Predictive MGMT status in a homogeneous cohort of IDH wildtype glioblastoma patients

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Abstract

Methylation of the O(6)-Methylguanine-DNA methyltransferase (MGMT) promoter is predictive for treatment response in glioblastoma patients. However, precise predictive cutoff values to distinguish “MGMT methylated” from “MGMT unmethylated” patients remain highly debated in terms of pyrosequencing (PSQ) analysis. We retrospectively analyzed a clinically and molecularly very well-characterized cohort of 111 IDH wildtype glioblastoma patients, who underwent gross total tumor resection and received standard Stupp treatment. Detailed clinical parameters were obtained. Predictive cutoff values for MGMT promoter methylation were determined using ROC curve analysis and survival curve comparison using Log-rank (Mantel-Cox) test. MGMT status was analyzed using pyrosequencing (PSQ), semi-quantitative methylation specific PCR (sqMSP) and direct bisulfite sequencing (dBiSeq). Highly methylated (> 20%) MGMT correlated with significantly improved progression-free survival (PFS) and overall survival (OS) in our cohort. Median PFS was 7.2 months in the unmethylated group (UM, < 10% mean methylation), 10.4 months in the low methylated group (LM, 10-20% mean methylation) and 19.83 months in the highly methylated group (HM, > 20% mean methylation). Median OS was 13.4 months for UM, 17.9 months for LM and 29.93 months for HM. Within the LM group, correlation of PSQ and sqMSP or dBiSeq was only conclusive in 51.5% of our cases. ROC curve analysis revealed superior test precision for survival if additional sqMSP results were considered (AUC = 0.76) compared to PSQ (cutoff 10%) alone (AUC = 0.67). We therefore challenge the widely used, strict PSQ cutoff at 10% which might not fully reflect the clinical response to alkylating agents and suggest applying a second method for MGMT testing (e.g. MSP) to confirm PSQ results for patients with LM MGMT levels if therapeutically relevant.

Keywords: Glioblastoma, Temozolomide (TMZ), O(6)-Methylguanine-DNA methyltransferase (MGMT), Methylation specific PCR (MSP), Pyrosequencing (PSQ), IDH (isocitrate dehydrogenase)
**Introduction**

Glioblastoma (GBM) is the most common and most aggressive primary brain tumor. The histological examination of neurosurgical tumor specimens as well as the immunohistochemical or molecular determination of the IDH1/2 status remain the gold standard for diagnosis of GBM [13]. Despite aggressive therapy, the survival of patients with GBM is approximately 15–17 months [21]. The current standard GBM therapy usually consists of neurosurgical resection, radiotherapy and additional chemotherapy with temozolomide (TMZ), an alkylation agent. But, chemosensitivity to TMZ strongly depends on epigenetic silencing by methylation of the O(6)-Methylguanine-DNA methyltransferase (MGMT) promoter [15]. Different randomized trials have shown that methylation of the MGMT promoter in GBM patients is associated with significantly higher survival rates if treated with radiotherapy and TMZ [4]. At the stage of recurrent disease, a TMZ rechallenge seems only reasonable in patients with clear methylation of the MGMT promoter based on the results of the DIRECTOR trial [24]. Recent data from the NOA-09 trial showed that newly diagnosed GBM patients with methylated MGMT promoter might benefit from a more intense first-line treatment regimen with CCNU in combination with TMZ [8], accepting an increased toxicity for an improved prognosis. These trials emphasize the importance of reliable MGMT status assessment and the need for predictive cutoff levels for clinical decision-making.

The methylation status of the MGMT promoter is widely determined by quantitative pyrosequencing (PSQ) [12, 28]. PSQ analysis uses a defined cutoff value to classify cases as “methylated” or “unmethylated” [1]. In many neurooncological centers, the biological cutoff is 10% [27]. However, a very strict cutoff value might not fully reflect the clinical response to TMZ therapy. Various previous studies that focused on the technical assessment of the MGMT status have suggested higher predictive cutoff levels above 10% [14, 17, 18].

Here, we aimed to determine a predictive cutoff level for clinical decision-making on the basis of a well-defined patient cohort of 111 IDH wildtype GBM patients. Three methylation groups were identified, which showed a very distinct clinical course in terms of PFS and OS: unmethylated 0–9% (UM), low methylated 10–20% (LM), and highly methylated > 20% (HM).

