Extent and Patterns of MGMT Promoter Methylation in Glioblastoma- and Respective Glioblastoma-Derived Spheres

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Abstract

**Purpose:** Quantitative methylation-specific tests suggest that not all cells in a glioblastoma with detectable promoter methylation of the O6-methylguanine DNA methyltransferase (MGMT) gene carry a methylated MGMT allele. This observation may indicate cell subpopulations with distinct MGMT status, raising the question of the clinically relevant cutoff of MGMT methylation therapy. Epigenetic silencing of the MGMT gene by promoter methylation blunts repair of O6-methyl guanine and has been shown to be a predictive factor for benefit from alkylating agent therapy in glioblastoma.

**Experimental Design:** Ten paired samples of glioblastoma and respective glioblastoma-derived spheres (GS), cultured under stem cell conditions, were analyzed for the degree and pattern of MGMT promoter methylation by methylation-specific clone sequencing, MGMT gene dosage, chromatin status, and respective effects on MGMT expression and MGMT activity.

**Results:** In glioblastoma, MGMT-methylated alleles ranged from 10% to 90%. In contrast, methylated alleles were highly enriched (100% of clones) in respective GS, even when 2 MGMT alleles were present, with 1 exception (<50%). The CpG methylation patterns were characteristic for each glioblastoma exhibiting 25% to 90% methylated CpGs of 28 sites interrogated. Furthermore, MGMT promoter methylation was associated with a nonpermissive chromatin status in accordance with very low MGMT transcript levels and undetectable MGMT activity.

**Conclusions:** In MGMT-methylated glioblastoma, MGMT promoter methylation is highly enriched in GS that supposedly comprise glioma-initiating cells. Thus, even a low percentage of MGMT methylation measured in a glioblastoma sample may be relevant and predict benefit from an alkylating agent therapy.

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Introduction

O6-Methylguanine-DNA methyltransferase (MGMT) is a cellular DNA repair protein ubiquitously expressed in normal human tissues (1). The MGMT protein rapidly reverses alkylation (e.g., methylation) at the O6 position of guanine, thereby neutralizing the cytotoxic effect of alkylating agents (2). Hence, expression of MGMT in tumor cells represents a key mechanism of resistance for alkylating agent therapy (3).

Epigenetic silencing by aberrant promoter methylation of MGMT (4) has become the first predictive marker for benefit from alkylating agent therapy in glioblastoma (5–7). As combined chemoradiotherapy comprising the alkylating agent, temozolomide, has become the new standard of care (8), there has been growing interest to use MGMT promoter methylation status for individual patient management, and for patient stratification or selection in clinical trials, and novel methods to assess MGMT status have been developed (9). Most methylation-specific (MS) assays are based on bisulfite conversion of the tumor-derived DNA that converts unmethylated cytosine, but not 5-methyl-cytosine to uracil, followed by qualitative or quantitative determination using methylation-specific PCR (MSP) or sequencing (10–12). Quantitative evaluation of methylated MGMT in tumors; for example, by quantitative MSP or MS clone sequencing suggests that not all cells in a given "methylated
Translational Relevance

Glioblastoma is the most common malignant primary brain tumor with a dismal prognosis of 15 months. Combined chemoradiotherapy comprising the alkylating agent, temozolomide, has led to a modest increase of survival. The demonstration that benefit from the alkylating agent, temozolomide, is largely restricted to patients whose glioblastoma contains a methylated O6-methylguanine DNA methyltransferase (MGMT) promoter has stirred up the field. Consequently, clinical trials now stratify or select glioblastoma patients according to the MGMT methylation status. However, the best technique of MGMT status determination, cutoff definition between methylated and unmethylated, and the relevance of extent of methylation are all subject to ongoing controversy. Our study provides molecular data to disentangle these issues, suggesting that in MGMT-methylated glioblastoma, cells with MGMT methylation are enriched in the glioblastoma-derived sphere fraction that comprises glioma-initiating cells. Hence, measurement of low levels of MGMT methylation may be relevant for potential benefit from alkylating agent therapy.

Materials and Methods

Glioblastoma samples

Tumor tissues were collected from patients operated in the Service of Neurosurgery at the University Hospital Lausanne, with written informed consent of the patients and approval of the local ethics committee (protocol F25/99). Tumors were histologically classified according to the WHO classification. Patient characteristics are detailed in Table 1.

