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MGMT promoter methylation status testing to guide therapy for glioblastoma: refining the approach based on emerging evidence and current challenges

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Abstract

Glioblastoma (GBM) is the most common primary malignant brain tumor, with a universally poor prognosis. The emergence of molecular biomarkers has had a significant impact on histological typing and diagnosis, as well as predicting patient survival and response to treatment. The methylation status of the O^6-methylguanine-DNA methyl-transferase (*MGMT*) gene promoter is one such molecular biomarker. Despite the strong evidence supporting the role of *MGMT* methylation status in prognostication, its routine implementation in clinical practice has been challenging. The methods and optimal cutoff definitions for *MGMT* status determination remain controversial. Variation in detection methods between laboratories presents a major challenge for consensus. Moreover, consideration of other clinical and genetic/epigenetic factors must also be incorporated into treatment decision making. In this review, we distill the available evidence to summarize our position on the optimal use of available assays, and propose strategies for resolving cases with equivocal methylation status and a framework for incorporating this important assay into research and clinical practice.

Key words

diagnostic | glioma | methylation | MGMT | molecular markers | prognostic

Glioblastoma (GBM) is the most common primary malignant brain tumor in adults.[1] Due to the variability of patient response to the current standard of care, there is a need for better prognostication and individualization of treatment regimens. The methylation status of the O^6-methylguanine-DNA methyl-transferase (*MGMT*) gene

promoter has been demonstrated as an important biomarker of tumor response to temozolomide (TMZ) chemotherapy.[2–4] However, despite the positive prognostic role of *MGMT* promoter methylation and the pressing need for the identification of prognostic biomarkers for patients with high-grade gliomas, the routine implementation of this biomarker in clinical practice has been challenging.

In this review, we describe the assays used to determine *MGMT* methylation status and discuss some of the potential reasons for the variability of applying this biomarker in clinical practice. Based on an overview of the latest evidence, we propose a possible approach to *MGMT* promoter methylation testing and subsequent therapeutic decision making.

The Role of MGMT Promoter Methylation in GBMs

The *MGMT* gene resides on chromosome 10 (10q26), and because in the majority of GBMs one allele of this chromosome is commonly lost, the remaining gene copy drives function.[5] Alkylating chemotherapeutic agents such as TMZ induce cytotoxic cell death in tumor cells by alkylating DNA at multiple sites. Repair of the most toxic event, alkylation of the O^6 group of guanine, is dependent on MGMT.

The *MGMT* gene encodes a repair protein (MGMT; formerly also termed alkyl guanine alkyltransferase[6]) that reverses this alkylation process. In this process, the methyl moiety is transferred onto the MGMT protein, which is thus consumed.[2,7] Epigenetic modification of the cytosine-phosphate-guanine (CpG) island at specific CpG sites within the *MGMT* promoter silences the gene, leading to inefficient repair of DNA alkylation and enhanced response to TMZ.[8–10]

Original evidence demonstrating the role of *MGMT* methylation status in response to TMZ emerged from the Stupp trial.[4] The predictive/prognostic role of MGMT promoter methylation has since been demonstrated in several other studies as well. Select examples of these studies are illustrated in Table 1.[2,11–17]

Withholding TMZ in unmethylated patients and exclusive treatment with TMZ for methylated tumors have been investigated in prospective randomized trials in elderly patients.[12,14,18] The NOA-08 and Nordic Elderly trials found that among patients ≥65 years and >60 years, respectively, who received TMZ alone, *MGMT* promoter methylation was associated with significantly longer survival than "unmethylated" tumors, while TMZ therapy was detrimental in patients with an unmethylated tumor.[12,14] Similarly, trials replacing TMZ by investigational agents in unmethylated tumors have demonstrated that omission of the alkylating agent chemotherapy is not detrimental.[19–21]

