Linking epigenetic signature and metabolic phenotype in IDH mutant and IDH wildtype diffuse glioma

Yannick Braun1 | Katharina Filipski1,2,3 | Simon Bernatz1 | Peter Baumgarten1,4 | Bastian Roller1 | Jenny Zinke1 | Pia S. Zeiner1,5 | Elena Ilina1 | Christian Senft4 | Michael W. Ronellenfitsch2,3,5,6 | Karl H. Plate1,2,3,6 | Oliver Bähr5 | Elke Hattingen6,7 | Joachim P. Steinbach2,3,5,6 | Michel Mittelbronn1,8,9,10,11 | Patrick N. Harter1,2,3,6

1Neurological Institute (Edinger Institute), University Hospital, Goethe University Frankfurt am Main, Frankfurt, Germany
2German Cancer Consortium (DKTK), partner site Frankfurt/Mainz, Frankfurt, Germany
3German Cancer Research Centre (DKFZ), Heidelberg, Germany
4Department of Neurosurgery, University Hospital, Goethe University Frankfurt am Main, Frankfurt, Germany
5Dr. Senckenberg Institute for Neurooncology, University Hospital, Goethe University Frankfurt am Main, Frankfurt, Germany
6Frankfurt Cancer Institute (FCI), Frankfurt, Germany
7Department of Neuroradiology, University Hospital, Goethe University Frankfurt am Main, Frankfurt, Germany
8Luxembourg Centre of Neuropathology (LCNP), Luxembourg
9Luxembourg Centre for Systems Biomedicine (LCSB), University of Luxembourg, Luxembourg
10National Centre of Pathology (NCP), Laboratoire national de santé (LNS), Luxembourg
11Department of Oncology (DONC), Luxembourg Institute of Health (LIH), Luxembourg

Correspondence
Patrick N. Harter, Neurological Institute (EdingerInstitute), University Hospital, Goethe University Frankfurt am Main, Frankfurt, Germany.
Email: patrick.harter@kgu.de

Abstract
Aims: Changes in metabolism are known to contribute to tumour phenotypes. If and how metabolic alterations in brain tumours contribute to patient outcome is still poorly understood. Epigenetics impact metabolism and mitochondrial function. The aim of this study is a characterisation of metabolic features in molecular subgroups of isocitrate dehydrogenase mutant (IDHmut) and isocitrate dehydrogenase wildtype (IDHwt) gliomas.

Methods: We employed DNA methylation pattern analyses with a special focus on metabolic genes, large-scale metabolism panel immunohistochemistry (IHC), qPCR-based determination of mitochondrial DNA copy number and immune cell content using IHC and deconvolution of DNA methylation data. We analysed molecularly characterised gliomas (n = 57) for in depth DNA methylation, a cohort of primary and recurrent gliomas (n = 22) for mitochondrial copy number and validated these results in a large glioma cohort (n = 293). Finally, we investigated the potential of metabolic markers in Bevacizumab (Bev)-treated gliomas (n = 29).

Results: DNA methylation patterns of metabolic genes successfully distinguished the molecular subtypes of IDHmut and IDHwt gliomas. Promoter methylation of lactate dehydrogenase A negatively correlated with protein expression and was associated with IDHmut gliomas. Mitochondrial DNA copy number was increased in IDHmut tumours and did not change in recurrent tumours. Hierarchical clustering based on metabolism panel IHC revealed distinct subclasses of IDHmut and IDHwt gliomas with an impact on patient outcome. Further quantification of these markers allowed for the prediction of survival under anti-angiogenic therapy.

Conclusion: A mitochondrial signature was associated with increased survival in all analyses, which could indicate tumour subgroups with specific metabolic vulnerabilities.

KEYWORDS
glioma, metabolism, mitochondria, DNA methylation, IDH mutation
INTRODUCTION

Diffuse gliomas include isocitrate dehydrogenase mutant (IDHmut) astrocytic and oligodendrogial tumours, as well as IDHmut and IDH wildtype (IDHwt) glioblastoma (GB). These tumours account for over 60% of primary brain tumours in the adult population in the United States [1]. The combined incidence is 4.2 per 100,000 capita. The 5-year survival rates range from 81.3% for oligodendroglioma to 29.8% for anaplastic astrocytoma and 5.5% for GB [1,2]. Initially, these gliomas were defined only by histology. Molecular alterations, such as IDH mutational status and co-deletions of chromosomal arms 1p and 19q can predict patient prognosis [3–5]. Both IDHmut and IDHwt gliomas are characterised by their diffuse infiltrative behaviour. However, these tumours display distinct epigenetic, genetic and transcriptional characteristics, which result in different outcomes in patients [4,6–9].

