

# Integrated analysis of pediatric glioblastoma reveals a subset of biologically favorable tumors with associated molecular prognostic markers

Andrey Korshunov · Marina Ryzhova · Volker Hovestadt · Sebastian Bender · Dominik Sturm · David Capper · Jochen Meyer · Daniel Schrimpf · Marcel Kool · Paul A. Northcott · Olga Zheludkova · Till Milde · Olaf Witt · Andreas E. Kulozik · Guido Reifenberger · Nada Jabado · Arie Perry · Peter Lichter · Andreas von Deimling · Stefan M. Pfister · David T. W. Jones

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**Abstract** Pediatric glioblastoma (pedGBM) is amongst the most common malignant brain tumors of childhood and carries a dismal prognosis. In contrast to adult GBM, few molecular prognostic markers for the pediatric counterpart have been established. We, therefore, investigated the prognostic significance of genomic and epigenetic alterations through molecular analysis of 202 pedGBM (1–18 years) with comprehensive clinical annotation. Routinely prepared formalin-fixed paraffin-embedded tumor samples were assessed for genome-wide DNA methylation

profiles, with known candidate genes screened for alterations via direct sequencing or FISH. Unexpectedly, a subset of histologically diagnosed GBM ( $n = 40$ , 20 %) displayed methylation profiles similar to those of either low-grade gliomas or pleomorphic xanthoastrocytomas (PXA). These tumors showed a markedly better prognosis, with molecularly PXA-like tumors frequently harboring BRAF V600E mutations and 9p21 (*CDKN2A*) homozygous deletion. The remaining 162 tumors with pedGBM molecular signatures comprised four subgroups: H3.3 G34-mutant (15 %), H3.3/H3.1 K27-mutant (43 %), IDH1-mutant (6 %), and H3/IDH wild-type (wt) GBM (36 %). These subgroups were associated with specific cytogenetic aberrations, *MGMT*

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A. Korshunov · D. Capper · J. Meyer · D. Schrimpf · A. von Deimling  
Clinical Cooperation Unit Neuropathology (G380), German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

A. Korshunov · D. Capper · J. Meyer · D. Schrimpf · A. von Deimling  
Department of Neuropathology, Heidelberg University Hospital, Im Neuenheimer Feld 224, 69120 Heidelberg, Germany

A. Korshunov · D. Capper · D. Schrimpf · A. E. Kulozik · G. Reifenberger · P. Lichter · A. von Deimling · S. M. Pfister  
German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

M. Ryzhova  
Department of Neuropathology, NN Burdenko Neurosurgical Institute, 5-th Tverskaya\_Yamskaya str. 16, Moscow, Russia

V. Hovestadt · P. Lichter  
Division of Molecular Genetics (B060), German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

S. Bender · D. Sturm · M. Kool · P. A. Northcott · S. M. Pfister · D. T. W. Jones (✉)  
Division of Pediatric Neurooncology (B062), German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany  
e-mail: davidjones@cantab.net; david.jones@dkfz.de

D. Sturm · T. Milde · O. Witt · A. E. Kulozik · S. M. Pfister  
Department of Pediatric Hematology and Oncology, Heidelberg University Hospital, Im Neuenheimer Feld 430, 69120 Heidelberg, Germany

O. Zheludkova  
Department of Neuro-Oncology, Russian Scientific Center of Radiology, Profsoyuznaya str. 36, Moscow, Russia

T. Milde · O. Witt  
Clinical Cooperation Unit Pediatric Oncology (G340), German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

G. Reifenberger  
Department of Neuropathology, Heinrich Heine University, Moorenstr. 5, 40225 Düsseldorf, Germany

methylation patterns and clinical outcomes. Analysis of follow-up data identified a set of biomarkers feasible for use in risk stratification: pedGBM with any oncogene amplification and/or K27M mutation ( $n = 124$ ) represents a particularly unfavorable group, with 3-year overall survival (OS) of 5 %, whereas tumors without these markers ( $n = 38$ ) define a more favorable group (3-year OS ~70 %). Combined with the lower grade-like lesions, almost 40 % of pedGBM cases had distinct molecular features associated with a more favorable outcome. This refined prognostication method for pedGBM using a molecular risk algorithm may allow for improved therapeutic choices and better planning of clinical trial stratification for this otherwise devastating disease.

**Keywords** Glioblastoma · Pediatric · Brain tumor · Methylation · Prognostic · Subgroup · Survival · *CDKN2A* · *BRAF*

## Introduction

Glioblastoma (GBM) is the most common primary malignant brain tumor and has an extremely dismal prognosis, with fewer than 5 % of patients surviving beyond 5 years after diagnosis [28, 30]. It remains incurable with current treatment strategies, which typically consist of surgical tumor removal followed by radiotherapy in combination with concurrent and maintenance temozolomide (TMZ) [10, 44, 51]. Incidence of GBM increases with age, accounting for about half of all malignant brain tumors in adults, but 10–15 % of pediatric CNS tumors (when also considering DIPG and high-grade glioma NOS) [30]. Although childhood and adult GBM share a related histopathological appearance and comparable clinical outcomes, it is now becoming apparent that these tumors are molecularly distinct entities with differing genomic and epigenomic landscapes [5, 15, 20, 21, 33, 45, 46]. For example, recently identified recurrent somatic mutations in *H3F3A* (encoding the histone variant H3.3) and *H3.1* genes (*HIST1H3B* and *HIST1H3C*) are frequent in pediatric

