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Intratumor DNA methylation heterogeneity in glioblastoma: implications for DNA methylation-based classification

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Background. A feature of glioblastoma (GBM) is cellular and molecular heterogeneity, both within and between tumors. This variability causes a risk for sampling bias and potential tumor escape from future targeted therapy. Heterogeneous intratumor gene expression in GBM is well documented, but little is known regarding the epigenetic heterogeneity. Variability in DNA methylation within tumors would have implications for diagnostics, as methylation can be used for tumor classification, subtyping, and determination of the clinically used biomarker O^6-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation. We therefore aimed to profile the intratumor DNA methylation heterogeneity in GBM and its effect on diagnostic properties.

Methods. Three to 4 spatially separated biopsies per tumor were collected from 12 GBM patients. We performed genome-wide DNA methylation analysis and investigated intratumor variation.

Results. All samples were classified as GBM isocitrate dehydrogenase (*IDH*) wild type (wt)/mutated by methylation profiling, but the subclass differed within 5 tumors. Some GBM samples exhibited higher DNA methylation differences within tumors than between, and many cytosine-phosphate-guanine (CpG) sites (mean: 17 000) had different methylation levels within the tumors. *MGMT* methylation status differed in *IDH* mutated patients (1/1).

Conclusions. We demonstrated that intratumor DNA methylation heterogeneity is a feature of GBM. Although all biopsies were classified as GBM *IDH* wt/mutated by methylation analysis, the assigned subclass differed in samples from the same patient. The observed heterogeneity within tumors is important to consider for methylation-based biomarkers and future improvements in stratification of GBM patients.

Key Points

1. Methylation differences can be higher within tumors than between.
2. Multiple DNA methylation subclasses exist in GBM.
3. Intratumor DNA methylation heterogeneity is a feature of GBM.

Importance of the Study

GBM is a heterogeneous diagnostic category with poor survival despite multimodal treatment, highlighting the need for patient stratification and adapted treatment. DNA methylation is a valuable tool for classification and subgrouping of tumors with distinct differences in prognosis, and could potentially contribute to improving diagnostics. Considering the variety of tumor clones and transcriptomic expression profiles previously described within GBM tumors, it is of utmost importance to characterize the intratumor DNA methylation heterogeneity and how it affects methylation-based biomarkers and classification. We demonstrate a high intratumor methylation heterogeneity, but methylation-based classification assigns the correct tumor type (GBM *IDH* wild type/mutated). However, the subtype differs within 5 tumors, and the *MGMT* promoter methylation status, frequently used for treatment decision in elderly patients and inclusion in clinical trials, differs in one *IDH* mutated tumor. The methylation heterogeneity we demonstrate within tumors should be considered for methylation-based biomarkers and improvements in GBM subtyping.

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults[1] and is characterized by an extensive heterogeneity both between and within tumors on a regional as well as single-cell level regarding genomic aberrations and transcriptomic expression.[2–4] Heterogeneity adds to the complexity of treating GBM, as the therapeutic response to radiation and chemotherapy varies between tumor clones.[5–7] The current treatment for GBM is maximal safe surgical resection followed by concomitant and adjuvant temozolomide (TMZ) and radiotherapy, which still only results in a median survival of 15 months.[8] O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme, which counteracts the damage induced by TMZ.[9] This gene can be silenced by methylation in the promoter,[10] and a survival benefit has been demonstrated for patients with methylated *MGMT*.[11] *MGMT* is frequently used clinically for treatment allocation (e.g. TMZ or radiotherapy) in elderly patients[12] and for stratification or selection to clinical trials.[13]

Molecular markers were implemented for the first time in the World Health Organization (WHO) 2016 classification,[14] and we are moving toward more objective tools for classification—for instance, DNA methylation.[15–17] DNA methylation can also be used to estimate the methylation age of tissues, which is accelerated in tumors,[18,19] and to predict mortality in healthy subjects.[20] Intertumor DNA methylation heterogeneity in GBM has been established by the existence of GBM subclasses with distinct prognosis,[17] as well as for gliomas of different grades[21] based on isocitrate dehydrogenase 1 (*IDH1*) mutation and glioma cytosine-phosphate-guanine (CpG) island methylator phenotype (G-CIMP).[22] Considering the substantial heterogeneity within tumors described for gene expression, treatment response, etcetera, we asked whether intratumor heterogeneity in GBM exists also at the level of DNA methylation. This knowledge is essential for future improvements in tumor classification and methylation-based biomarkers, to avoid misdiagnosis due to tumor heterogeneity and sampling bias. We therefore sampled 3 to 4 biopsies, spatially separated, per tumor from GBM patients and processed the samples on Infinium EPIC methylation arrays and investigated variations within individual tumors. The 38 included biopsies (from 12 patients) were homogeneously classified as GBM *IDH* wild type (wt) or mutated by methylation profiling despite the heterogeneity we found within tumors regarding CpG sites with variable methylation values, methylation age, and GBM subclass (5/12). This demonstrates the potential for DNA methylation profiling for diagnosis of single samples in the clinic, but also highlights that the observed DNA methylation heterogeneity within tumors should be considered for biomarkers and future improvements in patient stratification.

Materials and Methods

Patients and Samples

The study was approved by the regional ethics committee (Dnr 604–12) and carried out in accordance with the relevant guidelines and regulations. Three to 4 spatially separated biopsies per tumor were collected from 12 adult GBM and 3 meningioma (MNG) patients undergoing primary tumor resection during 2016–2018 at Sahlgrenska University Hospital (Gothenburg, Sweden) after signed informed consent. Biopsies were taken as early as possible in the surgical procedure to reduce brain shift, which is known to reduce accuracy of neuronavigation. Clearly spatially separated biopsies (e.g. center vs periphery or diametric opposite peripheries) as determined by the neurosurgeon were collected after confirmation of fluorescence based on 5-aminolevulinic acid (5-ALA). The location of each biopsy was tagged in the neuronavigation system and based on MRI differentiated into the following categories: *center*, central in the tumor; *periphery*, clearly within tumor but with more peripheral location than center; *border*, in the border between contrast and normal (non–contrast enhanced) tissue; *outside*, outside contrast-enhancing tumor.

Histology

Samples were processed for histology (see Supplementary Materials) and tumor content was assessed (blinded) by a specialist in clinical neuropathology.

Bisulfite Conversion and DNA Methylation Arrays

DNA was extracted from the biopsies using DNeasy Blood and Tissue Kit and TissueLyser (Qiagen), and 500 ng DNA was bisulfite converted with the EZ DNA Methylation Kit

(Zymo Research) as previously described.[23] DNA methylation levels were analyzed with Infinium MethylationEPIC BeadChip (Illumina) according to protocols supplied by the manufacturer.

Data Analysis

Methylation data were processed and normalized with Noob using the statistical software R with the package ChAMP.[24] The generated methylation data are available at Gene Expression Omnibus, accession number GSE116298. The reliability and technical reproducibility of the EPIC array are well established in the literature,[23,25,26] and we examined technical replicates from a recent publication[25] to determine a robust $\Delta\beta$ threshold for calling differentially methylated probes (DMP) in our cohort. $\Delta\beta > 0.3$ yielded less than 25 DMP between 2 sets of the aforementioned technical replicates and was therefore determined as the threshold in our study to avoid calling DMP due to reproducibility issues. For tumor classification/subtyping of GBM samples we used the MethPed classifier[16] and another published classifier.[15] The methylation status of the *MGMT* promoter was predicted with the package MGMTSTP27.[27,28] Methylation age was determined with Horvath age[18] and epiTOC.[19] A stricter pre-processing method was applied with the R package SeSAMe[29] to reduce artifactual detection of methylation in deleted or hyperpolymorphic regions of the genome for certain analyses as indicated in the results section. Tumor deconvolution of the methylation data accounting for tumor purity was performed using the R package InfiniumPurify.[30,31] Please see the Supplementary Methods for further details on the processing of methylation data.