IDH mutations result in the conversion of ketoglutarate (KG) to D-2-hydroxylglutarate (D2HG), which inhibits KG-dependent dioxygenases, such as ten-eleven translocation (TET) enzymes as well as Jumonji C domain-containing histone-lysin demethylases [10–13]. This leads to DNA and histone hypermethylation, which ultimately produces a hypermethylation phenotype known as the 'glioma CpG-island methylator phenotype' (G-CIMP) [14,15]. Differences in DNA methylation patterns allow for artificial intelligence-assisted tumour classification [16]. IDHwt GB has been further subdivided into three molecular subgroups: proneural (PN), classical (CL) and mesenchymal (MES). Initially, these subgroups were defined according to distinct gene expression profiling. In addition, DNA methylation analyses enable subdivision into these subgroups [16,17]. In the context of DNA methylation analyses, the subgroups are referred to as RTK I (PN), RTK II (CL) and mesenchymal.

Current treatment standards of IDHmut and IDHwt gliomas are similar. These treatments rely on tumour resection and combined radiochemotherapy with temozolomide or other DNA alkylating agents [18,19]. Trials of targeted therapeutic approaches have produced disappointing results [20–24].

Metabolic reprogramming is a characteristic hallmark of cancer [25]. Normalisation of deregulated cellular energy metabolism in tumours has been hypothesised to be a beneficial factor [25,26]. Pharmacological therapy either directly (e.g. ketogenic diet) or indirectly (e.g. anti-angiogenic treatment) targets cellular energy homoeostasis [27–29]. Anti-angiogenic therapy in GB induces an adaptation towards anaerobic metabolism and induces a hypoxic microenvironment [29,30]. In an IDHwt GB patient-derived xenograft model, anti-angiogenic therapy also led to reduced mitochondrial biomass in tumour cells [31].

Metabolic changes in malignant tumours compared to non-Neuroplastic tissues were first described almost 100 years ago by the German biologist Otto Warburg [32]. He discovered that tumour cells metabolised glucose to lactate via glycolysis despite the presence of oxygen. This phenomenon of aerobic glycolysis is termed the Warburg effect [33,34]. Alterations in glucose metabolism and mitochondrial respiration in cancer have been intensively studied over recent decades. Their contribution to malignant transformation or progression across different cancer entities is undisputed. However, the extent to which metabolic alterations are necessary to sustain the malignant phenotype and the stage of tumour progression at which they occur are unclear. Examining these mechanisms will help to understand the unsuccessful therapeutic attempts of metabolic normalisation in gliomas.

To gain further insights into metabolic regulation of gliomas, we investigated the DNA methylome profiles of IDHmut and IDHwt gliomas focussing on glucose metabolism and mitochondrial respiration. Additionally, to assess mitochondrial biomass, we analysed mitochondrial DNA copy number profiles. We validated these results using an immunohistochemistry (IHC)-based metabolism panel of three glioma patient cohorts, including patients who had undergone anti-angiogenic treatment. To investigate the influence of the immune microenvironment on our results, we analysed the immune cell content and composition by epigenetic data deconvolution and IHC. The findings revealed that DNA methylation patterns of key metabolic genes are strongly associated with molecular subgroups of gliomas and that IDHmut gliomas are enriched for a respiratory mitochondrial signature. A mitochondrial signature in IDHwt glioblastomas was also associated with improved patient survival. Finally, patients with a relatively increased mitochondrial signature displayed an improved response to anti-angiogenic treatment.

2 | MATERIALS AND METHODS

2.1 | Patient material and patient cohorts

DNA methylation analyses were performed using samples from 57 patients with glial brain tumours. All samples were collected from formalin-fixed and paraffin-embedded (FFPE) blocks from the University Cancer Center Frankfurt (UCT) Biobank from 2017 to 2019. The tissue was used for either DNA isolation as detailed in the corresponding segment or generation of tissue microarrays (TMAs). Further information regarding this patient cohort is summarised in File S1 (EPIC-Cohort).

mtDNA copy number analyses were performed in 22 patients with IDHwt or IDHmut primary and recurrent gliomas. DNA was extracted from 48 FFPE tissue samples from 22 patients and RT-qPCR was performed. Additional clinical information regarding these patients is summarised in File S2 (mtDNA-Cohort).

For further analyses, we chose a historical cohort of 293 patients with glioma graded according to the 2007 4th edition of the World Health Organization classification, including stratification with an antibody against IDH1R132H. All FFPE samples were processed as TMAs. All samples were collected as paraffin blocks from the UCT Biobank. Further data for this patient cohort and the analysed specimens are summarised in File S3 (Historical Glioma Cohort).

From all cohorts, we extracted a fourth cohort of 29 patients who received bevacizumab (Bev) during the course of their disease. All samples were collected as paraffin blocks before Bev treatment.
Further clinical data regarding this cohort are summarised in File S4 (Bev-Cohort).

The study was approved by the local ethics committee (GS 4/09, SNO-11-2017).