**Methods and Material**

**Tissue samples, clinical and patient data**

Two hundred ninety patients with newly diagnosed, previously untreated GBM (WHO grade IV) patients have been diagnosed between 2010 and 2015 at the Departments of Neurosurgery and Neuropathology, Charité Berlin, Germany. GBM diagnosis was confirmed by at least two experienced neuropathologists after surgical resection or stereotactic biopsy. According to the current WHO classification of CNS tumors [13], IDH mutation status was determined by IDH1 R132H immunohistochemistry (IHC) and bidirectional Sanger sequencing of exon 4 of the IDH1 as well as IDH2 gene for all GBM patients younger than 55 years [13]. Gliosarcoma, epithelioid glioblastoma, giant cell glioblastoma and IDH mutant tumors were excluded. The following clinical data were assessed: age at diagnosis, Karnofsky performance status (KPS), tumor localization, extent of resection and residual tumor volume, type and timing of adjuvant therapy, second-line therapy at recurrence, follow-up time, progression-free (PFS) and overall survival (OS) in months. The extent of tumor resection was determined by measuring the contrast-enhancing tumor volume in mm³ on T1-subtraction MRI imaging pre- and 48 hours postoperatively using the Brainlab iMRI software (Brainlab AG, Munich, Germany). Gross total resection (GTR) was defined as residual tumor volume < 2% [22]. PFS was assessed according to RANO criteria [25]. We identified 205 IDH wildtype GBM patients who matched the criteria mentioned above. Three long-term survivors (LTS; OS > 5 years) were identified in our cohort. For two LTS cases, DNA was sufficient to perform a genome-wide methylation analysis (EPIC array) which confirmed the diagnosis of GBM, IDH wildtype (Additional file 1: Figure S3 and Figure S4).

**Ethical statement**

This study was conducted according to the ethical principles of medical research involving human subjects according to the Declaration of Helsinki. The clinical data were assessed and anonymized for patients’ confidentiality. Ethical approval (EA2/064/17) was granted by the institutional ethics board of the Charité Ethics Committee.

**DNA extraction, bisulfite treatment and analysis of MGMT promoter methylation status in tumor samples**

Areas of high tumor cell content (≥ 80%) were chosen and macro-dissected for further analysis (Additional file 1: Figures S1a, dashed line; 1 b). Genomic DNA was extracted from formalin-fixed and paraffin embedded (FFPE) samples using the Qiagen DNeasy blood and tissue DNA extraction kit according to the manufacturer’s protocol (Qiagen, Hilden, Germany). The DNA was sodium bisulfite-modified using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA).

**Pyrosequencing (PSQ)** Quantitative methylation analyses were performed using the PyroMark Q24 MGMT kit (Qiagen, Hilden, Germany) and an automated PyroMark Q24 System (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Data was analyzed with...
the PyroMark Q24 Software 2.0 (Qiagen, Hilden, Germany). The percentage of methylated alleles was calculated as the mean value of the methylation percentage obtained. The cutoff value ≥10% was defined to classify MGMT methylated vs. unmethylated cases, which is commonly used and has been validated for routine clinical diagnostics [27]. Standardized positive and negative controls were included in every PSQ run. The PSQ results were evaluated by at least two experienced neuropathologists.

**Semi-quantitative methylation-specific PCR (sqMSP)**

sqMSP was performed with primers specific for either “methylated” or “unmethylated” DNA as previously described [5]. Original MSP PCR gels are shown in Additional file 1: Figure S2. Primers and PCR programs are listed in the methods and material section of Additional file 1. Semi-quantitative analysis of the optical band intensity (I) was performed using ImageJ (National Institutes of Health, Bethesda, USA). The following equation was used:

\[
\text{Band intensity methylated in}\% = \left( \frac{I_{\text{meth}} + I_{\text{unmeth}}}{I_{\text{meth}}} \right) \times 100
\]

\[
\text{Band intensity unmethylated in}\% = \left( \frac{I_{\text{meth}} + I_{\text{unmeth}}}{I_{\text{unmeth}}} \right) \times 100
\]

**Direct Bisulfite Sequencing (dBiseq)**

dBiseq was carried out as previously described [16] with minor adaptations. Primers and PCR program are listed in the methods and material section of Additional file 1. Sequencing was performed at Eurofins Genomics, Ebersberg, Germany. Sequenced samples were returned as .ab1 files, which were then analyzed using Chromas [9] (software program for PC, available at http://www.techneleysium.com.au/chromas.html).

**Analysis of MGMT promoter methylation status in positive and negative controls**

Both, positive and negative controls (listed in Additional file 1: Table S1) were assessed by PSQ, sqMSP, and dBiseq. Samples of non-neoplastic brain tissue and one samples with genomic DNA extracted from whole peripheral blood served as negative controls. The primary cell line SF126 and 7 tumor samples with clear MGMT promoter methylation levels >30% were used as positive controls.