Glioblastoma-derived spheres (GS)

Fresh glioblastoma tissue was mechanically and enzymatically dissociated into a single cell suspension using papain 30 U/mL (Worthington Biochem.; LS003119) and DNase I 40 μg/mL (Roche, 1284932) and subjected to magnetic bead cell sorting based on CD133 according to the manufacturer’s instructions (Miltenyi Biotec.). Cells were cultured under stem cell conditions using DMEM-F12 medium (Invitrogen; 10565-018) supplemented with human recombinant EGF (epidermal growth factor) and human recombinant basic FGF (fibroblast growth factor; Peprotech; AF-100-15 and 100-18B), 20 ng/mL of each, and 2% of B27 (Invitrogen; 17504). If not otherwise stated, 50% of the medium was substituted twice weekly. The tumorigenicity of GS was evaluated by orthotopic injection into the brain of five 6- to 9-week-old female Swiss nu/nu mice as described previously (14). GS-induced tumors were recovered at sacrifice, a part was preserved for histologic evaluation, and GS were rederived without selection in stem cell medium. The protocol was approved by the local veterinary authorities (VD11181-3).

DNA and RNA isolation

DNA and RNA were isolated from frozen tissue or cells using the AllPrep DNA/RNA Kit (Qiagen; 80204). DNA from paraffin sections were isolated as described before (11). Nucleic acids were quantified by UV spectrophotometric analysis using Nanodrop 1000 Spectrophotometer (Thermo Scientific).

Bisulfite treatment and gel-based and quantitative Methyltransferase-Specific PCR

A total of 600 ng of genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research; D5001). After the bisulfite treatment step, purified DNA was subjected to gel-based MSP using a 2-step approach with nested primers as previously described (15, 16), using presence or absence of PCR products derived from methylated and unmethylated MGMT on agarose gels as readout. The initial MGMT status of GS was determined between 2 and 6 months in culture, with the exception of 1871 that was first tested at 12 months. All other GS were retested between 2 and 4 times over the observation period of over 12 months. Quantitative MSP was performed by MDxHealth Inc. (former Oncomethylome Sciences).
as described (11). The copy number of methylated MGMT, the copy number of beta-actin (ACTB) that serves as reference gene for normalization, and the ratio “methylated MGMT*1000/ACTB” were reported.

Methylation-specific clone sequencing

PCR products from the first round of nested MSP (+75–364 from the transcription start site, TSS; Fig. 1B) encompassing 28 CpG sites were cloned into a pCR2.1-TOPO vector according to the manufacturer’s instructions (TOPO TA Cloning; Invitrogen). The plasmid was then used to transform TOP10 competent cells. Ten to twenty cloned fragments per sample were sequenced using M13 primers (Sanger method, Microsynth CH).

Real-time quantitative PCR

Real-time quantitative PCR was performed with Fast SYBR Green Master Mix (Applied Biosystem) using Rotor Gene 6000 Real-Time PCR system (Corbett Life Science).

PCR reactions were run as triplicates. The temperature profile was as follows: 95°C (100 seconds) followed by 40 cycles at 95°C (3 seconds) to 60°C (20 seconds). The quality of the products was controlled by a melting curve. For quantification, standard curves were established by amplification of serial dilutions of cDNA for both, the target gene and the endogenous reference (GAPDH). The primer sequences have been published previously (17, 18) and are listed in Supplementary Table S2.

MGMT activity

MGMT activity was measured in frozen samples on the basis of a radioactive assay that measures the transfer of tritium-labeled methyl groups from the O6 position of guanine to the MGMT protein as detailed elsewhere (19, 20). The mean of 3 independent measurements were reported. The limit of detection was at 1 fmol/mg of protein. Extracts of HeLa S3 (750 fmol/mg of protein)
and HeLa MR (no detectable MGMT protein) cells served as positive and negative control, respectively.

**Array CGH analysis**

Array comparative genomic hybridization (CGH) was performed using the Agilent Human Genome CGH Microarray Kit 44A as described (21). The array was analyzed with the Agilent scanner and the Feature Extraction software (v.9.5.3.1). Graphical overview was obtained using the CGH analytics software (v3.5.14) according to Hg18 genome assembly (March 2006).

