MGMT Promoter Methylation Status Is Not Related to Histological or Radiological Features in IDH Wild-type Glioblastomas

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Abstract

O6-methylguanine DNA methyltransferase (MGMT) promoter methylation is an important favorable predictive marker in patients with glioblastoma (GBM). We hypothesized that MGMT status could be a surrogate marker of pretreatment tumor biology observed as histopathological and radiological features. Apart from some radiological studies aiming to noninvasively predict the MGMT status, few studies have investigated relationships between MGMT status and phenotypical tumor biology. We have therefore aimed to investigate such relationships in 85 isocitrate dehydrogenase (IDH) wild-type GBMs. MGMT status was determined by methylation-specific PCR and was assessed for associations with 22 histopathological features, immunohistochemical proliferative index and microvessel density measurements, conventional magnetic resonance imaging characteristics, preoperative speed of tumor growth, and overall survival. None of the investigated histological or radiological features were significantly associated with MGMT status. Methylated MGMT status was a significant independent predictor of improved overall survival. In conclusion, our results suggest that MGMT status is not related to the pretreatment phenotypical biology in IDH wild-type GBMs. Furthermore, our findings suggest the survival benefit of MGMT methylated GBMs is not due to an inherently less aggressive tumor biology, and that conventional magnetic resonance imaging features cannot be used to noninvasively predict the MGMT status.

Key Words: Angiogenesis, Glioblastoma, Histopathology, Magnetic resonance imaging, MGMT promoter methylation, Tumor growth.

INTRODUCTION

Glioblastomas (GBMs) are the most common of the primary malignant brain tumors in adults (1). The overall survival is only 14–16 months despite standard treatment of surgical resection and adjuvant concomitant radiation and chemotherapy (temozolomide) (2, 3). GBMs are biologically highly complex and aggressive tumors, illustrated by their rapid growth (4) and heterogeneous histological and molecular pathology (5–7).

O6-methylguanine DNA methyltransferase (MGMT) promoter methylation is an important predictive biomarker of improved response to temozolomide in GBMs (8, 9). MGMT is a DNA-repair enzyme that removes alkylated guanine residues on DNA, and hence counteracts the effect of alkylating agents, such as temozolomide (10). Methylation of the MGMT promoter leads to inactivation of the enzyme, which is believed to cause the predictive effect (10). However, it is not yet established whether it is purely a predictive marker or in part prognostic by itself, as previous studies have shown conflicting results regarding its prognostic value among patients who did not receive chemotherapy (8, 11–15). As MGMT promoter methylation status guides treatment decisions regarding chemotherapy (9), several radiological studies have sought to...
noninvasively predict the methylation status. However, results from these studies have also been conflicting (16).

We hypothesized that MGMT promoter methylation status could be a surrogate marker of pretreatment phenotypical tumor biology assessed by histopathology and magnetic resonance imaging (MRI) in GBMs. Apart from some radiological studies, few studies have investigated such relationships. By exploring these potential relationships using tissue material and MRI scans collected before treatment, we aimed to discover if there are differences in the inherent aggressiveness between MGMT methylated and unmethylated patients (i.e. a prognostic value). Moreover, such potential biological differences may also partially explain the different responses to chemotherapy. Our study could also further elucidate whether MGMT status can be predicted from preoperative MRI scans. In a cohort of treatment-naïve, isocitrate dehydrogenase (IDH) wild-type (wt) GBMs previously assessed for preoperative growth characteristics (4), we have therefore aimed to investigate whether MGMT status was associated with histological and radiological features.

