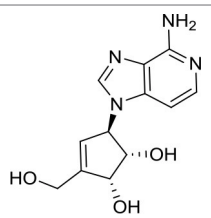
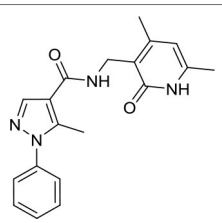
(Continued)

TABLE 1 | Continued

Compound	Structure	Target	Results	Combination
HDiB		HDAC1/2	Block GLI1/2 activities and SHH MB growth (16)	–
Curcurmin		HDACs	Increased survival in the Smo/Smo transgenic MB mouse model (17)	–
MAZ1863		Class IIa HDACs	Only very weak effects on MYC-MB cells (10)	–
MAZ1866		Class IIa HDACs	Only very weak effects on MYC-MB cells (10)	–
TH34		HDAC 6/8/10	Induced caspase-dependent programmed cell death in various MB cell lines (18)	–
Nicotinamide		SIRTs	SIRT1 inhibition might be a double edge sword in MB treatment (24)	–
JQ-1		BETs	Decreased proliferation and tumor growth in SHH MB via reducing the expression of. GLI1 and GLI2 (27) Active in a human group 3 MB xenograft model via MYC downregulation (28–30)	Effective combination with the CDK inhibitor milciclib, as both regulate the MYC function in MB via different actions, prolonging survival in a MB animal model (32)
I-BET151		BETs	Inhibition of the SHH pathway in SHH-MB cells as well as in a MB mouse model (31)	–

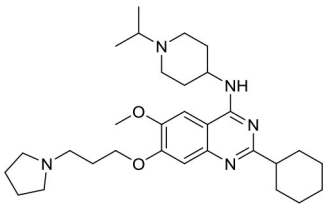
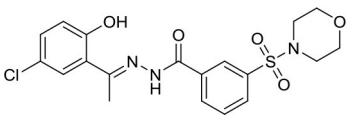
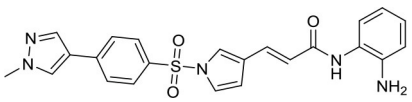
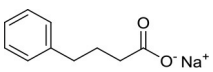
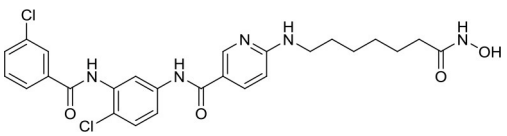
(Continued)

TABLE 1 | Continued

Compound	Structure	Target	Results	Combination
Decitabine		DNMTs	Found to be quite inactive in DAOY (13, 38) and UW228 MB cells (38) as well, differently D283med cells were quite sensitive (13)	Synergistic effect in D283med cells, but not in DAOY with decitabine (41). Newer study for both cell lines (13). Triple combination of decitabine/irradiation and abacavir turned out to work effectively in various MB cell lines (41) Phenylbutyrate in combination with decitabine and the tyrosine kinase inhibitor Gleevec induced apoptosis in DAOY and UW228 3 MB cell lines (38).
Zebularine		DNMTs	Inhibits the expression of SHH pathway components, such as SMO and GLI1, in DAOY and ONS-76 MB (39)	DNMTi zebularine has been tested in combination with vincristine in SHH MB cells, displaying a synergistic effect (39)
MC2840 (compound 2)		DNMTs	Impaired MB-SC growth led to high MB-SC differentiation rates (40)	–
MC3343 (compound 5)		DNMTs	Significantly impaired the MB-SC growth rate (40)	–
3-Deazaneplanocin A, DZNep		EZH2	Indirect and rather unspecific EZH2i in MB (33)	–
MC3629		EZH2	Reduces in a MB xenografted mice the tumor volume, stemness and cell proliferation and lastly induces apoptosis (34)	–

(Continued)

TABLE 1 | Continued

Compound	Structure	Target	Results	Combination
UNC0638		G9a	Reduces DAOY proliferation via controlling the USP37 expression mediated by G9a (35)	–
SP2509		LSD1	Disruptor of the CoREST–LSD1 complex active in various MB cell lines (36)	–
4SC-202		HDAC1/2/3 and LSD1	–	Active in various MB cell lines (36)
Sodium Phenylbutyrate		HDACs	–	Phenylbutyrate in combination with decitabine and the tyrosine kinase inhibitor imatinib induced apoptosis in DAOY and UW228 3 MB cell lines (38)
NL-103		HDAC/HH	–	NL-103 is a dual inhibitor of the HDACs and HH pathway with potential activity in MB (44)

prolonged survival rates (28). Similar results, corroborating the potential of JQ-1 in downregulating MYC expression, have been obtained by two other independent research groups (29, 30). Furthermore, JQ-1 has been demonstrated to block stem cell-associated signaling and was able to induce cell senescence in a MYC-MB cellular model as well as in xenograft mice (30).

Another BETi, namely I-BET151, has been shown to provide biological effects similar to JQ-1 in SHH MB. More precisely, this compound reduced the BRD4 binding to the GLI1 gene locus, thus resulting in the inhibition of the SHH pathway in SHH MB cells as well as in a MB mouse model (31).

Currently, JQ-1 is not in clinical trials for MB treatment due to its poor pharmacokinetic and pharmacodynamic properties (32). It is quite surprising that other BETi similar to JQ-1, such as RG6146 (TEN-010) or OTX105, which are currently evaluated in clinical trials for other cancer types [ClinicalTrials.gov NCT01987362, NCT02259114], have never been tested in MB even in preclinical studies. Nevertheless, BETi represent a promising strategy to follow the development of novel MB therapies.

Histone (De)methylation Modifiers

EZH2 Inhibitors (EZH2i)

Enhancer of zeste homolog 2 (EZH2) is a histone lysine *N*-methyltransferase involved in the PRC2 (Polycomb Repressive Complex 2), which has been widely studied in cancer including MB. One of the first published studies on MB used the rather toxic DZNep, an inhibitor of S-adenosyl-L-homocysteine hydrolase, as an indirect and quite unspecific EZH2i (33). Recently, our research group published the pyrazole compound MC3629 as a simplified analog of the two different SAM-competitive EZH2i EPZ005687 and GSK2816126. This particular compound was not only active in human SHH MB cancer cell models, where it significantly impaired H3K27me3 and PCNA protein levels leading to apoptosis, detected as an increased level of cleaved caspase 3, but also, to our knowledge for the first time, in a SHH MB murine model. Importantly, MC3629 better penetrated the blood-brain barrier *in vitro* and *in vivo,* when compared to the parent compound GSK2816126. This might explain at least in part why MC3629, despite its lower *in vitro* potency, efficiently reduced H3K27me3 levels in brain and cerebellum of MB xenografted mice

leading to decreased tumor volume, reduced stemness and cell proliferation ability, and, lastly, induction of apoptosis (34). These encouraging results confirm the importance of EZH2 in MB.

G9a Inhibitors

The deubiquitylase USP37 was identified as a target of REST, one of the main regulatory complexes in brain development and neurogenesis with aberrant overexpression in MB (5). Dobson et al. showed that the downregulated USP37 in human MB could be re-expressed after G9a inhibition. In more details, G9a catalyzes mono- and di-methylation of histone H3K9, and its histone methyltransferase activity correlated with gene repression of USP37 in MB. The USP37 promoter in MB possesses a significant level of histone H3K9 trimethylation, which was considerably diminished upon treatment of the DAOY cells with the G9a inhibitor UNC0638. This has been the first and unique pivotal study highlighting the importance of G9a inhibition, leading to arrest of MB cell proliferation via control of the USP37 expression (35). However, this is only the first step toward a G9a-based MB treatment, as this target needs to be further validated not only in other MB models but also in opportune *in vivo* studies.

LSD1 Inhibitors (LSD1i)

Lysine-specific demethylase 1 (LSD1), also known as KDM1A, has been the first of several protein lysine demethylases to be discovered. The modulation of this enzyme has also been studied in MB. Recently, it has been shown that SP2509 inhibited the enzymatic activity of LSD1 rather than acting as a protein-protein disruptor of the CoREST–LSD1 complex. SP2509 was able to block the growth of various human MB cell lines (DAOY, D283med, and ONS-76) through direct LSD1 inhibition (36). This study has a pivotal role since it could be used as a starting point for deeper mechanistic studies as well as for a novel therapeutic approach in MB.

DNMT Inhibitors (DNMTi)

DNA methyltransferases (DNMTs) are a family of enzymes that catalyze the transfer of a methyl group to the C5-cytosine residue of DNA. Aberration of DNA methylation leads to a wide variety of diseases, including cancer. DNMTi are one of the most studied epigenetic modulators after HDACi in cancer. The nucleoside analogs azacytidine and decitabine have been approved by FDA mainly in hematological malignancies (37). These compounds inhibit DNMTs after being incorporated into the DNA, leading to reduced methylation levels often resulting in enhanced tumor suppressor gene expression and finally in increased apoptosis (37). Decitabine was found to be quite inactive in DAOY (13, 38) and UW228 MB cells (38); differently, D283med cells seemed to be quite sensitive to the treatment with this inhibitor (13). Another nucleoside inhibitor, zebularine, inhibits the expression of SHH pathway components, such as SMO and GLI1 in DAOY and ONS-76 MB cell lines, leading to inhibition of their proliferation and to increase of apoptosis rates (39). The main problems of these nucleoside analogs are their poor chemical stability and high toxicity (37). Relatively few non-nucleoside inhibitors are known to date. One of them, developed in our research group has also been tested in MB. Compound 5 and compound 2, both structural isomer of the SGI-1027, have been tested for the first time as non-nucleoside inhibitors in mouse MB stem cells (MB-SC), expressing high levels of DNMT1. Compound 5 arrested the cell clonogenic activity impairing MB-SC growth rate, evaluated by quantification of PCNA levels, and induced cell adhesion and differentiation, evaluated by βIII-tubulin. In these assays, compound 5 displayed the highest growth arrest, while compound 2 induced higher differentiation already after treatment with lower doses (40). Both compounds are interesting tools for further *in vivo* validation, but also a starting point for further drug development.

COMBINATIONS CONTAINING AT LEAST ONE EPIGENETIC MODULATOR AND HYBRID COMPOUNDS

Chemoresistance is one of the key reasons why drug combinations are applied in therapy. Targeting a disease by just one active principle often results in drug resistance. This problem might be overcome by using two different drugs that target two different molecular pathways involved in the same disease. In MB, this strategy has also been used to various epigenetic modulators in combination with other molecules either targeting epigenetic pathways or non-epigenetic ones. Furthermore, we shed light on novel, innovative hybrid compounds targeting at least one epigenetic molecule as following.

Combinations of Two (or More) Epigenetic Modulators

Patties et al. published in a first study the effects of combination of several epigenetic modulators, such as the DNMTi decitabine, VPA and vorinostat as HDACi, in MB and later they extended the previous study with the use of irradiation, a common physical therapy approach to fight various cancers (41).

They discovered that the treatment of D283med cells with vorinostat and decitabine produced a synergistic effect in reducing tumor cell viability, whereas the exposure of DAOY cells to the same compounds did not have a synergistic effect (41). However, a more recent study by Yuan et al. resulted in a synergistic effect in both cell lines (13). This example shows that the precise assay conditions as well as concentrations of the drugs, are crucial for the outcome of a study. The last researchers also tested parthenolide in combination with decitabine obtaining a synergistic effect (13). Despite the numerous evidences of synergism by HDACi and DNMTi co-treatment, the precise mechanism of their interplay still needs to be elucidated (13, 41).

Interestingly, also the combination of VPA or vorinostat with irradiation showed similar effects compared to decitabine/irradiation treatments on the mentioned cell lines, even though to a lower extent (42). The latter most powerful combination deserves *in vivo* validation. Also, the combination experiments without irradiation might provide a promising

alternative therapeutic strategy, lowering the possibility of resistance.

Combinations of Epigenetic and Non-Epigenetic Modulators

Patties et al. did not only evaluate the combination of several epigenetic modulators, but also combined them with abacavir, a nucleoside analog HIV reverse transcriptase inhibitor, with or without irradiation (41, 42). Abacavir is not only known as an approved drug for HIV-treatment, but possesses also potent anti-cancer effects due to its ability to inhibit the telomerase activity, often overexpressed in several cancers (41). The triple combination of decitabine, abacavir, and irradiation turned out to work effectively in all three tested cell lines (DAOY, MEB-Med8a, D283med), warranting further *in vivo* investigations (41). Vorinostat has also been tested in association with the aurora kinase inhibitor MLN8237, leading to proliferation arrest in Group 3 MB cell lines (43).

Marino et al. evaluated the pan-HDAC inhibitor 4-phenylbutyrate in combination with the DNMTi decitabine and the tyrosine kinase inhibitor imatinib. The co-treatment reduced global methylation and induced apoptosis in DAOY and UW228 3 MB cell lines (38).

The DNMTi zebularine has been tested in combination with the well-known anti-cancer agent vincristine, able to interact with microtubules and tubulin, in SHH MB cells, displaying a synergistic effect (39). All the aforementioned studies are combining approved drugs or compounds which have already been extensively studied in the preclinical and clinical stage for other malignancies.

However, also newer chemical entity combinations have been tested in MB. As reported earlier, MYC is an important player in Group 3 and Group 4 MB. JQ-1 is influencing this pathway via BET inhibition and has been combined with the CDK inhibitor milciclib, because CDKs regulate events in MYC function as well. This combination was well-tolerated, reduced tumor cell growth, and significantly prolonged survival in MB animal model (32). In the future, the combination between BETi and CDKi could be further evaluated using in combination with milciclib, already in clinical trials [NCT01011439, NCT01301391], a BETi more drug-like than JQ-1.

However, for the treatment of MB all the aforementioned combinations are still in their early stage and need to be carefully evaluated before proceeding to the clinical area.

Hybrid Compounds

Hybrid compounds are single chemical entities hitting more than one target. Some of these innovative compounds have been also tested in MB. Inui et al. evaluated the dual HDAC1/2/3 and LSD1 inhibitor 4SC-202 in various MB cell lines (DAOY, D283med, and ONS-76). This compound proved to be active targeting both enzymes in the CoREST-HDAC-LSD1 complex. This study is one of the first examples using dual epigenetic inhibitors in MB (36). 4SC-202 deserves a deeper study regarding its detailed mechanism of action as well as further evaluation as a novel innovative therapy weapon.