GBM (approximately 40 % of tumors) but rare in adults [1, 7, 14, 22, 27, 41, 46, 48, 52, 53]. Conversely, prototypic alterations of adult primary GBM (e.g., *EGFR* amplification, *CDKN2A/B* homozygous deletions, *PTEN* mutations) are very infrequent in childhood [4, 5, 25, 32–34, 36, 37, 39, 45]. In addition, *IDH1* and *IDH2* mutations, which are found in ~90 % of secondary GBM, are also rare in pediatric tumors [31, 33, 46, 54].

While a multitude of molecular prognostic markers have been proposed for adult GBM (e.g., [50]), prognostic factors for their pediatric counterpart are yet to be precisely elaborated. Studies investigating pediatric GBM (pedGBM) for the prognostic value of mutations, gene expression patterns and copy number aberrations have tended to be inconsistent or contradictory, which may be explained by the relatively small cohort sizes and/or heterogeneity of the applied treatment modalities [4, 8, 11, 13, 16, 25, 26, 34, 36, 42, 47]. Cohort size is of particular importance in light of the extensive biological heterogeneity of these tumors, as highlighted by a recent methylome analysis of a pedGBM series that found distinct subgroups correlating with specific genetic alterations and clinical patterns [46], and by further recent in-depth genomics studies (e.g., [14, 53]). In the current study, we investigated the prognostic significance of genomic and epigenetic events assessed through a comprehensive molecular analysis of 202 pedGBM uniformly treated with surgery and radio-chemotherapy, and identified a robust set of markers that may improve the future clinical stratification of pedGBM patients.

## Materials and methods

### Patient population

Tissue samples (mostly formalin-fixed paraffin-embedded) were obtained from 202 pediatric patients (age 1–18 years) with a histological diagnosis of “glioblastoma, WHO grade IV” (Table 1). The median age was 12 years/months (95 % CI Y–Z), and the median follow-up was 16 months. At the time of this analysis, 133 patients (66 %) have died. None of these patients had a clinical history of previous gliomas, and most were treated in the Burdenko Neurosurgical Institute (Moscow) between 1998 and 2013. Informed consent was obtained from all patients or parents. According to neuroradiological and intraoperative data, just over half of the tumors was located in the cerebral hemispheres (112, 55 %), with 83 tumors (41 %) in the thalamus/basal ganglia region and seven (3 %) in the pons/brain stem (i.e., diffuse intrinsic pontine gliomas). Patients older than 3 years ( $n = 187$ ) were treated with surgery (gross total or subtotal tumor resection) followed by radiotherapy (limited field fractionated external beam radiotherapy with a dose

N. Jabado  
Division of Experimental Medicine, Department of Human Genetics, McGill University, Place Toulon, PT-239, Montreal, QC H3Z 2Z3, Canada

A. Perry  
Department of Pathology, Brain Tumor Research Center, University of California, 505 Parnassus Avenue, CA 94143-0102 San Francisco, USA

A. Perry  
Department of Neurological Surgery, Brain Tumor Research Center, University of California, 505 Parnassus Avenue, CA 94143-0102 San Francisco, USA

**Table 1** Clinical and molecular variables in a set of 202 pediatric gliomas originally diagnosed as GBM

Variable	H3.3 G34 ( <i>n</i> = 24)	H3.3/H3.1 K27 ( <i>n</i> = 69)	H3/IDH WT ( <i>n</i> = 59)	IDH1 ( <i>n</i> = 10)	PXA-like ( <i>n</i> = 27)	LGG-like ( <i>n</i> = 13)
Age ≤3	0 %	1 %	7 %	0 %	11 %	54 %
Median age	14	10	12	15.5	11	1
Range	9–17	3–18	1–18	12–18	1–18	1–14
Gender M vs. F	71/29 %	42/58 %	51/49 %	70/30 %	56/44 %	69/31 %
Hemispheric	100 %	1 %	81 %	100 %	70 %	77 %
Midline	0 %	99 %	19 %	0 %	30 %	23 %
GTR vs. STR	58/42 %	26/74 %	47/53 %	30/70 %	37/63 %	15/85 %
Radiotherapy	100 %	99 %	95 %	100 %	89 %	62 %
3-year PFS	18 %	0 %	15 %	70 %	31 %	59 %
3-year OS	37 %	0 %	26 %	90 %	70 %	91 %
Metastatic dissemination	13 %	54 %	14 %	0 %	7 %	8 %
Amplifications	46 %	42 %	71 %	20 %	4 %	0 %
<i>EGFR</i>	17 %	3 %	31 %	0 %	0 %	0 %
<i>MYC/N</i>	8 %	9 %	29 %	0 %	4 %	0 %
<i>PDGFRA</i>	13 %	23 %	25 %	0 %	0 %	0 %
<i>CCND/CDK</i>	29 %	20 %	41 %	20 %	0 %	0 %
9p homo del	17 %	1 %	24 %	60 %	30 %	0 %
<i>BRAF</i> mutations	0 %	4 %	2 %	0 %	48 %	31 %
10q loss	50 %	36 %	41 %	70 %	15 %	0 %
<i>MGMT</i> methyl	75 %	3 %	19 %	90 %	22 %	15 %

