

# Loss of 5-hydroxymethylcytosine and intratumoral heterogeneity as an epigenomic hallmark of glioblastoma

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**Abstract** Glioblastoma (GBM) is the most malignant neoplasm with predominant astrocytic differentiation and the most frequent primary brain tumor of the adult. Here, we investigated 170 human GBM specimens deriving from 162 patients, as well as 66 healthy control tissue specimens deriving from 27 patients, and analyzed the amount of 5-hydroxymethylcytosine (5hmC) in GBMs compared to normal brain and tumor infiltration zones. Additionally, we correlated the amount of 5hmC with two different proliferation markers, Ki67 and H3S10p. Genetic characterization of GBMs enabled us to analyze the effect of isocitrate dehydrogenase 1 (*IDH1*) mutations, O6-methylguanine-DNA-methyltransferase (MGMT) promoter methylation, and loss of heterozygosity of chromosome 1p and 19q (LOH1p/19q) on 5hmC amount. We found that GBMs show a tremendous loss of 5hmC, and we observed that even the infiltration zones show reduced amounts of 5hmC. Interestingly, the amount of 5hmC was inversely proportional to the two investigated proliferation markers, Ki67 and H3S10p. Correlation of 5hmC amount and molecular genetic markers of GBMs showed that there are no correlations of 5hmC amount and *IDH1* mutations, MGMT promoter methylation, and LOH1p/19q. Furthermore, we evaluated the intratumoral distribution of 5hmC in compact and infiltrating areas and found that the

quantification of the 5hmC amount is a useful tool in evaluation of tumor infiltration. In summary, our data emphasize that GBMs show a disturbed hydroxymethylome that is disrupted by *IDH1* independent pathways, and that loss of 5hmC shows astonishing intratumoral heterogeneity.

**Keywords** Epigenetic · 5-hydroxymethylome · 5hmC · Brain tumor · Glioblastoma · GBM

## Introduction

Glioblastoma (GBM) is the most malignant neoplasm with predominant astrocytic differentiation and the most frequent primary brain tumor, accounting for approximately 12 to 15 % of all intracranial neoplasms and 60 to 75 % of all astrocytic tumors [1]. In North America and Europe, the incidence is in the range of 3 to 4 new cases per 100,000 population per year [1]. Despite progress in radiation and chemotherapy, the prognosis is very poor; less than half of the patients survive more than 1 year [1–3]. Thus, GBM is classified as highly malignant World Health Organization (WHO) grade IV brain tumor.

In the recent years, studies revealed that intratumoral heterogeneity likely is the key to understanding low treatment response [4–6]. Sottoriva et al. performed an integrated genomic analysis of GBM that uncovered extensive intratumoral heterogeneity demonstrating that most patients display different GBM subtypes within the same tumor [4]. Furthermore, they characterized the clonal origin of each tumor fragment and detected multiple coexisting cell lineages [4]. These data reveal that the understanding of intratumoral heterogeneity is the basis for efficient therapeutic approaches.

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Although the molecular mechanisms leading to GBM are not yet understood, there is increasing evidence that genetic and epigenetic factors contribute to tumor formation: One crucial factor is the methylation of the O6-methylguanine-DNA-methyltransferase (MGMT) promoter. This epigenomic hallmark is directly associated with the response to chemotherapy and overall survival: While GBM with unmethylated MGMT promoter show a very low response rate to chemotherapy using temozolomide, methylated GBM show a very good response rate [1]. Furthermore, there is a small fraction of GBMs that show mutations in the isocitrate dehydrogenase 1 (*IDH1*) gene, a gene that is associated with the citrate cycle and with the generation of 5-hydroxymethylcytosine (5hmC) [7].