NL-103, a dual inhibitor of the HDACs and SHH pathway, shows a hybrid structure merging those of vismodegib, a smoothened receptor (SMO) inhibitor approved by FDA for other solid cancers, and vorinostat, which is known to target the SHH pathway by influencing the acetylation status of GLI1 and GLI2 (16). This innovative compound, with its unique dual-targeted activities, was able to inhibit SHH signaling pathway acting on two different targets, in the oncogene fibroblast model cell line NIH-3T3, where it was more effective than the treatment with single targeting compounds. Thus, in this study such a hybrid was proposed as an attractive candidate to be tested in HH-sensitive MB, however it remains still elusive since no further studies have been published so far (44).

CONCLUSIONS AND PERSPECTIVES

In this review, we have summarized and highlighted epigenetic modulators as promising drug targets in MB. However, there is still a long way to go: mainly not very specific, or older modulators have been used and often the brain permeability has not been well-evaluated. The molecular genetics and detailed epigenetic modulation of the various MB subgroups need to be further studied. As the MB subgroup is a key factor in choosing the right treatment, the development of personalized medicine with highly specific modulators could be a key in improving the poor survival rates of some MB subgroups. Therefore, research should not only focus on the design of more specific and selective epigenetic modulators, but also should study deeper the more biologically oriented factors, such as the tumor molecular genetics, the functional analysis of epigenetic factors and their potential modulation. In both cases, epigenetic modulators can be useful not only as tools to better understand the molecular mechanisms in MB, but also as novel potential drugs for innovative personalized treatments.

AUTHOR CONTRIBUTIONS

The literature research was conducted by all authors and the manuscript was written thereafter by the contribution of all authors. AM read, corrected, and supervised and coordinated all the work. All contributors read and approved the manuscript.

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Long Noncoding RNAs: Emerging Players in Medulloblastoma

Pietro Laneve[1], *Jessica Rea*[2] *and Elisa Caffarelli*[1]*

[1] Institute of Molecular Biology and Pathology, National Research Council, Rome, Italy, [2] Department of Biology and Biotechnology, Sapienza University of Rome, Rome, Italy

Central Nervous System tumors are the leading cause of cancer-related death in children, and medulloblastoma has the highest incidence rate. The current therapies achieve a 5-year survival rate of 50–80%, but often inflict severe secondary effects demanding the urgent development of novel, effective, and less toxic therapeutic strategies. Historically identified on a histopathological basis, medulloblastoma was later classified into four major subgroups—namely WNT, SHH, Group 3, and Group 4—each characterized by distinct transcriptional profiles, copy-number aberrations, somatic mutations, and clinical outcomes. Additional complexity was recently provided by integrating gene- and non-gene-based data, which indicates that each subclass can be further subdivided into specific subtypes. These deeper classifications, while getting over the typical tumor heterogeneity, indicate that different forms of medulloblastoma hold different molecular drivers that can be successfully exploited for a greater diagnostic accuracy and for the development of novel, targeted treatments. Long noncoding RNAs are transcripts that lack coding potential and play relevant roles as regulators of gene expression in mammalian differentiation and developmental processes. Their cell type- and tissue-specificity, higher than mRNAs, make them more informative about cell- type identity than protein-coding genes. Remarkably, about 40% of long noncoding RNAs are expressed in the brain and their aberrant expression has been linked to neuro-oncological disorders. However, while their involvement in gliomas and neuroblastomas has been extensively studied, their role in medulloblastoma is still poorly explored. Here, we present an overview of current knowledge regarding the function played by long noncoding RNAs in medulloblastoma biology.

Keywords: nervous system, pediatric tumor, medulloblastoma, long noncoding RNAs, oncogenes, tumor suppressors, diagnostic biomarkers, therapeutic targets

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Andrea Carai,
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Anshu Malhotra,
Emory University, United States
Francesca Del Bufalo,
Bambino Gesù Children Hospital (IRCCS), Italy

***Correspondence:**
Elisa Caffarelli
elisa.caffarelli@uniroma1.it

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INTRODUCTION

Medulloblastoma (MB), with an estimated 5000–8000 cases/year worldwide (1, 2), is an aggressive tumor arising in the cerebellum. It mainly affects children and is a major cause of mortality in pediatric oncology (3). While the previous classification of MB by the World Health Organization (WHO) was largely based on histological features (4), the new classification in 2016 exploited molecular parameters to catalog the large variety of tumors of the Central Nervous System (CNS) (5). Rational molecular-based classification was supported by the advancement of sequencing technologies allowing extensive genomic/transcriptomic studies. This classification benefits from the integration between histological and molecular parameters and led to no longer considering MB as a unique pathology.

Several subclasses of MB have been unveiled, each displaying dysregulated genes—the driver genes—altered by single nucleotide mutations, somatic copy-number aberrations, or by defects in transcriptional or post-transcriptional gene regulation.

In this review, we highlight the link between MB tumors and the emerging class of regulatory long noncoding RNAs (lncRNAs), and their potential as promising cancer biomarkers and novel therapeutic agents.

Medulloblastoma

Recent genomic and transcriptomic analyses on a large cohort of primary tumors assigned MBs to four molecularly distinct subgroups (6, 7). They include the extensively characterized WNT and SHH subgroups, and the Group 3 (G3) and Group 4 (G4), whose pathogenesis and signaling pathways are poorly defined.

WNT

Approximately 10% of all MB patients belong to this subgroup, characterized by the most favorable prognosis with 95% of survival (7, 8). WNT tumors, which exhibit classic histology, are recognizable by a WNT gene expression signature. Nuclear accumulation of β-catenin is considered a biomarker for WNT signaling pathway activation. This subgroup often carries heterozygous *TP53* mutations, as well as mutations in the DEAD-box helicase gene *DDX3X* and in chromatin modifiers genes, such as *SMARCA4* and *CREBBP*, indicating the implication of altered epigenome in the development of this disease. Integration of gene expression and DNA methylation profiles indicated that WNT subgroup comprises at least two subtypes, WNTα, mainly enriched for children and characterized by monosomy 6, and WNTβ, including mainly adults without monosomy 6 (9).

SHH

SHH MBs represent approximately 30% of all MB cases, characterized by an intermediate prognosis, with survival rates ranging from 60 to 80% (7). SHH tumors, mainly exhibiting desmoplastic histology, display an aberrant activation of the SHH signaling, due to mutations of negative regulators of SHH pathway, such as *PTCH1* and *SUFU,* and copy number aberrations of SHH target genes, such as *MYCN* and *GLI2* (7). *TP53* mutations are found in about 30% of childhood SHH MBs and are associated with extremely poor outcomes. Recent analyses suggest that SHH subgroup consists of four distinct subtypes. It includes SHHα, enriched for *MYCN* and *GLI2* amplifications, with the worst prognosis; SHHβ, harboring *PTEN* gene deletions and frequently metastatic; SHHγ displaying scarce copy number aberrations and SHHδ, that is enriched for *TERT* gene promoter mutations and has a favorable prognosis (9).

Group 3

G3 is the most aggressive subgroup accounting for about 25% of all MBs, about half of them being metastatic at diagnosis (10). These tumors display a MYC signature, being characterized by amplification of the *MYC* proto-oncogene and exhibiting aberrant *MYC* expression in almost all cases (7). G3 shows intra-tumoral heterogeneity, including three further subtypes: G3α and G3β with a more favorable prognosis compared to G3γ, which frequently harbors increased *MYC* copy number (9).

Group 4

G4, the most common subtype, accounts for 35% of all MBs. These tumors are often metastatic at diagnosis and have intermediate prognosis. It is the most enigmatic subgroup, characterized by a neuronal gene expression signature, resembling that of glutamatergic neurons (7). Common alterations pertain to inactivating mutations in *KDM6A* gene, duplication of *SNCAIP* gene, and amplification of *MYCN* and *CDK6* proto-oncogenes. G4 has been re-classified into G4α, characterized by *MYCN* and *CDK6* amplifications, G4β, strongly enriched for *SNCAIP* duplications and putative *PRDM6* overexpression, and G4γ enriched for focal *CDK6* amplification (9).

LONG NONCODING RNAS

RNA is considered as the most "rediscovered" biological macromolecule (11, 12) since, starting from the informational role assigned to mRNAs in 1961 (13, 14), novel unexpected functions have been attributed to RNA in the last three decades. In the 1980s, its capacity to catalyze biochemical reactions was associated with its ability to fold into complex tridimensional structures (15). In the early 1990s, regulatory functions were attributed to two long RNAs lacking protein-coding capacity, *H19* (16, 17), and *XIST* (18, 19). Since then, a huge number of noncoding RNAs, both short and long in size, was discovered in parallel with the finding that more than half of the transcriptome encodes non-proteinogenic transcripts. Among them, the lncRNAs number in the tens of thousands and include also circular RNAs, covalently closed RNA circles derived from back-splicing of linear transcripts (20). LncRNAs are >200 nucleotides and represent very versatile molecules for their unique ability to specifically recognize both nucleic acids and protein partners via base-pairing and modular tridimensional structures, respectively. They are flexibly involved in important biological processes, such as development, cell differentiation and growth, thanks to their main functions of gene expression regulators and the genome structure architects.

Mechanisms of Action

LncRNAs may be engaged in fine-scale modulation of gene expression as well as in large-scale control of developmental programs. They may act through a variety of mechanisms, depending on their cellular localization. Some of them are exclusively localized in the nucleus, others in the cytoplasm, others change their localization during development or differentiation, and still others show both localizations. In the latter case, a single lncRNA might have multiple molecular functions.

Nuclear lncRNAs

Nuclear lncRNAs (**Figure 1A**) can be found in the nucleoplasm or associated with chromatin (21). Typically, these latter are supposed to control protein-coding gene expression at the

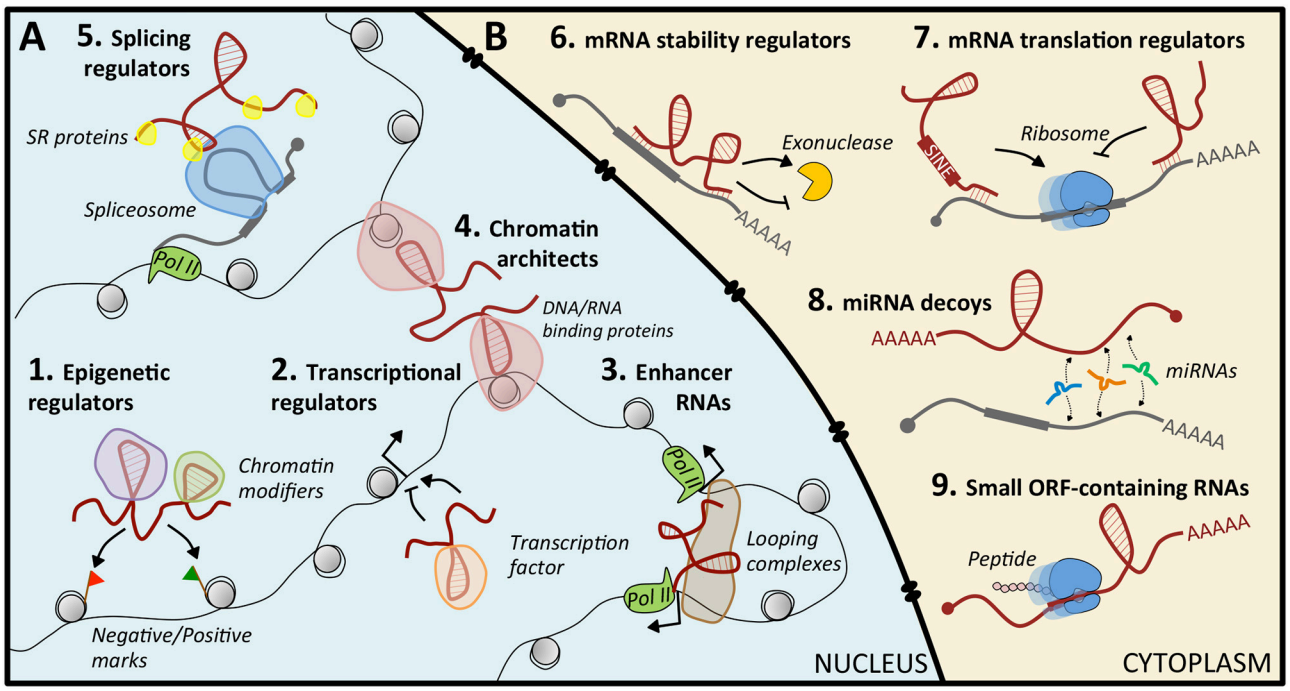

FIGURE 1 | Mechanisms of action of lncRNAs. **(A)** In the nucleus, lncRNAs can regulate gene expression by guiding epigenetic (1) or transcription (2) factors to specific loci, by acting as enhancers (3), by structuring the three-dimensional conformation of chromatin (4), or by recruiting splicing factors (5). **(B)** In the cytoplasm, they act by modulating mRNA stability (6) or translation (7), by sequestering miRNAs (8), or by coding small peptides (9).

epigenetic level by recruiting chromatin modifiers to specific genomic loci. This is achieved through their scaffolding activity, by which they interact simultaneously with distinct protein complexes, and through their capability to act as "molecular guides," that ensure the specificity of target recognition (21). This function can be carried out in *cis* or in *trans*. The *cis*-acting RNAs are typically low-abundant, and regulated genes are located in the proximity of their transcription site; *trans*-acting RNAs are more abundant and can modulate the expression of genes at independent loci (21). Notably, perturbations of the epigenetic regulation were recognized as causative of malignancies (22), and some cancer-related lncRNAs, such as *XIST* (23), *HOTAIR* (24–26), *NBAT* (27), and *LINC-PINT* (28), were reported to direct epigenetic modifications (29).

Nuclear lncRNAs can also act as regulators of transcriptional programs, by recruiting transcription activators or repressors to specific loci (30, 31), as enhancer RNAs that exert enhancer-like functions (32, 33), as chromosome architects and nuclear organizers that contribute to the formation of specific sub-nuclear structures (21, 34, 35), or as regulators of alternative splicing (36).

Cytoplasmic lncRNAs

Cytoplasmic lncRNAs (**Figure 1B**) regulate gene expression at the post-transcriptional level, often exploiting their sequence complementarity with transcripts deriving from the same genomic locus or from independent loci. Upon specific target recognition, they are able to modulate mRNA stability, both positively as *BACE1-AS* (37) and *TINCR* (38), and negatively as *½-sbsRNAs* (39), or translation, as *lincRNA p21* (40). Another role is that of decoys for microRNAs (miRNAs): in this case, the lncRNA functions as a competing endogenous RNA (ceRNA) that sequesters miRNAs from their mRNA targets, causing translational de-repression. This activity is based on regulatory crosstalk between multiple transcripts (41, 42). Notably, lncRNA-mediated ceRNA networks in cancer are continuously emerging (43, 44). However, only for a very limited number, such as *Gas5* (45), *linc-RoR* (46, 47), *NORAD* (48), and *linc-NeD125* (49), this function has been characterized: their aberrant enrichment or local increased concentration in pathological conditions can culminate in tumorigenesis. Finally, some lncRNAs may contain short open reading frames producing small, functional peptides (50).