**Quantitative chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) assay was performed using the MAGnify Chromatin Immunoprecipitation System (Invitrogen) according to the manufacturer’s protocols. Briefly, proteins from cell extracts of 3 × 10^6 cells were cross-linked to DNA by addition of formaldehyde to a final concentration of 1% for 10 minutes at room temperature. Cells were sonicated to yield fragments with an average size of 200 to 600 bp using the sonicator sonoplus mini20 (Bandelin electronic). The soluble chromatin fraction was collected, and 10% of the supernatant was used for input normalization. Equivalent amounts of either anti-Histone-H3 (Positive control; Abcam; AB1791), anti-Trimethyl-Histone H3 (Lys 4; Cell Signaling Technology; C42D8), anti-Trimethyl-Histone H3 (Lys 27; Cell Signaling Technology; C36B11) and normal rabbit IgG (negative control, Invitrogen) were added and incubated according to the protocol. Purified eluted DNA was quantified by quantitative real-time PCR. Primers for MGMT (22), and the 2 controls, GAPDH as representative of a gene with an open chromatin state (EZ ChIP; Chromatin Immunoprecipitation Kit) and MYOD1 as representative of a gene with a closed chromatin state (23) are listed in Supplementary Table S1. The experiments have been repeated 3 times with 2 biological replicates.

**Immunohistochemistry and estimation of tumor content**

Formaldehyde-fixed, paraffin-embedded tissue sections were immunostained for MGMT (MT3.1, dilution 1:50; NeoMarkers; Fremont), glial fibrillary acidic protein (GFAP), CD45 (M0701; DAKO), CD68 (ref. M0814; 1:100; DakoCytomation), and p53 (RM 9105-SO; dilution 1:500; NeoMarkers). Heat-induced epitope retrieval (HIER) was applied using citrate buffer of pH 1 = 6.0 (MGMT, 15 minutes; CD68, 5 minutes; CD45, p53, 10 minutes). The primary antibody was incubated overnight at 4°C. Secondary antibodies were applied using the Elite Vectastain Kit (Vector Laboratories) according to the manufacturer’s instructions. Immunoreactivity was visualized with 3,3′-diaminobenzidine as the chromogen. All sections were counterstained with hematoxylin. Negative controls were carried out by omission of the primary antibody.

The tumor cell content was estimated semi-quantitatively by the neuropathologist, Robert Janzer, by evaluating 4 representative high-power fields within the area macroplasmin for molecular analyses based on H&E (hematoxylin and eosin) staining, and in a second step, including the immunohistochemistry (IHC) for GFAP, identifying reactive astrocytes by their stellate and fine cell processes, and CD45 and CD68, visualizing microglia and macrophages. All cells expressing CD45 or CD68 were considered as nontumoral. Overall, cells excluded as non tumoral comprised vascular and intravascular cells, lymphocytes, macrophages, microglia, and reactive astrocytes.

**Results**

Ten paired samples of GS and their corresponding original glioblastoma tissues were evaluated for the extent and density of methylation by clone sequencing 28 of 97 CpGs in the CpG-island of the MGMT promoter (Fig. 1B). This region of the promoter encompasses the enhancer element and according to reporter assays is associated with complete silencing of the gene when fully methylated (24). Hence, most assays interrogate CpG methylation in this region (9).

These 10 samples investigated are highly selected in the sense that they were obtained from patients for whom we received large amounts of tissue (≥1 g) and who had mostly complete resections (Table 1). In our hands, 50% of glioblastoma subjected to the CD133 selection procedure to enrich for GICs eventually yield glioma-derived spheres that can be maintained in stem cell culture for further experiments. CD133-positive glioma cells have been shown to be enriched for GICs (25), although it has become clear that not all GICs are glioma-derived spheres (26). More recently, other markers or specific phenotypic properties have been proposed for the isolation of GICs (27).