5hmC is a DNA modification that was described in 2009 by Kriaucionis and Heintz in Purkinje neurons of the cerebellum [8]. Thereby, 5hmC is generated by oxidation of 5-methylcytosine (5mC). Currently, two possible functions of 5hmC are discussed: (1) It may act as an intermediate in active demethylation pathways, and/or (2) it may regulate gene expression by modifying transcription activity [9–13]. There is increasing evidence that both functions may be of biological significance depending on the tissue type. In stem cells, 5hmC may play a role in active demethylation and conversion of 5hmC to the further cytosine derivatives, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), that can subsequently be converted to unmodified cytosine [10, 14, 15]. Yldirim et al. have demonstrated that 5hmC is also of regulatory importance: As methyl-CpG-binding domain protein 3 (MBD3) co-localizes with ten-eleven-translocation 1 (TET1) and 5hmC in vivo, Yldirim et al. showed that a knockdown of MBD3 subsequently affects the expression of genes marked by 5hmC [16]. Another evidence is the high affinity of methyl-CpG-binding protein 2 (MeCP2) to 5hmC and 5mC: As 5hmC is enriched in active genes in central nervous system (CNS) tissue, 5hmC and MeCP2 may regulate chromatin structure and gene expression in the CNS [17]. Recently, Spruijt et al. identified dynamic readers for 5mC and 5hmC, as well as other oxidative derivatives of 5mC, indicating specific biological roles of 5mC and 5hmC [12]. But the distinct function of 5hmC still remains unclear.

In previous work, we have shown that 5hmC is significantly reduced in brain tumors compared to non-neoplastic brain [7]. Here, we focused on GBM and investigated a large cohort of 162 human GBM samples and correlated 5hmC data with genetic characteristics of the tumors: isocitrate dehydrogenase 1 (*IDH1*) mutations, O6-methylguanine-DNA-methyltransferase (MGMT) promoter methylation, and loss of heterozygosity of chromosome 1p and 19q (LOH1p/19q). Furthermore, we evaluated the intratumoral distribution of 5hmC in compact and infiltrating areas and found that quantifying the 5hmC amount is a useful tool in evaluation of tumor infiltration.

## Material and methods

### Selection of glioblastoma and control samples

In this study, we selected 170 human glioblastoma samples deriving from 162 different patients. All samples were obtained from the Brain Tumor Bank of the Center for Neuropathology, LMU Munich. All patients were treated at the Hospital of the University of Munich and were enrolled in quality assurance strategies. Written informed consent was obtained according to the guidelines of the local ethics committee. Surgical samples were snap frozen or fixed with 4 % buffered formalin, paraffin embedded, and subjected to routine stains including hematoxylin and eosin (H&E), Elastica van Gieson (EvG), Periodic acid–Schiff (PAS), and Gomori silver stain. Additionally, we performed immunohistochemistry using antibodies against glial fibrillary acidic protein (GFAP, Clone 6F2, Dako), microtubule-associated protein 2 (MAP2, Clone HM.2, Sigma), and Ki67 (Clone MIB1, Dako). In order to investigate solid tumor regions, we used only those samples with at least 90 % of viable tumor cells. The mean age of patients was 59 years with a median age of 62 years. Thirty-six percent of patients were female, 64 % were male. Further information on cases can be found in Table 1, Supplementary Table S1 and Supplementary Fig. S1.

Tumor infiltration regions were identified by histological and immunohistochemical evaluations. We considered cellular morphology, pleomorphism, vascular proliferation, and mitotic activity in routine stained sections, as well as findings in immunohistochemistry using antibodies against GFAP, MAP2, and Ki67. Detailed information can be found in Table 1 and in Supplementary Table S1.

As control samples, we selected either epilepsy surgical brain tissue or brain tissue provided by the BrainBank Munich. Written informed consent was obtained according to the guidelines of the local ethics committee. All 66 samples deriving from 27 control cases used in this study did not show any neurological deficits, insult, or tumor in the relevant brain region. We used either frozen (stored at  $-80^{\circ}\text{C}$ ) or FFPE tissue samples of cerebral grey and white matter and cerebellar cortex. The mean age of patients was 62 years with a median age of 61 years. Thirty-seven percent of patients were female, 63 % were male. Detailed information on cases can be found in Table 1 and in Supplementary Table S2.