THE ROLE OF LNCRNAS IN CNS

The CNS of mammals is a very sophisticated system in which neuronal and glial cells structurally and functionally interact to guarantee the proper brain activity. Numerous evidence correlates the evolutionary increase in human brain complexity with the expanding number of lncRNAs (51, 52). Accordingly, 40% of human annotated lncRNAs are expressed in the brain, where they display neuro-anatomical and/or cell-type specific expression, and about 30% of lncRNAs appears to be primate-specific (31, 53). Notably, compared to lncRNAs from other tissues, the brain-specific lncRNAs are: (i) the most evolutionarily

conserved species, (ii) predicted to retain conserved secondary structures, and (iii) preferentially adjacent to protein-coding genes involved in neuronal differentiation and function (54). Overall, these findings indicate that brain-specific lncRNAs likely possess conserved functions and are crucially implicated in higher-order cognitive abilities as well as in establishing neural cell-type diversity and function. This hypothesis is sustained by their spatiotemporal expression, which is exquisitely regulated during NS development (55) and in response to neuronal activity (56). So far, a growing body of literature shows that lncRNAs influence every step of neurodevelopment, from early stages of differentiation to synaptogenesis (57–59). *In vitro* studies revealed some lncRNAs, such as *RMST* (60), *TUNA* (61), *DALI* (62), and *PAUPAR* (63), that control complex gene expression programs underlying the neurogenic commitment of pluripotent embryonic stem cells. This is mainly achieved through their action of "guide" RNAs that convey transcriptional and/or epigenetic factors on the promoters of neuronal genes. *In vivo* analyses identified other species such as *GOMAFU* (56, 64, 65), *EVF2* (66), *PNKY* (67), and *linc-BRN1B* (68) that, through the recruitment of epigenetic, transcriptional, or splicing factors, govern the balance between self-renewal and neuronal differentiation. LncRNAs also contribute to synaptogenesis and neuronal plasticity, which underlies learning, memory, and cognition, by regulating crucial proteins that control neurite elaboration (69), translation in synapses (70, 71), and ion channel subunits (72).

LNCRNAS IN NEURO-ONCOLOGICAL DISORDERS

Based on their crucial role in NS development and function, lncRNA qualitative and/or quantitative alterations may profoundly impact on different neurological pathologies, including neurodevelopmental, neurodegenerative, neuro-immunological, and neuro-oncological disorders (73, 74). In the latter settings, lncRNAs have drawn extensive attention as molecules that may drive tumorigenesis. In addition, they can serve as predictors of cancer sub-types as well as potential therapeutic targets.

It is widely understood that mutations, epigenetic alterations or somatic copy number aberrations in the noncoding portion of the genome underlie cancer pathology (75). Accordingly, recent studies indicated that lncRNAs are highly deregulated in cancer, where they participate as tumor-suppressors or oncogenes in tumor initiation and progression. Notably, most lncRNAs displaying aberrant expression are cancer-type unique (76). However, despite the identification of a large number of lncRNAs in neurological cancers, only for a few of them mechanisms of action have been experimentally clarified.

Extensive studies have been carried out in gliomas, the most prevalent types of primary intracranial carcinoma (77). Several lncRNAs associated with glioma stemness (78–80), proliferation, and migration (81–84) have been identified, and most of them function as miRNA decoys (81).

Neuroblastoma (NB) is a pediatric tumor of the sympathetic NS, accounting for more than 7% of childhood malignancies (85). The molecular link between deregulated lncRNA expression and NB tumorigenic features is emerging (86), and several deregulated lncRNAs during NB pathogenesis have been uncovered (87–92).

Our knowledge of lncRNA function in MB physiopathology is still fragmentary. Genome-wide association studies may help to understand how genetic polymorphisms in lncRNA loci contribute to MB predisposition (93). Furthermore, in spite of the numerous high-throughput expression studies carried out so far, lncRNAs have been largely disregarded. However, re-annotation of array-based data and integration of cancer phenotype associations allowed prioritizing disease-related lncRNAs in tumors, including MB (94), demonstrating the potential of data re-analyses. In another study, a *de-novo* genome-wide inspection of MB subgroup-specific chromosomal alterations identified the first G3 MB gene fusions (6). They involve the 5′-end of *PVT1*, a lncRNA hosting the putative MB oncogene miR-1024 (95, 96). In the *PVT1-MYC* fusion, the induction of miR-1024 and the associated malignant phenotype may be explained through an oncogenic positive feedback-loop, established by MYC on its response elements on PVT1 promoter (6).

Other studies focused on the role played in MB by previously identified noncoding oncogenes. Among them, *UCA1* (97) and *CRNDE* (98, 99) are upregulated in MB samples. *UCA1* knockdown in MB cells results in the arrest of cell cycle progression, suppression of cell migration, and proliferation (100). Similarly, *in vitro* downregulation of *CRNDE* blocked cell cycle, inhibited proliferation and aggregation, while increasing apoptosis. Tumor growth was also reduced in MB mouse models silenced for CRNDE (101). Inversely, the lncRNA *HOTAIR* (102) is downregulated in MB samples, whereas its target genes *HOXD8* and *HOXD10* are upregulated (103). The misbalance of these crucial developmental genes may partially account for the embryonic origin and the pediatric onset of MB. However, their mechanisms of action are presently unknown.

Mechanistic insights into the role of lncRNAs in MB biology have been carried out only for a very few species, as discussed below.

Mechanisms of Action of lncRNAs in Mb

The colon cancer upregulated transcript *CCAT1* (104) is a prototype of oncogenic lncRNA, associated with several carcinomas, where it promotes cell proliferation, invasion, migration, and chemoresistence (105–107). In MB, its expression is upregulated in 20 unstratified tumor samples and also in at least four MB cell lines (108). *CCAT1* knockdown in MB cells causes the decrease of cell proliferation rate, (depending on *CCNA* and *CDK2* gene repression), cell migration, and invasion. Its *in vivo* depletion reduces the volume of subcutaneous tumors of xenotransplanted mice (108). *CCAT1* has been proposed to play its oncogenic role by altering the phosphorylated, active status of components of the tumorigenic MAPK pathway. In combination with previous reports indicating *CCAT1* as a miRNA sponge (109–111), this study suggests that *CCAT1* may control tumorigenesis through multiple activities.

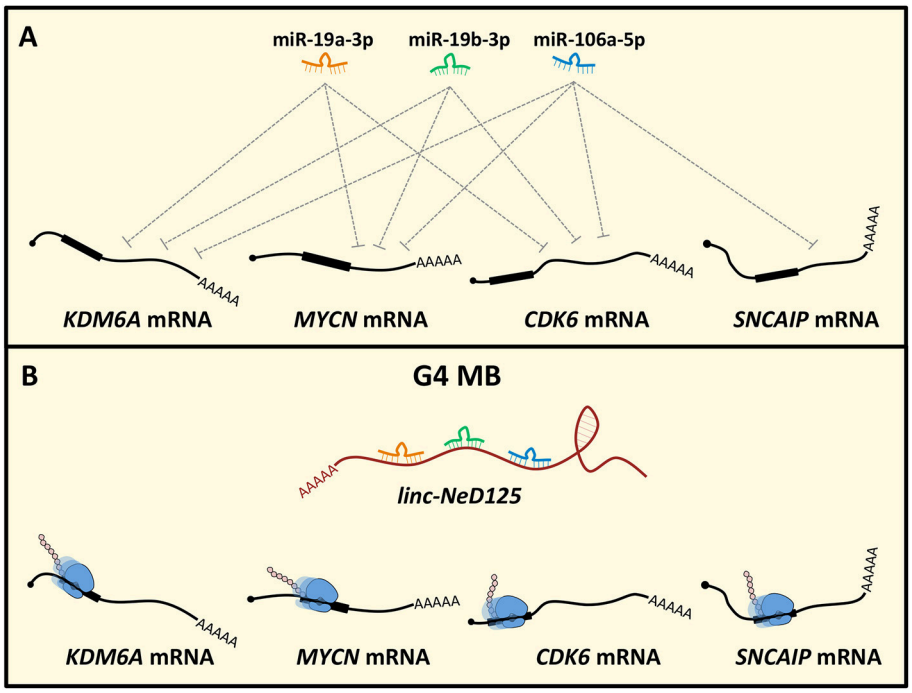

FIGURE 2 | *Linc-NeD125*-based network in MB. **(A)** MiR-19a-3p, miR-19b-3p, and miR-106a-5p pleiotropically control *KDM6A*, *MYCN*, *CDK6*, *SNCAIP* gene expression. **(B)** In G4 MB, *linc-NeD125* is upregulated and, functioning as a decoy for the three miRNAs, causes translational derepression of the G4 MB driver genes *KDM6A*, *MYCN, CDK6*, *SNCAIP*.

Another lncRNA implicated in MB is *ANRIL* (112), which plays a pivotal role in multiple cancers as an epigenetic regulator of its neighbor tumor-suppressors *CDKN2A/B* (113, 114). *ANRIL* expression is upregulated in MB cells, where its knockdown lowers cell viability and migration while increasing apoptosis, by deranging the expression of several apoptotic factors (115). *ANRIL* has been shown to act as a decoy for miR-323, a miRNA identified in neurons (116) and characterized as a glioma tumor-suppressor (117, 118). Consistently, miR-323 silencing counteracted the abovementioned *ANRIL*-dependent cell phenotypes. This regulative axis impinges on *BRI3*, a miR-323 target gene (119) encoding for a brain-expressed transmembrane factor (120). *BRI3* activates MAPK, AKT and WNT signaling cascades, already associated with MB progression (121–123), through a double mechanism: *BRI3* upregulation enhances the phosphorylation of p38, MAPK, ERK, and AKT kinases and stimulates the accumulation of Wnt3a, Wnt5a, and β-catenin. The dysregulation of such pathways may partially explain the apoptotic phenotypes observed upon imbalance of *ANRIL*/miR-323/*BRI3* module (115).

More recently, the lncRNA *LOXL1-AS1*, the antisense transcript to the LOXL1 genomic locus, whose variants are strongly associated with the exfoliation syndrome (124), was found to be overexpressed in MB tissues. *In vitro* and *in vivo* experiments revealed that it controls cell viability, proliferation, cell cycle, and metastasis by activating the PI3K-AKT pathway (125).

Recently, the ceRNA mechanism has emerged as a crucial pathogenic pathway in MB. *Linc-NeD125* was the first ceRNA identified in MB and, generally, in tumors of the CNS (49). It was identified in NB cells as the precursor of miR-125-b1 (126), a neuronal-enriched miRNA (127) involved in neural cell differentiation (128), function (129) and NB and MB cell proliferation, and apoptosis (130, 131). Notably, *linc-Ned125* is significantly and specifically upregulated in primary G4 MBs, compared to the other subgroups. In this context, it functions as a miRNA decoy. *Linc-NeD125* interacts with miR-19a-3p, miR-19b-3p, and miR-106a-5p that pleiotropically control the expression of four G4 MB driver genes, namely *KDM6A*, *MYCN*, *CDK6,* and *SNCAIP* (7) (**Figure 2**). Through this mechanism, *linc-NeD125* causes the driver gene translational de-repression, contributing to G4 MB tumorigenesis and/or to the maintenance of cancer cell identity. This study highlighted *linc-NeD125* as a novel potential G4 driver gene, as well as a specific biomarker and a potential therapeutic target. Accordingly, its knockdown in G4-derived cells caused a significant reduction of cell proliferation, migration, and invasion (49).

The second example of ceRNA in MB is the lncRNA *Nkx2-2as*, that behaves as a tumor-suppressor in SHH MB subgroup. It is highly down-regulated in MB cells derived from a SHH mouse model and it suppresses the malignant phenotype of MB cells, functioning as a sponge for miR-103/107 and miR-548 m. This activity causes the depression of the tumor-suppressor genes *BTG2/Tis21/PC3* and *LATS1/2*, promoting tumor growth *in vitro* and *in vivo* (132).

FUTURE DIRECTIONS

The main challenges in fighting cancer are the identification of specific biomarkers, for timely diagnosis and prognosis, and novel tumor-driver genes, which can be therapeutically targeted for suppressing tumor growth. The former function would help the choice of pre-operative treatments and facilitate the tumor follow-up examinations. Unfortunately, very few biomarkers are known for pediatric tumors (133) and in MB <20 protein-coding genes have been characterized as promising candidates. However, most of these biomarkers were identified from single studies and from heterogeneous tumor types, lacking tumor-specificity (133). The recent categorization of MB into at least four subtypes, with distinct features, led the scientists to consider them as distinct pathologies with likely different responses to therapy. This new perspective triggered the search for novel MB-subgroup specific biomarkers and therapeutic targets. For both issues lncRNAs are very challenging (75). Since many of them are uniquely expressed in specific cancer types, they may function as powerful cancer biomarkers (134). In addition, for their ability to fold into complex tridimensional structures that increase their stability, they can be easily detected into body fluids as urine, blood, and cerebrospinal fluids, making the tumor diagnosis less invasive (75). Notably, lncRNAs are also considered new relevant targets for cancer therapy as highly tissue-specific drivers of cancer phenotypes. Finally, in this search for lncRNAs as novel molecules that distinguish clinically relevant cancer subtypes and predict tumor behavior, the circular RNAs are proving to be effective cancer biomarkers for their abundance, stability, and specificity (135).

AUTHOR CONTRIBUTIONS

PL and EC reviewed the literature and wrote the manuscript. JR prepared the figures and contributed to the text discussion.