2.2 | IHC, microscopy, scoring and semi-automated digital quantification of immune cell infiltration

All TMA cores were punched from representative non-necrotic tumour areas. Three-micrometre thick sections of the TMAs were made with a microtome. The sections were placed on SuperFrost-Plus slides (Thermo Fisher Scientific, Waltham, MA, USA) and stored overnight in a 37°C incubator. IHC was performed using antibodies against the following antigens: semi-purified mitochondrial preparation anti-mitochondrial antibody (clone MTC02, dilution 1:100, Abcam, Cambridge, MA, USA), mitochondrial cytochrome c oxidase I (MT-CO1 (1:200 dilution, Cell Signaling Technologies, Danvers, MA, USA) and lactate dehydrogenase A (LDHA, 1:100 dilution, Cell Signaling Technologies). Throughout the text, MTC02 is used to denote the anti-mitochondrial antibody. Stained TMA slides were examined using a model BX41 microscope and images were taken with an attached DP72 camera (both from Olympus, Tokyo, Japan).

For semi-quantitative evaluation, the H-Score was applied as described previously [35]. The relationships between the metabolic surrogate markers were investigated using quartile ratios of each tumour and the corresponding marker (1. quartile 0-25%, 2. quartile 25-50%, 3. quartile 50-75%, 4. quartile 75-100%). Immune cell infiltration of the EPIC-Cohort was assessed histologically by IHC against CD8 (clone C8/144B, 1:100 dilution; DAKO, Glostrup, Denmark), CD68 (1:200 dilution, M0876, DAKO) and Iba1 (019-19741, 1:1000 dilution; Wake, Neuss, Germany). For semi-automated digital quantification of immune cell content, whole slide scanning of CD8, CD68 and Iba1 staining was performed using an Axio Scan.Z.1 (Carl Zeiss Microscopy GmbH, Jena, Germany) with a 20× objective lens. Scanned images in the czi file format were uploaded into HALO™ Next-Generation Image Analysis software (Version 3.0.311.287, Indica Labs, Albuquerque, NM, USA). Segmentation of TMA cores and exclusion of tumour necrosis, larger vessels and artefacts were performed using implemented software tools. Furthermore, the multiplex IHC module was employed to count total cells and positive cells for CD8, CD68 and Iba1.

2.3 | DNA isolation for mtDNA copy number analyses

Tissue specimens from FFPE samples were examined using haematoxylin and eosin staining to determine non-necrotic regions of tumour tissue. One or two cores 2 mm in diameter and 3 to 5-mm thick were punched out from the selected regions of the paraffin blocks. DNA was extracted using the Stratagene Genomic DNA Kit (Stratec, Berlin, Germany) according to the manufacturer’s protocols.

2.4 | Real-time quantitative PCR (RT-qPCR)

Isolated DNA was diluted to a concentration of 10 ng/µl. RT-qPCR was performed using SYBR Green Master Mix (Thermo Fisher Scientific) on a CFX96Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA). Beta-2 microglobulin primers were used as a nuclear DNA amount reference (forward: gcttggagtcttacaat-gttcca; reverse: ccatgtactaaatgtctaaatggt) and compared to two pairs of primers targeting the D-Loop of the mitochondrial DNA (mtDNA) genome (MtDLoop1: forward: gtgaatacatatccggccaagaag; reverse: ttagcctgaagggaaaccagca; MtDLoop2: forward: caccagcttaacaggttca; reverse: aatgggagagggaggac). Analyses were performed in triplicate. Cycle threshold (CT) values were determined. Relative mtDNA copy number was calculated using the following previously described formulae [36]: ΔCT = (nucDNA CT - mtDNA CT) and Relative mitochondrial DNA content = 2 × 2ΔCT.

2.5 | EPIC DNA methylation analysis

DNA from 57 patient samples was isolated as described above. CpG sites illustrated in the text were analysed with the EPIC 850 K Array (Illumina, San Diego, CA, USA). After standardised DNA processing, bisulphite treatment and further processing and hybridisation were performed as recommended by the manufacturer and previously described [16]. The resulting data were processed using Illumina Genome Studio, JMP14 (SAS Institute, Cary, NC, USA) and Partek Genomic Suite software (Partek Incorporated, St. Louis, MO, USA). The chosen gene sets and CpG sites included for analyses are listed in Supplementary Information 5 + 6. Only CpG sites with a potential single nucleotide polymorphism with minor allele frequency (MAF) <0.01 (1%) were included in the analyses. Promoter-associated CpGs were chosen based on their provided information (regulatory feature group). Ten thousand CpGs with the highest average deviation were chosen for main clustering (File S6). Shared CpGs between the chosen gene set and the 10 000 most variable CpGs were investigated using quartile ratios of each CpG site illustrated in the text were analysed with the EPIC 850 K Array (Illumina, San Diego, CA, USA). After standardised DNA processing, bisulphite treatment and further processing and hybridisation were performed as recommended by the manufacturer and previously described [16]. The resulting data were processed using Illumina Genome Studio, JMP14 (SAS Institute, Cary, NC, USA) and Partek Genomic Suite software (Partek Incorporated, St. Louis, MO, USA). The chosen gene sets and CpG sites included for analyses are listed in Supplementary Information 5 + 6. Only CpG sites with a potential single nucleotide polymorphism with minor allele frequency (MAF) <0.01 (1%) were included in the analyses. Promoter-associated CpGs were chosen based on their provided information (regulatory feature group). Ten thousand CpGs with the highest average deviation were chosen for main clustering (File S6). Shared CpGs between the chosen gene set and the 10 000 most variable CpGs were investigated using the online tool provided by VIB/UGent Bioinformatics & Evolutionary Genomics http://bioinformatics.psb.ugent.be/webtools/Venn/. All patient samples were classified according to the previously described molecular classification [16].