### Immunohistochemistry

FFPE tissue was used for immunohistochemistry (IHC). Standard protocols were followed as described previously [7]. As primary antibodies, we used polyclonal rabbit anti-5-hydroxymethylcytosine antibodies (1:1000, Active Motif, Rixensart, Belgium), monoclonal mouse anti-Ki67 antibodies (1:500, Dako, Hamburg, Germany),

**Table 1** Overview of GBM and control patients. We investigated 170 GBM tissue specimen of 162 patients and 66 control tissue specimen of 27 patients. Mean age of GBM patients was 59 years with a median age of 62 years. Sixty-four percent of patients were male and 36 % were female

Group	Number of specimen (patients) ( <i>n</i> )	Age (mean, median) (year)	Sex (m, f, m/f)
Glioblastoma	170 (162)	59, 62	64 %, 36 %, 1.7
Control	66 (27)	62, 61	63 %, 37 %, 1.7

rabbit anti-H3S10p antibodies (1:100, Cell Signaling Technology, Danvers, MA, USA), and monoclonal mouse anti-IDH1<sup>R132H</sup> antibodies (1:20, Dianova, Hamburg, Germany). In case of 5hmC, we used the Super Sensitive Polymer-HRP Detection System (BioGenix, San Ramon, USA) and 3,3'-diaminobenzidine (DAB). All other stains were performed on a Ventana Benchmark System (Roche, Basel, Switzerland). The identification of representative tumor regions was performed by light microscopy: We first evaluated the whole tumor tissue in both routine stains and IHC. Then, we selected representative tumor regions and took pictures with 10×, 20×, and 40× magnification. Depending on the size of the tumor specimens (gross surgery or stereotactic biopsy), we selected a single tumor region or multiple representative tumor regions for subsequent analysis. Immunofluorescence staining was performed according to standard protocols. As primary antibodies, we used polyclonal rabbit anti-5-hydroxymethylcytosine antibodies (1:1000, Active Motif, Rixensart, Belgium) and monoclonal mouse anti-Ki67 antibodies (1:500, Dako, Hamburg, Germany). As secondary antibodies, we used Alexa Fluor 488 goat anti-rabbit IgG (1:300, Invitrogen, Darmstadt, Germany) and Alexa Fluor 546 goat anti-mouse IgG (1:300, Roth, Karlsruhe, Germany). Nuclear staining was performed using 4',6-diamidin-2-phenylindol (DAPI, Roth, Karlsruhe, Germany). Sections were coverslipped using Dako Fluorescence Mounting Medium (Dako, Hamburg, Germany).

### Extraction of genomic DNA

DNA extraction was performed using micro-dissection. Target regions (solid tumor regions or tumor infiltration zones) were microscopically identified using H&E-stained slides. Tumor tissue was subsequently detached from serial slides.

In case of control tissue, cerebral cortex, white matter, and cerebellar cortex were labeled using representative H&E-stained slides. Target region was subsequently detached from serial slides using micro-dissection.

DNA extraction was performed using a Maxwell 16 system (Promega, Madison, USA) in combination with the Maxwell 16 FEV FFPE kit (Promega, Madison, USA) following the manufacturer's protocol.

with a male to female ratio of 1.7. Mean age of controls was 62 years with a median age of 61 years. Sixty-three percent of patients were male and 37 % were female with a male to female ratio of 1.7

### Analysis of *IDH1/2* mutations, MGMT promoter methylation, and LOH1p/19q

To identify isocitrate dehydrogenase 1/2 (*IDH1/2*) mutations, we sequenced (1) an 88-bp long fragment of the *IDH1* locus including codon 132 and (2) an 83-bp long fragment of the *IDH2* locus including codon 172. We applied the pyrosequencing technique as described previously [7]. In case of *IDH1*, we used the primer pair 5'-biotin-AAAAATATCCCC CGGCTTG-3' (forward) and 5'-TGCCAACATGACTTAC TTGATCC-3' (reverse) for PCR amplification of the target region and the sequencing primer 5'-TGATCCCCATAAGC A-3'. In case of *IDH2*, we used the primer pair 5'-TCCCAC GCCTAGTCCCTG-3' (forward) and 5'-biotin-CTCTCCAC CCTGGCCTACC-3' (reverse) for PCR amplification of the target region and the sequencing primer 5'-AGCCCATC ACCATTG-3'. In addition to pyrosequencing, we used the IDH1<sup>R132H</sup> antibody to detect *IDH1* mutations. MGMT promoter methylation was determined as described previously [18]. Loss of heterozygosity of chromosome 1p and 19q (LOH1p/19q) was detected as described previously [19].

### Statistical analysis

The 5hmC amount was determined quantitatively by cell counting using the Image J cell counting software. Statistical evaluations were performed using GraphPad Prism 5. As statistical tests, we used unpaired *t* test to compare two groups and one-way ANOVA with Newman-Keuls post-hoc test to compare multiple groups, respectively. A *p* value of <0.05 was considered as statistically significant.