**Frequency and methylation density of methylated alleles in GBM and respective GS**

Using qualitative MSP visualized on a gel, 7 of the 10 samples were considered MGMT methylated (Supplementary Fig. S1 and Table 1). For all glioblastoma with MGMT methylation, a band for MGMT unmethylated alleles was also detectable. In accordance, subsequent MS clone sequencing of the original glioblastoma tissue revealed that 10% to 90% of all clones sequenced showed dense methylation (Figs. 1 and 2B), arbitrarily defined as at least 4 consecutive CpGs methylated in a given interrogated clone. In contrast, in the respective GS, all alleles were methylated regardless of the gene copy number with 1 exception (Figs. 1and 2B). In this sample, GS_2683, the methylated alleles were also enriched, however, exhibited methylation in only 40% of the clones. This is in accordance with a normal chromosome 10 copy number as determined by aCGH (Supplementary Fig. S4), comprising a methylated and an unmethylated allele, respectively. However, we cannot exclude to have different populations with a distinct methylation status.

The density of MGMT promoter methylation, defined as the number of methylated CpGs over 28 interrogated
CpGs, ranged from 25% to 90%, never reaching 100%, and showing a characteristic pattern in a given tumor (Figs. 1 and 2C). The pattern was retained in the respective GS cultured under stem cell conditions. Furthermore, the pattern was even maintained after spheres were rederived from respective tumors induced orthotopically in nude mice exemplified for GS_2669 and GS_2540 (Fig. 1A).

Characteristic features of the GS-derived tumors indicative for the presence of GICs, such as highly invasive growth and resemblance to the original glioblastoma is displayed in Supplementary Figure S5. These are properties that have been attributed to GIC-derived tumors in previous reports and are in stark contrast to glioblastoma cell line–derived intracranial tumors (28, 29).

Figure 2. MGMT promoter methylation and MGMT expression in GBM and GS. The correlation of MGMT methylation and expression is visualized for the original glioblastoma tissue (GBM, blue) and respective GS (red): A, visualizes the ratio of meth, MGMT/1000/ACTB by qMSP; B, the percentage of clones with dense MGMT methylation is represented; 10 or more clones were evaluated by MS clone sequencing; C, the methylation density is defined as percentage of methylated CpGs of 28 CpGs interrogated; D, MGMT expression as evaluated by real-time quantitative PCR. The molecular characterization of the GS was performed between 2 and 6 months in culture, with the exception of GS_1871 that was evaluated at 12 months. PBL, peripheral blood lymphocytes.
For the 3 paired cases with unmethylated MGMT, MSP was repeated at several time points for the GS fractions to rule out the possibility that MGMT promoter methylation is acquired in the GS as an in vitro artifact. This was further confirmed by MS sequencing in late passages (GS_1871, 360 days, GS_2108, 165 days; GS_2638, days 120). MS clone sequencing of GS_2638, 1 of the 3 unmethylated GS lines, identified individual scattered methylated CpG sites in some clones, confirming the unmethylated status as determined by all other methods utilized (Fig. 1A and Supplementary Figs. S1 and S2).

qMSP confirmed the observed "enrichment" of methylated alleles in GS as compared with the original glioblastoma (Fig. 2A). In 2 cases, however, 2207 and 2683, the assay did not adequately reflect the frequency of methylated MGMT alleles (ratio of meth_MGMT normalized with ACTB). Both cases, exhibited a methylation pattern not favoring hybridization of the primers of the qMSP assay. In fact, in both the cases, a signal for MGMT methylation was measured in the original glioblastoma and was enriched in the respective GS. The measured meth_MGMT copy number would be visible on a gel [2207: GBM (glioblastoma) 34 copies, GS 342 copies; 2683: GBM 22 copies, GS 86 copies], as also shown for the gel-based MSP result (Supplementary Fig. S1). However, the high copy number measured for ACTB that does not suffer from changes in PCR efficiency neutralized the signal in the normalization step.

To estimate the frequency of such false-negative cases, we looked at the raw data from diagnostic MGMT tests performed by MDxHealth Inc. for glioma patients treated in Lausanne in the last 3 years. Of 76 tests, only 2 cases showed a low, but measurable meth_MGMT copy number with a normalized ratio (ACTB) corresponding to unmethylated MGMT. In fact, 1 corresponded to the patient sample GBM_2683 characterized here. The second, glioblastoma 2558, was subsequently subjected to clone sequencing for characterization of the methylation pattern. Indeed, clone sequencing revealed methylated alleles in 20% of the clones with a density of 30% (Supplementary Fig. S3). The pattern of methylation was unfavorable for detection by qMSP and the gel-based assay. The latter revealed a band in 2 of 4 replicas, thus the test was at the limit of detection. Only formalin-fixed material was available for this case.

