J Neuropathol Exp Neurol
Vol. 79, No. 8, August 2020, pp. 855–862
doi: 10.1093/jnen/nlaa060

ORIGINAL ARTICLE

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

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Abstract

O^6-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).

O^6-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

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V.E.M. and A.L.S. have received research scholarships from NTNU-Norwegian University of Science and Technology. O.S. has received research salaries from the National Competence Centre for Ultrasound and Image Guided Therapy, and E.M.B. has received research funding from the Central Norwegian Brain Tumor Registry. The funding sources had no role in the design of the study, writing of the manuscript, or collection, analysis, and interpretation of data.

O.S. is a previous unpaid member of a National Advisory Committee on Treatment Guidelines for Brain Tumors. All other authors have no duality or conflicts of interest to declare.

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 Stensjøen et al in which the preoperative growth dynamics of GBMs were investigated (4). Patients were retrospectively selected from all patients >18 years operated for newly diagnosed GBMs at St. Olav's University Hospital, Trondheim, Norway between January 2004 and May 2014 (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 previously 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

compartments, and estimation of speed of tumor growth have previously been accounted for (4, 18). The software BrainVoyager QX (Brain Innovation, Maastricht, the Netherlands) was used for the volume segmentations (4). The tumor volumes were segmented from 2 preoperative T1wGd MRI scans from each patient (first scan taken at radiological diagnosis and the second preoperative scan for neuronavigation). The MRI characteristics assessed for associations with *MGMT* status were segmented from the second, preoperative scan. Total tumor volume was defined as the combined volume of the contrast enhancing rim and the noncontrast enhancing (necrotic) core. Preoperative speed of growth was estimated from the total tumor volumes at both scans and the interval between them (4, 18). A fitted Gompertzian growth curve from 106 patients was used to dichotomize the patients into having tumors growing faster or slower than expected, as previously described (18).

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.

Ethics

This study was approved by the Regional Ethics Committee (Central) as part of a larger project (reference numbers 2011/974 and 2013/1348) in accordance with the 1964 Helsinki declaration and later amendments. Most of the patients had provided written informed consent to be included (reference 2011/974), and the Regional Ethics Committee waived informed consent for retrospective evaluation of patient data for the remaining patients.

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

TABLE 1. *MGMT* and Clinical and Radiological Factors. Distributions of Clinical and Radiological Parameters Within the *MGMT* Methylated and Unmethylated Patient Groups

	Methylated *MGMT* (n = 31)	Unmethylated *MGMT* (n = 54)	p Value	Test Performed
Mean age (SD)	65 (10.4)	63 (11.2)	0.230	Two-sample *t*-test
Male	71%	69%	0.814	Chi-square
Median preoperative total tumor volume (range)	28.8 mL (1.0–243.5)	31.6 mL (1.0–153.0)	0.777	Mann-Whitney *U*
Median preoperative contrast enhancing volume (range)	16.6 mL (1.0–215.4)	18.3 mL (0.9–63.9)	0.913	Mann-Whitney *U*
Median preoperative necrotic core volume (range)	9.7 mL (0.0–89.9)	8.1 mL (0.1–106.5)	0.695	Mann-Whitney *U*
Median preoperative percentage necrosis (range)	27.8% (1.4–78.6)	34.9% (3.5–69.9)	0.204	Mann-Whitney *U*
Fast-growing tumors[a]	48%	50%	0.886	Chi-square
Median KPS (range)	7.5 (4–10)	8 (5–10)	0.552	Mann-Whitney *U*
GTR	35%	26%	0.352	Chi-square
Chemotherapy	81%	81%	0.924	Chi-square
Radiotherapy	90%	94%	0.664	Fisher's exact
Median survival	15.9 months 95% CI (12.5–26.2)	10.2 months 95% CI (8.6–13.7)	0.048*	Log-rank

MGMT, O^6-methylguanine DNA methyltransferase; SD, standard deviation; KPS, Karnofsky performance status; GTR, gross total resection; CI, confidence interval.

[a]Speed of tumor growth was estimated from segmented tumor volumes from 2 preoperative MRI scans and the interval between them. A fitted Gompertzian growth curve based on the volume data was used to dichotomize the tumors into growing faster or slower than expected from the curve (18).

*Statistically significant, $p \leq 0.05$.