## Results

### GBMs show significantly less 5hmC compared to normal brain regions

As a first approach to identify epigenetic variations in GBM samples compared to normal brain, we analyzed the amount of 5hmC in GBMs and cerebral cortex, white matter, and cerebellum using immunohistochemistry (IHC). We found that the number of 5hmC-positive cells is significantly lower in GBMs

compared to various brain regions: Healthy cerebral cortex showed mean amounts of 73.2 % 5hmC-positive cells, healthy cerebral white matter showed mean amounts of 45.2 % 5hmC-positive cells, and healthy cerebellar cortex showed mean amounts of 50.1 % 5hmC-positive cells. In contrast to healthy brain regions, the amount of 5hmC-positive cells was significantly decreased in GBMs showing only 2.23 % of 5hmC-positive cells ( $p < 0.001$ , ANOVA and Newman-Keuls post-hoc test, Fig. 1a).

### Intratumoral variation of 5hmC and association of 5hmC with low cellular proliferation

We next focused on tumor infiltration and 5hmC amount. Comparing solid GBM tissue and infiltration zones with normal brain regions (cerebral cortex, white matter, cerebellum), we saw that infiltrating tumor regions exhibited significantly fewer 5hmC-positive cells (16.27 %) compared to cerebral cortex (73.2 %), white matter (45.2 %), and cerebellum (50.1 %), but significantly more 5hmC compared to solid tumor regions (2.23 % 5hmC-positive cells,  $p < 0.001$ , ANOVA and Newman-Keuls post-hoc test) (Fig. 1b).

Focusing on the intratumoral distribution of 5hmC and the correlation with markers for tumor proliferation (Ki67 and phosphorylated histone H3), we found astonishing intratumoral heterogeneity of the hydroxymethylome. Determining the amount of 5hmC-positive cells in areas of solid tumor, we found only very few 5hmC-positive cells detectable (mean of 2.23 %; Fig. 2a, b). In contrast to solid tumor, infiltration zones showed more 5hmC-positive cells (mean of 16.27 %; Fig. 2c, d). Especially at the edge of solid tumor

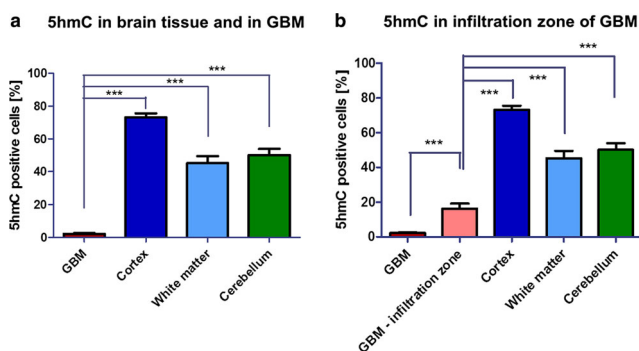
and the transition to infiltration, the change in the amount of 5hmC-positive cells from 2.23 % in solid tumor to 16.27 % in infiltration zone was very impressive (Fig. 2c, solid tumor can be found at the upper left corner, infiltration zone at the lower right corner of the picture).

Additionally, we performed evaluations of Ki67-positive nuclei. Ki67 is a nuclear protein that is associated with cellular proliferation. We found that regions of low 5hmC amount (Fig. 2e, f) showed high numbers of cells that stain positive for Ki67 (30.1 %). In contrast to these findings, regions of higher 5hmC amount (Fig. 2g, h) showed lower numbers of Ki67 positive cells (3.9 %).