MGMT methylation is associated with no or low MGMT expression in GS

Evaluation of MGMT RNA expression in the paired samples revealed no or very low expression of MGMT in any GS with dense MGMT methylation, regardless of the pattern that is significantly lower than in the unmethylated GS samples (P = 0.03; Fig. 2D). Several of the respective original glioblastoma tissues (GBM_2207, GBM_2288, GBM_2540; Fig. 2D) however, displayed MGMT expression, which in some cases reached similar levels of unmethylated cases (GBM or GS), normal brain or peripheral blood lymphocytes. Physiologic expression levels of MGMT vary in a cell-type–specific manner, with normal liver expressing more than 10× the expression of normal brain and lymphocytes (Fig. 2D). In accordance with lack of expression, MGMT activity was below the limit of detection in the GS of completely methylated cases (Fig. 3). Moderate activity was measured in GS_2683 that...
has a methylated and an unmethylated allele. And the unmethylated GS_2638 showed the highest activity.

MGMT activity was measurable in all respective original tissues including the glioblastoma of the methylated cases. The source of MGMT expression and MGMT activity in the methylated cases is likely nontumoral tissue as will be discussed later.

MGMT methylation is associated with a closed chromatin structure

Next, we addressed the question whether the observed CpG methylation in the MGMT promoter was associated with a repressive chromatin structure. Hypermethylation of CpG islands in the promoter region leads to gene silencing either by direct inhibition of transcription factor binding, or by attracting methylated DNA-binding proteins, recruiting other transcriptional repressors such as histone acetylases (HDACs) and histone methyl transferases. Respective modifications of histones lead to changes in the chromatin structure resulting in transcriptionally inactive chromatin (reviewed in ref. 30). A mark for transcriptionally repressed chromatin is trimethylation of histone H3 at lysine 27 (H3K27m3), whereas trimethylation at lysine 4 (H3K4m3) is associated with transcriptionally active chromatin. Indeed, qChIP analysis provided evidence that dense MGMT methylation in GS was associated with presence of histone marks for inactive chromatin (H3K27m3) and absence of histone marks for active chromatin (H3K4m3; Fig. 4). In accordance with lack of MGMT expression and MGMT activity, this pattern for closed chromatin was also observed for GS_2207 that exerted a methylation density of only 35%, and was therefore falsely classified as unmethylated by qMSP. GS_2683 with a methylated and an unmethylated MGMT allele accordingly displayed both, marks for active and inactive chromatin. Interestingly, GS_2638 with an exclusively unmethylated MGMT promoter revealed marks for active, but also inactive chromatin that may explain the relatively low MGMT expression observed for this particular case (Figs. 2D and 4). In contrast, lymphocytes expectedly showed only marks for active chromatin.

Origin of MGMT expression in methylated GBM

In contrast to the original clinical tumor samples, we found good accordance in the GS between loss of MGMT expression, lack of MGMT activity, and marks for a repressive chromatin structure on one hand, and MGMT promoter methylation on the other. The detection of unmethylated MGMT alleles and expression and activity of MGMT in an otherwise methylated tumor can be explained by the presence of contaminating/infiltrating nontumoral cells that we and others have shown to express MGMT RNA, protein or display MGMT activity, like normal brain (Figs. 2 and 3), tumor blood vessels, tumor-infiltrating lymphocytes, and tumor-associated macrophages or microglia (9, 31). For macrophages or activated microglia that are morphologically not always easy to recognize, we have shown MGMT expression by double staining with CD68 (9). Furthermore, presence of a nonmethylated allele in a glioblastoma balanced for chromosome 10 may explain MGMT expression. Semi-quantitative estimation of the tumor cell content based on morphology on
H&E-stained slides ranged from 62% to 96% (Fig. 5A). However, subsequent evaluation taking into account IHC for several markers, including GFAP and CD45, and in particular CD68 (Fig 5B) that recognizes activated macrophages and microglia, suggested that the tumor content was dramatically overestimated in some cases for example, GBM_2669. Normal gene dosage at the MGMT locus, with presence of a methylated and an unmethylated allele, explained why less than 50% of methylated clones were detected in GS_2683. However, in the other 3 cases with a balanced MGMT locus, both alleles were methylated, indicative for complete silencing of MGMT.