**Genome-wide DNA methylation analysis**

DNA methylation signature analysis was performed using the Illumina Infinium Methylation EPIC array as previously described [2].

**IDH1 and IDH2 Sanger sequencing**

Bidirectional Sanger sequencing of exon 4 of IDH1 and IDH2 was performed in IDH R132H IHC-negative or equivocal cases in all patients <55 years of age. PCR primers for the genomic regions corresponding to IDH1 exon 4 (codon R132) and IDH2 exon 4 (codon R172) and the flanking intronic sequences are displayed in the methods and material section of Additional file 1. Sequencing was performed at Eurofins Genomics, Ebersberg, Germany.

**Immunohistochemical procedures**

Immunohistochemical stainings were performed on a Benchmark XT autostainer (Ventana Medical Systems, Tuscon, AZ, USA) with standard antigen retrieval methods (CC1 buffer, pH8.0, Ventana Medical Systems, Tuscon, AZ, USA) using 4-μm-thick, FFPE tissue sections (Additional file 1: Figures S1 c-f). The following primary antibodies were used: polyclonal rabbit anti-GFAP (1:2000, Dako), monoclonal mouse anti-MIB1 (Ki-67, 1:100, clone M7240, Dako), polyclonal rabbit anti-ATRX (1:200, Sigma), mouse monoclonal anti-IDH1 R132H (1:20, clone H09, Dianova). The iVIEW DAB Detection Kit (Ventana Medical Systems, Tuscon, AZ, USA) was used according to the manufacturer’s instructions. Sections were counterstained with hematoxylin, dehydrated in graded alcohol and xylene, mounted and coverslipped. IHC stained sections were evaluated by two independent, experienced neuropathologists. When no agreement was reached, the sections were reviewed by our team of neuropathologists at our department (Charité) and further molecular diagnostics (e.g. IDH1/IDH2 bidirectional Sanger sequencing, genome-wide DNA methylation analysis (EPIC analysis)) was performed.

**Statistical analysis**

Statistical analysis was performed in cooperation with the Charité’s Institute for Biometrics and Clinical Epidemiology using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Kaplan-Meier survival curves were obtained and differences in PFS and OS were tested for statistical significance using the log-rank test. Significance level was set at \( p < 0.05 \).

ROC analysis was used for diagnostic test evaluation. The true positive rate (Sensitivity) was plotted as a function of the false positive rate (100-Specificity) for different cutoff points. The area under the ROC curve (AUC) measured the accuracy. An AUC of 1 represents a perfect test; 0.8-0.9 a good test, 0.7-0.8 a fair test, 0.6-0.7 a poor test, and an area of ≤0.5 represents a worthless test.

**Results**

**Study cohort**

Heterogeneity of the patient cohort (e.g. in terms of the IDH status) has been a major point of criticism in
Table 1 Patients’ characteristics of our study cohort

| Study cohort n=111 | n | % |
|-------------------|---|---|
| Gender            |   |   |
| Female            | 48 | 43 |
| Male              | 63 | 57 |
| Age in years      |   |   |
| Mean              | 58.9 | - |
| Median            | 61.2 | - |
| Range             | 18.85-4 | - |
| MGMT              |   |   |
| Meth. (mean ≥10%) | 56 | 51 |
| Unmeth. (mean < 10%) | 55 | 49 |
| Toxicity during 1st line therapy |   |   |
| CTG °III-IV       | 4 | 4 |
| 2nd line therapy  |   |   |
| mTMZ              | 38 | 34 |
| TMZ rechallenge   | 7 | 6 |
| CCNU+Procarbazine | 4 | 4 |
| BEV               | 3 | 3 |
| Re-irradiation    | 4 | 4 |
| TTFields          | 5 | 5 |
| Re-resection      | 33 | 30 |
| Follow up in months |   |   |
| Mean              | 19.4 | - |
| Median            | 15.4 | - |
| Range             | 0.3-90 | - |
| PFS in months     |   |   |
| Mean              | 12 | - |
| Median            | 7.8 | - |
| Range             | 0.3-56 | - |
| OS in months      |   |   |
| Mean              | 19.8 | - |
| Median            | 15.5 | - |
| Range             | 0.5-90 | - |

BEV bevacizumab, CCNU lomustin, CTG common toxicity criteria, GTR gross total resection, MGMT O6-methylguanine-DNA-methyltransferase, meth methylated, mTMZ metronomic temozolomide, OS overall survival, PR partial resection, TMZ temozolomide, TTFields tumor treating fields, unmeth unmethylated
cases, additional dBiseq data was available (representative results in Fig. 2e). PSQ and dBiseq showed identical results in only 45.5% (n=10/22), MSP and dBiseq in 90% (n=18/20) of cases. The detailed results are displayed in Additional file 1: Table S1. In general, in cases with PSQ ≥ 16%, we observed a very high consistency between PSQ, MSP and dBiseq results.