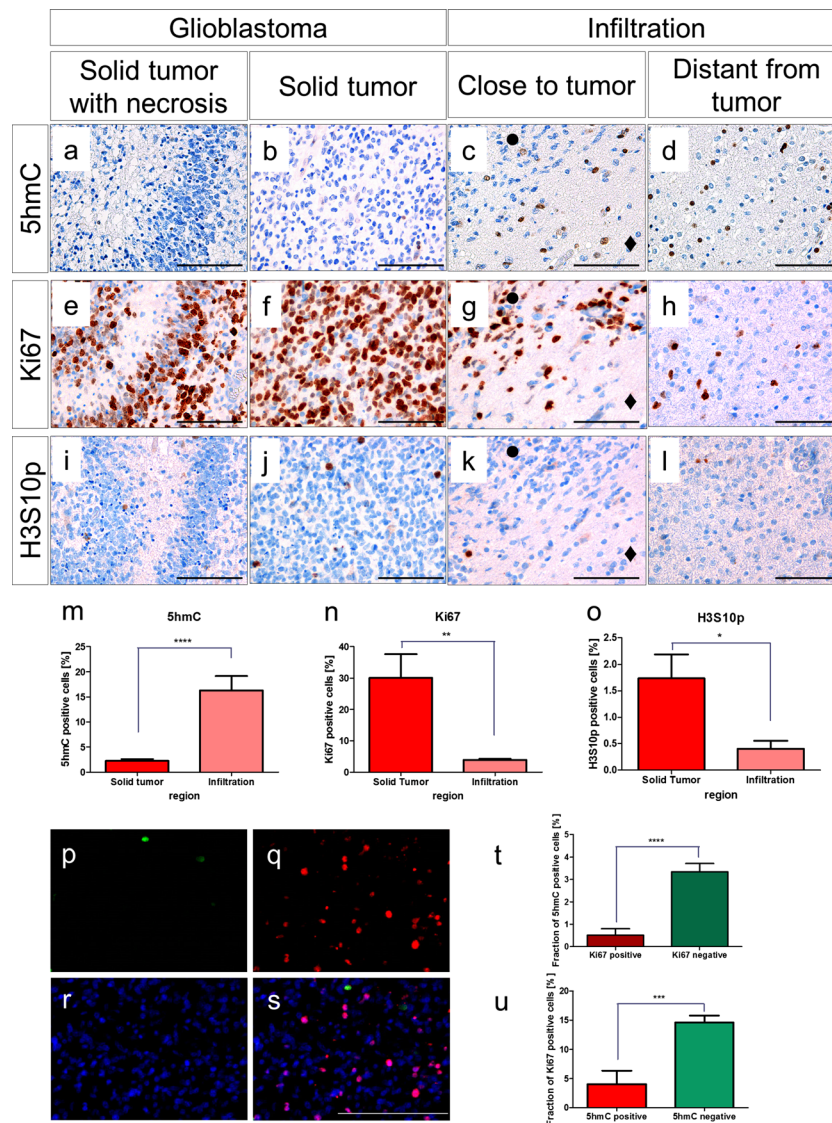
To further characterize the regions of solid tumor growth and infiltration zones, we used antibodies against the phosphorylated histone H3 (H3S10p), a marker for mitotic activity. We found that in regions with low amounts of 5hmC-positive cells (Fig. 2i, j), there were frequently H3S10p-positive nuclei (1.74 %). In regions with higher amounts of 5hmC-positive cells (Fig. 2k, l), there were only very few positively stained nuclei (0.41 %).

Quantifications of 5hmC amount, Ki67-positive cells and H3S10p-positive cells in solid tumor regions and infiltration zones revealed significant differences of both 5hmC amount and markers for cellular proliferation (Ki67 and H3S10p). In regions of solid tumor regions, there were significantly less 5hmC-positive cells (2.23 %) compared to infiltration zones (16.27 %,  $p < 0.0001$ , unpaired  $t$  test) (Fig. 2m). In case of Ki67-positive cells, there was a significantly greater proportion of Ki67-positive cells in solid tumor regions (30.1 %) compared to infiltration regions (3.9 %,  $p < 0.01$ , unpaired  $t$  test) (Fig. 2n). By quantifying the number of H3S10p-positive cells in solid tumor tissue and infiltration zones, we found a significantly greater proportion of H3S10p-positive cells (1.74 %) in solid GBM tissue compared with infiltration zones (0.41 %,  $p < 0.05$ , unpaired  $t$  test) (Fig. 2o).