Although these trials support withholding TMZ in unmethylated tumors, in current clinical practice this is reserved for clinical trials or for the treatment of elderly patients (in an effort to limit toxicity and burden from combined modality therapy). A survey of specialists caring for neuro-oncology patients showed that 77% would consider assessment of *MGMT* promoter methylation status in the management

of elderly patients with GBM.[22] Further, the 2017 European Association for Neuro-Oncology (EANO) guidelines for the treatment of malignant gliomas recommended that *MGMT* testing be considered as standard practice in elderly patients (>65–70 y).[23] These guidelines suggest that *MGMT* methylated patients should receive TMZ as part of their regimen, whereas patients with unmethylated tumors should receive hypofractionated radiotherapy alone. Nevertheless, the definition of 'elderly' varies in clinical trials and practice.[12,14,24] The recent Canadian Cancer Trials Group CE.6 clinical trial (CE.6 study) reported by Perry et al, comparing a short course of radiation therapy (RT) alone versus a short course of RT combined with TMZ in adults ≥65 years old with GBM demonstrated a notable increase in survival in elderly patients with an unmethylated MGMT status, though this difference was not statistically significant (10.0 vs 7.9 mo, $P = 0.08$).[17] This was the first study to examine the benefit of a combined chemo-radiation strategy in elderly patients, whereas the NOA-08 and Nordic trials directly compared radiation-based regimens with TMZ, and the pivotal European Organisation for Research and Treatment of Cancer (EORTC) 26981/National Cancer Institute of Canada (NCIC) CE.3 trial had an upper age limit of 70 years. We conclude that in fit elderly patients, hypofractionated radiotherapy with TMZ remains the treatment of first choice; however, in more fragile or very old patients, exclusive therapy with TMZ may be better for tumors that are MGMT methylated, while for patients with an unmethylated promoter, RT should be considered.

MGMT methylation status alone is not the sole predictor of response to treatment. Hegi et al observed that even among unmethylated patients treated with radiation and TMZ, progression-free survival (PFS) was significantly improved, while increase in overall survival (OS) approached significance.[2] In all trials there are occasional patients who appear to benefit from TMZ chemotherapy despite the absence of *MGMT* promoter methylation (Table 1). This is due in part to the fact that trials are reported as intent to treat and may thus include misclassified tumors or other histologies, as, for example, revealed in the report on the central pathology review of EORTC 26981/NCIC CE.3 trial.[25] Other factors such as assay variability, cutoff definitions, or other molecular aberrations should also be considered. The median age in the Hegi et al study was 57 years, and as expected a notable proportion (7%; 9/130 analyzed) of patients had been isocitrate dehydrogenase 1 (*IDH1*) mutated,[25] portending favorable prognostic factors not accounted for by the study.[2] In addition, almost all patients with the CpG island methylator phenotype (G-CIMP) also had *MGMT* promoter methylation.[26] Posttranscriptional modifications of *MGMT* mRNA affect the expression of the gene product as well and these have been comprehensively reviewed elsewhere.[24] Other molecular changes may also correlate with *MGMT* promoter methylation. In anaplastic oligodendrogliomas, van den Bent et al have shown a strong correlation between *MGMT* promoter methylation and 1p/19q codeletion, though this may in part be confounded by the high correlation between *IDH1* mutation status.[27] This has been supported recently by the observation that almost all high-risk low-grade glioma patients with *IDH* mutation, with

Table 1 Landmark trials establishing the predictive and prognostic role of *MGMT* promoter methylation