MATERIALS AND METHODS

Patients and Samples

The selection of patients was based on the previous work by Stensjoen et al in which the preoperative growth dynamics of GBMs were investigated (4). Patients were retrospectively selected from all patients (n = 262) (4). Patients with ≥2 preoperative contrast-enhancing T1-weighted (T1wGd) MRI scans taken ≥14 days apart were eligible, and patients without contrast enhancement and/or gliomatosis cerebri were excluded (4). All cases were microscopically revised and IDH mutation status assessed according to the 2016 World Health Organization (WHO) Classification of Tumors of the Central Nervous System (17). IDH mutation status was first assessed using immunohistochemistry (18), and all immune-negative patients <55 years (18 patients) were additionally sequenced using Sanger sequencing according to previous described methods (using the BigDye Terminator v3.1 cycle sequencing kit and the 3130 genetic analyzer from Applied Biosystems, Foster City, CA) (19). Three patients were IDH mutated and were therefore excluded from the study. In 5 patients, IDH2 could not be sequenced; however, these were all IDH1 wt on sequencing. Due to the very low frequency of IDH2 mutations in newly diagnosed GBMs (20, 21), these were categorized as IDH wt and included in the study. The collection of clinical data regarding survival, treatment, sex, age at diagnosis, and Karnofsky performance status have previously been accounted for (18). Furthermore, of the 106 patients (4) analyzed for MGMT promoter methylation status, 18 were excluded (17%) due to inconclusive results. Hence, 85 patients were included in the current study.

DNA Extraction and MGMT Methylation-Specific PCR

For DNA isolation, an area of central tumor morphology (visually 100% tumor cells) was marked on hematoxylin and eosin (H&E) slides from formalin-fixed paraffin-embedded (FFPE) tissue blocks for each patient. Necrotic areas were avoided. Due to a lack of tumor material, 4 cases had a tumor cell content of 40%–70% in the marked areas. The marked areas were manually dissected, and tumor DNA then extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). QIAcube (Qiagen) was used for automated spin column process of DNA purification following the manufacturer’s instructions.

Methylation-specific PCR (MSP) was performed following bisulfite treatment of the isolated DNA using the EpiTect Fast Bisulfite Conversion kit (Qiagen). According to the method by Esteller et al (22), PCR amplification was performed using specific primers covering methylation of the MGMT promoter and exon 1 region. Methylated and unmethylated PCR products were detected with 4% agarose gel. An MGMT methylation-positive and a negative tissue control were applied during the whole process. The investigator who analyzed and interpreted the MSP data was blinded to other data.

Histopathology and Immunohistochemistry

All available H&E-stained FFPE sections from each case were assessed for the presence of 22 histopathological features. Definitions of each feature can be found in our previous publication (23). Most features were defined as either present or absent, while cellular density and atypia were semiquantitatively graded. Mitoses were counted in 10 high-power fields in hotspots. In 32 cases (38%), the amount of tissue on H&E slides has previously been subjectively categorized as sparse (23). This semiquantitative categorization was based on the collective area of viable (i.e. nonnecrotic) tumor tissue on all available H&E slides from each patient. Sparse tissue amount was often due to the patient being biopsied or having extensive necrosis in the resected material (23).

The immunohistochemical examinations of IDH1 R132H (monoclonal, IDH1 R132H/H09, 1:100, Dianova, Hamburg, Germany) (18), Ki-67/MIB-1 (monoclonal, Ki-67/MIB-1, 1:800 or 1:50, Dako, Glostrup, Denmark) (18), and CD105/endoglin (monoclonal, CD105/endoglin/SN6h, 1:50, Dako) (24) have previously been done and described in detail. The proliferative index (PI) of Ki-67/MIB-1 was quantified as described in our previous publication (18). The degree of angiogenesis has previously been quantified using microvessel density measurements of endoglin/CD105 (24). In short, the microvessel density was computed as the mean count of the number of vascular structures within a grid for 3 high-power fields in hotspots at ×400 magnification.