2.6 | DNA methylation-based immune cell deconvolution

The deconvolution was performed using RnBeads 2.0, which includes the leukocytes unmethylation for purity (LUMP) algorithm. This algorithm estimates the immune cell content of tumours based on 44 non-methylated immune-specific CpG sites [37,38].
2.7 Data visualisation and statistical analyses

All statistical analyses were performed using either JMP11/14 (SAS Institute) or R (R Core Team, 2019). Epigenomic and IHC data visualisation were performed with R utilising a ComplexHeatmap-package [39] to generate heat maps and hierarchical clustering, and JMP11/14. JMP add-in with the R-package Rtsne was used to generate t-distributed stochastic neighbour embedding (t-SNE) plots.

3 RESULTS

3.1 DNA methylation profiling defines distinct metabolic clusters of diffuse gliomas.

In the first approach, analysis of 57 patients in the EPIC-Cohort, for the 10,000 most variable CpG sites revealed strong clustering into IDHmut and IDHwt tumours, as well as the strong association of clusters with established molecular subclasses (File S7). To determine the epigenetic regulation of cellular bioenergetics, we defined gene subsets based on their involvement in glycolysis and oxidative phosphorylation either directly (e.g. facilitation of specific reactions) or indirectly (e.g. initiation of transcription of the mitochondrial genome). For gene set information, please see File S5. Furthermore, we added nuclear-encoded genes involved in the electron transport chain, which resulted in 2542 investigated ‘Metabol’ CpG sites of these genes represented on the EPIC Array (Figure 1, File S6). Mitochondria-encoded genes and potential DNA methylation of these genes were not represented in the applied EPIC Array.

Hierarchical cluster analyses based on beta values of the corresponding CpG site as well as t-SNE analyses clearly differentiated tumours according to their IDH mutation status (Figure 1A,B). The results indicated a correlation of the methylation status of metabolism-associated genes with the IDH mutation status (Figure 1A,B). Although IDHmut gliomas displayed a CpG site hypermethylation phenotype as compared to IDHwt gliomas, this did not entirely refer to the investigated ‘Metabol’ CpG site gene cluster. In contrast, methylation profiles appeared similar to a certain extent. Only a small subset of CpG sites seemed to determine the different clusters. Interestingly, clustering can not only separate IDHmut from IDHwt tumours, but also suggests subclustering into known epigenetic subgroups [16,17].

Next, we assessed the most relevant shared CpGs between the groups of most variable CpGs and ‘Metabol’ CpGs. Only 17 CpG sites were shared from both the ‘Metabol’ CpG site cluster and from the 10,000 most variable CpGs in the EPIC-Cohort (see the Venn diagram presented in Figure 1C). The genes with the most represented shared CpGs were the glycolytic gene PFKP, encoding the enzyme phosphofructokinase and the ATP5G2 gene, which encodes a subunit of the mitochondrial ATP synthase (Figure 1D). These 17 CpG sites were able to discriminate IDHmut from IDHwt tumours (File S7).

3.2 LDHA promoter-associated CpGs are highly regulated in different glioma subtypes

LDHA is upregulated in IDHwt glioma as compared to IDHmut glioma [40]. Furthermore, a recent study reported the increased hypermethylation of LDHA promoters during malignant progression in IDHmut gliomas [41]. To further investigate potential functionally relevant methylation patterns in gliomas, we investigated only annotated promoter-associated CpGs of the group of shared CpGs (Figure 1D), including LDHA promoter-associated CpGs (Figure 2A). The analysis led to the investigation of 65 CpG sites in the EPIC-Cohort. The resulting clusters were again almost exclusively linked to the IDH mutation status of the samples. Many of these promoter-associated CpGs were hypomethylated. Only promoter-associated CpGs of ATP5G2 and LDHA showed obvious differential methylation in the molecular subgroups (Figure 2A). Interestingly, LDHA promoter-associated CpG sites were hypermethylated in almost all IDHmut tumours, while some showed hypermethylation only in a subset of CpG sites. Interestingly, none of the 1p/19q co-deleted tumours showed a fully hypomethylated LDHA promoter, whereas some IDHmut astrocytomas and high-grade astrocytomas presented with LDHA promoter hypomethylation. All IDHwt gliomas showed a hypomethylated LDHA promoter. These collective observations implicated LDHA promoter-associated CpG site hypomethylation as a feature associated with glioma aggressiveness and potentially associated with malignant progression of high-grade IDHmut astrocytomas. The ATP5G2 promoter-associated CpG site methylation patterns were not entirely associated with IDH mutation status. Nevertheless, IDH mutant tumours showed a hypermethylated phenotype, which was also observed in some IDHwt tumours (Figure 2A). In contrast, the promoter-associated CpGs of NDUFV2A, which encodes the NADH-ubiquinone oxidoreductase complex (complex I) of the mitochondrial respiratory chain, was unmethylated in any sample from the EPIC-Cohort, suggesting that these genes are essential for cellular energy homeostasis.