Taken together, the percentage of methylated alleles in a glioblastoma with a methylated MGMT status was in general in good accordance with the tumor content adjusted for CD68-positive cells and gene dosage at the MGMT locus, with the exception of GBM_2459. In this tumor, only 10% of the clones were methylated, in contrast to 100% of the clones in the respective GS. The discrepancy could not be explained by the tumor cell content, which was estimated at 60%. This suggests that a tumor cell subpopulation overlapping with the GS fraction is MGMT methylated in this glioblastoma.

Our experiments demonstrate that the unavoidable presence of variable amounts of nontumoral cells in clinical samples of glioblastoma precludes measurement of MGMT expression or MGMT activity as biomarker. As the small numbers of cases prohibited statistical evaluation of differences in MGMT expression between methylated and unmethylated samples in the present study, we compared MGMT mRNA expression in a larger set of glioblastoma from the EORTC-NCIC study for which gene expression profiles and the MGMT methylation status were available (32). Although, we found a significant difference of MGMT expression between MGMT methylated and unmethylated samples \( P < 0.002; \) Mann–Whitney test), there was no significant association with outcome when either considering only the 42 patients in the TMZ/RT arm or all 68 patients (Cox analysis, corrected for age, \( P > 0.4 \) or \( P > 0.2 \), respectively). This is not surprising given the large
<table>
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<th>MMS (&lt;27, 27–30)</th>
<th>Perform status</th>
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Abbreviations: MTR, macroscopically total resection (>98%); STR, subtotal resection (90%–98%); PR, partial resection (50%–90%); MMS, minimental status; OS, overall survival; meth, methylated; and unmeth, unmethylated.

<sup>a</sup> Recurrent glioblastoma.

<sup>b</sup> No MRI within 24 hours.

<sup>c</sup> Overall survival from first surgery 47 months, from second surgery 5 months.

<sup>d</sup> Patient received TMZ as second-line treatment.
overlap of expression levels observed between the 2 populations (Supplementary Fig. S6). In contrast, the MGMT methylation status was significantly ($P < 0.0001$) associated with outcome in either of the evaluated subpopulations (32).

**Discussion**

The clinical utility of the MGMT methylation status as a biomarker for benefit from alkylating agent therapy in gliomas has led to an ongoing debate on how the effect of MGMT should be assessed and which specific procedure would be the best for routine clinical application.

Comparison of the methylated allele frequency estimated by MS clone sequencing in the original glioblastoma tissues and respective GS and evaluation of the tumor cell content of the respective macrodissected paraffin sections suggest that in MGMT-methylated glioblastoma, the apparent level of methylation is strongly influenced by the presence of nontumoral tissue. However, the methylation pattern determined in the original tumor tissue was always retained in the respective GS, and dense methylation was associated with very low MGMT expression and lack of MGMT activity. This was in concordance with the presence of histone marks for inactive chromatin (H3K27m3) and absence of histone marks for active chromatin (H3K4m3). As postulated, these patients with a methylated MGMT gene had longer survival (Table 1; $P = 0.004$; log-rank test), although the small numbers preclude proper statistical evaluation. In contrast, estimates of MGMT expression and activity levels in the original tumor tissue were confounded by prominent "contamination" of the tumor tissue with CD68+ microglial cells and macrophages expressing MGMT (9) and maybe responsible for false-positive results. This might explain the lack of association of MGMT expression in glioblastoma, as measured by IHC, with survival of patients treated in a phase III trial with temozolomide, whereas the MGMT methylation status in the same tumor samples was a strong predictor of outcome ($n = 122$; $P = 0.0001$; ref. 33). Hence, this as well as previous studies certainly discourage the use of protein expression or enzyme activity in clinical samples of glioblastoma to evaluate the MGMT status for patient selection or stratification (9, 31, 33). For tests evaluating the promoter methylation status, a particularly tricky point for all tests is the definition of both a technically and clinically relevant cutoff for MGMT promoter methylation. Despite some uncertainty of the perfect cutoff, the majority of samples will likely be classified similarly by most tests. Individual samples may be "misclassified" due to incomplete methylation of the CpGs interrogated by a given test or presence of undue amounts of nontumoral tissue diluting the signal in a quantitative test normalizing for input (e.g., ACTB).