MRI Characteristics and Preoperative Tumor Growth

The MRI segmentations of total tumor volumes, volumes of the contrast enhancing and noncontrast enhancing...
Statistical Analyses

Statistical analyses were performed using Stata version 16 (StataCorp LLC, College Station, TX). Statistical significance was set at \( p \leq 0.05 \). Associations between MGMT status and categorical variables were analyzed using Chi-square/Fisher’s exact tests, while associations between MGMT status and continuous variables were assessed using Mann-Whitney U analyses. A Kaplan-Meier plot and the log-rank test were used for the univariable analyses between MGMT status and overall survival and a Cox proportional hazard model was used for multivariable survival analyses. The selection of variables in the multivariable model has previously been accounted for (18). All included variables followed the proportional hazard assumption, which was tested using Schoenfeld residuals in Stata.

**RESULTS**

MGMT and Clinical and Radiological Factors

In the 85 included patients, the distributions of age, sex, Karnofsky performance status, Ki-67/MIB-1 PI, and microvessel density of CD105 corresponded to previous reports (18, 24). The relationships between MGMT status and clinical and radiological factors are shown in Table 1. MGMT status was not significantly associated with any of the clinical factors or MRI volumetrics (Table 1). There was no significant association between MGMT status and MRI assessed preoperative speed of growth (Table 1).

**MGMT and Histological Features**

Distributions of the 22 histopathological features and the immunohistochemical markers (Ki-67/MIB-1 PI and microvessel density of CD105) in the MGMT methylated and unmethylated groups are presented in Table 2. There were no significant associations between MGMT status and any of the histological features assessed (Table 2). The difference in the presence of microvascular proliferation in the MGMT methylated and unmethylated groups was likely confounded by sparse tissue amount. In our previous work, we found that microvascular proliferation was significantly less present in...
cases with sparse tissue amount \( (p < 0.001, \text{Chi-square test, unpublished student thesis}) \). In addition, there was a near-significant trend of more MGMT unmethylated cases in cases with sparse tissue material \( (p = 0.088, \text{Chi-square test}) \) in the current study. To avoid this confounding effect, microvascular proliferation was redefined to only include well-sampled cases (Table 2). Microvascular proliferation was significantly associated with methylated MGMT status when not corrected for tissue amount \( (p = 0.018, \text{Chi-square test}) \).

**MGMT and Survival**

The median overall survival was 13.3 months (95% confidence interval [CI] 9.9–15.7). Methylated MGMT status was significantly associated with overall survival both in the univariable analysis (Table 1; Fig. 1) and in the multivariable Cox model (Table 3).

### DISCUSSION

MGMT promoter methylation is a pivotal predictive marker in IDH wt GBMs. However, we did not find any significant associations between the MGMT promoter methylation status and several biological parameters in treatment-naïve patients. These parameters included 22 histopathological features, proliferative activity, degree of angiogenesis, quantitative MRI volumetrics, and preoperative speed of radiological tumor growth. Altogether, these findings suggest

### TABLE 2. MGMT and Histological Features. Distributions of the Histological Features Within the MGMT Methylated and Unmethylated Patient Groups