MGMT Pyrosequencing

Pyrosequencing of 20 ng bisulfite converted DNA was performed using the PyroMark PCR kit, PyroMark Q24 CpG MGMT kit, and PyroMark Q24 Advanced CpG reagents on PyroMark Q24 Advanced (Qiagen) according to the manufacturer's instructions. A total of 7 CpG sites were investigated; chromosome 10 129467243-129467275 (UCSC hg38). The reader is referred to the Supplementary Methods for further details.

Sanger Sequencing

Fifty ng of DNA from biopsies classified as *IDH* mutated by DNA methylation profiling was PCR amplified with *IDH1* primers and purified with ExoSAP-IT PCR Product Cleanup Reagent (Affymetrix), and PCR products were Sanger sequenced (GATC Biotech).

Results

Heterogeneous DNA Methylation Subclasses within GBM Tumors

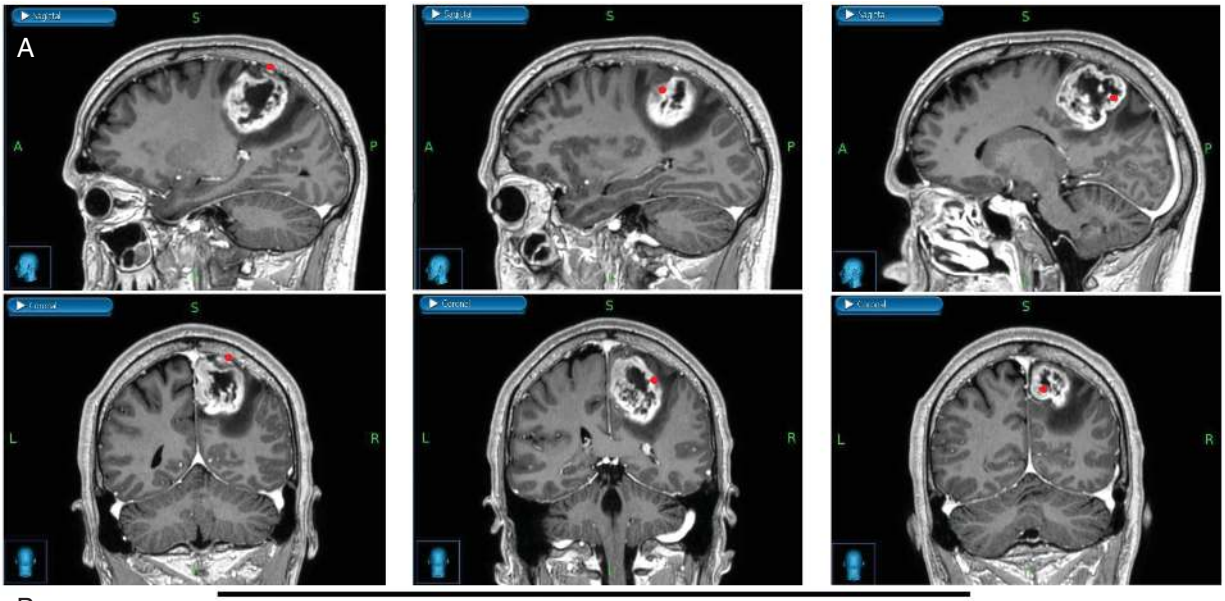
We sampled 3–4 spatially separated biopsies, fluorescent with 5-ALA, per tumor during tumor resection of GBM patients using neuronavigation and classified the biopsies as border, peripheral, and central (Figure 1A). Illumina methylation arrays were used to profile the genome-wide methylation pattern of the samples. Tumor content was evaluated by a neuropathologist by histology. Samples with ≥70% tumor content were included for further analysis, which left 38 biopsies from 12 GBM patients (Figure 1B).

We and others have previously developed DNA methylation-based diagnostic classifiers[15,16] and we employed these to investigate the intratumor heterogeneity of the biopsies regarding diagnostic classification. All samples were classified as GBM with a high score by MethPed (>0.7; max 1.0) and as a match for GBM *IDH* wt or *IDH* mutant by the classifier by Capper et al[15] (Supplementary Table 1). However, the subclass varied within 5 of 12 tumors; 3 had mesenchymal and receptor tyrosine kinase II (RTKII) subclasses, and 2 contained both RTKI and RTKII (Figure 1C). This demonstrates that several DNA methylation subclasses, according to the current classifier, exist intratumorally. There was no significant correlation in our cohort between the assigned GBM subclass and the regions that the biopsies were sampled from (data not shown). G-CIMP, which is associated with *IDH* mutations,[32] can be used to predict length of survival and subtyping of glioma of different grades.[21,22] One patient in the cohort (GU-HGG-216) was *IDH* mutated and G-CIMP positive according to methylation profiling, and both markers were consistent in all biopsies (Figure 1D).

GBM Biopsies Can Be More Similar by DNA Methylation to Other Tumors than within Tumors

To further evaluate heterogeneity in GBM, we included homogeneous intratumor MNG samples to use as comparison. Multiple samples, as described above, were analyzed from 3 MNG WHO grade I patients (tumor content ≥80%; Supplementary Table 2), which we, based on the benign nature of this tumor,[14] expected to be homogeneous. The patient identity of all samples was first verified by single-nucleotide polymorphism clustering (Supplementary Figure 1A). The genome-wide heterogeneity in methylation was then investigated with a multidimensional scaling (MDS) plot based on all CpG sites (Figure 2A) and unsupervised hierarchical clustering based on the top 5000 deviating CpG sites (Figure 2B). As expected, the MNG samples formed a tight cluster in both analyses, while the GBM samples demonstrated a larger spread with biopsies from the same tumor interspersed with biopsies from other tumors, reflecting a higher similarity to samples from other tumors compared with their intratumor samples. We hypothesized that this could be dependent on the region the biopsy was sampled from or the GBM subclass, but neither showed any clear association in our cohort (Supplementary Figure 1B–C).

Copy-number alterations (CNAs) have been shown to differ within GBM tumors,[3] which is in agreement with our study where hierarchical clustering based on the mean value of CNA segments showed that one patient (GU-HGG-204, who did not cluster by methylation either) did not cluster together and additional patients had biopsies at different branching levels (Figure 2C). The main intratumor CNA differences in GU-HGG-204 were loss of chromosomes 4, 6, and 8 (Supplementary Figure 2). We applied a stricter



B

Patient ID	Age (years)	Gender (M/F)	Diagnosis	Biopsy 1	Biopsy 2	Biopsy 3	Biopsy 4
GU-HGG-154	60	M	GBM, IDH wt	B, 80%	C, 90%	O, 80%	B, 90%
GU-HGG-182	66	M	GBM, IDH wt	P, 70%	-	C, 90%	C, 70%
GU-HGG-185	81	M	GBM, IDH wt	B, 80%	B, 80%	C, 95%	-
GU-HGG-200	54	F	GBM, IDH wt	B, 90%	B, 90%	B, 80%	-
GU-HGG-204	74	F	GBM, IDH wt	P, 70%	P, 80%	C, 70%	-
GU-HGG-216	71	F	GBM, IDH mut	C, 90%	P, 90%	B, 80%	-
GU-HGG-224	63	F	GBM, IDH wt	P, 80%	C, 70%	P, 70%	-
GU-HGG-225	67	M	GBM, IDH wt	P, 70%	P, 80%	C, 90%	-
GU-HGG-260	70	M	GBM, IDH wt	B, 90%	P, 90%	B, 75%	-
GU-HGG-269	65	F	GBM, IDH wt	B, 75%	P, 70%	P, 90%	-
GU-HGG-271	52	M	GBM, IDH wt	B, 90%	P, 90%	P, 80%	-
GU-HGG-287	65	F	GBM, IDH wt	P, 80%	P, 90%	C, 90%	P, 90%