To assess whether the epigenetic switch of LDHA promoter methylation and demethylation has direct effects on the protein level, we investigated LDHA expression via IHC (Figure 2B). LDHA expression at the immunohistochemical level was significantly correlated with LDHA promoter methylation status (Figure 2C,D). The findings directly linked epigenetic regulation to protein expression.

3.3 Copy number of mtDNA is linked to IDH mutational status

The foregoing data showed that several promoters of genes that might be needed for cellular energy homeostasis were not differentially methylated between IDHwt and IDHmut tumours, including NDUFV2. Thus, we were interested in determining whether the total mitochondrial biomass varied between these glioma subgroups. To gain insights into mitochondrial biomass, we used mtDNA copy number as a surrogate in our mtDNA-Cohort. The mtDNA copy number was higher
FIGURE 1  (A) Hierarchical clustering of 57 glioma patients (EPIC-Cohort) and 2542 CpG sites belonging to metabolic genes. (B) t-SNE plot of the same patients and 2542 CpG sites. (C) Venn diagram of 10,000 most variable CpG sites and 2542 metabolic CpG sites among the 57 glioma patients. Seventeen CpG sites were shared in both cohorts. (D) Gene symbols of genes corresponding to the shared 17 CpG sites. Number of CpG sites per gene are depicted in the pie chart.
in IDHmut tumours than in IDHwt tumours (Figure 3A). Furthermore, when comparing primary with matched pair recurrent tumours, mtDNA copy number was not statistically significantly different, although some patients showed differences in mtDNA copy number between primary and recurrent tumours (Figure 3B). To extend these results to the proteomic level, we employed two immunohistochemical biomarkers. One was the antibody to MTC02, which recognises a 60-kDa non-glycosylated protein component of mitochondria. We used this as a surrogate for mitochondrial biomass. We also used MT-CO1, which is essential for oxidative phosphorylation as an integral part of the electron transport chain (Complex IV). MT-CO1 serves as a surrogate for mitochondrial respiratory capacity (Figure 3C).

Correlation of the relative expression of these proteins with mtDNA copy number revealed a positive correlation between mtDNA copy number and MTC02, or between MTC02 and MT-CO1 (Figure 3D). The findings implicated MTC02 as a useful surrogate biomarker for mitochondrial biomass but also suggested that pure mitochondrial biomass might not be directly linked to functionality. Finally, a higher relative mtDNA copy number was associated with better patient overall survival (Figure 3E).

### 3.4 | IHC of metabolic surrogate biomarkers reveals strong association with IDH mutation status and mesenchymal signature in IDHwt gliomas

Next, we were interested in further comparing our epigenetic findings of the EPIC-Cohort with protein expression of metabolic surrogate biomarkers of the mtDNA-Cohort. As metabolic surrogates,
we used the aforementioned targets of LDHA (glycolysis), MTC02 (mitochondrial biomass) and MT-CO1 (electron transport chain/oxidative phosphorylation).

In the first approach, we were interested in the expression of these metabolic surrogates in epigenetically defined gliomas. LDHA expression was significantly lower in IDH

mut subclasses than in IDH

wt glioma subclasses (Figure 4A). LDHA expression was not different within the epigenetic subclasses of IDH

wt tumors. In contrast, MTC02 showed very low expression in the RTKII subclass of IDH

wt gliomas. This class represents the classical GB molecular signature. MTC02 levels were significantly higher in mesenchymal IDH

wt gliomas, IDH

mut 1p/19q co-deleted oligodendrogliomas and IDH

mut astrocytomas (Figure 4B).