|                         | Methylated MGMT (n = 31) | Unmethylated MGMT (n = 54) | p Value     | Test Performed |
|-------------------------|--------------------------|-----------------------------|-------------|----------------|
| Necrosis                |                          |                             |             |                |
| Small                   | 84%                      | 81%                         | 0.781       | Chi-square     |
| Large                   | 94%                      | 89%                         | 0.705       | Fisher’s exact |
| Palisades\(^a\)         | 84%                      | 72%                         | 0.206       | Chi-square     |
| Microvascular proliferation\(^b\) | 100%                 | 90%                         | 0.249       | Fisher’s exact |
| High cellular density   | 42%                      | 33%                         | 0.428       | Chi-square     |
| Severe atypia           | 16%                      | 26%                         | 0.297       | Chi-square     |
| Median mitotic count (range) | 16.0                  | 11.5                        | 0.109       | Mann-Whitney U |
| (0–43)                  | (0–65)                   |                             |             |                |
| Vascular features       |                          |                             |             |                |
| Thrombosis              | 81%                      | 87%                         | 0.534       | Fisher’s exact |
| Hemorrhage              | 87%                      | 78%                         | 0.290       | Chi-square     |
| Pseudorosettes\(^c\)    | 29%                      | 25%                         | 0.726       | Chi-square     |
| Secondary structures of Scherer\(^d\) | 70%                 | 71%                         | 0.011       | Chi-square     |
| Desmoplasia             | 65%                      | 67%                         | 0.840       | Chi-square     |
| Leukocytes              |                          |                             |             |                |
| Macrophages             | 97%                      | 91%                         | 0.049       | Fisher’s exact |
| Lymphocytic infiltrates | 58%                      | 76%                         | 0.085       | Chi-square     |
| Small cell glioblastoma | 23%                      | 17%                         | 0.502       | Chi-square     |
| Cell types              |                          |                             |             |                |
| Gemistocytes            | 29%                      | 19%                         | 0.263       | Chi-square     |
| Small cells             | 29%                      | 22%                         | 0.483       | Chi-square     |
| Sarcomatous cells       | 13%                      | 20%                         | 0.385       | Chi-square     |
| Myxomatoïd             | 6%                       | 17%                         | 0.314       | Fisher’s exact |
| Giant cells             | 6%                       | 11%                         | 0.705       | Fisher’s exact |
| Primitive neuronal component | 6%                   | 11%                         | 0.705       | Fisher’s exact |
| Oligodendroglial cells  | 10%                      | 6%                          | 0.664       | Fisher’s exact |
| Median Ki-67/MIB-1 PI (range) | 17.5              | 13.2%                        | 0.333       | Mann-Whitney U |
| (4.3–40.7)              | (1.4–57.3)               |                             |             |                |
| Median microvessel density count of CD105\(^e\) (range) | 15.2                  | 11.8                        | 0.216       | Mann-Whitney U |
| (4–42.7)                | (0.7–50)                 |                             |             |                |

MGMT, O6-methylguanine DNA methyltransferase; PI, proliferative index.

\(^a\)Includes only tumors with central tumor morphology in the analysis \( (n = 84) \) (23).

\(^b\)Tumors with sparse tissue amount were excluded from the analysis \( (53 \text{ included cases}) \), because sparse tissue amount was likely a confounder of the association between microvascular proliferation and MGMT status.

\(^c\)Includes only tumors with paraffin sections with viable central tumor morphology \( (n = 82) \) (23).

\(^d\)Recorded as present when \( \geq 1 \) of the following features were observed: Perineuronal satellitosis, angiocentric structures, or subpial clustering, as previously defined (23). Only recorded in tumors containing infiltration zones into gray matter \( (n = 55) \).

\(^e\)Includes only tumors with enough tissue amount or adequate morphology for the microvessel density assessment \( (n = 82) \) (24).
that MGMT status is not a surrogate marker of the pretreatment phenotypical biology of IDH wt GBMs.

Tumor biology has been extensively studied using experimental models; however, these models will never fully mimic the unique micro-environment of human GBMs (25). In this study, tissue samples were obtained from the first surgical intervention and only preoperative MRI scans were assessed. Hence, the assessed biological features were unaffected by radiochemotherapy. Nevertheless, 82% (n = 70) were preoperatively treated with corticosteroids, and there was a nonsignificant trend (p = 0.144) of more corticosteroid use in MGMT methylated tumors (data not shown). Therefore, we cannot entirely exclude corticosteroid use as a confounding factor. In summary, our study enabled us to study links between the phenotypical biology and MGMT status occurring during the natural history of human IDH wt GBMs.