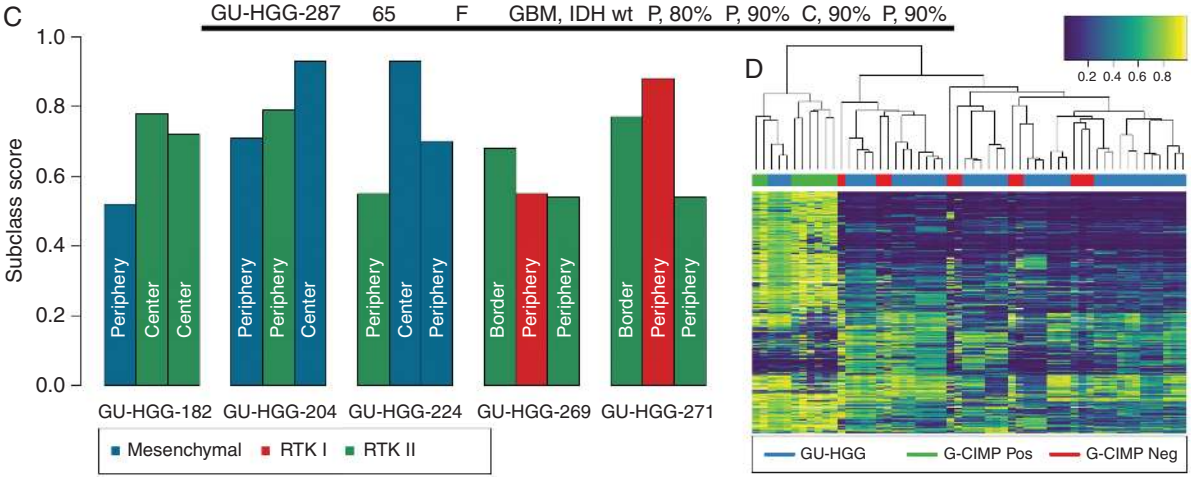


Fig. 1. Diagnostics by DNA methylation profiling. (A) 5-ALA and neuronavigation were used to sample 3–4 biopsies per tumor from spatially separated regions categorized as border (B; left), peripheral (P; middle), and central (C; right) illustrated here with sagittal (top) and coronal (bottom) planes. (B) Data for the 12 included glioblastoma (GBM) patients including sampling region and histological tumor content of the biopsies. The listed diagnosis is the histological diagnosis of the patient based on tumor material separate from the biopsies. (C) DNA methylation profiling classified all biopsies as GBM *IDH* wt/mutated but the subclass, according to a previously published classifier,[15] differed in 5 patients. (D) Unsupervised hierarchical clustering of the GBM biopsies (blue) and The Cancer Genome Atlas glioma samples, previously classified as positive for glioma CpG island methylator phenotype (G-CIMP; green) or G-CIMP negative (red), respectively.[22] Three GBM biopsies (all GU-HGG-216) clustered as G-CIMP positive.

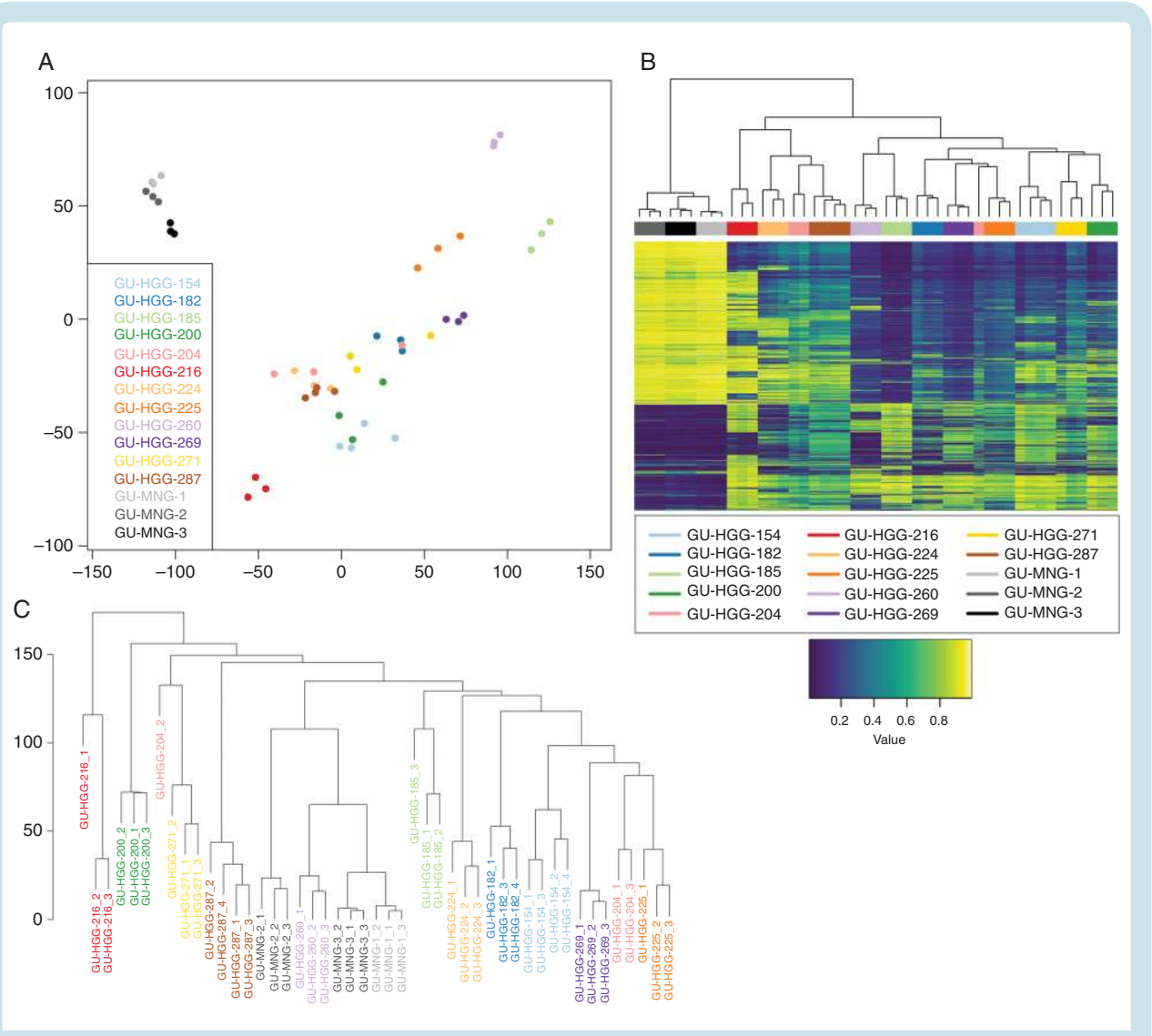


Fig. 2. Genome-wide heterogeneity in glioblastoma. (A) Multidimensional scaling (MDS) plot based on all sites on the EPIC methylation array shows intratumor GBM heterogeneity as several samples are located closer, and are thus more similar, to samples from other tumors than their intratumor samples. (B) Unsupervised hierarchical clustering of the top deviating 5000 CpG sites on the methylation array clusters biopsies from each patient together for all patients except one. (C) Copy-number alterations (CNAs) were inferred from methylation data and hierarchically clustered.

pre-processing method with the R package SeSAMe[29] to reduce artifactual detection of methylation in regions with CNA and repeated the MDS and cluster analysis (Supplementary Figure 3A–B). The MDS plot based on all CpG sites was very similar to Figure 2A, suggesting that CNA differences within the tumors overall do not influence the heterogeneity in DNA methylation. GU-HGG-204, however, clustered together after the stricter pre-processing, thus indicating that the intratumor CNA differences were underlying the heterogeneity in the previous clustering (Figure 2B).