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 (0–43)	11.5 (0–65)	0.109	Mann-Whitney *U*
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%	0.911	Chi-square
Desmoplasia	65%	67%	0.840	Chi-square
Leukocytes				
Macrophages	97%	91%	0.409	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
Myxomatoid	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% (4.3–40.7)	13.2% (1.4–57.3)	0.333	Mann-Whitney *U*
Median microvessel density count of CD105[e] (range)	15.2 (4–42.7)	11.8 (0.7–50)	0.216	Mann-Whitney *U*

MGMT, O^6-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 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 ≥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).

cases with sparse tissue amount ($p < 0.001$, 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 ($p = 0.088$, 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 ($p = 0.018$, 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

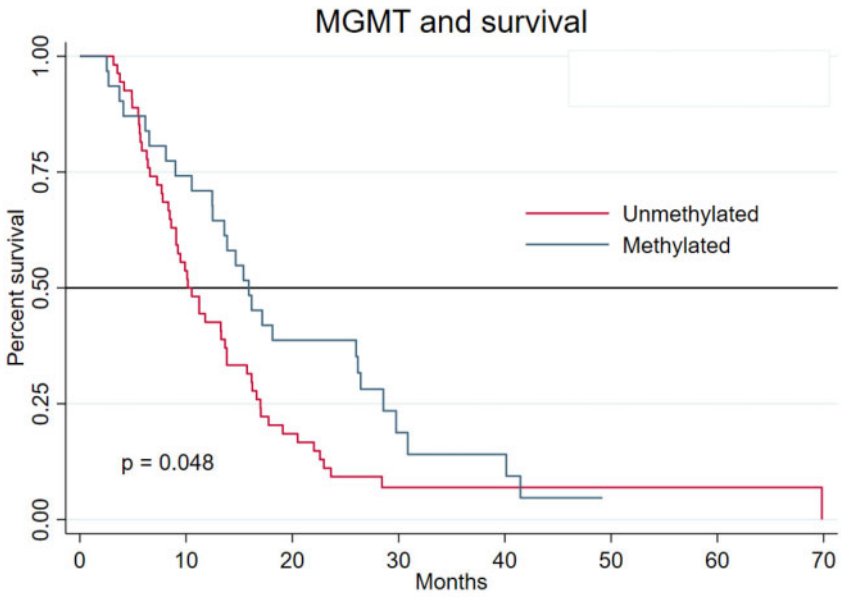

FIGURE 1. Kaplan-Meier plot of overall survival of *MGMT* methylated (blue line) and *MGMT* unmethylated (red line) patients. *MGMT* methylated patients survived significantly longer than unmethylated ($p = 0.048$, log-rank test). MGMT, O^6-methylguanine DNA methyltransferase.

TABLE 3. Survival Analyses. Univariable and Multivariable Cox Analyses

	Univariable HR (95% CI)	p Value	Multivariable HR (95% CI)	p Value
Age	1.01 (0.99–1.03)	0.332	1.00 (0.98–1.03)	0.790
KPS	0.68 (0.57–0.82)	<0.001*	0.75 (0.60–0.95)	0.017*
Preoperative tumor volume	1.01 (1.00–1.01)	0.042*	1.00 (0.99–1.01)	0.956
GTR	0.69 (0.42–1.14)	0.150	0.66 (0.37–1.18)	0.162
Chemotherapy	0.17 (0.09–0.32)	<0.001*	0.27 (0.13–0.58)	0.001*
Radiation	0.05 (0.02–0.14)	<0.001*	0.12 (0.04–0.38)	<0.001*
Methylated *MGMT* status	0.62 (0.39–1.00)	0.051	0.60 (0.37–0.97)	0.038*

HR, hazard ratio; CI, confidence interval; KPS, Karnofsky performance status; GTR, gross total resection; MGMT, O^6-methylguanine DNA methyltransferase.
*Statistically significant, $p \leq 0.05$.

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 *IDH* 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 GMBs. 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 inductor 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-naïve patients with a population-based referral and the preoperative MRI assessments are the main strengths of the study. Important limitations are potential selection biases, preoperative corticosteroid treatment, sampling errors and interobserver variability of the histological assessments, and the explorative nature of the statistical analyses. We chose not to correct for multiple statistical testing despite the increased risk of false positive findings. Interestingly, 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-naïve *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.

ACKNOWLEDGMENTS

We would like to thank Mona Jonsson, Tore Skaldebø, Tone Furre, Ingeborg Engesvold, and Torild Gladsø for the excellent laboratory work with the methylation-specific PCR analyses of the MGMT promoter methylation status at the Department of Pathology, St. Olav's University Hospital. We would also like to thank the staff at the Cellular and Molecular Imaging Core Facility (CMIC), NTNU-Norwegian University of Science and Technology for outstanding laboratory work with the histological and immunohistochemical analyses. Open Access funding provided by NTNU-Norwegian University of Science and Technology.

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