**MGMT and Histology**

We could not find any significant associations between MGMT status and the histopathological features or immunohistochemically assessed degree of proliferation and angiogenesis (Table 2). Few previous studies have investigated relationships between MGMT status and histological features. However, Hegi et al investigated such relationships by looking at 13 morphological features in newly diagnosed GBM patients (26). Yet, they only found a significant association between methylated MGMT status and higher Ki-67/MIB-1 PI. However, this association is limited by various aspects of the assessments of Ki-67/MIB-1 PI (23, 27, 28). Pistollato et al found a higher MGMT expression (corresponding to unmethylated tumors) in the hypoxic, inner core of GBMs (29). They also found that cells derived from these areas were more resistant to temozolomide, which was further related to the higher MGMT expression (29). In our previous studies, we found that
thromboses independently predicted faster tumor growth, which indicated that hypoxia drives faster tumor growth (23, 24). Because our previous publications included IDH1 mutant tumors and that thromboses have been found to associate with IDH1 wt status (30), we reanalyzed the data from our previous publications while including only IDH wt GBMs. The reanalysis showed similar results, suggesting that thromboses promote aggressiveness also among IDH wt GBMs. Interestingly, neither thromboses nor faster preoperative growth were associated with MGMT status in the current study (Tables 1 and 2). Previous experimental studies have also linked MGMT expression to increased hypoxia (31–33) and decreased angiogenesis (34) in GBM cell lines. However, these results are conflicting, as hypoxia is known to be an important inducer of angiogenesis (35). Furthermore, a recent comprehensive genomic study showed considerable differences in mRNA expression profiles and DNA methylation profiles between GBM patient material and the in vitro and in vivo models derived from it (36). These findings illustrate challenges in extrapolating findings from experimental models on MGMT methylation status and expression. Altogether, the inconsistent results from previous pathological and experimental studies are in line with our findings, which suggest MGMT methylation status is not linked to pretreatment histology in GBMs.

**MGMT and MRI**

We found no significant associations between MGMT status and total tumor volumes, contrast enhancing volumes, necrotic volumes, the percentage of necrosis, or preoperative speed of growth (Table 1). As mentioned, previous radiological studies have aimed to noninvasively predict MGMT status using conventional and advanced MRI characteristics. However, results have been conflicting and derived no expert consensus (16). Still, most studies have found significant associations between unmethylated MGMT status and MRI parameters indicating increased aggressiveness, such as more necrosis (37) and higher vascularity. Higher vascularity was in these studies measured as (i) ring enhancement (37, 38), (ii) higher normalized relative cerebral blood volume (39), (iii) higher relative cerebral blood flow (16), (iv) more edema (40), and (v) lower apparent diffusion coefficient (also indicating increased cellularity) (16, 41). On the contrary, others have found methylated MGMT status to significantly associate with necrosis (16), lower apparent diffusion coefficient (42), and higher relative cerebral blood volume (43). In line with our study, others found no significant associations between MGMT status and conventional MRI features (44–47). Nevertheless, machine learning approaches might be a way to advance and have thus far shown both promising (47–50) and negative results (45). In summary, our results along with the previous conflicting studies indicate that MGMT status cannot yet be noninvasively predicted from MRI scans.

**MGMT and Survival**

**MGMT** promoter methylation was an independent predictor for improved survival when adjusted for several clinical factors in the multivariable analyses (Table 3). However, this does not necessarily mean that methylated MGMT status is an independent prognostic factor, as MGMT status may have affected the temozolomide use in the studied patients. As defined by Clark, a prognostic factor is “associated with clinical outcome in the absence of therapy or with the application of a standard therapy that patients are likely to receive” (51). Hence, it is a feature of the natural history of the disease. A predictive factor is “associated with response or lack of response to a particular therapy” (51). Ideally, predictive factors should be studied in randomized controlled trials (RCTs), isolating the effect of the potential biomarker related to treatment. Prognostic factors are better studied in cohort studies where treatment is not dependent on the studied biomarker. In our study, most patients received chemotherapy regardless of the MGMT status (Table 1). However, among elderly GBM patients, the Stupp protocol is more seldom given and patients with MGMT methylated tumors may be selected for chemotherapy alone (12). Second-line chemotherapy is also more likely to be given to patients with MGMT methylated lesions. Thus, since MGMT status is to some extent used for treatment decisions, the seemingly independent effect of MGMT status on survival in the multivariable analyses may be colored by the use of MGMT status for treatment selection.