Intratumor Heterogeneity in DNA Methylation Age and Median Methylation

The global DNA methylation heterogeneity in GBM and MNG was assessed by examining the median methylation values within each tumor (Figure 3A) and the coefficient of variation (CV) of it. The CV between GBM tumors was 15% and only 3% for MNG. The CV within the individual tumors ranged 0.1–2% for MNG, while it was 0.5–6.5% for GBM, demonstrating that GBM can vary more intratumorally than MNG interpatient. Another proposed method to assess heterogeneity is to determine the proportion of intermediate methylation (PIM) score, since methylation has a bimodal distribution, and an intermediate value consequently evaluates heterogeneity within the sample (biopsy in this case). The PIM varied intertumor for GBM (range: 10–27% PIM) as well as intratumor (range: 0.2–5 percentage Δ PIM) while the PIM was similar between MNG tumors (range: 13–16%), but varied less intratumor than GBM (0.2–1.5 percentage Δ PIM; Figure 3B).

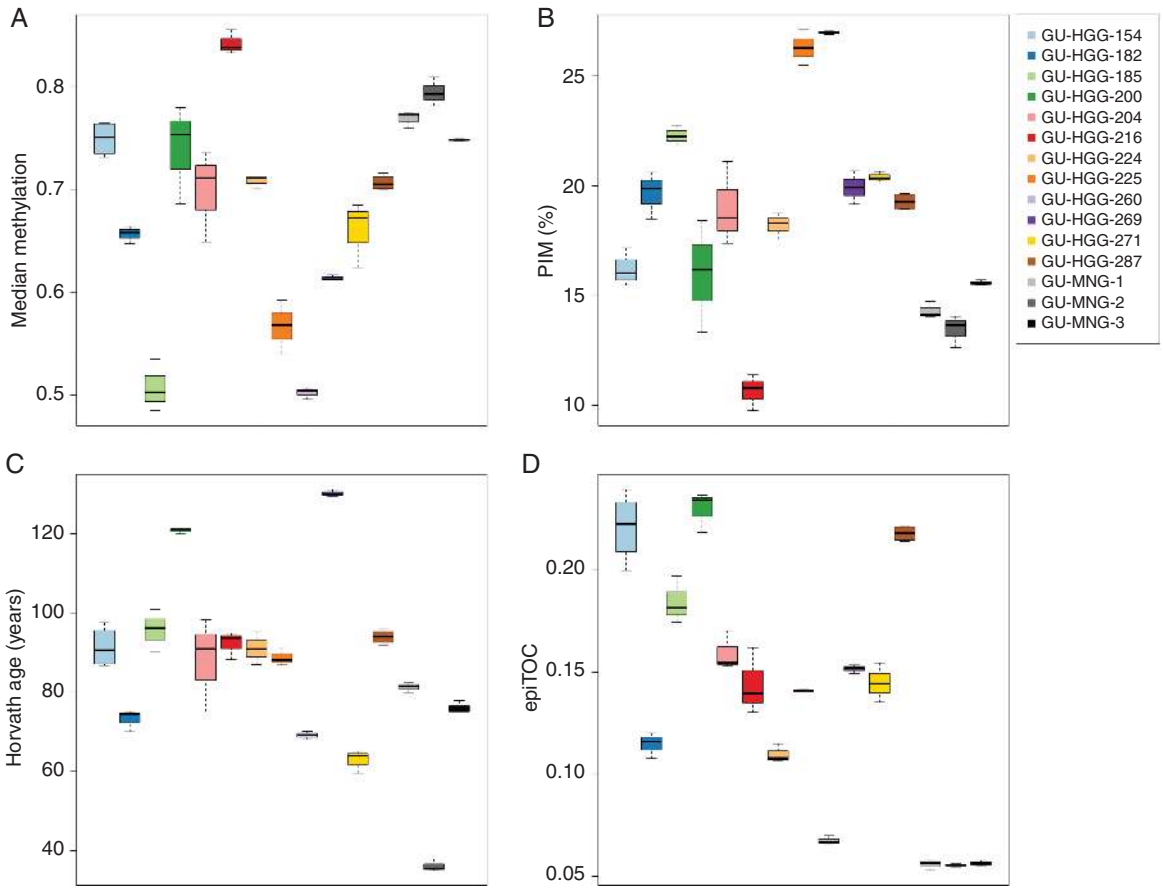


Fig. 3. DNA methylation age varies among biopsies from the same glioblastoma tumor. (A) Median methylation values of the biopsies and (B) the proportion of intermediate CpG sites (PIM) vary more in GBM than MNG. The legend applies to all subparts of this figure. (C) Horvath and (D) epiTOC methylation age varies intratumorally for several GBM tumors but not for MNG.

Accelerated methylation age (higher DNA methylation age than chronological age) varies across GBM subclasses[18] and has been suggested as a biomarker in glioma.[33] A biomarker should ideally be homogeneous and we therefore investigated the methylation age within GBM tumors and compared it with MNG. The methylation age was assessed by Horvath age[18] and epiTOC[19] and the scores were in good agreement (Pearson correlation 0.61, *P*-value $6*10^{-6}$; Supplementary Figure 4A). Differences within GBM tumors were detected both with Horvath age (mean ΔHorvath age = 7 y) and epiTOC (mean ΔepiTOC = 0.015), while the MNG tumors were more homogeneous (mean ΔHorvath age = 3 y; mean ΔepiTOC = 0.003; Figure 3C–D). Accelerated aging was seen in 45/47 GBM and MNG biopsies with the Horvath age (Δmean = 25 and 13 y, respectively; Supplementary Figure 4B).

Individual CpG Sites Demonstrate Intratumor Heterogeneity

Next, we characterized the number of DMP ($\Delta\beta > 0.3$) occurring between different patients in our cohort to use as a comparison for the intratumor DMP. The mean number of DMP between GBM patients was 86 000 (Figure 4A) and 21 000 between MNG patients. However, the mean number of DMP within GBM tumors was 17 000 (range: 522–50 684), but only 110 for MNG (range: 94–131) highlighting the intratumor heterogeneity in GBM (Figure 4B). GU-HGG-204, which did not cluster by CNA, was not significantly enriched for DMP on the chromosomes with differing copy number intratumorally compared with the other tumors. We also repeated the DMP analysis after applying the stricter pre-processing pipeline in SeSAMe[29] to reduce erroneous detection of methylation in regions with CNA. This reduced the mean number of intertumor DMP slightly in GBM (72 000 instead of 86 000 previously) and MNG (17 000 instead of 21 000). The number of intratumor DMP was reduced in a similar manner; average of 13 000 DMP in GBM (17 000 without CNA correction) and in MNG (50 instead of 110; Supplementary Figure 5), showing that the majority of intratumor DMP were not influenced by differences in CNA.