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Direct Involvement of Cranial Nerve V at Diagnosis in Patients With Diffuse Intrinsic Pontine Glioma: A Potential Magnetic Resonance Predictor of Short-Term Survival

Giovanna Stefania Colafati[1†], Ioan Paul Voicu[1,2†], Chiara Carducci[1], Massimo Caulo[3], Maria Vinci[2], Francesca Diomedi-Camassei[4], Pietro Merli[2], Andrea Carai[5], Evelina Miele[2], Antonella Cacchione[2], Paolo Tomà[6], Franco Locatelli[2] and Angela Mastronuzzi[2]*

[1] Department of Imaging, Neuroradiology Unit, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [2] Department of Onco-haematology, Cell and Gene Therapy, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [3] Department of Neuroscience and Imaging, ITAB-Institute of Advanced Biomedical Technologies, University G. d'Annunzio, Chieti, Italy, [4] Pathology Unit, Department of Laboratories, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [5] Neurosurgery Unit, Department of Neuroscience and Neurorehabilitation, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [6] Radiology Unit, Department of Imaging, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

Background: Diffuse intrinsic pontine glioma (DIPG) has a dismal prognosis. Magnetic resonance imaging (MRI) remains the gold standard for non-invasive DIPG diagnosis. MRI features have been tested as surrogate biomarkers. We investigated the direct involvement of cranial nerve V (CN V) in DIPG at diagnosis and its utility as predictor of poor overall survival.

Materials and Methods: We examined MRI scans of 35 consecutive patients with radiological diagnosis of DIPG. Direct involvement of CN V was assessed on the diagnostic scans. Differences in overall survival (OS) and time to progression (TTP) were analyzed for involvement of CN V, sex, age, tumor size, ring enhancement, and treatment regimen. Correlations between involvement of CN V and disease dissemination, magnet strength and slice thickness were analyzed. Statistical analyses included Kaplan-Meier curves, log-rank test and Spearman's Rho.

Results: After excluding six long-term survivors, 29 patients were examined (15 M, 14 F). Four patients presented direct involvement of CN V. Histological data were available in 12 patients. Median OS was 11 months (range 3–23 months). Significant differences in OS were found for direct involvement of CN V (median OS: 7 months, 95% CI 1.1–12.9 months for involvement of CN V vs. 13 months, 95% CI 10.2–15.7 for lack of involvement of CN V, respectively, $p < 0.049$). Significant differences in TTP were found for the two treatment regimens (median TTP: 4 months, 95% CI 2.6–5.3 vs. 7 months, 95% CI 5.9–8.1, respectively, $p < 0.027$). No significant correlation was found between involvement of CN V and magnet strength or slice thickness ($r = -0.201$; $p =$ NS). A trend toward positive correlation was found between direct involvement of CN V at diagnosis and dissemination of disease at follow-up ($r = 0.347$; $p < 0.065$).

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***Correspondence:**
Giovanna Stefania Colafati
gstefania.colafati@opbg.net

† These authors have contributed equally to this work and are listed as co-authors

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Conclusions: In our cohort, direct involvement of CN V correlated with poor prognosis. Based on our data, we suggest that in DIPG direct involvement of CN V should be routinely evaluated on diagnostic scans.

Keywords: child, MRI, DIPG = diffuse intrinsic pontine glioma, cranial nerves, biomarkers

INTRODUCTION

Diffuse Intrinsic Pontine Glioma (DIPG) remains the pediatric brain tumor with the worst prognosis (1). Despite recent advances in detailing the biology of DIPG (2), <5% of affected children survive at 3 years irrespective of histological grading and median overall survival (OS) from diagnosis is 9–12 months (3).

The most common clinical features include cranial nerve palsy, ataxia, and long tract signs.

MRI is the gold standard non-invasive method for DIPG diagnosis (4, 5) based on both major and minor diagnostic criteria (6–8) in the context of a typical clinical presentation. Typical MRI findings consist of a large expansive pontine lesion that is hypointense or isointense on T1 weighted (T1w) imaging, hyperintense on T2 weighted (T2w) and fluid-attenuated inversion recovery (FLAIR) imaging, and of variable enhancement with gadolinium contrast agent (4).

Conventional and advanced MRI parameters (3, 9–16) have been tested in different studies in an effort to predict survival at the time of diagnosis. Ring contrast enhancement has been suggested to inversely correlate to OS (17).

Direct cranial nerve involvement in brainstem gliomas has been suggested to represent an extra-axial extension of the tumor. This feature seems to be related to glial cells extending from cranial nerve nuclei to the root entry zone (18–20). Cranial nerves V, VII and VIII have been described to be predominantly involved, but no correlation to tumor grade has been demonstrated.

In the present study we describe the direct involvement of CN V at diagnosis in patients with DIPG in a consecutive single institution series and correlate this imaging feature with clinical variables to test its value as prognostic biomarker.

MATERIALS AND METHODS

Patients

The Institutional Review Board approved this research waiving the need for informed consent from patients or their parents/legal guardians for this specific retrospective analysis. The study was conducted in agreement with the principles contained in the Declaration of Helsinki.

We collected data from 35 consecutive patients diagnosed with DIPG in our Institution, who underwent MRI from 1st January 2003 to 1st February 2018. Diagnosis was based on clinical and radiological criteria consistent with DIPG (6, 7).

Patients with either, radiological findings inconsistent with DIPG, and/or a diagnosis of neurofibromatosis, and/or diagnostic exams which did not include the entire neuraxis were excluded. Patients with an overall survival longer than 24 months were not included in the survival analysis, although their characteristics were reported in detail.

Magnetic Resonance Imaging

MR examinations were performed on a 3 Tesla (3 T) magnet (Siemens Magnetom Skyra, Erlangen, Germany) or on a 1.5 Tesla (1.5 T) magnet (Siemens Magnetom Vision Plus, Erlangen, Germany). All patients underwent at least T1w sagittal, T1w and T2w axial sequences, FLAIR, T2w coronal and diffusion (DWI) sequences. These sequences were used for image analysis. Two different acquisition protocols were implemented on the two scanners. Slice thickness of the acquired sequences was 4 mm on the 1.5 T magnet and 3 mm on the 3 T magnet. All patients received scan of the entire neuraxis. A contrast medium agent was used in all studies. Patients deemed unable to cooperate for age-related and/or clinical status-related reasons were imaged under general anesthesia.

For each patient, radiological and clinical data were collected. In order to reduce the possibility of an interpretation bias, patient scans were first anonymized and then transferred to a separate workstation.

Two expert neuroradiologists from different institutions, both with 20 years of experience, reviewed patient scans in consensus to collect radiological data. This method was used for all MRI analyses unless otherwise specified.

The radiological criteria for DIPG diagnosis (T1 hypointense and T2 hyperintense lesions with poorly defined margins involving more than 50% of the pons) were first assessed.

Diagnostic scans were then reviewed to investigate direct involvement of CN V by the tumor. Direct involvement of CN V was defined by the thickening and/or abnormal signal intensity involving the root entry zone and the proximal cisternal course of the cranial nerve, contiguous with the tumor, visible on at least two sequences (**Figure 1**). The absence of contrast enhancement did not exclude the diagnosis of involvement of CN V. Exclusion of leptomeningeal spread was deemed necessary to confirm the diagnosis of direct involvement of CN V. Leptomeningeal spread was defined as the region of pathologic contrast enhancement consistent with disease localization confined in any region of the neuraxis and not contiguous with the tumor.

In order to investigate if in our cohort other MRI features could affect patient survival we collected other two known potential MRI surrogate biomarkers: tumor size and ring enhancement.

Tumor size was defined as the product of the longest perpendicular diameters of the tumor, measured on an axial plane on FLAIR sequences. This method has been evaluated to assess tumor response to therapy (21) and has been debated in literature

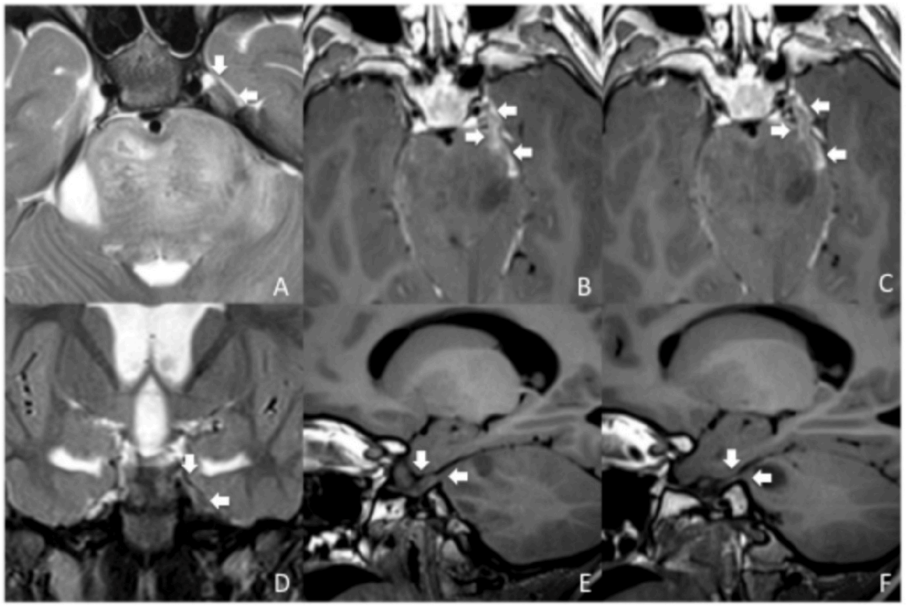

FIGURE 1 | MRI diagnosis of direct involvement of CN V by DIPG in a 4-years-old boy. Axial TSE T2 **(A)**, para-axial reformatted T1 post-contrast **(B,C)**, coronal TSE T2 **(D)** and para-sagittal reformatted T1 images **(E,F)** show thickening of the left CN V causing filling of the left Meckel cave, consistent with direct cranial nerve involvement from the tumor (**A–E**, white arrows). The pathologic thickening of the left CN V is associated with contrast enhancement (**B,C**, white arrows). Pathologic thickening of CN V is better delineated when comparing it with the contralateral normal V cranial nerve (**F**, arrows).

(22). The presence of ring enhancement was assessed on T1 post-contrast images.

For each patient, all follow-up scans were evaluated for the presence of involvement of CN V and dissemination of the disease. All patients underwent a fixed follow-up protocol. Controls at follow-up were performed 30 days after radiotherapy, and thereafter every 3 months, unless new symptoms occurred.

Since both a 1.5 Tesla magnet and a 3 Tesla magnet were employed in our study with two different protocols, potential correlations between the involvement of CN V with the magnet strength and with slice thickness were also analyzed.

An experienced neuro-oncologist blinded to image analysis collected clinical data. The neuroradiologists were blinded to the results of clinical data collection.

Statistical Analysis

Quantitative variables were reported as median value and ranges, while categorical variables were expressed as absolute values and percentages.

Overall survival (OS) was defined as the length of time from the date of diagnosis to the date of death or last follow-up. Time to progression (TTP) was defined as the length of time from the date of diagnosis until disease progression. Tumor progression was defined as radiological progression, associated with worsening of pre-existing symptoms or the appearance of new symptoms. Radiological progression was defined as an increase >25% in tumor size, based on the RAPNO criteria (21), and/or metastatic progression of the disease. Metastatic progression was defined as new distant signal alterations and/or leptomeningeal enhancement, consistent with disease. Probability of OS and TTP were calculated according to the Kaplan and Meier method (23) and expressed as median OS and median TTP, respectively. All results were expressed as probability or cumulative incidence (%) and 95% confidence interval (95% CI).

Differences in OS and TTP were estimated with the log–rank test (Mantel–Cox). Direct cranial nerve involvement at diagnosis, the presence of ring enhancement and sex were used as categorical variables. Tumor size and patient age were stratified for values superior and inferior to their median values.

Spearman's rho was used to evaluate the relationship between the involvement of CN V at diagnosis with the magnet strength, and the slice thickness, as well as with the tumor dissemination at follow-up.

P-values < 0.05 were considered to be statistically significant; *p*-values between 0.05 and 0.1 were not considered statistically significant but were reported in detail in the text; *p*-values ≥ 0.1 were reported as non-significant (NS).

Statistical analysis was performed using SPSS statistics (version 20). Survival analysis used 31/08/2018 as reference date.

RESULTS

For this study, we initially considered 35 patients radiologically diagnosed with DIPG. Six patients presented an OS of more than 24 months and were not included in the survival analysis, but their characteristics were reported in detail. Clinical, radiological and histological characteristics of long-term survivors are shown in **Table 1**.

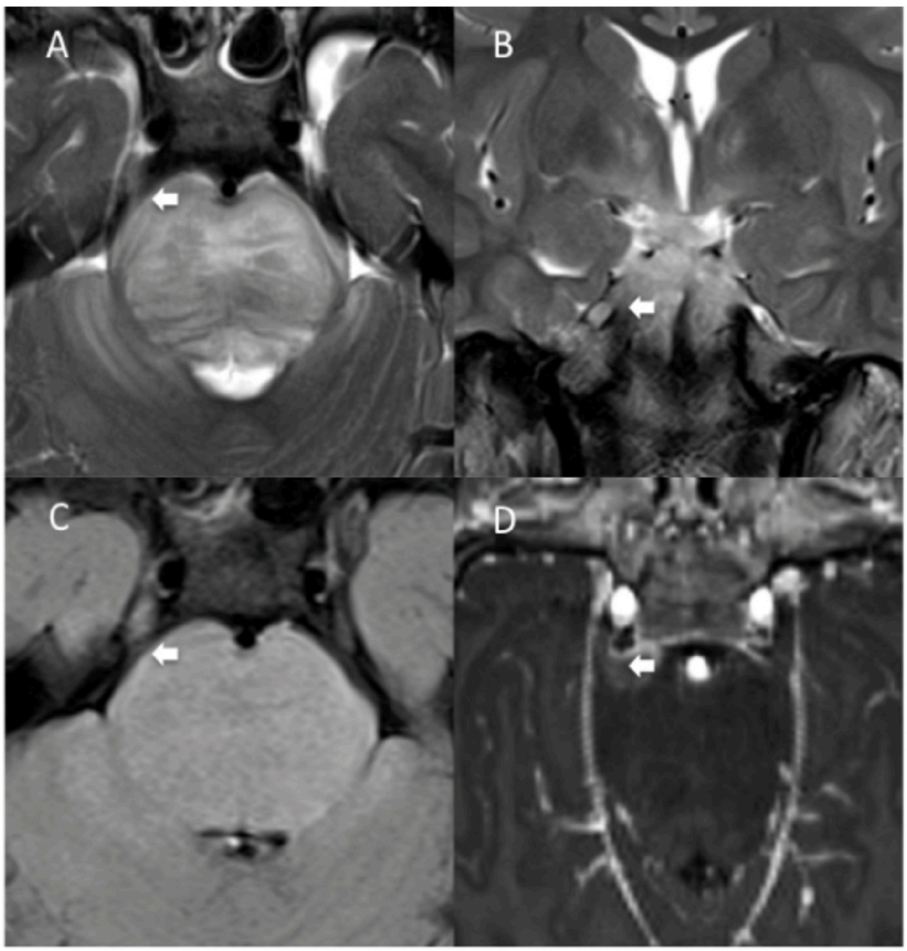

FIGURE 2 | Another MRI diagnosis of direct involvement of CN V by DIPG in a 7-years-old boy. TSE T2 axial **(A)** and coronal **(B)**, axial FLAIR **(C)**, and T1 post-contrast para-axial **(D)** reformatted MRI images show thickening and abnormal signal intensity involving the root entry zone and the cisternal course of the right V cranial nerve, contiguous with the tumor, (**A–C**, white arrows). Findings are consistent with direct right V cranial nerve involvement by the tumor. There is associated contrast enhancement (**D**, white arrow).