54–59.4 Gy on the tumor bed) and adjuvant chemotherapy with TMZ [10]. Fifteen patients 3 years or younger were treated initially with chemotherapy alone after surgery, on the basis of the Baby POG protocol [12]. Recurrent tumors were treated with polychemotherapy using various regimens. Patients receiving biopsy only were not included in this study, since sufficient material for molecular analysis was a prerequisite. Histological diagnosis was based on the current WHO criteria for GBM—an astrocytic glioma with brisk mitotic activity, microvascular proliferation and/or necrosis [28]. Full details of the cohort are given in Supplementary Table 1.

#### Molecular analysis

DNA was extracted from tumors and analyzed for genome-wide methylation patterns using the Illumina HumanMethylation450 BeadChip (450k) array as previously described [17, 46]. Processing of DNA methylation data and detection of copy number aberrations were performed with custom approaches described in Sturm et al. [46]. For unsupervised hierarchical clustering we selected the most variably methylated probes across the dataset (according to standard deviation thresholds). Samples were clustered using 1-Pearson correlation coefficient as the distance measure and average linkage (*x*-axis). Methylation probes were

reordered by hierarchical clustering using Euclidean distance and average linkage (*y*-axis).

To evaluate the methylation status of the *MGMT* promoter region, we used the *MGMT\_STP27* logistic regression model [2]. For a small number of cases that did not have sufficient DNA remaining for 450k analysis (*n* = 12), *MGMT* promoter methylation was measured using the CpG WIZ™ MS-PCR Kit (Merck Millipore, Germany). In addition, mutational analysis for *H3F3A* (H3.3), *HIST1H3B* and *HIST1H3C* (H3.1), *IDH1*, and *BRAF* genes was performed using targeted Sanger sequencing. Primer sequences are available on request. FISH analyses were performed using commercially available probes to human oncogenes *EGFR*, *MYC*, *MYCN*, *PDGFRA*, *CDK4* (all from Abbott), and *MET*, *CDK6*, *CCND1* and *CCND2* (all from Abnova; Supplementary Table 2).

#### Statistics

The distribution of overall survival (OS) was calculated according to the Kaplan–Meier method. OS was calculated from the date of diagnosis until death of patient from disease or last contact for patients who were still alive. For multivariate analysis, Cox proportional hazards regression models were used. Estimated hazard ratios are provided with 95 % confidence intervals and a *p* value from the Wald test. Tests with a *p* value below 0.05 were considered significant.

## Results

Hierarchical clustering of genome-wide DNA methylation data showed four tumor subsets within the overall patient cohort (Fig. 1). Three of these clusters were defined by K27M or G34R mutations in H3.3 (or rarely H3.1 for K27M) or *IDH1* mutations, as expected [46]. None of the *IDH1*-mutant tumors showed evidence for a precursor lesion, unlike in adults where these mutations typically arise in secondary GBM. The group of samples lacking any of these mutations, however, showed a much greater degree of heterogeneity in their methylome profiles. We, therefore, re-analyzed the H3/*IDH* wild-type (wt) tumors, to delineate potential additional subgroups. Given the unexpectedly long survival of some of the patients in this cohort and the broad histological spectrum of pediatric gliomas, we additionally included 10 low-grade gliomas (LGGs; namely pilocytic astrocytoma) and 10 pleomorphic xanthoastrocytomas, as well as 12 normal brain samples, for comparison. A notable fraction of tumors with a histological appearance of GBM was found to more closely resemble either the PXAs or LGGs (27/202 with PXA, 13 % and 13/202 with LGG, 6 %; Supplementary Figure 1). A blinded review of 18 of these tumors by an experienced neuropathologist (A. Perry) confirmed their histological appearance as GBM in 15 cases, with only three tumors thought to have features of PXA or LGG upon re-examination (example histologies per group shown in Supplementary Figure 2). Tumors with DNA methylation patterns similar to those of low- or intermediate-grade lesions typically occurred in younger patients (median age 7 vs. 12 years;  $p = 0.0005$ , two-tailed  $t$  test), including 10 of the 15 patients aged 3 years or younger (a full list of tumor and patient characteristics is given in Supplementary Table 1). The genomic alterations observed in these two subsets also stood out compared with the remainder of the samples. In the molecularly LGG-like group, 4/13 tumors (31 %) harbored a BRAF V600E mutation, and all showed generally balanced genomes in terms of copy number (no focal oncogene amplifications detected, and no loss of the *CDKN2A/B* locus at 9p21). In the group resembling PXA on the basis of DNA methylation, 13/27 tumors (48 %) displayed a V600E mutation, and 8/27 (30 %) showed homozygous 9p21 deletion, with three carrying both lesions. Both genetic alterations have previously been described as frequent events in PXA [24, 49]. Only four of the remaining 162 samples (2 %) were BRAF V600E mutant. Importantly, tumors displaying a molecularly lower grade signature carried a much more favorable prognosis than those with molecular high-grade features, with 3-year OS of 91 % for the LGG-like samples and 70 % for the PXA-like tumors (Fig. 2).