The GBM intratumor DMP were mostly only present between 1 biopsy pair or 2 biopsy pairs, but not shared between all pairs of the biopsies (Figure 4C). The location of the intratumor DMP in GBM was significantly

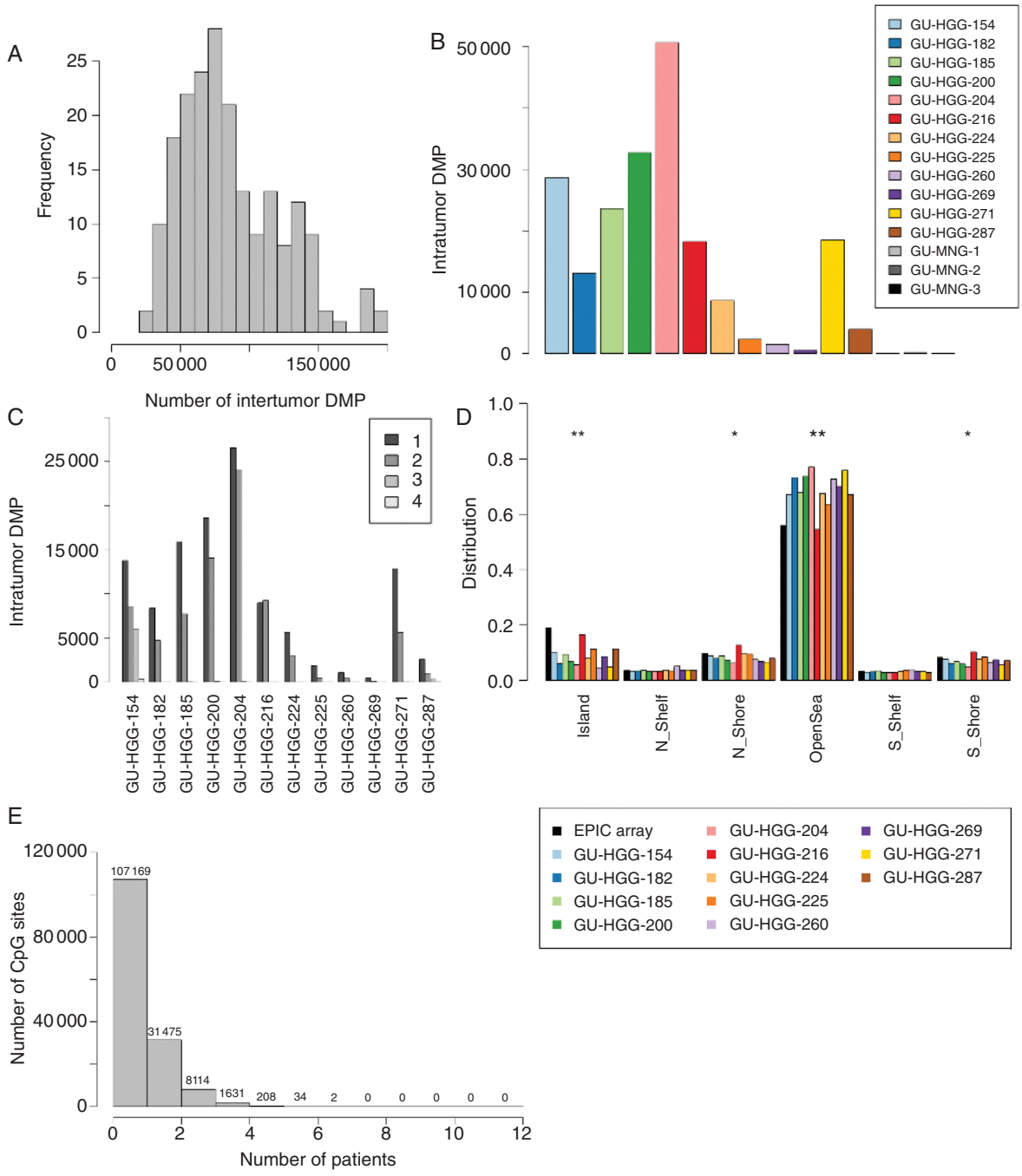


Fig. 4. CpG site-specific intratumor heterogeneity in glioblastoma. (A) The frequency of the number of intertumor differentially methylated probes (DMP; $\Delta\beta > 0.3$) in the GBM cohort. (B) The number of intratumor DMP in each tumor respectively and (C) between how many intratumor biopsy pairs the DMP occurred. (D) Distribution of where the intratumor DMP occurred and the black bar (EPIC) illustrate the distribution of all CpG sites on the methylation array as a point of reference; if the proportion of DMP in a certain region exceeds this bar there is an enrichment of DMP in this region. * denotes significant change of the GBM intratumor DMP compared with the distribution on the array, *P*-value < 0.05. ** denotes *P*-value < 0.01. (E) Depiction of the number of intratumor DMP occurring in multiple GBM tumors.

enriched in open sea regions (*P*-value 0.001) and reduced in CpG islands, N-Shore and S-Shore (*P*-value $5*10^{-4}$, 0.03 and 0.01) compared with the distribution of CpG sites on the array (Figure 4D). Given the pattern of enrichment in open sea regions, we investigated whether specific CpG sites were frequently altered in multiple tumors. However, this was not the case, as the same DMP was rarely detected in several tumors (only 36 CpG sites shared between 6/12 GBM tumors; Figure 4E).

A

Sample	Probability (%)	Predicted state (M/U)	Sample	Probability (%)	Predicted state (M/U)	Sample	Probability (%)	Predicted state (M/U)
GU-HGG-154_1	99	M	GU-HGG-204_1	100	U	GU-HGG-260_1	99	U
GU-HGG-154_2	99	M	GU-HGG-204_2	96	U	GU-HGG-260_2	94	U
GU-HGG-154_3	99	M	GU-HGG-204_3	98	U	GU-HGG-260_3	96	U
GU-HGG-154_4	99	M	GU-HGG-216_1	98	M	GU-HGG-269_1	87	U
GU-HGG-182_1	99	U	GU-HGG-216_2	95	U	GU-HGG-269_2	97	U
GU-HGG-182_3	98	U	GU-HGG-216_3	99	U	GU-HGG-269_3	95	U
GU-HGG-182_4	97	U	GU-HGG-224_1	88	M	GU-HGG-271_1	77	M
GU-HGG-185_1	100	U	GU-HGG-224_2	62	M	GU-HGG-271_2	80	M
GU-HGG-185_2	98	U	GU-HGG-224_3	72	M	GU-HGG-271_3	67	U
GU-HGG-185_3	98	U	GU-HGG-225_1	98	U	GU-HGG-287_1	98	U
GU-HGG-200_1	97	U	GU-HGG-225_2	78	U	GU-HGG-287_2	99	U
GU-HGG-200_2	99	U	GU-HGG-225_3	86	U	GU-HGG-287_3	99	U
GU-HGG-200_3	98	U				GU-HGG-287_4	99	U

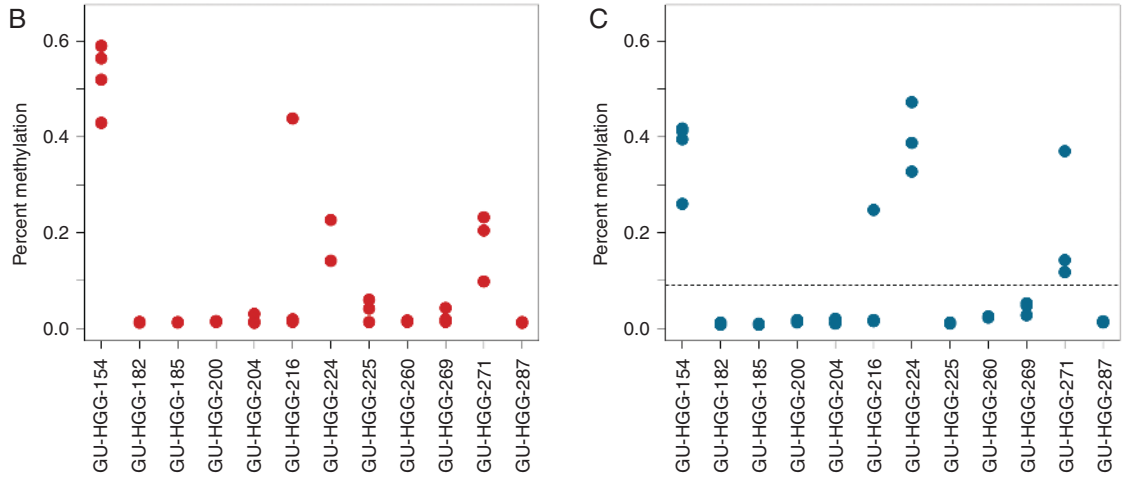


Fig. 5. Intratumor heterogeneity in methylation of the *MGMT* promoter. (A) Predicted methylation state (M = methylated, U = unmethylated) and probability for the *MGMT* promoter based on methylation array values show heterogeneity for 2 patients (GU-HGG-216 and GU-HGG-271). (B) Average percent methylation from methylation arrays of the 2 CpG sites (cg12434587 chr 10 129466945-129466946, cg12981137 chr 10 129467311-129467312) in the *MGMT* promoter used by the MGMT-STP27 R package for estimating the methylation status of *MGMT*. (C) Average methylation over 4 CpG sites (chr 10 129467255-129467273, hg38) in the *MGMT* promoter measured by pyrosequencing verifies intratumor heterogeneity in GU-HGG-216. Each circle represents one biopsy and the dashed line indicates the limit used in the clinic for methylated vs unmethylated (9%).