The final cohort consisted of 29 patients: clinical, radiological and histological data are shown in **Table 2**. Among them, there were 15 boys (M = 51.7%) and 14 girls (F = 48.3%). Median age at diagnosis was 5.7 years (range 2.5–14.4). Histological confirmation was also obtained in a subgroup of 12 patients, as since December 2015 robot-assisted trans-frontal stereotactic needle biopsy has routinely been proposed at diagnosis to patients with DIPG referred to our Institution (24). All patients undergoing histological confirmation presented a H3K27M mutation. Before 2011, all patients received first-line radiation and chemotherapy according to the Stupp protocol (25). After 2011, all patients received first-line treatment with radiation, Nimotuzumab and Vinorelbine (26).

Ring enhancement was present in nine patients (3 M; 31% of the cohort). Median tumor size at diagnosis was 1,736 mm^2 (range 850–2,552 mm^2).

Direct trigeminal nerve involvement at diagnosis was found in four patients (3 M, 1 F, 13.8% of the cohort, **Figure 2**). Two of them (50%) did not show contrast enhancement. Median age at diagnosis in the subgroup with CN V involvement was 4.8 years (range 3–5.6).

In two cases (50%), direct involvement of CN V was also confirmed at follow-up. In one of these children trigeminal nerve involvement was bilateral (**Figure 3**).

Dissemination at follow-up was observed in five patients (17.2%) of the general cohort, two of them belonging to the direct CN V involvement group. At follow-up, one of the two patients with involvement of CN V (50%) also presented dissemination (50%).

Median OS in our cohort was 11 months (range 3–23 months). Median TTP was 7 months (range 2–18 months).

Log-rank test revealed significant differences in OS between the group with direct involvement of CN V (median OS: 7 months, 95% CI 1.1–12.9 months) and the group without (median OS: 13 months, 95% CI 10.2–15.7; $p < 0.049$, **Figure 4**). No significant differences in OS were found in relation to ring enhancement, tumor size, treatment protocol, age, and sex (p = NS).

TABLE 1 | Clinical, radiological, and available histological characteristics in long-term survivors with DIPG from our cohort.

Patient ID	Sex	V cranial nerve involvement	Status	Tumor size	Ring enhancement	Treatment	Histological diagnosis	Age at diagnosis	Progression	Time to progression	Dissemination at follow up	Overall survival
8	F	Yes	DOD	2,035	No	B	Low grade astrocytoma, H3K27 wild type	104	Yes	7	Yes	32
15	M	No	DOD	1,564	No	B	Diffuse midline glioma, H3K27 mutant	79	Yes	9	No	27
16	M	No	AWD	2,070	Yes	B	Pilocytic astrocytoma	24	Yes	63	No	68
24	M	No	AWD	2,296	No	A	Not available	45	No	183	No	183
26	F	No	DOD	1,692	No	B	Diffuse midline glioma, H3K27 mutant	46	Yes	13	No	25
28	M	No	DOD	1,800	No	A	Not available	42	Yes	6	No	31

ID, patient number; sex, male (M) or female (F); V cranial nerve involvement, direct V cranial nerve involvement at diagnosis present (yes) or absent (no); Status, patient outcome including alive with disease (AWD), dead of disease (DOD) or lost at follow-up (LAF); Tumor size, tumor size expressed in mm^2; Ring enhancement, ring enhancement present (yes) or absent (no) at diagnosis; Treatment, patient first line of treatment with radiotherapy and Temozolomide (A) or with radiotherapy, Nimotuzumab and Vinorelbine (B); Histological diagnosis, available histological diagnosis, according to the WHO 2016 classification; Age at diagnosis, age at diagnosis expressed in months; Progression, the presence (yes) or absence (no) of clinical and/or radiological signs of disease progression; Time to progression, the length of time from the date of diagnosis of DIPG until the radiological and/or clinical progression of the disease, expressed in months; Dissemination at follow-up, distant localization of the tumor present (yes) or absent (no) on the follow-up scans; Overall survival, overall survival expressed in months.

Sex, age at diagnosis, tumor size at diagnosis, ring enhancement and involvement of CN V did not significantly affect TTP (p = NS). Significant differences in TTP were found for different treatment protocols (median TTP: 4 months, 95% CI 2.6–5.3 months for radiotherapy and Temozolomide vs. 7 months, 95% CI 5.9–8.1 for radiotherapy, Nimotuzumab and Vinorelbine, respectively, $p < 0.027$, **Figure 5**). No significant correlation was found between direct involvement of CN V at diagnosis with magnet strength (p = NS) and slice thickness (p = NS).

A trend toward a positive correlation was found between direct involvement of CN V at diagnosis and dissemination of disease at follow-up ($r = 0.347$; $p < 0.065$).

DISCUSSION

In this study we investigated the MRI finding of direct involvement of CN V at diagnosis as a potential biomarker for short-term progression in patients with DIPG. CN V involvement was significantly associated to a shorter OS (**Figure 4**).

To our best knowledge, there are no studies in the literature specifically focusing on the direct involvement of CN V in DIPG. The reasons for this may be mainly due to the difficulty to objectively assess the direct nerve involvement and the limited available information about the clinical impact of such finding. Due to their anatomical location, DIPGs present a very high probability of direct cranial nerve infiltration. From a clinical standpoint, in these patients cranial nerve palsies are a common manifestation of the disease. However, clinical examination may not be predictive of direct cranial nerve extension of the tumor. Therefore, radiological evaluation could prove valuable were cranial nerve involvement a clinically significant variable in children with DIPG.

We hypothesized that direct involvement of CN V would be more frequent in tumors with a higher propensity to infiltrate and, possibly, to disseminate at follow-up. Since the majority of DIPG show local progression, being able to identify a subgroup of patients with a higher dissemination risk at follow-up may lead to different tailored treatment strategies, i.e., craniospinal radiation.

In our study, patients with direct CN V involvement at diagnosis showed a shorter sOS (7 vs. 13 months, $p < 0.049$) and a higher rate of tumor dissemination at follow-up ($r = 0.347$, $p < 0.065$). Although the small number of patients represents a considerable limit to our analysis, we believe these promising results are worthy of further investigation.

In principle, it is possible that other cranial nerves, such as the VII–VIII, could be infiltrated in patients with DIPG. It has to be considered, though, that due to the characteristics of DIPG it may be difficult to distinguish between nerve involvement and tumor bulging at that level. Therefore, we decided to focus only on the involvement of CN V, where the finding was deemed unequivocal. Our findings (four patients with involvement of CN V, 13.7% of our cohort) suggest that direct involvement of CN V may not be so rare in patients with DIPG (**Figures 1–3**).

TABLE 2 | Clinical, radiological, and histological characteristics of the patients with DIPG from our cohort.

Patient ID	Sex	Status	Age at diagnosis	Treatment	Available histology	Histological diagnosis	Time to progression	Overall survival	CN V diagnosis	CN V follow-up	Tumor size	Ring enhancement	Dissemination at follow up	Scanner	Slice thickness
1	M	DOD	81	B	No	n/a	14	18	No	no	2,000	No	Yes	3 T	3 mm
2	M	DOD	54	A	No	n/a	2	4	Yes	Yes	1,768	No	Yes	3 T	3 mm
3	M	DOD	100	A	No	n/a	3	5	No	No	1,116	No	No	1.5 T	4 mm
4	F	DOD	57	A	No	n/a	3	10	No	No	851	No	No	1.5 T	4 mm
5	F	DOD	112	B	Yes	Diffuse midline glioma, H3K27M mutant	11	20	No	No	1,845	Yes	No	3 T	3 mm
6	M	DOD	51	A	No	n/a	3	7	Yes	No	1,925	No	No	1.5 T	4 mm
7	M	DOD	154	B	Yes	Diffuse midline glioma, H3K27M mutant	7	8	No	No	999	No	No	3 T	3 mm
9	M	DOD	61	B	No	n/a	9	13	No	No	1,344	No	No	3 T	3 mm
10	F	LAF	166	B	Yes	Diffuse midline glioma, H3K27M mutant	6	6	No	No	1,000	No	No	3 T	3 mm
11	F	DOD	49	B	No	n/a	7	14	No	No	1,739	No	No	3 T	3 mm
12	F	DOD	50	A	No	n/a	7	13	No	No	1,344	No	No	1.5 T	4 mm
13	M	DOD	76	A	No	n/a	8	10	No	No	1,462	Yes	No	1.5 T	4 mm
14	M	DOD	51	B	No	n/a	5	8	No	No	1,152	No	Yes	3 T	3 mm
17	M	DOD	81	A	No	n/a	7	16	No	No	2,552	No	Yes	1.5 T	4 mm
18	F	DOD	46	B	No	n/a	3	7	No	No	2,160	No	No	3 T	3 mm
19	F	DOD	36	A	No	n/a	4	10	Yes	Yes	1,764	Yes	No	1.5 T	4 mm
20	F	DOD	49	B	No	n/a	2	3	No	No	1,161	No	No	3 T	3 mm
21	M	DOD	72	B	No	n/a	8	7	No	No	2,262	Yes	No	3 T	3 mm
22	F	DOD	81	B	Yes	Diffuse midline glioma, H3K27M mutant	7	9	No	No	850	No	No	3 T	3 mm
23	F	DOD	51	A	No	n/a	4	23	No	No	1,800	Yes	No	1.5 T	4 mm
25	M	DOD	173	B	No	n/a	8	12	No	No	1,364	No	No	3 T	3 mm
27	M	DOD	67	B	Yes	Diffuse midline glioma, H3K27M mutant	8	12	Yes	No	1,824	No	Yes	3 T	3 mm
29	F	DOD	68	B	Yes	Diffuse midline glioma, H3K27M mutant	8	11	No	No	1,102	Yes	No	3 T	3 mm
30	M	AWD	73	B	Yes	Diffuse midline glioma, H3K27M mutant	5	15	No	No	1,736	Yes	No	3 T	3 mm
31	F	DOD	79	B	Yes	Diffuse midline glioma, H3K27M mutant	3	15	No	No	1,575	No	No	3 T	3 mm
32	M	DOD	65	B	Yes	Diffuse midline glioma, H3K27M mutant	6	13	No	No	2,200	Yes	No	3 T	3 mm
33	F	AWD	111	B	Yes	Diffuse midline glioma, H3K27M mutant	6	13	No	No	1,519	Yes	No	3 T	3 mm
34	F	AWD	30	B	Yes	Diffuse midline glioma, H3K27M mutant	18	18	No	No	1,980	No	No	3 T	3 mm
35	M	DOD	72	B	Yes	Diffuse midline glioma, H3K27M mutant	7	8	No	No	1,833	No	No	3 T	3 mm

ID, patient number; sex, male (M) or female (F); Status, patient outcome including alive with disease (AWD), dead of disease (DOD) or lost at follow-up (LAF); Age at diagnosis, age at diagnosis expressed in months; Treatment, patient first line of treatment with radiotherapy and Temozolomide (A) or with radiotherapy, Nimotuzumab and Vinorelbine (B); Available histology, available (yes) or non-available (no) histological diagnosis, according to the WHO 2016 classification; Histological diagnosis, diffuse midline glioma, H3K27M mutant, according to the WHO 2016 or n/a, not available; Time to progression, the length of time from the date of diagnosis of DIPG until the radiological and/or clinical progression of the disease, expressed in months; Overall survival, overall survival expressed in months; CN V diagnosis, direct cranial nerve V involvement at diagnosis present (yes) or absent (no); CN V follow-up, direct cranial nerve V involvement at follow-up present (yes) or absent (no); Tumor size, tumor size expressed in mm^2; Ring enhancement, ring enhancement present (yes) or absent (no) at diagnosis; Dissemination at follow-up, distant localization of the tumor present (yes) or absent (no) on the follow-up scans; Scanner, magnet strength, expressed as 3 Tesla (3 T) or 1.5 Tesla (1.5 T); Slice thickness, slice thickness of the sequences contained in the imaging protocol, respectively 3 millimeters (3 mm) or 4 millimeters(4 mm).

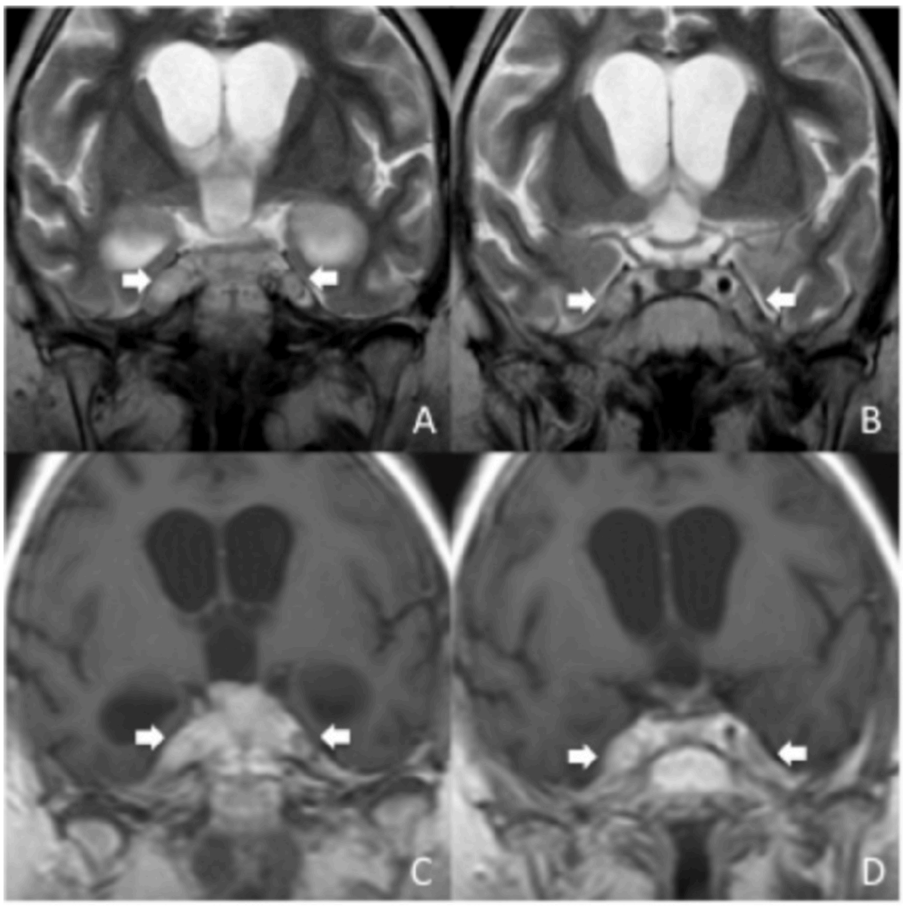

FIGURE 3 | MRI findings of V cranial nerve involvement at follow-up in a 4-years-old girl previously diagnosed with DIPG. Coronal TSE T2 **(A,B)** and post-contrast coronal T1 **(C,D)** images show extensive thickening of both V cranial nerves and abnormal bulging of the Meckel caves, consistent with direct infiltration by the tumor (**A–D**, white arrows). There is associated contrast-enhancement (**C,D**, white arrows).