Our further analyses focused on the 162 tumors with pedGBM molecular signatures based on 450k array

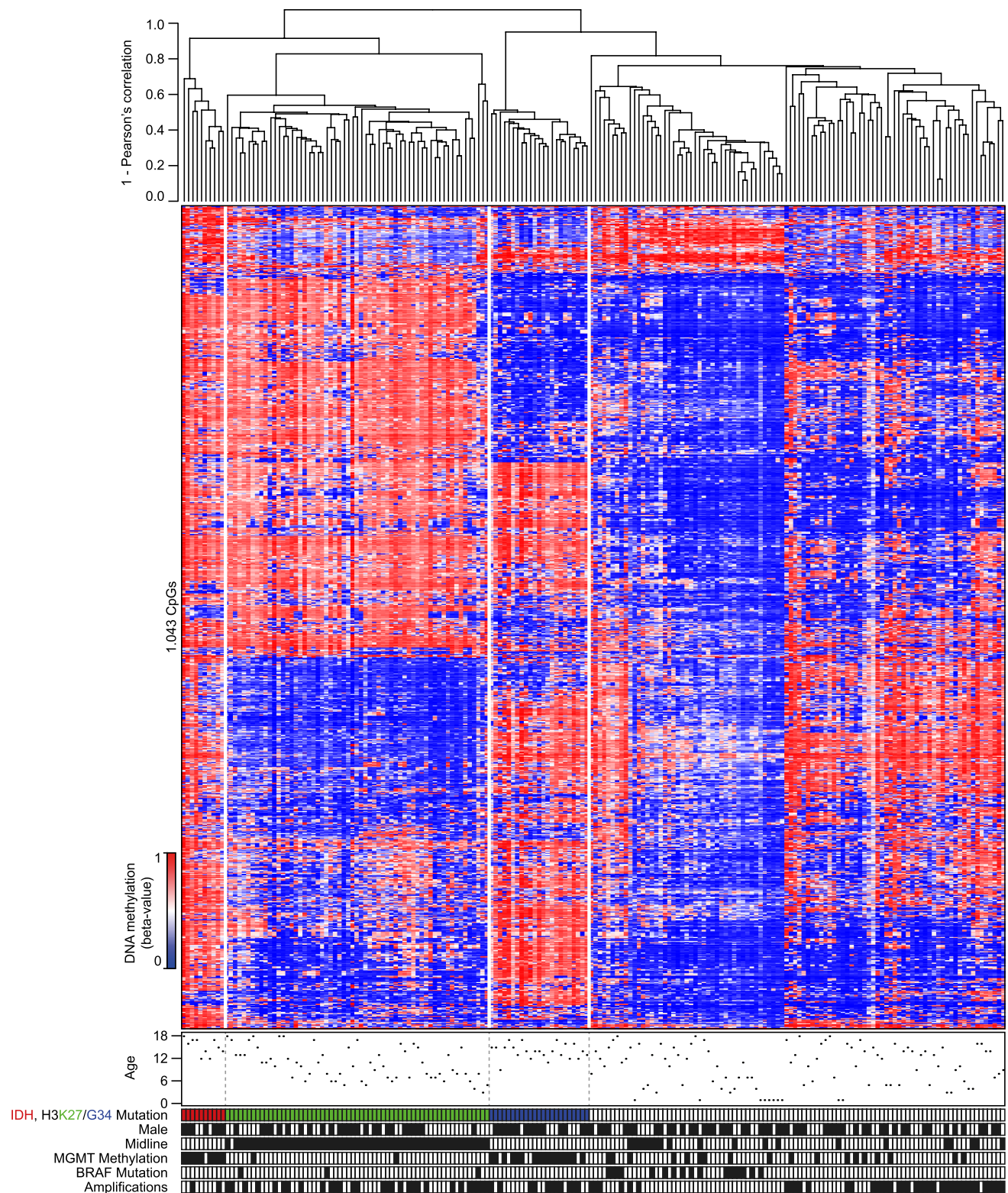
profiling. These could be further classified into four distinct methylation subgroups: H3.3 G34-mutant ( $n = 24$ , 15 %), H3.3/H3.1 K27-mutant ( $n = 69$ , 43 %), *IDH1*-mutant ( $n = 10$ , 6 %), and H3/*IDH* wt-GBM ( $n = 59$ , 36 %). The predicted mutations by DNA methylation subgrouping were confirmed in all cases by direct sequencing of *H3F3A*, *HIST1H3B/HIST1H3C* and *IDH1*, with *G34R*, *K27M* (65 *H3.3* and four *H3.1*), and *IDH1*-mutant tumors exclusively distributed to the corresponding methylation subgroups. Eighty-four pedGBM (52 %) disclosed amplifications of one or more oncogenes, with higher frequency in wt-GBM (71 vs. 46 % in G34, 42 % in K27 and 20 % in *IDH1* mutants, respectively) and differences in the types of amplifications observed in different molecular subgroups (Table 1). The most commonly amplified gene was *PDGFRA* (30/162, 19 %), followed by *EGFR* (26/162, 16 %) and *MYCN* (17/162, 10 %). The overall frequency of selected oncogene amplifications is listed in Supplementary Table 3. In addition to focal amplification, deletions of the *CDKN2A/B* locus and of chromosome arm 10q were also examined. Homozygous 9p21 deletion was found in 25 tumors overall (15 %), and was more frequent in *IDH1*-mutant pedGBM (6/10, 60 %) but very rare in K27M tumors (1/69, 1 %). Heterozygous loss of 10q was found in 42 % of pedGBM (68/162), and was distributed across all molecular subtypes.