DNA Methylation Heterogeneity within the Same Tumor Occurs in the MGMT Promoter

The methylation status of the *MGMT* promoter is currently used clinically for treatment allocation in elderly patients.[12] We predicted *MGMT* methylation based on methylation array values with the MGMT-STP27 package,[27,28] and found that the only *IDH* mutated patient in our cohort (GU-HGG-216) was heterogeneous for *MGMT*, while 1/11 *IDH* wt were heterogeneous (Figure 5A–B). The MGMT-STP27 prediction, which is based on 2 CpG sites, is frequently used in clinical trials.[13,34] In clinical diagnostics, however, other sites are more commonly analyzed, using pyrosequencing[35](Supplementary Figure 6A). We therefore also performed pyrosequencing on 4 and 7 commonly used CpG sites (chr 10 129467255-129467273 and 129467243-129467275, respectively) according to thresholds previously suggested.[36] The results verified the heterogeneity in GU-HGG-216 and classified all *IDH* wt homogeneously (Figure 5C; Supplementary Figure 6B).

DNA Methylation Heterogeneity within Tumors Is Not Caused by Differing Tumor Content

The heterogeneity of GBM tumors also includes cell composition with a varying mix of tumor cells, normal cells, immune cells, etc. Differences in tumor content within individual tumors could as such affect methylation values. We therefore used the R package InfiniumPurify[29] to estimate tumor purity differences within tumors and deconvolute the methylome accounting for tumor purity. The tumor purity difference within tumors correlated significantly with the number of intratumor DMP prior

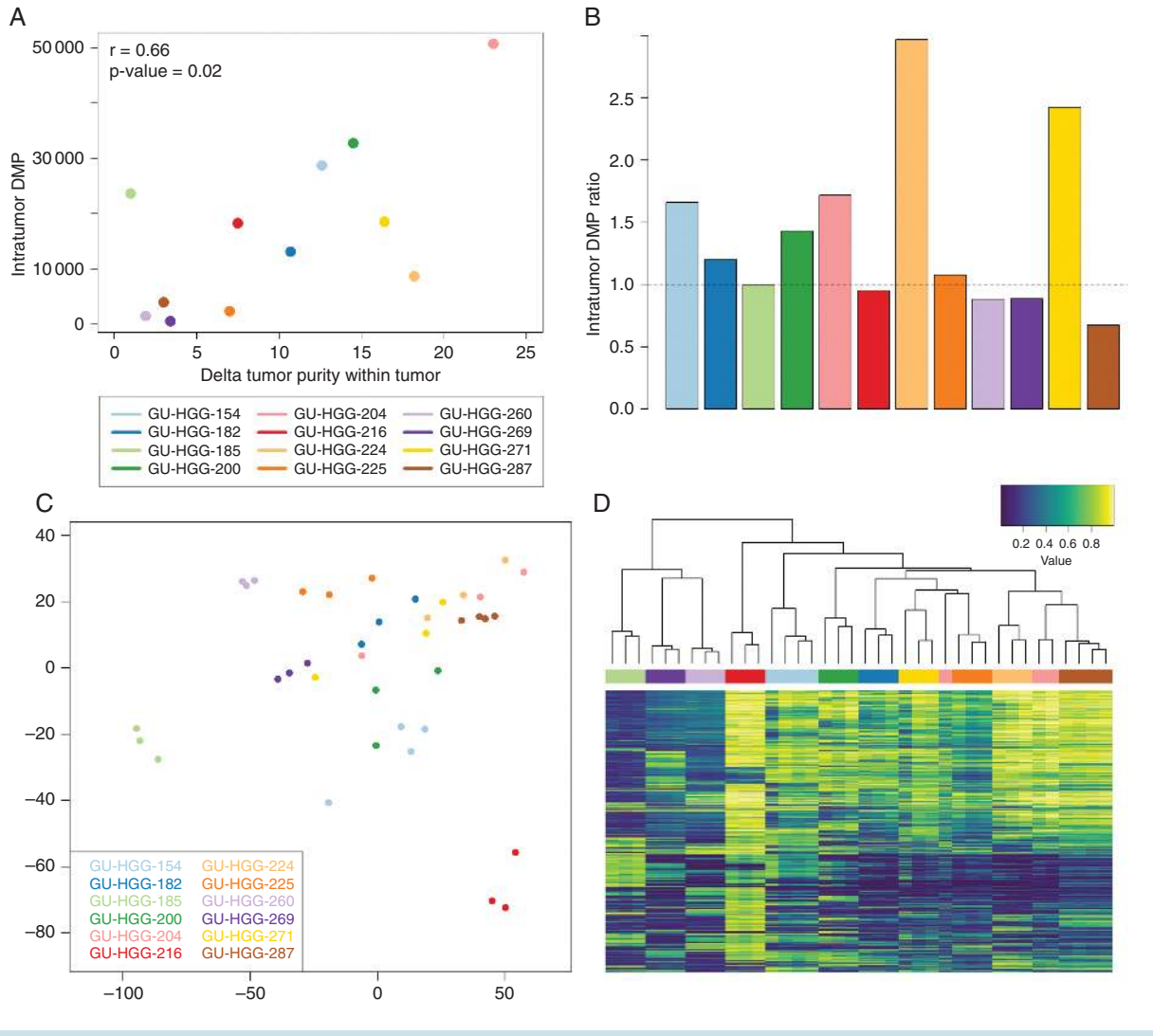


Fig. 6. Differing tumor content within the tumors is not the cause of intratumor methylation heterogeneity. (A) Tumor purity was estimated with the R package InfiniumPurify,[29] and purity differences within tumors correlated with the number of intratumor DMP. (B) Methylation data were deconvoluted accounting for tumor purity and the number of intratumor DMP after deconvolution compared with before deconvolution showed increases for some tumors and decreases for some tumors, but overall the change was not significant (paired Wilcoxon test, *P*-value 0.09). (C) MDS plot based on all CpG sites and (D) unsupervised hierarchical clustering of the top 5000 deviating CpG sites of deconvoluted methylation values demonstrated similar results as without deconvolution (Figure 2A–B).

to deconvolution (Figure 6A). The number of intratumor DMP after tumor deconvolution increased for some samples and decreased for some (Figure 6B), but overall there was no significant difference (*P*-value 0.09, paired Wilcoxon test). Further, the MDS plot based on all CpG sites after tumor deconvolution still showed a high intratumor methylation heterogeneity as several biopsies were closer, and thus more similar, to samples from other tumors than samples from the same tumor (Figure 6C). Further, the cluster after tumor deconvolution (Figure 6D) also looked similar, which taken together with the results from the MDS plot and DMP analysis demonstrated that differences in tumor content within individual tumors is not the cause of the observed intratumor methylation heterogeneity.