Since CN V is a small structure (the nerve has a caliber of <3 millimeters), spatial resolution of the diagnostic images is important. For this reason, it is possible that magnet strength and/or the slice thickness may influence the detection rate of CN V involvement, and that high-resolution images obtained from 3 T magnets could allow higher detection rates. In our cohort, however, we found no significant correlation between the diagnosis of CN V involvement and magnet strength ($r = -0.201$, $p =$ NS) or slice thickness ($r = -0.201$, $p =$ NS).

Follow-up scans in our cohort revealed direct involvement of CN V in two of four cases (**Table 2**). Interestingly, one of the two patients with CN V involvement at follow-up also presented leptomeningeal spread of the disease (50%). Although our population is small, these findings seem consistent with our hypothesis that CN V involvement is more frequent in tumors with a higher propensity to disseminate at follow-up. However, when assessing the predictive value of direct involvement of CN V, different factors should be taken into account, mainly therapy-related tumor changes, and the low number of observed patients with involvement of CN V, which limited further analyses.

Other MRI parameters have been tested for survival prediction (9–14, 17). Among different MRI and clinical features, we analyzed if tumor size, ring enhancement, age, and sex influenced OS in our cohort. Utility of calculating tumor size was debated. Some authors have emphasized the relevance of inter-observer variability (27) and stressed the importance of reproducibility of the measures and single reader measurement of tumor size on DIPG scans. In our cohort, greater tumor size at diagnosis did not modify overall survival, which is consistent with the findings in the literature (15, 16). Some authors have also tested and found a discrepancy between longitudinal volumetric measures and metabolic evolution of the tumor measured by spectroscopy, the latter being better suited to response assessment strategies (28). Ring contrast enhancement has shown a negative correlation with OS in a large retrospective study (17). In our cohort, a trend toward shorter OS for patients without ring enhancement was observed ($p < 0.78$). The reasons behind this result may be due to our small sample size. In the same study age <3 years was correlated to a longer OS (17). Since in our cohort there were only two patients <3 years, we

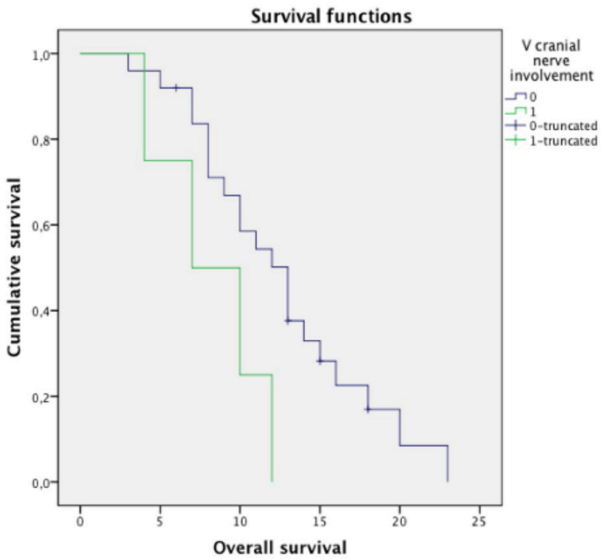

FIGURE 4 | Survival analysis with Kaplan-Meier curves for patients in our cohort presenting with V cranial nerve involvement at diagnosis. Patients with direct involvement of CN V (label = "1," green line, median OS: 7 months, 95% CI 1.1–12.9 months) presented significantly lower overall survival (OS) compared to the group without involvement of CN V (label = "0," violet line, median OS: 13 months, 95% CI 10.2–15.7; log rank test: $p < 0.049$). The other parameters (not shown) were not associated with significant differences in OS (p = NS).

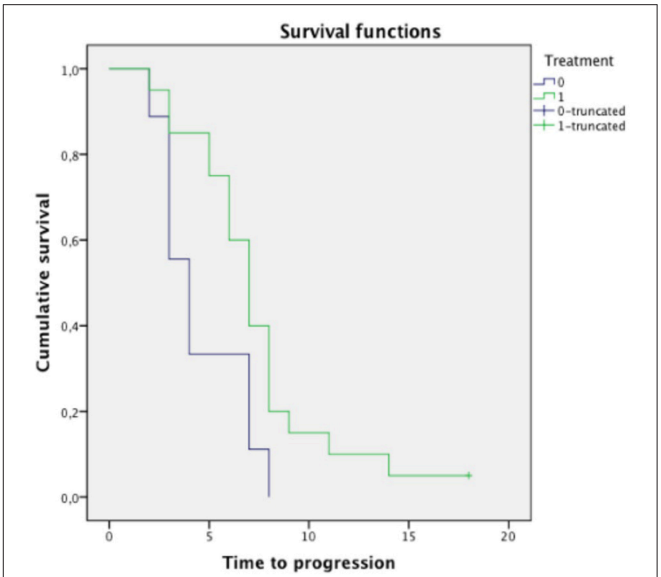

FIGURE 5 | Survival analysis with Kaplan-Meier curves for the type of treatment administered to the patients in our cohort. Patients treated with radiotherapy, Nimotuzumab and Vinorelbine (label = "1," green line, median TTP: 7 months, 95% CI 5.9–8.1 months) presented significantly higher time to progression (TTP) than patients treated with radiotherapy and Themozolomide (label = "0," violet line, median TTP: 4 months, 95% CI 2.6–5.3 months, log rank test: $p < 0.027$). The other parameters (not shown) were not associated with significant differences in TTP (p = NS).

decided to stratify age to the median (5.7 years). As such, age did not show significant correlations with OS. Sex did not show significant correlations with OS, which was also consistent with the findings in literature (17).

Significant differences in TTP were found for patient treatment (median TTP: 4 months, 95% CI 2.6–5.3 months for radiotherapy and Temozolomide vs. 7 months, 95% CI 5.9–8.1 for radiotherapy, Nimotuzumab and Vinorelbine, $p < 0.027$). This suggests that the new therapeutic regimen lengthened the time to progression of disease, even if it did not change OS. Our findings seem to confirm that treatment with radiotherapy in combination with Nimotuzumab and Vinorelbine represents an interesting therapeutic option (26, 29).

No differences in TTP were found for direct involvement of CN V (p = NS). Further research should be performed on the correlation between this finding and the results reported for OS for direct involvement of CN V. Sex, age, tumor size and ring enhancement also did not significantly affect TTP (p = NS).

We excluded long-term survivors with an OS of more than 24 months in the statistical analysis. The rationale for our decision is that long-term survivors in our cohort significantly differed from the other patients in terms of heterogeneous clinical, histological and radiological characteristics (**Table 1**).

More specifically, in the long-term survivor cohort:

- Two of the six patients (PT 16 and 24) are still alive with disease (with a median OS of 68 and 183 months, respectively). They also presented a pattern of progression, which is not typical for the disease (PT 16 progressed after 63 months, and PT 24 never progressed);
- Two of the six patients (PT 8 and 16) did not harbor the H3K27M mutation;
- After review, four of these six patients (PT 8, 16, 24, and 28) had radiological features, consistent with atypical DIPG (well-defined borders and exophytic components).

The small group of long-term survivors probably deserves to be included in a larger multicentric study, in order to identify consistent trends and relevant features (30).

Recent advances in the molecular characterization of DIPG have begun to disclose biological signatures possibly associated to outcome and response to treatment (31). The term DIPG itself is slightly out-dated, as it has been shown that most of these tumors present distinct mutations, most notably the H3K27M. In the 2016 revision of the WHO brain tumor classification, these neoplasms were termed as diffuse midline glioma, H3K27M-mutant (32). Imaging studies are currently directing toward highlighting correlations between the relevant imaging findings and the new molecular findings (33, 34). If involvement of CN V was confirmed to be a predictor of poor survival in DIPG, it could be hypothesized that such finding could also have prognostic significance for the newer entity of the diffuse midline glioma H3K27M mutant. In our cohort, we can report the presence of the H3K27M mutation associated to the involvement of CN V, only for one patient. For the other patients, the biopsy had not been performed and so the mutational status could not be tested.

This study has some limitations. Specifically, we acknowledge the limit of a single institution, retrospective study, employing a low number of patients. Clinical practice has shown that a higher order of collaboration between professionals is required in order to improve our understanding and better progress toward treatment of this disease (30, 35). Another limit of the study was the methodology employed for assessment of direct involvement of CN V, which was made by two neuroradiologists in consensus, even if we tried to mitigate that issue by employing two neuroradiologists from different institutions. Furthermore, for 17 out of 29 patients in our cohort, tissue samples for histological confirmation of the H3K27M mutation were not available. This is because the stereotactic biopsy procedure was introduced in our institution from 2015.

In conclusion, our data suggest that direct involvement of CN V is a surrogate biomarker of poor survival in patients with DIPG, which could be assessed on conventional images. Further studies on larger cohorts, possibly associated with correlation to histological and molecular findings, should be performed to cross validate our results and further test our hypotheses.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

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

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CASE REPORT
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Vemurafenib Treatment of Pleomorphic Xanthoastrocytoma in a Child With Down Syndrome

Giuseppe Petruzzellis[1], Diletta Valentini[2], Francesca del Bufalo[1], Giulia Ceglie[3], Andrea Carai[4], Giovanna Stefania Colafati[5], Emanuele Agolini[6], Francesca Diomedi-Camassei[7], Tiziana Corsetti[8], Iside Alessi[1], Angela Mastronuzzi[9], Franco Locatelli[1,10] and Antonella Cacchione[9]*

[1] Department of Hematology, Oncology and Stem Cell Transplantation, Bambino Gesù Children's Hospital (IRCCS), Rome, Italy, [2] Pediatric and Infectious Disease Unit, Bambino Gesù Children's Hospital (IRCCS), Rome, Italy, [3] University Department of Pediatrics, Bambino Gesù Children's Hospital, University of Rome Tor Vergata, Rome, Italy, [4] Neurosurgery Unit, Department of Neuroscience and Neurorehabilitation, Bambino Gesù Children's Hospital (IRCCS), Rome, Italy, [5] Neuroradiology Unit, Department of Imaging, Bambino Gesù Children's Hospital (IRCCS), Rome, Italy, [6] Medical Genetics Laboratory, Bambino Gesù Paediatric Hospital (IRCCS), Rome, Italy, [7] Department of Pathology, Bambino Gesù Children's Hospital (IRCCS), Rome, Italy, [8] Hospital Pharmacy Unit, Children's Hospital and Research Institute 'Bambino Gesù' (IRCCS), Rome, Italy, [9] Neuro-oncology Unit, Department of Hematology, Oncology and Stem Cell Transplantation, Bambino Gesù Children's Hospital (IRCCS), Rome, Italy, [10] Department of Gynecology/Obstetrics and Pediatrics, Sapienza University of Rome, Rome, Italy

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United States

***Correspondence:**
Angela Mastronuzzi
angela.mastronuzzi@opbg.net

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Brain tumors are the most common solid neoplasms of childhood, but they are very rarely reported in children with Down Syndrome (DS), who develop more commonly different types of malignancies. In particular, we hereby report the case of an 8-years-old child with DS that presented to our attention for neurological and endocrinological issues. Brain imaging revealed the presence of a mass that was partially resected revealing a histological diagnosis of Pleomorphic Xanthoastrocytoma (PXA), a rare WHO grade II tumor extending from the diencephalic region into the surrounding brain tissue. These tumors can harbor the *BRAF* mutation p.V600E, targetable by the specific inhibitor Vemurafenib. After confirming the presence of the mutation in the tumor, the patient was treated with Vemurafenib. The treatment proved to be effective, leading to a partial response and a stabilization of the disease. Usually, in patients with DS a reduction of the dose of chemotherapeutic drugs is necessary. Vemurafenib was instead well-tolerated as the only observed adverse effect was grade I skin toxicity. This is, to our knowledge, the first case of a PXA reported in a child with DS and the first DS patient treated with Vemurafenib.

Keywords: brain tumor, down syndrome, BRAF V600E mutation, pleomorphic xanthoastrocytoma, vemurafenib

BACKGROUND

Down syndrome (DS) is the most common chromosome abnormality among live births, with an incidence of ∼1 every 800 live births (1). It is characterized by a common phenotype that can result from three main types of cytogenetic abnormalities: the most common is Trisomy 21 non-disjunction, accounting for ∼95% of cases; in about 3–4% of the cases, a Robertsonian translocation involving chromosome 21 is found; the less common known abnormality is represented by Trisomy 21 mosaicism (47, +21/46), responsible for the remaining 1–2% (2).

Children with DS exhibit a wide variety of medical conditions, such as cognitive impairment, dysmorphic features, congenital heart diseases, gastrointestinal abnormalities, reduction in visual acuity and accomodation, hearing loss, endocrine, and immune deficiencies (3).

Even though life expectancy in these patients has significantly increased over the last decade, children with DS still have a higher risk of neonatal and infant mortality when compared to children without DS, because of the above mentioned comorbidities (4).

One of the most severe conditions associated with DS is the development of malignant tumors, mainly acute leukemia. On the other hand, solid tumors, and, in particular, brain neoplasms are rarely reported in patients with DS, and therefore the biological behavior and natural history of these tumors are not well-described and understood. Whereas, several oncogenes have been identified as responsible for the development of acute leukemias in DS, little is known about the molecular basis of solid tumors in this population.