*MGMT* promoter methylation was found in 40/162 pedGBM (25 %), with a marked variability amongst the molecular subgroups. This epigenetic event was most frequently found in *IDH1*- (90 %) and G34-mutant (75 %) subtypes and occasionally in wt tumors (19 %), whereas K27 tumors very rarely showed *MGMT* methylation (3 %).

In line with previous reports, the pedGBM subgroups showed specific patterns of tumor location in the CNS. K27 tumors were almost exclusively located in midline structures (thalamus and brain stem, with only one exception), whereas the other tumor subgroups arose almost exclusively in the cerebral hemispheres, with the exception of a small subset of wt-GBM detected in the basal ganglia or pons. Over half of K27-mutant GBM developed post-treatment CNS tumor dissemination, whereas for other pedGBM molecular subsets the frequency of craniospinal metastases was much lower. No tumors displayed evidence of gross metastatic spread at initial presentation.

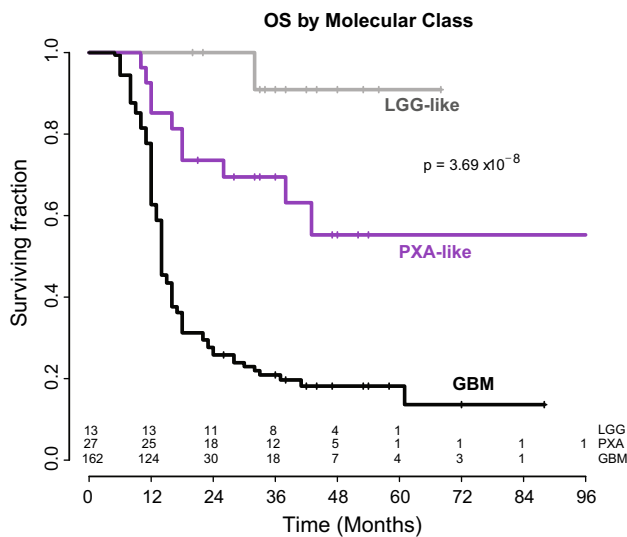
Correlation of pedGBM subgroups and other clinical or molecular parameters with patient outcome revealed a number of significant associations. *IDH1*-mutant tumors showed a significantly longer survival than the other subgroups ( $p = 6.5 \times 10^{-5}$ ), while K27M GBM showed a significantly worse outcome ( $p = 7.6 \times 10^{-13}$ ) (Fig. 3a). G34R-mutant tumors showed a trend towards increased survival, but this was not significantly different from WT tumors ( $p = 0.16$ ). Univariate overall survival analysis for various clinical and





**Fig. 1** Unsupervised hierarchical clustering of DNA methylation of the full tumor cohort based on the 1043 most variable methylation probes ( $SD > 0.30$ ). *Black bars* positive for given variable; *white bars* negative for given variable. ‘*Amplifications*’ the presence of any

oncogene amplification as detailed in the text. IDH1, H3 K27 and H3 G34-mutant groups are clearly distinguished, with a more heterogeneous H3/IDH wild-type group on the *right-hand side*