Discussion

The WHO classification of brain tumors relies heavily on histopathological criteria, but high intra- and inter-observer variability has been demonstrated.[37] Several DNA methylation-based classifiers have therefore been developed and successfully reclassified[15,16] and stratified patients into subgroups with distinct survival times.[17,38]The unbiased diagnosis offered by the DNA methylation-based classifiers is consequently considered for diagnostic use. One important factor to evaluate is whether the classification is homogeneous within tumors, particularly for heterogeneous tumors such as GBM. Heterogeneity in gene expression,

genomic aberrations, microenvironment, etcetera between GBM patients as well as within single tumors has been extensively studied, but intratumor DNA methylation heterogeneity has not been investigated. We therefore sampled 3–4 biopsies per GBM tumor, processed the samples on IlluminaEPIC methylation arrays and analyzed the intratumor DNA methylation heterogeneity in GBM and its implications for DNA methylation-based diagnostics.

Thirty-eight GBM biopsies (from 12 patients) with ≥70% tumor content were included in the study and all were classified as GBM by the DNA methylation-based classifiers. This demonstrates that DNA methylation profiling accurately provides homogeneous intratumor diagnoses. However, we demonstrated the existence of multiple GBM methylation subclasses, according to the current subtyping,[15] within a single tumor as 5 out of 12 tumors had varying subclasses, which has previously been shown with gene expression as well.[4] Three of these tumors had combinations of RTKII and mesenchymal, suggesting a similar biology and/or need for further refinement of these subclasses, which has been mentioned previously.[15] We also noted in a proportion of these tumors that the biopsy with a lower tumor content coincided with a switch to the mesenchymal subtype, potentially indicating that this subclass reflects tumors with lower tumor content rather than a separate entity. GBM is a diffusively infiltrating tumor where the tumor content consequently varies both between and within tumors, as does the amount of immune cells and macrophages.[39] Differing tumor content between samples can influence methylation results and potentially mask methylation differences between "pure" tumor methylomes or create differences. We therefore only included samples with ≥70% tumor content as recommended by Capper et al[40] and performed tumor deconvolution of methylation values accounting for tumor purity with InfiniumPurify.[30] The intratumor heterogeneity observed by DMP and MDS analyses was not significantly altered after tumor deconvolution. This demonstrated that differing tumor content within tumors did not cause the observed intratumor methylation heterogeneity in this study. We also verified that intratumor CNA differences overall did not affect the methylation results by repeating analyses after applying the SeSAMe package[29] in pre-processing to filter away artifactual methylation values in areas with CNA differences.

The heterogeneity of GBM between tumors was assessed by the CV of the median methylation, and the value, 15%, differed from the previously reported 3%, but that was calculated from only 4 patients using reduced representation bisulfite sequencing data, which could explain the difference.[41] The intratumor CV of our GBM samples was lower (range: 0.5–6.5%) than the CV between tumors, but in some cases higher than the heterogeneity observed between different MNG tumors (3%), indicating a high intratumor heterogeneity in GBM. This was also seen regarding DMP within GBM tumors (average: 17 000), which was around 20% of the number of DMP between patients (average: 86 000). However, MNG had extremely few DMP within tumors (average: 110), which is <1% of the DMP between tumors (average: 21 000). This illustrates the DNA methylation homogeneity within MNG WHO grade I tumors and the intratumor heterogeneity in GBM. The distribution of the intratumor DMP in GBM in relation to the promoter demonstrated significant enrichment in the open sea region and depletion mainly in CpG islands. A likely explanation is that conserved regions such as CpG islands are less likely to be altered by so-called passenger events compared with the open sea region. However, no specific CpG sites were altered within the open sea region, as very few sites were DMP in multiple tumors.

The intratumor heterogeneity in GBM was also revealed by a higher similarity between different tumors than within tumors in several cases on the MDS plot based on all sites. This demonstrated that GBM biopsies can be more similar DNA methylation-wise to different tumors than other regions of the same tumor. A recent study demonstrated that GBM samples were more similar by gene expression to samples derived from another tumor but from the same region than samples of the same tumor from a different region.[42] As DNA methylation reflects the cell of origin, it is tempting to speculate that this could hold true for DNA methylation as well, but we saw no clear association in our cohort to either tumor region or GBM subclass.

Methylation of the *MGMT* promoter correlates with prolonged patient survival[11] and is used clinically for treatment allocation in elderly patients.[12] Intratumor heterogeneity of *MGMT* has previously been reported in some studies,[43,44] while others showed homogeneity.[45] Our pyrosequencing results showed intratumor heterogeneity in *MGMT* methylation for one patient, which was the only *IDH* mutated patient in our cohort. *MGMT* methylation is less explored for this patient group, and further studies with larger cohorts are required to determine the relevance of *MGMT* methylation for *IDH* mutated patients. *MGMT* was homogeneous in our cohort for GBM *IDH* wt based on pyrosequencing, but one patient was heterogeneous according to the prediction from the methylation arrays. We noted, however, that the discordant biopsy (GU-HGG-271–3) had a lower probability score compared with the other biopsies.

Methylation age has been suggested as a biomarker in glioma,[33] and we therefore evaluated it in our cohort using 2 methods: Horvath age[18] and epiTOC.[19] The methods were in good agreement, and the Horvath age was accelerated in all except 2 samples, consistent with earlier studies.[18,33] Methylation age, assessed by both methods, was homogeneous in MNG, but heterogeneous within GBM tumors, thus questioning its value as a prognostic biomarker. A robust biomarker should ideally be homogeneous within tumors, a criterion that is often overlooked but needs to be considered in future studies of candidate biomarkers.

In conclusion, we showed that intratumor DNA methylation heterogeneity is a feature of GBM, and our results suggest that the alterations mainly occur in less conserved regions (open sea), but not altering specific CpG sites within that region. DNA methylation-based classification was still able to provide homogeneous intratumor diagnoses (GBM *IDH* wt and GBM *IDH* mutated, respectively). Caution should, however, be exercised regarding subgrouping of GBM, as we demonstrated that multiple methylation subclasses coexist within the same tumor. We further showed that a large number of CpG sites were differentially methylated within the GBM tumors and that

the intratumor heterogeneity affected the suggested biomarker methylation age. The clinically implemented biomarker *MGMT* promoter methylation was heterogeneous for the *IDH* mutated tumor, warranting further studies on this patient group. All *IDH* wt tumors were homogeneous for *MGMT* based on pyrosequencing, but one was heterogeneous according to the prediction from the methylation arrays necessitating further studies. The observed intratumor heterogeneity in DNA methylation in this study needs to be considered for methylation-based biomarkers and future stratification of GBM subtypes to improve diagnostic accuracy for this heterogeneous disease.

Supplementary Material

Supplementary data are available at *Neuro-Oncology* online.

Keywords

classification | DNA methylation | glioblastoma | heterogeneity | MGMT

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Authorship statement. AW, ASJ, and HC designed the study and HC coordinated it. ASJ performed the surgical procedures and provided clinical data and input to the project. AW performed all experiments except the histology and Sanger sequencing, which was done by SFV. TOB performed the histopathological evaluations. AW analyzed the data with assistance from TK. AW prepared the figures and tables and wrote the manuscript with input from HC. All authors revised and approved the final version of the manuscript.