Pleomorphic Xanthoastrocytoma (PXA) is a rare brain tumor that most commonly affects children and young adults. The prognosis is favorable when total resection is possible, but in patients not amenable to the eradication, chemotherapy, and/or radiotherapy are the only available therapeutic options. However, these regimes rarely lead to a control of the disease and the prognosis is generally poor (5). Between 60 and 65% of grade 2 and grade 3 PXAs are *BRAF* p.V600E mutated (6, 7) and treatment with Vemurafenib, a BRAF inhibitor approved for the treatment of *BRAF*—mutated metastatic melanoma, demonstrated efficacy in several cases of primary brain tumor, including PXAs (8).

We hereby report, to the best of our knowledge, the first case of a PXA harboring a *BRAF* p.V600E mutation in a patient with DS.

CASE PRESENTATION

An 8-year old boy with DS was referred to the DS outpatient care unit of the Bambino Gesù Children's Hospital for progressively impaired gait and signs of early puberty.

During neurological examination, a slight asymmetrical gait pattern was noted. This anomaly was firstly attributed to the general motor clumsiness typical of DS patients. When evaluating sexual development, a Tanner Stage of P2G2 was observed, with a bilateral testicular volume of 8 ml. To confirm the clinical suspect of early puberty, Gonadotropin-releasing hormone (GnRH) stimulation test was performed. The results showed: basal FSH of 0.7 mIU/mL and after LHRH administration: 3.78 mIU/mL; basal LH was 1.3 mU/mL, and after stimulation: 20.11 mU/mL; Testosterone basal level was 54.5 ng/dL, PRL, beta-HCG, DHEAS and thyroid function were all normal. These results confirmed the suspect of an early puberty of central origin and a brain Magnetic Resonance Imaging (MRI) was then performed. The brain imaging showed diffused pathological tissue, extending from the left diencephalic region and involving the cerebral peduncle caudally, the basal ganglia region cranially (globus pallidus, putamen and posterior arm of the internal capsule), the outer capsule laterally, the temporo-mesial cortex and subcortical white matter, which extended deeply to the anterior portion of the temporal lobe, to the optic chiasm and bilateral retrochiasmatic tract (**Figure 1**).

The patient underwent partial resection of the lesion and the histopathological examination was compatible with the diagnosis of WHO grade II Pleomorphic Xanthoastrocytoma. *BRAF* p.V600E mutation was then assessed by immunohistochemistry (**Figure 2**) and through Sanger sequencing of the *BRAF* gene, revealing positive. Based on these results, after the parents of the patient provided formal, informed consent and the therapy was approved by Institutional Review Board, treatment with the *BRAF* p.V600E inhibitor Vemurafenib was started. Initially, the lower dose proved to be active in adults was administered (i.e., 240 mg/day *per os* twice a day) and was later increased to 480 mg twice a day. After 32 months the therapy was discontinued, and the disease remained stable 3 months after the stop therapy.

The only side effect reported was a transient follicular truncal rash in the first month of administration with fickle subcutaneous nodules, treated with local topical corticosteroids. No ECG changes or/and suspected skin lesions developed.

A new brain MRI after 6 months of therapy demonstrated an important reduction of the lesion and a substantial reduction of enhancement, the last MRI (30 months after diagnosis) demonstrated a stable disease (**Figure 1**).

Clinical response, with gait and movements improvement, was also noted shortly after the beginning of therapy.

DISCUSSION

DS is associated with several hematological disorders occurring at different ages. Neonates with DS may present with transient asymptomatic blood count abnormalities such as neutrophilia, thrombocytopenia and polycythemia. Within 1–2 months of life, 3–10% of infants with DS develop transient myeloproliferative disease (TMD) (9). Despite a spontaneous regression in most of the cases, TMD can be fatal or lead to the subsequent development of myeloid leukemia in 20% of children with DS. Children with DS also have an increased risk of developing leukemia, their risk is, in fact, approximately 10 to 20 times higher compared with children without DS (10). They represent 2% of all pediatric acute lymphoblastic leukemias (ALL) and 10% of pediatric acute myeloid leukemias (AML). The presence of somatic mutations involving GATA1 gene is associated with acute megakaryoblastic leukemia (AMKL) in people with DS (11).

In a study performed on 2,814 individuals with DS, the authors have shown that the occurrence of cancer in DS is unique with a high risk of leukemia in children and a decreased risk of solid tumors, and amongst these brain tumors, in all age-groups (12, 13).

The molecular basis of this paradox of tumorigenesis in DS is not well-understood yet. Several experimental observations have shown that DS should be a cancer-prone condition: higher rate of whole chromosome and segmental chromosome instability, increased DNA damage and defective DNA repair, immunodeficiency and susceptibility to infections, oncogenes

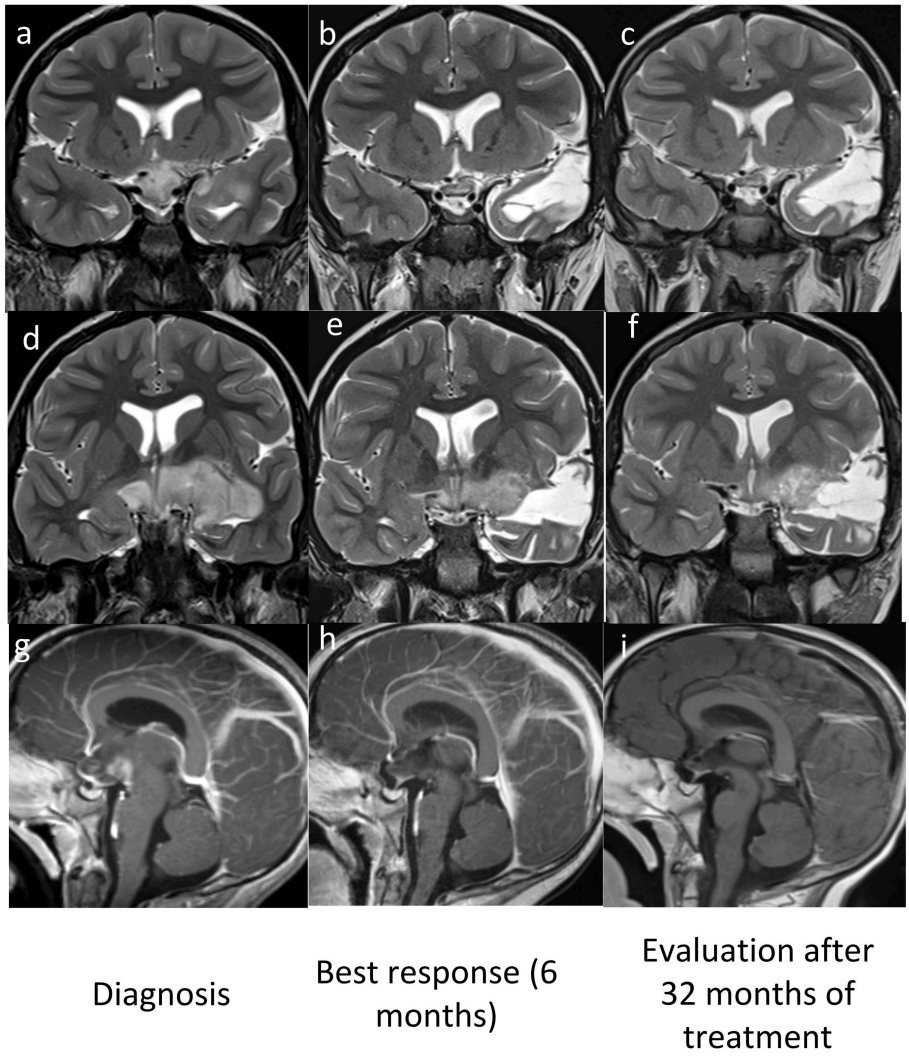

FIGURE 1 | MRI pre **(a,d,g)** and post Vemurafenib **(b,c,e,f,h,i)** coronal T2w **(a–f)** and sagittal Gd T1w **(g–i)** images show best response after 6 months **(b,e,h)** and stable disease after 32 months **(c,f,i)**.

on chromosome 21 (13). Also, abnormalities in DS related to the extra-copy of chromosome 21 include upregulation of pro-apoptotic and angiogenesis genes. For example, dysregulation of the Notch/Wnt pathway has been associated with premature aging processes in DS that may relate to leukemia (14).

On the other hand, however, cancer-inhibiting properties have been observed in other genes on chromosome 21: the *APP* gene, transcriptional factors such as ETS proto-oncogene 2 (*ETS2*), the angiogenesis suppressors Dscr1 and Dyrk1A and the *Collagen-18* gene whose fragment, endostatin, is an anti-angiogenic compound (15, 16).

Adiponectin and leptin are both involved in signaling pathways important in cancer in patients with DS. They have an opposite role, in fact leptin has many cancer-predisposing properties, such as pro-angiogenesis, reduction of apotosis, activation of Wnt, and Notch signaling (17) and it can also promote hematologic malignancies (18). Adiponectin, on the contrary, has several cancer-inhibiting properties: anti-angiogenesis, induction of apopotosis, activation of tumor-suppressors, and activation of cell signaling pathways (19–21). Several recent epidemiological studies (10, 22–27) showed that the risk of all major groups of solid tumors was decreased in patients with DS. Testicular tumors are an exception in this regard, as they seem to occur three times more often than expected in men with Down syndrome. The increased frequency of cryptorchidism (27) and testicular microlithiasis (28) may explain this evidence.

The association of DS with central nervous system tumors is extremely rare. In 1966, Miller (29) reported that among 56.199 individuals with DS only 5 developed brain tumors (estimated prevalence almost 9 per 100,000), while in the general population the estimated prevalence is 47.59/100,000 (22.31 for child, 48.49 for adolescents/young adults, and 57.75 for adult population) (30).

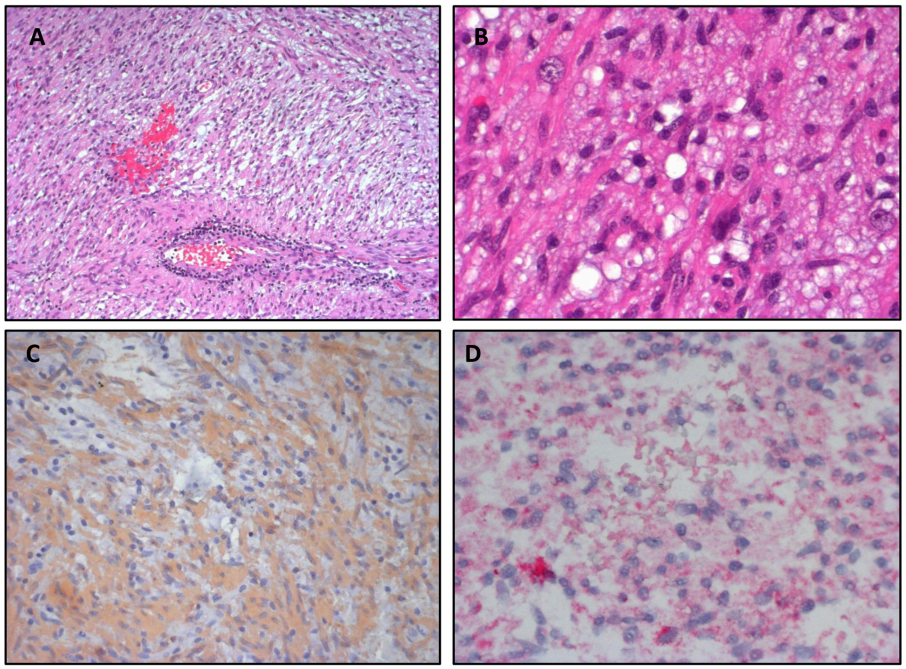

FIGURE 2 | Pleomorphic Xanthoastrocytoma. **(A,B)** Hematoxylin&Eosin staining shows a marked cellular pleomorphism, with the coexistence of several cell types. **(C)** Strong positivity for GFAP in immunohistochemistry. **(D)** Positivity for BRAF V600E in immunohistochemistry.

In the United States, brain tumors have been reported in 36 individuals with DS only, the vast majority of them represented by specific histological subgroups such as germ cells and mesenchymal tumors (13). Another study performed on post-mortem autopsies of children with DS revealed that only 3 out of 104 children presenting a malignancy had developed brain tumors (12).

One interesting example is Medulloblastoma. It is, in fact, the most common malignant brain tumor of childhood, but is particularly rare in the DS population and serves as perhaps the best example of the disparity of solid tumors in these individuals (31, 32). Similar to Medulloblastoma, Meningioma in DS was reported in only one case (33).

In this report we have described the first case, to the best of our knowledge, of Pleomorphic Xantoastrocytoma in a child affected by DS. PXA is a very rare tumor, its typical location is the temporal lobe and it usually involves both the superficial and the overlying meninges (34). Histologically, PXA is characterized by markedly pleomorphic cells, eosinophilic granular bodies, prominent reticulin deposition and a superficial meningocerebral location. PXA typically present a slow growth rate at presentation and the most common presenting symptoms are seizures. The overall survival rate is 80% at 5 years and 70% at 10 years.

Although PXAs are generally considered indolent neoplasms, they are associated with a higher frequency of recurrence, malignant transformation, and death, compared with other low-grade gliomas, such as pilocytic astrocytomas. The treatment of PXA typically involves surgical resection followed by radiological monitoring. Recurrent lesions or tumors that demonstrate anaplastic features at primary resection are treated with the same radiation and chemotherapeutic protocols used for high grade gliomas such as anaplastic astrocytoma and glioblastoma.

The mutation of the *BRAF* gene, which is typical of this tumor (7, 35), involves the activation of the MAP Kinase (MAPK) pathway, which has been shown to be the main molecular alteration present in Low Grade Gliomas (36). The most frequent mutation is the point mutation that occurs at codon 600 (*BRAF* p.V600E) that results in substitution of valine by glutamic acid.

Approximately 70% of pilocytic astrocytoma contain duplication of BRAF gene, which is rare in PXAs (37). The BRAF gene duplication leads to the formation of a fusion between the KIAA1549 locus and BRAF and the resulting protein displays a constitutively activated kinase activity, causing an aberrant activation of the downstream MAPK/ERK pathway.

Hsiao et al. found the *TMEM106B-BRAF* fusion in a case of PXA with anaplastic features in a 10-yr-old-female. This alteration results in replacement of the amino-terminal regulatory domain of BRAF with the amino-terminal region of TMEMB106B. They demonstrated that the fusion results in aberrant activation of *BRAF* signaling, with activation of MAPK/ERK pathway (38).