The extent of methylation required and the sets of CpGs that are crucial for complete silencing are unknown due to the complexity of gene regulation (34). Methyl-CpG binding proteins, such as methyl CpG binding protein 2 (MeCP2) and methyl-CpG binding domain protein 2 (MBD2), bind to aberrantly methylated sequences, leading to alterations of chromatin structure and preventing binding of transcription factors, thereby silencing the gene (24).

The density and pattern of MGMT methylation may lead to underestimation of methylated alleles; this is due to location of the primers or the fidelity requirements of the polymerase with the respective methylation-specific primers. On the other hand, the evaluation of a set of CpGs, as opposed to individual CpGs, allows identification of dense methylation required for silencing. Thus, in qMSP, specificity to recognize dense methylation may sometimes be on the cost of sensitivity. Conversely, MS-pyro-sequencing recognizes the integral of methylation at individual CpG sites (35). In extreme, this could lead to a call of "methylated" in a case with only scattered methylated CpGs that never reaches the necessary density in a given allele to result in silencing, hence, a false call. A similar test result would be obtained with few densely methylated alleles coming from a fraction of cells. Identification of cases presenting a methylated and an unmethylated allele would be a challenge for any test on tumor tissue due to the confounding signal from normal tissue. Interestingly, we found in 3 of 4 GS with a normal copy number of the MGMT locus that both alleles were methylated.

Given the complexity of the biological relationship between promoter methylation and gene silencing and the difficulties to integrate these features into a test that is in addition complicated by presence of nontumoral tissue, there are unavoidable drawbacks for any technology attempting to predict loss of MGMT expression for potential benefit from alkylating agent therapy. Key to introducing tests for diagnostics is their prospective validation. At present, most clinical trials use qMSP for prospective patient stratification or patient selection (36, 37). Results for validation are expected in early 2011 from the Radiotherapy Oncology Group (RTOG) 0525/EORTC 26052-22053 Intergroup trial (http://clinicaltrials.gov; NCT00304031) investigating 2 temozolomide chemotherapy regimens. In this study, all patients’ tumors were tested before stratified randomization for the MGMT status according to a technical cutoff based on qMSP including a gray zone around the cutoff termed "indetermined" (11, 36). Expected from the bimodal distribution of methylated and unmethylated cases, only a small percentage of cases were close to the technical cutoff (indeterminate; $n = 62$, 6.2%; ref. 36). On the basis of our present study, we expect that the samples classified as "indeterminate" comprise samples with distinct features, such as "difficult" methylation patterns inefficiently recognized by the test, or a small subpopulation of cells displaying a methylated promoter. Either group may or may not respond to alkylating agent therapy giving the impression of a dose-dependent effect of MGMT methylation on prognosis of glioblastoma patients treated with temozolomide and radiotherapy as proposed by Dunn et al. (35). In the first, it is not clear if the methylation pattern leads to silencing; in the latter group, the question is if the subpopulation with a methylated...
promoter is relevant to the malignant behavior of the tumor for example, overlaps with GICs. These considerations do not take into account other possibly prevailing molecular and clinical factors for resistance or tumor growth.

In conclusion, although most methylation-specific tests will be able to discriminate clearly methylated from clearly unmethylated cases that have an association with gene silencing, there is a gray zone comprising samples with different features recognized to a different degree by distinct technologies. The frequency of these difficult to classify subgroup is hard to estimate and may comprise around 5% of glioblastoma patients; but, most importantly may contain a fraction of patients that will benefit from alkylating agent chemotherapy. This view is supported by our finding that methylated MGMT alleles were enriched in GS, a tumor-relevant cell subpopulation, even when the original glioblastoma exerted only a minority of methylated alleles. For these gray-zone patients, standard temozolomide chemotherapy or concomitant chemoradiotherapy should not be withheld until better treatment options emerge. On the basis of these considerations, the EORTC 26082/22081 trial (http://clinicaltrials.gov, NCT01019434), selecting for MGMT unmethylated patients based on a qMSP assay, has introduced a respective safety margin (lower bound of the 95% confidence interval of the technical cutoff) in order not to withhold temozolomide from a patient group potentially benefitting from it.

Disclosure of Potential Conflicts of Interest

ME Hegi is a paid consultant for MDxHealth Inc.

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