These observations indicated pronounced differences in mitochondrial biomass within the molecular subgroups of IDH

wt GB. Mesenchymal GBs displayed the highest MT-CO1 levels among the IDH

wt gliomas and in the whole EPIC-Cohort. Significant differences compared to RTKI (proneural) IDH

wt gliomas and

FIGURE 3 (A) Relative mtDNA copy number of IDH

mut and IDH

wt gliomas (mtDNA-Cohort). (B) Comparison of relative mtDNA copy number of primary tumours and corresponding recurrent tumours (p-values of nonparametric Wilcoxon tests are depicted in (A) and (B)). (C) MTC02 and MT-CO1 protein expression in gliomas showing high and low expression. (D) Correlation analyses of MTC02 and MT-CO1 protein expression as well as relative mtDNA copy number. (E) Kaplan–Meier survival chart of mtDNA-Cohort with regard to high or low-relative mtDNA copy number (dichotomised after median split).
FIGURE 4  H-Scores of LDHA (A), MTC02 (B) and MT-CO1 (C) in the epigenetically characterised EPIC-Cohort (n = 56; in one case staining results were non-conclusive, thus this case was excluded from analyses). (D) Cluster analysis of EPIC-Cohort with regard to LDHA, MTC02 and MT-CO1 H-Score expression. (E) MT-CO1/LDHA quartile ratios of the EPIC-Cohort. (F) Cluster analysis of the 2542 CpG sites belonging to metabolic genes with special regard to epigenetic subtype of IDHwt gliomas and MT-CO1/LDHA quartile ratios (high versus low; single case with missing IHC data is marked with ‘X’).
**DNA METHYLATION AND METABOLISM OF DIFFUSE GLIOMAS**

**3.5 | Immune cell content is associated with epigenetic subclass but not with MT-CO1/LDHA ratio**

To evaluate the impact of immune cell composition on the detected signatures, we employed tumour deconvolution analyses based on the EPIC Array data (LUMP algorithm) as well as immune cell quantification using IHC and semi-automated analysis of CD8, CD68 and Iba1. Immune cell infiltration was highest in IDHwt glioma subclasses compared to IDHmut specimens with the highest immune cell content in the mesenchymal subclass (Figure 5A). The predominant immune cell type was microglia (Figure 5B). CD8-positive T-lymphocytes were scarce in the analysed specimen, with mean frequencies ranging from 0.08% in 1p/19q co-deleted oligodendroglioma to 0.39% in mesenchymal subtype glioblastomas (Figure 5B). Immune cell content detected by epigenetic deconvolution strongly correlated with immune cell content detected by IHC and semi-automated analyses (Figure 5C). From all analysed metabolic markers, only LDHA expression was associated with immune cell content (Figure 5D). We did not detect statistically significant differences in cell type-specific immune cell content between MT-CO1/LDHA high versus low tumours (Figure 5E).

**3.6 | Metabolic surrogate biomarkers are associated with patient prognosis in a large population-based glioma cohort**

To test whether our results are applicable in a routine histopathological setting of non-epigenetically classified gliomas, we performed IHC analyses of LDHA, MTC02 and MT-CO1 in a historical Glioma Cohort of 293 patients.

Hierarchical clustering of LDHA, MTC02 and MT-CO1 H-Scores led to four major clusters. Cluster 2 was mainly enriched for IDHmut tumours, although it was not an exclusive IDHmut cluster (Figure 6A,B). Clusters 1 and 4 mainly represented two distinct immunohistochemical subgroups of IDHwt GB with only single IDHmut gliomas in both groups. Cluster 4 was generally defined by high LDHA expression and lower MTC02 and MT-CO1 expression. Cluster 4 was exclusive to GB (either IDHmut or IDHwt) and anaplastic IDHmut astrocytomas. IDHmut oligodendrogliomas or IDHmut astrocytomas were not present in this cluster (Figure 6B). To investigate whether this immunohistochemical signature impacted patient survival, we performed Kaplan-Meier analyses of all clusters. Cluster 4 displayed significantly worse survival compared to all other clusters when including all glioma subgroups (Figure 6C). To exclude bias induced by IDH mutation status, we performed the same analyses focusing only on IDHwt GB. Survival was worse in Cluster 4 patients than in Cluster 1 patients (Figure 6D).

**3.7 | MT-CO1/LDHA quartile ratio is a biomarker for therapy response in Bev-treated gliomas**

Finally, we hypothesised that the distinct metabolic status of gliomas would lead to different responses to a metabolically effective treatment strategy, such as anti-angiogenic treatment with Bev. We investigated glioma patients who received Bev as third-line therapy after standard treatment. Interestingly, higher levels of MT-CO1 as a single biomarker and in relation to LDHA expression was associated with a significantly better progression-free and overall survival after Bev treatment (Figure 7). These findings were independent of IDH mutational status and MGMT promoter methylation status in a multivariate parametric survival analysis (effect likelihood ratio progress-free survival: IDH1R132H status \( p = 0.2431 \), MGMT promoter methylation status \( p = 0.1090 \), MT-CO1/LDHA ratio \( p = 0.0286 \); effect likelihood ratio overall survival: IDH1R132H status \( p = 0.0621 \), MGMT promoter methylation status \( p = 0.1668 \), MT-CO1/LDHA ratio \( p = 0.0016 \)).