Inhibitors of MAPK pathway have been considered as a potential target therapy for these tumors. Among such inhibitors, Vemurafenib, a competitive small molecule that selectively recognizes the ATP binding domain of the *BRAF* p.V600E mutant, has proved to be effective in the treatment of metastatic melanoma, a neoplasm frequently associated with

BRAF mutations. More recently, an activity of this drug was proved also in pediatric *BRAF* p.V600E mutated malignant astrocytomas (39).

In the case presented the mutation *BRAF* p.V600E was screened in immunohistochemistry and later confirmed by Sanger sequencing. This finding allowed us to treat the patient with Vemurafenib. This is, to our knowledge, the first case of a DS patient treated with Vemurafenib. The therapy proved to be effective as the subsequent MRI staging evaluation revealed a partial response (according to RECIST criteria) and later controls proved a stabilization of the disease.

Children with DS often respond to chemotherapy treatment as well as children without DS. However, they are more likely to experience severe toxicity with standard chemotherapy regimens, particularly those requiring methotrexate, and this often leads to a reduction of the chemotherapy doses, leading to a less effective treatment (5, 40). The treatment has been well-tolerated by the patient without serious adverse effects, proving the safety of the drug. The only adverse effect reported was grade one skin toxicity.

The use of Vemurafenib has not yet been approved for pediatric patients affected by brain tumors and DS. Although central nervous system (CNS) tumors are the most common pediatric solid organ tumor, they are very rare in patients with DS. However, the possibility of development of brain tumors in DS should be kept in mind, especially in case of unusual neurological and endocrinological symptoms. In conclusion, we have shown the safety and efficacy of Vemurafenib in a pediatric patient with DS affected by PXA.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Internal Review Board of the Bambino Gesù Children's Hospital with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Internal Review Board of the Bambino Gesù Children's Hospital.

INFORMED CONSENT

The authors declare that written informed consent was obtained from the patient's parents for publication of this case report.

AUTHOR CONTRIBUTIONS

GP, FdB, AM, and FL designed the study. GP, FdB, GC, DV, AntC, and IA cured the collection of the data. FdB, GC, IA, AndC, AM, GSC, TC, and FD-C interpreted and analyzed the data. GP, DV, FdB, and GC drafted the manuscript. FD-C performed immunohistochemistry analysis. EA performed molecular analysis. AM and FL critically revised the manuscript for intellectual content.

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Elevated NLR May Be a Feature of Pediatric Brain Cancer Patients

Michal Yalon[1†], Amos Toren[1,2†], Dina Jabarin[2], Edna Fadida[3], Shlomi Constantini[3] and Ruty Mehrian-Shai[1]*

[1] Pediatric Hemato-Oncology, Edmond and Lilly Safra Children's Hospital and Cancer Research Center, Sheba Medical Center, Ramat Gan, Israel, [2] The Sackler School of Medicine, Tel-Aviv University, Tel Aviv, Israel, [3] Department of Pediatric Neurosurgery, Dana Children's Hospital, Tel-Aviv-Sourasky Medical Center, Tel Aviv, Israel

Pediatric brain tumors are the most common solid tumor type and the leading cause of cancer-related death in children. The immune system plays an important role in cancer pathogenesis and in the response to immunotherapy treatments. T lymphocytes are key elements for the response of the immune system to cancer cells and have been associated with prognosis of different cancers. Neutrophils on the other hand, which secrete pro-angiogenic and anti-apoptotic factors, enhance the ability of tumor cells to grow and develop into metastases. We conducted a retrospective study of 120 pediatric brain cancer patients and 171 elective pediatric patients hospitalized in Dana Children's Hospital and Sheba Medical Center. Data on age, sex, treatment, lymphocyte, neutrophil, and monocyte count were collected from routinely performed preoperative blood tests. Neutrophil-to-lymphocyte ratio (NLR), and the lymphocyte-to-monocyte ratio (LMR) were calculated and significance was determined by paired *T* test. $p < 0.05$ was considered as statistically significant. NLR was significantly higher in the pediatric brain cancer patients. The high NLR in pediatric brain cancer patients is the result of a combination of low lymphocytes and high neutrophils. Both of these factors can have a role in cancer development and propagation and also in response to therapy.

Keywords: pediatric brain cancer, neutrophils, lymphocytes, NLR, LMR

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Andrea Carai,
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Joseph Louis Lasky,
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Ute Katharina Bartels,
Hospital for Sick Children, Canada

***Correspondence:**
Ruty Mehrian-Shai
ruty.shai@sheba.health.gov.il

[†]These authors have contributed equally to this work

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INTRODUCTION

Pediatric brain tumors are the most common solid tumor type and the leading cause of cancer-related deaths in children aged 0–14 years (1). There is increasing evidence that malfunction of immune system may contribute to tumor development. Failure to eliminate tumor cells or keeping the tumor cells in a dormant state by the immune system are included in the "hallmarks of cancer" (2). Understanding these underlying mechanisms has provided a basis for the development of new immunotherapies against tumors.

Lymphocytes have, among others, an anti-tumor effect (3). T lymphocytes are key elements to eliminate cancer cells and their dysfunction have been associated with development of cancer (4) and prognosis for several cancer types.

Neutrophils and monocytes on the other hand, may promote tumor progression (5). Neutrophils which secrete pro-angiogenic and anti-apoptotic factors, enhance the ability of tumor cells to grow and develop into metastases (6). Elevated numbers of neutrophils in the peripheral blood is associated with poor outcome in several types of cancers (7). Tumor-associated neutrophils can acquire a pro-tumor phenotype, supporting tumor growth, and suppressing the antitumor

immune response (8). Myeloid-derived suppressor cells (MDSC) which are closely related to neutrophils and monocytes (9) play a major role in suppression of T cells (10, 11). Not only are levels of MDSC increased in the blood of patients with cancer, MDSC frequencies correlate to clinical cancer stage in several cancers including bladder cancer (12) and breast cancer (13).

Neutrophils, lymphocytes and monocytes have prognostic value in patients with a variety of common solid tumors (14). These patients are characterized by an increase in circulating neutrophil levels accompanied by a fall in circulating lymphocyte levels thus the neutrophil to lymphocyte ratio (NLR) is increased. In general, the blood NLR is high in patients with more advanced or aggressive cancers (15) and correlates with poor survival of patients with many solid tumors (16, 17).

Lymphocyte-to-monocyte ratio (LMR) has been suggested to be an important factor for predicting prognosis in patients with lung cancer, colon cancer and hematologic malignancies (18, 19). In a meta-analysis of adult solid tumors it was concluded that low pre-treatment LMR is an unfavorable prognostic factor (20).

In this study we investigated NLR and LMR in pediatric brain cancer patients on day of diagnosis before any treatment administration.

METHODS

Clinical data from pediatric brain cancer patients (M = malignant group) and elective pediatric patients hospitalized for hernia repair were retrospectively analyzed. Exclusion criteria were steroid treatment before the blood test and a history of concomitant diseases. Age, Sex, treatment, blood count plus differential were collected from blood tests performed before surgery in both groups. NLR, and the LMR were calculated and significance was determined by paired T-test. $p < 0.05$ was considered as statistically significant. ANOVA test was used to compare mean of NLR value in different brain tumor types.

RESULTS

Clinical data from 120 pediatric brain cancer patients and 171 elective pediatric patients hospitalized for hernia repair were retrospectively analyzed. The study was approved by the ethical review boards of both Sheba and Tel Aviv Sourasky Medical Centers and was consistent with the declaration of Helsinki. Cancer patients who received dexacort before the blood test were excluded. Children from the elective hernia surgery control group with known background diseases were also excluded. Ninety-nine cancer and 62 control patients that met the inclusion criteria and had all the data in the medical records were included in the analysis. Brain cancer patients consisted of 37 astrocytoma patients, 27 medulloblastoma patients, 25 patients with pilocytic astrocytoma, and 10 glioblastoma patients. Age, Sex, treatment, lymphocyte, Neutrophil count, and monocyte count were collected from routinely performed preoperative blood tests. NLR and the LMR were calculated and significance was determined by paired T-test. $P < 0.05$ was considered as statistically significant.

Male/female ratios in the study and control group were 64%/36% and 61%/29%, respectively. Mean age was 10.26 and 8.7 years old, respectively. No significant NLR and LMR differences were found between males and females in the patient group. **Table 1** summarizes the blood count results, differential, NLR, and LMR in both groups.

The mean percentage of neutrophils was higher in the patient group (64.3%) compared to the control group (56.11%) $p = 0.001$. In contrast, the mean percentage of lymphocytes was significantly higher in the control group (32.08%) compared to the patient group (27.11%) $p = 0.009$. As a result, the NLR was significantly higher in the patient group (4.59) compared to the control group (2.96) $p = 0.025$. Although the mean percentage of monocytes was higher in the patient group (6.1%) compared to the control group (3.3%) $p = 0.001$, there was no significant difference in LMR between the patient group (5.3) compared to the control group (4.2) ($p = 0.08$).

Table 2 summarizes the mean NLR results in different brain tumor types in children. Although NLR values of astrocytoma and pilocytic astrocytoma are lower than glioblastoma and medulloblastoma, no significant difference was found between the mean of NLR value in different tumor types by ANOVA test $\{F = 3.75, p = 0.13\}$.

DISCUSSION

Pediatric cancers and especially solid tumors can have devastating effects on young lives and their families. There is increasing evidence that failures of the immune system may contribute to tumor development. Lymphocytes have an important role in tumor cells elimination. Neutrophils and

TABLE 1 | Blood count results, differential, NLR, and LMR in brain cancer and hernia groups.

Parameter	Brain cancer	SD	Hernia	SD	*p*-value
White blood cells	10.2 X 109/L	5.3	11.2X109/L	10.9	0.550
Neutrophils	64.3%	16.5	56.1%	16.7	0.001
Lymphocytes	27.1%	14.3	32.0%	14.9	0.009
Monocytes	6.1%	2.9	3.3%	3.3	0.001
NLR	4.5	5.4	2.9	3.4	0.025
LMR	5.3	3.9	4.2	2.5	0.086

SD, Standard deviation.

TABLE 2 | NLR value results in different brain tumor types in children.

Tumor type	*N*	NLR
Astrocytoma	37	3.7
Medulloblastoma	27	6.0
Pilocytic astrocytoma	25	3.3
Glioblastoma	10	5.4

N, number of samples.

monocytes on the other hand, may promote tumor progression. In fact, suppression of the immune response to cancer cells is one of the impediments to develop effective immunotherapeutic approaches for glioma patients (21). Overcoming the suppression can be done by blocking the inhibitory signals of the immune system by anti CTLA4 and anti PD1/PDL1 therapy (22). On the other hand, persisting response of the inflammatory milieu paves the way to cancer (23). Neutrophils trigger and sustain a state of chronic inflammation (6) and high systematic immune-inflammation is significantly associated with a lower overall survival rate of patients with colon cancer (24), lung cancer (25), and breast cancer (26). Suppressing this arm of the immune response is necessary to limit proliferative signaling, angiogenesis, migration and invasion processes (2). Inhibitors of inflammatory pathways have been successful in pre-clinical tumor models and early clinical trials (27).

It has been shown that elevated NLR is predictive of poorer overall survival in patients with hepatocellular/ squamous cell carcinoma, GBM (28–30), pancreatic ductal adenocarcinoma (31), ovarian cancer (32) and pediatric sarcoma (33). In addition, preoperative elevated NLR reflects a systemic inflammatory response and is a predictor of poor survival in gastric cancer (16). Finally, high NLR is associated with poorer outcomes in patients receiving immune checkpoint inhibitors (34). In our study, pediatric brain cancer patients' blood samples present both increased neutrophil count and decreased lymphocyte count resulting in significantly higher NLR. Moreover, although no significant difference was found between the mean of NLR value in different tumor types in children, NLR values of astrocytoma and pilocytic astrocytoma are lower than glioblastoma and medulloblastoma. It is important to note that pediatric astrocytoma and pilocytic astrocytoma are with a more favorable prognosis compared to glioblastoma and medulloblastoma.

Using the body's own immune system to fight cancer is increasingly desirable treatment for cancer because of its' potential to specifically target the tumor while limiting damage to normal tissue. Because lymphocytes account for only 20–40% of the total WBC count, low levels of T cells may go unnoticed when WBC count is checked without a differential count of CD4+ (helper) T cells/CD8+ (suppressor) T cells/B cells. Reduced lymphocyte numbers may contribute to cancer development and may affect treatment, especially the current promising treatment of immunotherapy. Furthermore, the success of immunotherapy in tumors of the central nervous system depends on the immune microenvironment and lymphocyte tracking through the blood-brain barrier (BBB) into the central nervous system (35). The brain is presumably an immune-privileged organ due to the BBB (36). During gliomagenesis, the BBB is broken and circulating immune cells including T cells, B cells, macrophages, and MDSC cross the BBB (37). The resulting bi-directional communication between immune cells and glioma cells create an immunosuppressed microenvironment that promotes tumor survival and growth (38). Glioma-associated microglia and macrophages are attracted to the tumor (39) and can be polarized into M2 becoming tumor-supportive and immunosuppressive cells (40). The glioma immunosuppressive environment is further enhanced by elevated numbers of regulatory T cells (41). Immunophenotyping of pediatric brain tumors reveal immunosuppressive phenotype in higher grade tumors with more regulatory T cells present in these tumor types (42). Interestingly, it has been shown recently that intracranial tumors like GBM may cause depletion of mature T cells (43). The tumor imposed depletion of Sphingosine-1-phosphate receptor 1 from the T cell surface prevents their trafficking from lymphoid organs into the circulation. It may well be that in pediatric brain cancer patients the T cells are sequestered in the lymph nodes and can inversely be released to fight cancer by manipulations yet to be discovered. Thus, Immunotherapy via adoptive cell transfer, especially with T cells engineered to express chimeric antigen receptors, represents a promising approach (44). Recent evidence has demonstrated that systemic therapy for brain tumors is not limited by the BBB (45). Cellular therapy as well as reversing immune-suppression through immune checkpoint blockade are showing promising results for GBM (46). In light of these findings the prognostic as well as treatment response prediction value of the NLR warrants prospective validation in large cohorts of children with CNS tumors.

ETHICS STATEMENT

The study was retrospective study of clinical files. Clinical data from 120 pediatric brain cancer patients and 171 elective pediatric patients hospitalized for hernia repair were retrospectively analyzed. The study was approved by the ethical review boards of both Sheba and Tel Aviv Sourasky Medical Centers and was consistent with the declaration of Helsinki.

AUTHOR CONTRIBUTIONS

RM-S and DJ contributed to implementation, analysis, and interpretation of the data. RM-S, DJ, MY, AT, EF, and SC were involved in experimental design, in the writing of the manuscript, and have read and approved the final version.

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