We additionally investigated the survival profiles of all transition zone patients after combining PSQ and MSP results. First, we redistributed the LM patients to either the UM or HM category based on MSP testing. As expected, the differences between UM vs. HM were highly significant: PFS (**p<0.0001, HR 3.002, CI 1.886 to 4.778) and OS (**p<0.0001, HR 2.629, CI 1.729 to 3.997, Additional file 1: Figure S6 a, b). Next, we defined the following more detailed four groups to investigate if the integration of MSP resulted in a redistribution of LM patients to either the UM or HM category: UM, LM + MSP unmethylated, LM + MSP methylated, and HM. The results still clearly indicated a transition zone for median PFS and OS, which seemed independent of the MSP results (Additional file 1: Figure S6 c, d). Moreover, curve comparison between PSQ LM + MSP unmethylated and PSQ LM + MSP methylated showed no significant difference, most likely due to small sample size and presence of one LTS patients within the LM group.

Regarding the aforementioned results, we performed ROC curve analysis for prognostic test evaluation for PSQ (cutoff 10%) alone and for PSQ (cutoff 10%) combined with sqMSP results. LM cases that were considered MGMT unmethylated by sqMSP were therefore assigned to the UM group, LM cases that were considered MGMT methylated by sqMSP were therefore assigned to the HM group. ROC curve analysis revealed superior test precision with an AUC = 0.76 for PSQ (cutoff 10%) combined with sqMSP results compared to PSQ (cutoff 10%) alone (AUC = 0.67; Figure 2 a). Additionally, we performed step-wise cutoff testing for 10%, 12%, 15%, 17%, and 20% PSQ results. At a cutoff of 17%, highest test precision was reached with an AUC of 0.77 (Figure 2 b).

**Discussion**

We demonstrate that IDH wildtype GBM patients with low methylation of the MGMT promoter (mean 10-20%) represent a “transition zone” in terms of PFS and OS compared to clearly unmethylated (0-9%) and highly methylated (> 20%) patients. For patients with low methylated MGMT promoter (10-20%), PSQ results could be validated in only 51.5% (n=17/33 samples, Additional file 1: Table S1) by one other method (sqMSP or dBiseq) to be clearly methylated.

Both, MSP and PSQ, have independently been suggested as the “gold standard” for methylation analysis of the MGMT gene promoter [3, 11]. As to which method to use, the scientific community has not reached a consensus yet
Several studies have demonstrated the prognostic value of MSP. Nevertheless, MSP primers are generated to detect either unmethylated or fully methylated MGMT promoter sites, which may in turn result in a lower sensitivity of this method [10]. Furthermore, MSP lacks international standardization [19]. In contrast to MSP, PSQ provides information about the extent of methylation at each individual CpG site, which improves the sensitivity of analyzing heterogeneous methylation patterns within a tumor sample [10]. Nevertheless, the optimal cutoff value is still a matter of scientific debate [1]. The predictive cutoff is strongly influenced by i) interlaboratory differences, ii) technical challenges of MGMT testing, which are strongly dependent on successful bisulfite treatment of the DNA [6], and particularly iii) tissue processing, such as formalin-fixation and paraffin-embedding [17, 18]. Therefore, determining a “grey zone” seems to be a more reasonable approach than setting a very strict cutoff.

**Fig. 2** a: Receiver operating characteristics (ROC) for 10% PSQ cutoff (AUC = 0.67) and 10% PSQ cutoff corrected for sqMSP results (AUC = 0.76) in terms of overall survival. b: Receiver operating characteristics (ROC) for determination of optimal cutoff values for PSQ results at 10% (AUC = 0.67), 12% (AUC = 0.72), 15% (AUC = 0.74), 17% (AUC = 0.77) and 20% (AUC = 0.75). c: Representative methylation-specific PCR (MSP) shows results obtained from 15 different, representative GBM samples and one negative control (NC). The DNA was extracted from formalin-fixed and paraffin-embedded tissue. Methylated samples demonstrated PCR products with primers detecting the methylated (M, 89bp) and unmethylated (U, 93bp) MGMT promoter sequence. Clearly unmethylated samples showed PCR products only for the unmethylated MGMT promoter sequence (U). The pyrogram (d) demonstrates the pyrosequencing result of patient #19 (PSQ mean = 14%). The yellow areas correspond to the internal control of conversion (arrows). The blue areas indicate the polymorphism (T/C) as result of the bisulfite treatment and show the level of the methylation (%) of each CpG. An exemplary section of direct Bisulfite Sanger sequencing trace (forward) of patient #26 demonstrates CpGs 13-18 of 27 (arrows) which are partly (T/C) or fully converted (e).
Even though previous studies have identified 10% as the PSQ cutoff to distinguish methylated from unmethylated samples - often based on biological determinants comparing non-neoplastic to neoplastic tissue [4, 17, 27, 28] - several more recent studies have suggested to introduce a “transition” or “grey zone” [7, 17, 18, 26] for partly methylated tumors that perhaps cannot be assigned to either the methylated or unmethylated category. Many of these studies were criticized to small sample size and heterogeneous patient population [28] including different therapeutic regimens and IDH mutant as well as IDH wildtype GBM patients.