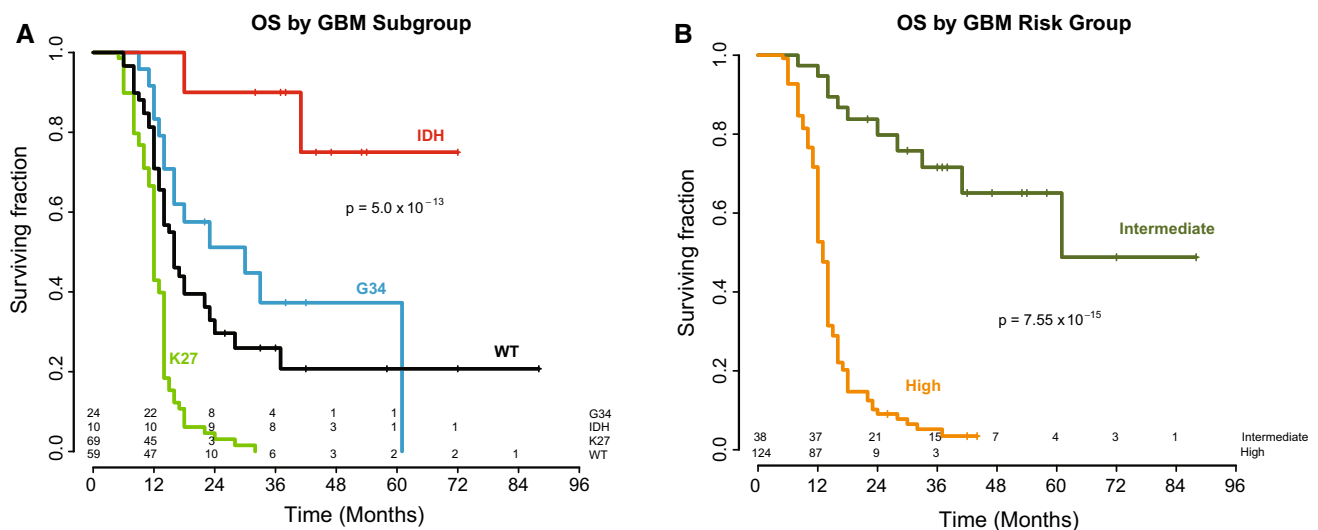
**Fig. 2** Survival analysis for various molecular tumor subsets defined by DNA methylation profiling in histologically diagnosed glioblastoma. Tumors with methylation patterns more closely matching low-grade glioma (*LGG-like*) or pleomorphic xanthoastrocytoma (*PXA-like*) have a significantly better prognosis. *OS* overall survival

molecular parameters revealed that midline tumor location, K27M mutation and presence of oncogene amplification(s) were each associated with poor outcome, whereas male gender, presence of G34 or *IDH1* mutations, 9p21 deletion and *MGMT* promoter methylation were associated with a more favorable clinical course (Table 2). Some variables were also found to be significant within particular GBM subsets. For G34-mutant tumors, male gender was associated with a

significantly better prognosis ( $p = 0.04$ ), while presence of an oncogene amplification was a negative prognostic marker ( $p = 0.001$ ). Amplifications were also associated with poor outcome in wt tumors ( $p = 0.002$ ; Supplementary Table 4). None of the tested clinical or molecular variables were significantly associated with outcome within the K27 or *IDH1* pedGBM groups. A small proportion of patients were found to survive  $\geq 36$  months after diagnosis, which would be considered as relatively ‘long-term’ survival with GBM. The clinical patient data and molecular features of these tumors are summarized in Supplementary Table 5.

Multivariate analysis identified the presence of amplified oncogene(s) and K27M mutation as independent significant prognostic markers for poor outcome amongst the overall pedGBM cohort (each at  $p < 0.01$ ), while *IDH1* mutation ( $p = 0.03$ ) was a significant independent marker of longer survival (Table 2).

The above analyses together led us to propose a model for stratification of pedGBM into high- or intermediate-risk groups, based on the presence of K27 mutation and/or oncogene amplifications. In this, tumors harboring any of the focal oncogene amplifications assessed by FISH, or with a K27M mutation would be considered as high risk, while tumors lacking these markers may be considered as intermediate risk. Applying this model, the high-risk group displayed a 3-year overall survival of 5 %, while in the intermediate group it was 72 % ( $p = 7.55 \times 10^{-15}$ ; Fig. 3b), suggesting potential clinical utility of this classification. The intermediate-risk group showed an enrichment for male patients, hemispheric location, G34/*IDH1* mutations and *MGMT* methylation, while  $>90$  % of cases displaying tumor dissemination through



**Fig. 3 a** Survival analysis for molecular subtypes of 162 pediatric glioblastomas according to *IDH1* and Histone 3 mutation status. Tumors with LGG-like or PXA-like profiles were excluded from this analysis. **b** Molecular stratification of pedGBM. pedGBM with K27

mutation and/or oncogene amplification ( $n = 124$ ) were designated ‘high’ risk, while those lacking these features were considered ‘intermediate’ risk ( $n = 38$ ). *OS* overall survival

**Table 2** Results of univariate and multivariate survival analysis for pediatric glioblastoma ( $n = 162$ )

Variable	Uni HR	<i>P</i>	Multi HR	<i>P</i>
Age: $\leq 3$ vs. $> 3$ years	0.68 (0.22–2.14)	0.51	NA	–
Gender: male vs. female	0.59 (0.41–0.85)	<b>0.005</b>	0.86 (0.59–1.25)	0.42
Location: midline vs. hemispheric	3.44 (2.35–5.03)	<b>&lt;0.001</b>	1.15 (0.57–2.35)	0.70
Resection: GTR vs. STR	0.81 (0.56–1.17)	0.26	NA	–
Amplifications: yes vs. no	1.89 (1.31–2.74)	<b>&lt;0.001</b>	1.84 (1.25–2.69)	<b>0.002</b>
9p21 homo deletion: yes vs. no	0.50 (0.29–0.88)	<b>0.02</b>	1.09 (0.59–2.02)	0.78
10q loss: yes vs. no	1.12 (0.78–1.60)	0.53	NA	–
<i>K27M</i> mutation: yes vs. no	3.88 (2.65–5.70)	<b>&lt;0.001</b>	2.84 (1.38–5.87)	<b>0.005</b>
<i>G34R</i> mutation: yes vs. no	0.49 (0.28–0.85)	<b>0.01</b>	0.92 (0.43–1.99)	0.84
<i>IDH1</i> mutation: yes vs. no	0.10 (0.02–0.41)	<b>0.001</b>	0.18 (0.04–0.85)	<b>0.03</b>
<i>BRAF</i> mutation: yes vs. no	1.61 (0.59–4.38)	0.35	NA	–
<i>MGMT</i> : methylated vs. unmeth.	0.32 (0.20–0.52)	<b>&lt;0.001</b>	0.79 (0.41–1.52)	0.48