Immunohistochemical co-staining of GBM tissue (Fig. 2p–s) using antibodies against 5hmC (Fig. 2p), Ki67 (Fig. 2q) and DAPI (Fig. 2r) revealed that almost all 5hmC-positive cells were Ki67 negative and vice versa (Fig. 2s). Quantifications of 5hmC and Ki67-positive cells revealed that only 0.5 % of Ki67-positive cells stained positive for 5hmC, and that a significantly greater proportion of Ki67-negative cells stained positive for 5hmC (3.3 %,  $p < 0.0001$ , unpaired  $t$  test) (Fig. 2t). In case of 5hmC-positive cells, only 4.1 % of 5hmC-positive cells stained positive for Ki67 and a significantly greater proportion of 5hmC-negative cells stained positive for Ki67 (14.6 %,  $p < 0.001$ , unpaired  $t$  test) (Fig. 2u). These findings indicate that 5hmC is associated with low proliferative activity of GBM cells.



**Fig. 1** Quantification of 5hmC in GBM and normal brain. **(a)** Using immunohistochemistry to quantify 5hmC in GBM and normal brain (cortex, white matter, cerebellum), we see that the number of 5hmC-positive cells is significantly decreased in GBM compared with normal brain ( $p < 0.001$ ). **(b)** Quantifying the amount of 5hmC-positive cells in GBM, infiltration zones, and normal brain, we see that infiltration zones show significantly increased numbers of 5hmC-positive cells compared to solid tumor regions ( $p < 0.001$ ), but significantly decreased numbers compared to normal brain ( $p < 0.001$ ). ANOVA with the Newman-Keuls post-hoc was applied. Indicated are mean and SEM. *n.s.* not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$



**Fig. 2** Immunohistochemical stains of GBM samples. Using antibodies against 5hmC, we see very few 5hmC-positive cells in solid tumor regions with (a) and without necrosis (b). At the transition from solid tumor (black circle) to infiltration (black diamond), there is an increase of 5hmC-positive cells (c). In infiltration regions, we see a further increase of 5hmC-positive cells (d). Staining for the proliferation marker, Ki67 shows high proliferative activity in solid tumor regions (e, f) compared with lower proliferative activity in infiltration zones (g, h). Using antibodies for the mitosis-associated phosphorylated histone H3 (H3S10p), we see a greater proportion of positively stained nuclei in solid tumor regions (i, j) compared to infiltration zones (k, l). Quantifications of the above findings (a–l) demonstrate significantly lower amounts of 5hmC ( $p < 0.0001$ ) (m), significantly higher amounts of Ki67 ( $p < 0.01$ ) (n), and significant higher amounts of H3S10p-positive

cells ( $p < 0.05$ ) (o) in solid tumor regions compared to infiltration zones. (p–s) Immunohistochemical co-staining of 5hmC (p), Ki67 (q), and DAPI (r) in solid GBM tissue reveal that Ki67-positive cells stain negative for 5hmC and vice versa (s). Quantifications show that there is a significantly greater proportion of Ki67-negative cells and 5hmC-positive cells (3.34 %) compared to Ki67-positive cells (0.51 %,  $p < 0.0001$ , unpaired  $t$  test) (t), and that there is a significantly larger proportion of 5hmC-negative cells that stain positive for Ki67 (14.57 %) compared to 5hmC-positive cells (4.05 %,  $p < 0.001$ , unpaired  $t$  test) (u). (a–l, p–s) (Black circle) solid tumor, (black diamond) infiltration zone. Scale bar 100  $\mu$ m. (m–o, t–u) As statistical test,  $t$  test was applied. Indicated are mean and SEM. *n.s.* not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$

### Genetic characterization of GBMs did not show significant correlations of 5hmC amount with *IDH1* mutations, MGMT promoter methylation, and LOH1p/19q

We next analyzed the genetic and epigenetic alterations that are associated with GBMs: isocitrate dehydrogenase 1 (*IDH1*)

mutations, O6-methylguanin-DNA-methyltransferase (MGMT) promoter methylation, and loss of heterozygosity of chromosome 1p and 19q (LOH1p/19q).

*IDH1* is an enzyme of the citrate cycle that is responsible for the generation of alpha-ketoglutarate (aKG). Especially in World Health Organization (WHO) grade II and grade III gliomas, there are frequently mutations at codon 132 of the

*IDH1* gene locus. These mutations are gain of function mutations leading to the production of 2-hydroxyglutarate (2HG), a known oncometabolite. Additionally, 2HG is a competitive inhibitor of ten-eleven-translocation (TET) proteins that convert 5mC to 5hmC.