Seeing that IDH mutant GBMs demonstrate a hypermethylator phenotype and show a favorable clinical course, the impact of MGMT methylation on survival may have been overestimated in those studies [23].

Clearly, our study also has some limitations that restrict the interpretation of our data. There are both, the retrospective character and the single center experience. Nevertheless, a key advantage of this study is that it provides a large data set (n=111) from both clinically and molecularly very well-documented and characterized subgroup of IDH wildtype GBM patients (according to the most recent WHO classification).

As the different methylation groups demonstrate a very distinct clinical course in terms of PFS and OS, and PSQ and sqMSP/sBiseq results are only concordant in 51.5% of LM patients - which might partly be explained by a heterogeneous methylation pattern and technique-dependent analysis of different CpG sites within the MGMT promoter [19] - we conclude that PSQ results in patients with low MGMT promoter methylation (10-20%) should be interpreted with caution. If therapeutically relevant, a second technique, e.g. MSP could be additionally used to substantiate the results in MGMT PSQ transitional (10-20%) cases. Our ROC curve analysis indicates that the combination of PSQ and MSP results is diagnostically beneficial in the LM patient cohort. Our results, furthermore, suggest 17% as the most accurate cutoff value for PSQ analysis. It has been the consensus in clinical practice to also treat patients with low level MGMT methylation as a potential benefit cannot be excluded. Nevertheless, further scientific investigation is necessary to establish this efficacy. Especially in elderly (≥70 years) or fragile GBM patients, a further stratification would be favorable as these patients have a higher risk of chemotherapy-related toxicity and demonstrate less survival benefit from alkylating agents if MGMT is unmethylated [19]. To conclude, we recommend the following classification system be used (particularly if FFPE samples are used): clearly unmethylated (<10%), low methylated (between 10-20%), and clearly methylated (>20%), which correlated with significantly improved PFS and OS in our cohort.

Additional file

**Additional file 1: Figure S1. Glioblastoma histology and representative IDH1 Sanger sequencing results. Figure S2. Original MSP PCR gels. Figure S3. Methyltyosition profiling report of GBM LTS patient #20 (GBM #20). Figure S4. Methyltyosition profiling report of GBM LTS patient #14 (GBM #14). Figure S5. IDH2 mutation. Figure S6. Kaplan-Meier curves for progression-free (PFS) and overall survival (OS) after combining PSQ and MSP results. Table S1. MGMT PSQ result of subgroup LM (10-20%) and corresponding sqMSP and sBiseq results. (DOXX 6001 kb)**

**Abbreviations**

CCNU: cyclonexyl-chloroethyl-nitrosourea; dBiSeq: direct bisulfite sequencing; FFPE: formalin-fixed and paraffin embedded; GTR: gross total resection; H&E: hematoxylin and eosin; HM: highly methylated; IDH: isocitrate dehydrogenase; LM: low methylated; meth: methylated; MGMT: O(6)-Methylguanine-DNA methyltransferase; OS: overall survival; PFS: progression-free survival; PSQ: Pyrosequencing; ROC: receiver operating characteristic; sqMSP: semi-quantitative methylation specific PCR; TMZ: Temozolomide; UM: unmethylated; unmeth: unmethylated

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**Authors’ contributions**

JR, AK and JO designed the study concept, performed data analysis, participated in data acquisition and wrote the manuscript. RK, ES, FP, and CH performed data analysis, participated in data acquisition, and revised the manuscript. KL performed the statistical data analysis and revised the manuscript. FF, MIA, FL, FH, and PV revised the manuscript and contributed to data analysis. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets supporting the conclusions of this article are included within the article and its additional file.

**Ethics approval and consent to participate**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Competing interests**

The authors declare that they have no competing interests.

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**Additional file 1:** Glioblastoma histology and representative IDH1 Sanger sequencing results.
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