Hazard ratios for tumors with a clear molecular GBM signature ( $n = 162$ ; excluding PXA-like or LGG-like samples) were calculated using a Cox proportional hazards regression model. Values in parentheses indicate 95 % confidence intervals. Significance *p* values are calculated according to the Wald test;  $p < 0.05$  was considered significant. Only variables which were significant upon univariate analysis (Uni HR) were taken into the multivariate model (Multi HR)

Bold values indicate  $p < 0.05$

**Table 3** Clinical and molecular variables in high- and intermediate-risk pediatric glioblastoma molecular subsets

Variable	High-risk pedGBM ( $n = 124$ )	Intermediate-risk pedGBM ( $n = 38$ )
Age $\leq 3$ years	2 %	5 %
Median age (range)	11 (3–18)	14 (1–18)
Gender: male/female	43/57 %	74/26 %
Hemispheric/midline	37/63 %	97/3 %
Resection GTR/STR	34/66 %	55/45 %
3-year OS	5 %	72 %
Metastatic dissemination	36 %	8 %
G34 mutant	9 %	34 %
IDH mutant	2 %	21 %
<i>MGMT</i> methylation	16 %	53 %
<i>BRAF</i> V600E mutation	3 %	0 %
9p21 homozygous deletion	11 %	29 %
10q loss	47 %	26 %

A summary of clinicopathological features according to molecular risk group in 162 tumors with molecular GBM signatures (PXA-like and LGG-like tumors excluded)

the CNS at recurrence were found in the high-risk group (Table 3). Therefore, together with the PXA/LGG-like cases, 78/202 (39 %) pedGBM had distinct molecular features associated with a more favorable clinical outcome.

## Discussion

Given that pediatric GBMs are clinically and biologically heterogeneous, improvements in therapy will require

patient stratification based upon their molecular hallmarks. In the current study, we have investigated a representative cohort of homogeneously treated primary pedGBM using genome-wide DNA methylation profiling and other targeted methods for verification. Array-based DNA methylation analysis has significant potential for modern molecular pathology, because it is technically applicable for everyday practice using small amounts of DNA extracted from routine FFPE samples [17]. Moreover, this analysis allows the simultaneous evaluation of various components of a tumor's molecular signature, including the global methylome (which may correlate with specific point mutations in, e.g., *IDH1* or *H3F3A*), target gene promoter methylation status (e.g., *MGMT*) and DNA copy number alterations [46].

Application of this technique allowed us to identify a set of tumors (almost 20 %) originally diagnosed as “GBM” (confirmed upon blinded histological re-evaluation in most cases), but which more closely resembled either PXA or LGG in terms of their DNA methylation profile. Patients suffering from these tumors showed significantly better OS in comparison to molecularly confirmed GBM, suggesting a distinct origin and less malignant nature of these tumors. There is known morphological overlap between GBM and ‘anaplastic’ PXA (aPXA) in particular, with anaplastic features portending a poorer prognosis [18]. There are currently no highly sensitive, specific and reproducible morphological criteria for the distinction of aPXA from other malignant gliomas. Some studies have shown that neuroimaging and histological characteristics of ‘epithelioid’, ‘rhabdoid’, and/or giant cell GBM overlap greatly with those described in aPXA [6, 23]. The frequency of *BRAF*

V600E mutation in ‘true’ GBM vs. PXA is also currently unclear, although it is reported as a much more common feature in the latter group [40]. It is notable that very few molecular GBMs in our series harbored this alteration (4/162, 2 %), while roughly half of the molecularly PXA-like tumors were V600E mutant (13/27, 48 %). Although their outcome was significantly better in terms of overall survival, the PXA-like tumors still exhibited a high rate of recurrence (18/27, 67 %, recurred within the follow-up period), which calls into question the efficacy of current GBM treatment protocols for these lesions. Since therapy with targeted BRAF and/or MEK inhibitors may represent a more rational regime for these (BRAF-mutated) PXA-like cases, it is very important to identify them accurately, regardless of their histological appearance [38]. Interestingly, a recent report highlighted a similar subset of patients with secondary high-grade glioma (sHGG) arising from a lower grade precursor lesion, that were enriched for BRAF V600E mutations and *CKDN2A* deletion [29]. These tumors showed a longer latency to malignant transformation and a better prognosis relative to BRAF-wild-type sHGG. We believe it likely that this group of tumors is biologically related to what we define as PXA-like tumors, with frequent BRAF V600E mutation and 9p21 deletion and intermediate outcomes between WHO Grade I lesions and clear molecular GBM. Further clarification as to the nature of this apparently distinct subset through detailed analysis of additional cohorts would, therefore, be of significant value.