In this study, we analyzed *IDH1* mutations using pyrosequencing and IHC. We did not find significant differences between the amount of 5hmC in GBMs with and without *IDH1* mutation (1.00 versus 2.03 % 5hmC-positive cells,  $p > 0.05$ , unpaired *t* test) (Fig. 3a).

MGMT is an enzyme that is responsible for DNA repair. Hegi et al. described in 2005 that an epigenetic silencing of MGMT by promoter methylation compromises DNA repair. By testing the relationship between MGMT silencing and the survival of GBM patients comparing radiotherapy alone with combined radiotherapy and concomitant or adjuvant treatment with temozolomide, Hegi et al. found that patients with a methylated MGMT promoter benefited from additional treatment with temozolomide, whereas those without a methylated MGMT promoter did not have such a benefit [20]. Thus the addition of temozolomide to radiotherapy for newly diagnosed GBMs results in a significantly better survival with only a minimum of additional toxicity [21, 22]. A 5-year follow-up of the phase III study of the EORTC-NCIC trial demonstrated in 2009 that the analysis of the MGMT methylation status identifies patients that benefit from the addition of temozolomide to radiotherapy [23, 24].

In the study presented here, we analyzed if there is a correlation between MGMT promoter methylation and the global amount of 5hmC. Using IHC, we did not find any significant differences of 5hmC amount in GBMs with (1.75 % 5hmC-positive cells) and without (1.48 % 5hmC-positive cells) methylation of the MGMT promoter ( $p > 0.05$ , unpaired *t* test) (Fig. 3b).

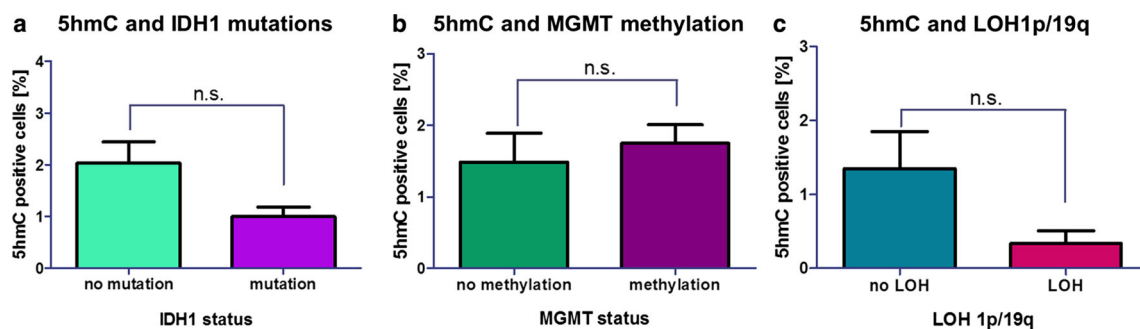
LOH1p/19q is another marker indicating molecular subtypes of GBMs. Thereby LOH1p/19q especially resembles

an oligodendroglial appearance in histology. Clinically, patients with LOH1p/19q show a significantly better response to chemotherapy and a better outcome [19, 25, 26]. In this study, we analyzed if LOH1p/19q correlates with 5hmC amount. We did not see any significant differences in GBMs with (0.34 % 5hmC-positive cells) and without (1.34 % 5hmC-positive cells) LOH1p/19q ( $p > 0.05$ , unpaired *t* test) (Fig. 3c).

## Discussion

### Intratumoral heterogeneity of the hydroxymethylome

In this study, we compared areas of solid tumor with areas of tumor infiltration. We then correlated the amount of 5hmC with the proliferation markers Ki67 and H3S10p (phosphorylated histone H3). Especially in solid tumor tissue, we see that most tumor cells do not show 5hmC in detectable amounts. Interestingly, the amount of 5hmC-positive cells increases significantly by moving away from solid tumor regions towards tumor infiltration. Nevertheless, the amount of 5hmC in tumor infiltration zones remains significantly lower compared with healthy brain tissue. Unaffected cerebral white matter, cortex, and cerebellar cortex exhibit significantly higher amounts of 5hmC compared to tumor infiltration zones. Thereby, 5hmC seems to be a more sensitive marker to determine infiltration zones compared with markers commonly used to detect proliferating cells, e.g., Ki67 protein and H3S10p [27–29]. As GBMs are highly infiltrative tumors [1], quantification of 5hmC amount may thus be a useful tool to determine infiltration zones. Nevertheless, further studies will be necessary to determine the benefit of 5hmC quantification in routine histology.