Our finding that MGMT status was not related to pretreatment phenotypical tumor biology indicates that methylated MGMT status is not associated with an intrinsically less aggressive tumor biology. This further suggests methylated MGMT status is not a prognostic factor by itself but merely a predictive marker. As mentioned, previous studies have shown conflicting results regarding the prognostic value of MGMT status among patients who were not treated with chemotherapy. Three RCTs on elderly patients (11–13) and a retrospective cohort from the preStupp area (14) did not find a significant difference in overall survival according to MGMT status in the radiotherapy-only treated group. Conversely, the EORTC-NCIC RCT on younger patients (8, 52) and a retrospective study by Rivera et al (15) found a prognostic value of MGMT status within the same patient group. However, second-line therapy with temozolomide was given to a higher percentage of the radiotherapy-only patients in the EORTC-NCIC trial (~60%) than in the 3 other RCTs (~30% in all) (11–13). Furthermore, in the EORTC-NCIC trial, they argue that the survival benefit is probably due to an effect of second-line chemotherapy, as the progression-free survival was short and the overall survival relatively long in the MGMT methylated cases in the radiotherapy-only group (8, 52). Moreover, Rivera et al found that methylated MGMT status also predicted an increased response to radiotherapy (15). They further speculated whether methylated MGMT status could represent a surrogate marker of improved treatment response in general or of undiscovered processes causing an inherently less aggressive tumor biology (15). However, our study suggests the latter speculation is not the case in IDH wt GBMs. Moreover, our results also indicate that the increased response to chemotherapy in MGMT methylated GBMs is not due to pretreatment differences in phenotypical tumor biology. Altogether, our findings along with previous studies indicate the increased survival of MGMT promoter methylated patients is due to an
increased response to therapy, and not due to an intrinsically less aggressive tumor biology.

Methodological Aspects

To date there is no consensus regarding the best assay for detecting the MGMT methylation status (53, 54). We used MSP, which has been related to survival in several pivotal clinical studies (8, 10, 22, 53). The finding of 36% MGMT methylated cases corresponds to the 30%–60% in previous studies (10). Interestingly, there was a near-significant trend of more MGMT unmethylated tumors when tumor material was sparse (p = 0.088). This association is perhaps due to the assay’s propensity toward more false negatives when the amount of isolated DNA is low. Intratumoral heterogeneity in MGMT status has also been reported (55, 56), which may contribute to a higher risk of false negative results in cases with sparse tissue. Further technical limitations of the MSP assay have been elaborated elsewhere (10, 54, 57). Still, the primers used in this study correspond to an area of the promoter found to best correlate with survival and MGMT expression in patients with GBM (58, 59).

Limitations regarding the collection of clinical data, the histopathological and immunohistochemical assessments, the segmentation of tumor volumes and different tumor compartments, and the estimation of growth rates have previously been described in detail (4, 18, 23, 24). The relatively large population of treatment-naive patients with a population-size of 0.8, the power was estimated to be conclusion that none of these tests were significant further supports our MGMT between one false positive finding of the 29 performed statistical tests based on the set p value of 0.05, one would expect at least one false positive finding of the 29 performed statistical tests between MGMT status and biological features. Hence, the fact that none of these tests were significant further supports our conclusion that MGMT status is not related to pretreatment phenotypical tumor biology. Assuming a standardized effect size of 0.8, the power was estimated to be ~90% for each analysis between MGMT status and the biological features. The results should be validated in future studies.

Conclusion

In conclusion, we did not find any significant associations between MGMT promoter methylation status and histological or MRI features in treatment-naive IDH wt GBM patients. These findings suggest MGMT status is not related to the pretreatment phenotypical biology in IDH wt GBMs, which indicate that the increased survival of MGMT methylated patients is not due to an inherently less aggressive tumor biology. Also, our findings suggest that preoperative conventional MRI characteristics cannot be used for noninvasive prediction of the MGMT status.

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