Tumors with GBM histology but an LGG molecular signature were highly enriched in infants, and were also associated with favorable overall survival. Some previous studies have shown that congenital and infant GBMs are chemotherapy-sensitive tumors and associated with good outcomes [10, 35]. In contrast, infant LGGs are often reported as having a markedly inferior prognosis compared with their older counterparts [3, 43]. These observations may indicate a distinct biological behavior of LGG and high-grade glioma (HGG) in infants compared with older children. It may also be the case, however, that there is simply a much higher degree of diagnostic uncertainty in gliomas of all grades in very young children, as tumors of infancy are diagnosed at an earlier, less differentiated growth phase. For example, it has been reported that more malignant-looking ‘pilomyxoid’ astrocytomas often display a more differentiated appearance upon recurrence [9]. Chemotherapy-induced differentiation of infant ‘GBM’ into histologically low-grade glioma has also previously been reported [19]. The mixing of true biological LGG and HGG into different histological grades could result in the observed discrepancies in terms of clinical outcome in infants. Our data indicate that a ‘low-grade’ epigenetic signature is a hallmark of some GBM-like infantile tumors,

which could be used to identify a set of patients with a more favorable prognosis. Closer examination of such infant gliomas with high-grade histology yet low-grade molecular signature (and vice versa) is clearly warranted in future clinical trials.

Exclusion of the above tumors enabled us to further characterize the subset of pedGBM with molecular high-grade signatures, where previous studies looking at prognostic markers may have been somewhat hampered by cohort sizes; heterogeneous treatment; a lack of stratification into biological subgroups, and potentially also by intermixing with unidentified molecularly lower grade lesions [4, 8, 11, 13, 16, 25, 26, 34, 36, 42, 47]. Median overall survival in this group was only 14 months, comparable to their adult GBM counterparts. We also confirmed the presence of four biological subgroups of GBM based on their global DNA methylation patterns, which strongly correlate with genomic alterations. More than half of pedGBM had hotspot mutations in histone H3-encoding genes, thus confirming their prominent biological significance in pedGBM tumorigenesis. Tumors allocated to the various molecular subgroups also showed clear differences in clinical features, including tumor location and prognosis, as previously reported (e.g., reviewed in [45]). In keeping with earlier reports, the current study confirmed K27 pedGBM as the most unfavorable molecular variant [46]. These tumors: (1) are associated with deep midline locations and, as a consequence, have limited options for tumor resection; (2) develop frequent craniospinal tumor dissemination after treatment and (3) lack *MGMT* promoter methylation in almost all cases, implying a low efficacy of TMZ-based therapy. While the frequency of tumor dissemination in the K27 group is not yet fully understood (it may, for example, be related to their close contact with the ventricular system), it suggests that these highly aggressive lesions will remain a particular clinical challenge, even if better methods for local tumor control are identified. This subset may, therefore, warrant further intensified craniospinal therapy, with the addition of targeted epigenetic modifiers as they become available. On the other end of this clinical spectrum are G34- and *IDH1*-mutant tumors, which carried a more favorable prognosis, as previously described [46]. It is possible to speculate that the almost universal *MGMT* methylation identified in these groups may correlate with enhanced responsiveness to TMZ and thus better outcomes. Particularly for G34-mutant GBM, where this link has not previously been established, assessment in future clinical trials is of critical importance. Wild-type GBMs occupy an intermediate prognostic niche with a wide range of clinical outcomes, where various oncogene amplifications could portend especially unfavorable prognosis and resistance to standard treatment modalities. A low proportion of *MGMT* methylation in the wt group (19 %) further suggests that TMZ may be of limited benefit,



with targeted RTK/MAPK/mTOR inhibitors possibly representing a more rational alternative.

In conclusion, this study demonstrates the significant molecular heterogeneity of histologically similar pediatric gliomas, as detected by global DNA methylation analysis. The recognition of a significant subset of tumors (particularly in infants) which are histologically malignant but molecularly and prognostically lower grade, is an important issue to be further assessed in additional cohorts. The utility of array-based DNA methylation profiling or other markers to identify such cases also requires further validation. Subgroups of true molecular high-grade tumors showed close correlations with clinical and biological parameters that may be predictive of response to therapy (e.g., *MGMT* methylation). Furthermore, a set of potential prognostic biomarkers was established that may be of use for the stratification of pedGBM into clinically relevant subsets. This grouping, applicable from minimal quantities of standard FFPE material, could enable rapid pedGBM prognostication in routine diagnostic laboratories. Practical and reliable prognostication of pediatric glioma through molecular risk algorithms will be essential for rational stratification in clinical trials and for guiding optimal targeted therapeutic regimes. In this context, our findings provide a framework for such stratification as a basis for verification in additional clinical cohorts.

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