**Fig. 3** Genetic analysis of *IDH1* mutations, MGMT promoter methylation, and LOH1p/19q and correlation with 5hmC amounts. Subgrouping GBMs according to the *IDH1* mutation status reveals no significant difference between 5hmC amount in tumor with and without *IDH1* mutation (mean of 1.0 % 5hmC-positive cells in GBMs with *IDH1* mutation and 2.03 % 5hmC-positive cells in GBMs without *IDH1* mutation,  $p > 0.05$ , unpaired *t* test) (a). Correlation of MGMT promoter

methylation with the amounts of 5hmC in GBMs shows no significant difference between GBMs with and without MGMT promoter methylation (b). An analysis of LOH1p/19q with the amounts of 5hmC in GBMs shows that tumors with and without LOH1p/19q do not exhibit significant differences of 5hmC amount (c). As statistical test, *t* test was applied. Indicated are mean and SEM. *n.s.* not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$

## Relationship of 5hmC and proliferation markers in GBMs and infiltration zones

Correlating the amount of 5hmC with commonly used proliferation markers (Ki67 and H3S10p), we see that solid tumor tissue with high proliferative activity (numerous Ki67-positive and H3S10p-positive cells) shows very few 5hmC-positive cells. To test if there is a correlation between markers for proliferation and 5hmC positivity, we next performed immunohistochemical co-staining and found that almost all 5hmC-positive cells were Ki67 negative and vice versa. These data are well in line with published data indicating 5hmC as a stable epigenetic marker for cell-type specific functions and low cellular proliferation [8, 17, 30]. Furthermore, our findings emphasize that cells undergoing malignant transformation lose the normal epigenome and constitute aberrant methylation and hydroxymethylation patterns.

## Low amounts of 5hmC in GBM do not correlate with MGMT promoter methylation, *IDH1* mutations, and LOH1p/19q

Investigating a total number of 162 human GBM samples, we were able to study the effects of MGMT promoter methylation, *IDH1* mutations, and LOH1p/19q on 5hmC amount.

MGMT promoter methylation is of primary importance for individualized treatment of glioblastoma patients as patients with methylated MGMT promoter benefit from additional treatment with temozolomide, whereas those without methylated MGMT promoter do not have such benefits [20]. Subsequently, the addition of temozolomide to radiotherapy results in a significantly better survival [21, 22]. Interestingly, the analysis of 5hmC amount and MGMT promoter methylation did not show significant correlations. Thus, it seems that MGMT promoter methylation is a phenomenon occurring independently from the loss of the hydroxymethylome in GBMs. Since tumors with LOH1p/19q show a significantly better response to chemotherapy using temozolomide [19, 25, 26], we analyzed if there is a correlation of LOH1p/19q with 5hmC. Interestingly, we did not find correlations of LOH1p/19q and 5hmC amount. Subsequently, we performed genetic analysis of GBMs to reveal if there is a correlation of 5hmC amount and *IDH1* mutations. Interestingly, we did not find a correlation of *IDH1* mutations and 5hmC amount. As mutated *IDH1* (R132H) leads to the production of 2HG, a competitive inhibitor of ten-eleven-translocation (TET) enzymes [31], our data emphasize that in *IDH1* wild type, GBMs alternative oncogenic pathways are activated leading to the loss of 5hmC [32, 33].

In summary, the data presented here show that there is a severe disturbance of the hydroxymethylome in GBM. As our comprehensive genetic analysis reveals that loss of 5hmC is independent from MGMT promoter methylation, *IDH1*

mutations, and LOH1p/19q, our data emphasize that in the majority of GBMs, the hydroxymethylome that is disrupted by *IDH1* independent oncogenic pathways. Thus, future studies investigating gene expression of enzymes responsible for methylation/demethylation pathways in *IDH1* wild type, GBMs may help to further understand molecular mechanisms occurring in tumorigenesis. Additionally, our findings show that 5hmC may be useful as a marker for tumor infiltration zones of GBMs. The data presented will be the basis for future studies in the field of glioma research and will further drive research on tumor epigenomics.

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**Conflict of interest** None.

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