**4 | DISCUSSION**

Since 2008, the increased understanding of tumour biology of diffuse gliomas with the discovery of IDH mutations has included the definition of new subclasses of gliomas [7]. Basic and translational studies have investigated the role of mutant IDH and discovered that IDH mutations result in a DNA and histone hypermethylation phenotype [10–15]. DNA methylation analyses permit precise tumour classification [16]. However, there is still a lack of understanding of the relationship between epigenetic changes and metabolism.

The aim of our study was to characterise the metabolic differences in diffuse gliomas with a special focus on epigenetic (DNA methylation) and proteomic (metabolic panel IHC) alterations, as
well as differences in mitochondrial biomass and genetics (metabolic panel IHC and D-Loop qPCR). We were able to confirm that IDHmut tumours showed a hypermethylated phenotype. The EPIC-Cohort displayed representative epigenomic alterations, which enabled us to further investigate metabolically relevant DNA methylation patterns. Hierarchical clustering of CpG sites belonging to metabolic genes was able to subclassify IDHmut and IDHwt tumours. Our analyses even showed a subgrouping of IDHwt gliomas in known epigenetic subclasses. In this context, the molecular subgroup of mesenchymal gliomas, which consists of GB with a mesenchymal signature, almost always contained a different metabolic signature as compared to the RTK I (proneural) and RTK II (classical) classes.

LDHA promoter-associated CpGs showed a hypermethylated phenotype in most IDHmut tumours, while all IDHwt gliomas were hypomethylated. To extrapolate the functional implications of these results, we showed that the methylation status of LDHA promoters was highly associated with LDHA expression at the protein level. This suggests epigenomic programming of LDHA, while general regulators of mitochondria were not differentially methylated in IDHmut
FIGURE 6  (A) Cluster analysis of LDHA, MTC02 and MT-CO1 H-Scores of the historical Glioma Cohort. Clusters 1-4 are highlighted by respective colours. (B) Contingency table showing distribution of Clusters 1-4 according to diagnosis. Panels (C) and (D) showing Kaplan–Meier charts according to Clusters 1-4. Panel (C) displays all gliomas, (D) only shows IDH1R132H non-mut glioblastomas. Tables on the right side illustrate the results of statistical analyses Clusters 1-4 versus Clusters 1-4. O: Oligodendrogliaoma, A: Astrocytoma, AA: Anaplastic Astrocytoma, GB: Glioblastoma
versus IDHwt tumours. Our findings confirm the recent proposal of the DNA methylation-based reprogramming of glycolytic enzymes in IDHmut gliomas [41].

ATP5G2 was identified as one of the most extensively regulated genes at the DNA methylation level. ATP5G2 encodes a subunit of the ATP Synthase complex V of the electron transport chain. We observed that most of the ATP5G2 promoter-associated CpG-islands were hypermethylated in the IDHmut gliomas. Only a small subset of IDHwt samples also showed increased methylation, but in fewer CpG sites. ATP5G2 has been previously described as being epigenetically regulated by DNA methylation in non-muscle invasive bladder cancer and renal cell carcinoma [42,43]. In these studies, ATP5G2 was identified as a hypermethylated potential tumour suppressor gene that was increasingly inactivated.

**FIGURE 7** Kaplan–Meier survival analyses of the Bev-Cohort. Progression-free (PFS) and Overall survival (OS) stratified by (A) LDHA and (B) MT-CO1 expression as well as (C) MT-CO1/LDHA quartile ratio. High and low values were grouped after median split. Dots display single patients (black dots IDH1R132H mut, grey dots IDH1R132H non-mut tumours). Investigated tissue corresponds to last resection/biopsy before Bev treatment was initiated.
with malignancy. Interestingly, in the EPIC-Cohort in patients with IDHwt tumours promoter-associated CpG sites were mostly hypomethylated. Another study found that ATP5G2 was upregulated in an ischaemic rat brain model. The authors described upregulated ATP5G2 gene expression 24 h after artery occlusion [44], suggesting that ATP5G2 gene expression is an early hypoxia/ischaemia response gene. The role of the expression of genes like ATP5G2 in gliomas needs further investigation.