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References

1. Ostrom QT, Gittleman H, Farah P, et al. CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006–2010. *Neuro Oncol*. 2013;15(Suppl 2):ii1–ii56.
2. Verhaak RG, Hoadley KA, Purdom E, et al.; Cancer Genome Atlas Research Network. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell*. 2010;17(1):98–110.
3. Sottoriva A, Spiteri I, Piccirillo SG, et al. Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. *Proc Natl Acad Sci U S A*. 2013;110(10):4009–4014.
4. Patel AP, Tirosh I, Trombetta JJ, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science*. 2014;344(6190):1396–1401.
5. Lee JK, Wang J, Sa JK, et al. Spatiotemporal genomic architecture informs precision oncology in glioblastoma. *Nat Genet*. 2017;49(4):594–599.
6. Lan X, Jörg DJ, Cavalli FMG, et al. Fate mapping of human glioblastoma reveals an invariant stem cell hierarchy. *Nature*. 2017;549(7671):227–232.
7. Segerman A, Niklasson M, Haglund C, et al. clonal variation in drug and radiation response among glioma-initiating cells is linked to proneural-mesenchymal transition. *Cell Rep*. 2016;17(11):2994–3009.
8. Stupp R, Mason WP, van den Bent MJ, et al.; European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005;352(10):987–996.
9. Gerson SL. MGMT: its role in cancer aetiology and cancer therapeutics. *Nat Rev Cancer*. 2004;4(4):296–307.
10. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res*. 1999;59(4):793–797.
11. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med*. 2005;352(10):997–1003.
12. Malmström A, Grønberg BH, Marosi C, et al.; Nordic Clinical Brain Tumour Study Group (NCBTSG). Temozolomide versus standard 6-week radiotherapy versus hypofractionated radiotherapy in patients older than 60 years with glioblastoma: the Nordic randomised, phase 3 trial. *Lancet Oncol*. 2012;13(9):916–926.
13. Wick W, Gorlia T, Bady P, et al. Phase II Study of Radiotherapy and Temsirolimus versus Radiochemotherapy with Temozolomide in Patients with Newly Diagnosed Glioblastoma without MGMT Promoter Hypermethylation (EORTC 26082). *Clin Cancer Res*. 2016;22(19):4797–4806.
14. Louis DN, Perry A, Reifenberger G, et al. The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta Neuropathol*. 2016;131(6):803–820.
15. Capper D, Jones DTW, Sill M, et al. DNA methylation-based classification of central nervous system tumours. *Nature*. 2018;555(7697):469–474.

16. Danielsson A, Nemes S, Tisell M, et al. MethPed: a DNA methylation classifier tool for the identification of pediatric brain tumor subtypes. *Clin Epigenetics*. 2015;7:62.
17. Sturm D, Witt H, Hovestadt V, et al. Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. *Cancer Cell*. 2012;22(4):425–437.
18. Horvath S. DNA methylation age of human tissues and cell types. *Genome Biol*. 2013;14(10):R115.
19. Yang Z, Wong A, Kuh D, et al. Correlation of an epigenetic mitotic clock with cancer risk. *Genome Biol*. 2016;17(1):205.
20. Chen BH, Marioni RE, Colicino E, et al. DNA methylation-based measures of biological age: meta-analysis predicting time to death. *Aging (Albany NY)*. 2016;8(9):1844–1865.
21. Ceccarelli M, Barthel FP, Malta TM, et al; TCGA Research Network. Molecular profiling reveals biologically discrete subsets and pathways of progression in diffuse glioma. *Cell*. 2016;164(3):550–563.
22. Noushmehr H, Weisenberger DJ, Diefes K, et al; Cancer Genome Atlas Research Network. Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma. *Cancer Cell*. 2010;17(5):510–522.
23. Kling T, Wenger A, Beck S, Carén H. Validation of the MethylationEPIC BeadChip for fresh-frozen and formalin-fixed paraffin-embedded tumours. *Clin Epigenetics*. 2017;9:33.
24. Morris TJ, Butcher LM, Feber A, et al. ChAMP: 450k chip analysis methylation pipeline. *Bioinformatics*. 2014;30(3):428–430.
25. Pidsley R, Zotenko E, Peters TJ, et al. Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling. *Genome Biol*. 2016;17(1):208.
26. Moran S, Arribas C, Esteller M. Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences. *Epigenomics*. 2016;8(3):389–399.
27. Bady P, Delorenzi M, Hegi ME. Sensitivity analysis of the MGMT-STP27 model and impact of genetic and epigenetic context to predict the MGMT methylation status in gliomas and other tumors. *J Mol Diagn*. 2016;18(3):350–361.
28. Bady P, Sciuscio D, Diserens AC, et al. MGMT methylation analysis of glioblastoma on the Infinium methylation BeadChip identifies two distinct CpG regions associated with gene silencing and outcome, yielding a prediction model for comparisons across datasets, tumor grades, and CIMP-status. *Acta Neuropathol*. 2012;124(4):547–560.
29. Zhou W, Triche TJ Jr, Laird PW, Shen H. SeSAMe: reducing artifactual detection of DNA methylation by Infinium BeadChips in genomic deletions. *Nucleic Acids Res*. 2018;46(20):e123.
30. Zheng X, Zhang N, Wu HJ, Wu H. Estimating and accounting for tumor purity in the analysis of DNA methylation data from cancer studies. *Genome Biol*. 2017;18(1):17.
31. Qin Y, Feng H, Chen M, Wu H, Zheng X. InfiniumPurify: an R package for estimating and accounting for tumor purity in cancer methylation research. *Genes Dis*. 2018;5(1):43–45.
32. Turcan S, Rohle D, Goenka A, et al. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature*. 2012;483(7390):479–483.
33. Liao P, Ostrom QT, Stetson L, Barnholtz-Sloan JS. Models of epigenetic age capture patterns of DNA methylation in glioma associated with molecular subtype, survival, and recurrence. *Neuro Oncol*. 2018;20(7):942–953.
34. Bell EH, Zhang P, Fisher BJ, et al. Association of MGMT promoter methylation status with survival outcomes in patients with high-risk glioma treated with radiotherapy and temozolomide: an analysis from the NRG oncology/RTOG 0424 trial. *JAMA Oncol*. 2018;4(10):1405–1409.
35. Bienkowski M, Berghoff AS, Marosi C, et al. Clinical Neuropathology practice guide 5-2015: MGMT methylation pyrosequencing in glioblastoma: unresolved issues and open questions. *Clin Neuropathol*. 2015;34(5):250–257.
36. Quillien V, Lavenu A, Ducray F, et al. Validation of the high-performance of pyrosequencing for clinical MGMT testing on a cohort of glioblastoma patients from a prospective dedicated multicentric trial. *Oncotarget*. 2016;7(38):61916–61929.
37. van den Bent MJ. Interobserver variation of the histopathological diagnosis in clinical trials on glioma: a clinician's perspective. *Acta Neuropathol*. 2010;120(3):297–304.
38. Taylor MD, Northcott PA, Korshunov A, et al. Molecular subgroups of medulloblastoma: the current consensus. *Acta Neuropathol*. 2012;123(4):465–472.
39. Darmanis S, Sloan SA, Croote D, et al. Single-Cell RNA-seq analysis of infiltrating neoplastic cells at the migrating front of human glioblastoma. *Cell Rep*. 2017;21(5):1399–1410.
40. Capper D, Stichel D, Sahm F, et al. Practical implementation of DNA methylation and copy-number-based CNS tumor diagnostics: the Heidelberg experience. *Acta Neuropathol*. 2018;136(2):181–210.
41. Sheffield NC, Pierron G, Klughammer J, et al. DNA methylation heterogeneity defines a disease spectrum in Ewing sarcoma. *Nat Med*. 2017;23(3):386–395.
42. Puchalski RB, Shah N, Miller J, et al. An anatomic transcriptional atlas of human glioblastoma. *Science*. 2018;360(6389):660–663.
43. Parker NR, Hudson AL, Khong P, et al. Intratumoral heterogeneity identified at the epigenetic, genetic and transcriptional level in glioblastoma. *Sci Rep*. 2016;6:22477.
44. Parkinson JF, Wheeler HR, Clarkson A, et al. Variation of O(6)-methylguanine-DNA methyltransferase (MGMT) promoter methylation in serial samples in glioblastoma. *J Neurooncol*. 2008;87(1):71–78.
45. Cao VT, Jung TY, Jung S, et al. The correlation and prognostic significance of MGMT promoter methylation and MGMT protein in glioblastomas. *Neurosurgery*. 2009;65(5):866–875; discussion 875.