Cellular metabolism and the provision of bioenergetics are not only restricted to glycolysis. Thus, we were interested in whether the mitochondrial biomass varied between IDHmut and IDHwt tumours. We investigated our mtDNA-Cohort for mitochondrial DNA copy number variations. Interestingly, mitochondrial DNA copy number was higher in IDHmut tumours than in IDHwt tumours, suggesting a relatively increased mitochondrial signature in IDHmut tumours. Additionally, we showed that mitochondrial copy number correlated with a surrogate parameter of mitochondrial biomass (antibody MTCO2) but not with protein expression of MT-CO1, a major contributor to oxidative phosphorylation. These findings underline that mitochondrial biomass should not be directly associated with mitochondrial functionality. In line with our findings, Navis and co-workers described higher amounts of mitochondria in an IDHmut glioma model [45]. Furthermore, in non-glial tumours, Farshidfar and co-workers identified an mRNA signature of IDH

When analysing DNA methylation signatures of tumour tissue, it should be considered that these signatures also reflect the non-tumour cell compartment. Cellular deconvolution revealed significant differences in immune cell infiltration in the distinct epigenetic glioma subclasses. In particular, mesenchymal GB showed high cellular heterogeneity with increased microglia/macrophage and lymphocyte infiltration, as previously reported [49,50]. Although there is a strong interaction between tumours and especially microglia, our data do not support the hypothesis that the metabolic signature is a pure immune cell signature. Our data indicate that key metabolic markers are not associated with immune cell content.

When we investigated metabolic aspects in our population-based historical Glioma Cohort, we detected two distinct subclasses of mainly IDHwt gliomas, while IDHmut gliomas were enriched in one cluster. Survival analyses revealed that the cluster of IDHwt tumours with high LDHA and low MT-CO1/MTCO2 expression showed worse patient prognosis. This again emphasised that, in case of IDHwt status, a mitochondrial signature might be associated with better patient survival.

To further explore our hypothesis that metabolic surrogate biomarkers are prognosticators in gliomas, we analysed glioma tissue from patients treated with Bev. Strikingly, patients who showed a mitochondrial signature in their tumour tissue had significantly better overall survival following Bev treatment. These findings are consistent with experimental data demonstrating that glioma cells without functional mitochondria are resistant to Bev [51]. Although this finding needs to be validated in larger cohorts of patients, these pilot data are compatible with the hypothesis that tumours with a mitochondrial signature are susceptible to anti-angiogenic therapy because they are less capable of adapting their metabolism to conditions of Bev-induced hypoxia.

In summary, the metabolism of IDHmut and IDHwt tumours differs substantially and relevant metabolic genes are regulated by DNA methylation. Furthermore, mitochondrial biomass is increased in IDHmut tumours and is stable during the clinical course. Importantly, even within IDHwt gliomas, a mitochondrial signature was associated with a better clinical outcome. At the epigenetic level, a mitochondrial signature was associated with the mesenchymal subclass in GB. Additionally, respiratory tumours may respond better to anti-angiogenic treatment than glycolytic tumours. The collective data indicate that for therapeutic regimens that either directly or indirectly affect tumour metabolism, the interrogation of multidimensional metabolic biomarkers is a promising strategy to define predictive signatures that can guide individualised treatment decisions.

**ACKNOWLEDGEMENT**

The authors thank Tatjana Starzetz and Maika Dunst for their technical support. Open access funding enabled and organized by Projekt DEAL.
CONFLICT OF INTEREST

JPS received grants from Merck and UCB as well as honoraria for lectures, travel, or advisory board participation from Abbvie, Bristol-Myers Squibb, Medac, Roche, Novocure and UCB. MWR has received research funding from the UCB. There are no conflict of interests specific to this manuscript.

AUTHOR CONTRIBUTIONS

YB and PNH designed the study; YB, KF, SB, BR, JZ, PB, PSZ, EI, MM and PNH performed the experiments; YB, BR, PB, PSZ, CS, MWR, OB, EH, MM and PNH involved in data acquisition and data collection; CS, KHP, EH and JPS provided material and clinical data; PNH involved in study supervision; All the authors involved in writing and editing the manuscript.

ETHICAL APPROVAL

The study was approved by the local ethics committee (GS 4/09, SNO-11-2017).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Pia S. Zeiner https://orcid.org/0000-0001-6626-9211
Patrick N. Harter https://orcid.org/0000-0001-5530-6910

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**File S1:** Clinical data of epic-Cohort (numbers in parentheses represent relative values). Histopathology of idhw t and idhmut tumours is depicted at the bottom of the table.

**File S2:** Clinical data of mtDNA-Cohort (numbers in parentheses represent relative values).

**File S3:** Clinical data of historical Glioma Cohort (numbers in parentheses represent relative values).

**File S4:** Clinical data of Bev-Cohort (numbers in parentheses represent relative values).

**File S5:** Mitochondrial metabolism genes.

**File S6:** CpGs included in the analyses.

**File S7:** (a) Hierarchical clustering of 57 glioma patients and 10,000 most variable CpG sites. (b) t-SNE plot of the same patients and 10,000 most variable CpG sites. (c) Hierarchical clustering of 57 glioma patients and 17 CpG sites that were shared between the 10,000 most variable CpG sites and 2542 metabolic CpG sites.

**How to cite this article:** Braun Y, Filipski K, Bernatz S, et al. Linking epigenetic signature and metabolic phenotype in IDH mutant and IDH wildtype diffuse glioma. *Neuropathol Appl Neurobiol.* 2021;47:379–393. https://doi.org/10.1111/nan.12669