Study	Design	MGMT Testing Assay	MGMT Status	Prognostic Value
Hegi et al, 2005 (EORTC 26981/22981- NCIC CE.3)[2]	Adults with WHO grade IV glioma Randomized to RT vs RT + TMZ (phase III) 573 patient enrolled → 307 evaluable samples→ methylation status determined in 206 (67% of evaluable samples)	MS-PCR	Methylated: 92/206 patients (44.7%) Unmethylated: 114/206 patients (55.3%)	Median OS: - RT = 15.3 months - RT + TMZ = 21.7 months OS HR (RT vs RT + TMZ): -0.51 (95% CI 0.31–0.84) Median PFS: - RT = 5.9 months - RT + TMZ = 10.3 months PFS HR (RT vs RT + TMZ): - 0.48 (95% CI 0.31–0.75) Median OS - RT = 12.7 months - RT + TMZ = 11.8 months OS HR (RT vs RT + TMZ): - 0.69 (95% CI 0.47–1.02) Median PFS - RT = 4.4 months - RT + TMZ = 5.3 months PFS HR (RT vs RT + TMZ): - 0.62 (95% CI 0.42–0.92)
Wick et al, 2009 (NOA-04)[13]	Adults with WHO grade III glioma Randomized to RT vs TMZ vs procarbazine/lomustine/vincristine (phase III) Only samples with 80% + tumor content were evaluated 274 patients enrolled → 202 evaluable samples → methylation status determined in 202 (100% of evaluable samples)	MS-PCR	Methylated: 123/202 patients (60.9%) Unmethylated: 79/202 patients (39.1%)	PFS HR (methylated vs unmethylated): - 0.59 (95% CI 0.37–1.1) TTF HR (methylated vs unmethylated): - 0.52 (95% CI 0.29–0.91)
Gilbert et al, 2013 (RTOG 0525)[11]	Adults with primary glioblastoma Randomized to RT + TMZ (standard) vs RT + TMZ (dose-dense) 1173 patients registered → 833 patients randomized → methylation status determined in 762 (91% of evaluable samples)	qMS-PCR	Methylated: 245/762 patients (32.1%) Unmethylated: 517/762 patients (67.9%)	Median OS (methylated): - 21.2 months (95% CI 17.9- 24.8) Median OS (unmethylated): - 14.0 months (95% CI 12.9- 14.7) OS HR (methylated vs unmethylated): - 0.58 (95% CI 0.48–0.69) Median PFS (methylated): - 8.7 months (95% CI 6.6–11.2) Median PFS (unmethylated): - 5.7 months (95% CI 5.1 to 6.1) PFS HR (methylated vs unmethylated): - 0.61 (95% CI 0.52–0.73)

Table 1   *Continued*

Study	Design	MGMT Testing Assay	MGMT Status	Prognostic Value
Wick et al, 2012 (NOA-08)[12]	Adults >65 years old with grades III and IV glioma Randomized to RT vs TMZ (phase III) Only samples with 80% + tumor content were evaluated 412 patients enrolled → 209 evaluable samples → methylation status determined in 209 (100% of evaluable samples)	qMS-PCR on 182 samples MS-PCR on all samples	Methylated: 73/209 patients (35%) Unmethylated 136/209 patients (65%)	OS HR (TMZ vs RT): - 0.69 (95% CI 0.35–1.16) EFS HR (TMZ vs RT): - 0.53 (95% CI 0.33–0.86) OS HR (TMZ vs RT): - 1.34 (95% CI 0.92–1.95) EFS HR (TMZ vs RT): - 1.95 (95% CI 1.41–2.69)
Malmstrom et al, 2012 (Nordic)[14]	Adults >60 years old with WHO grade IV astrocytoma (changed to >65 years after October 15, 2004) Randomized to TMZ vs hypofractionated RT vs standard RT (phase III) 343 patients enrolled → 291 patients randomized → methylation status determined in 203 (70% of evaluable samples)	qMS-PCR	Methylated: 91/203 patients (45%) Unmethylated: 102/203 patients (55%)	OS HR (TMZ vs any RT): - 0.64 (95% CI 0.39–1.04) OS HR (TMZ vs any RT): - 1.16 (95% CI 0.78–1.72)
Gilbert et al, 2014 (RTOG 0825)[15]	Adults with primary glioblastoma Randomized to RT + TMZ + bevacizumab vs RT + TMZ (phase III) 978 patients enrolled → 637 patients randomized (65% of evaluable samples)	qMS-PCR	N/A	Median OS (methylated): - 23.2 months (95% CI 20.1–28.3) Median OS (unmethylated): - 14.3 months (95% CI 13.6–15.3) OS HR (methylated vs unmethylated - 2.10 (95% CI 1.65–2.68) Median PFS (methylated): - 14.1 months (95% CI 10.5–16.1) Median PFS (unmethylated): - 8.2 months (95% CI 7.5–9.2) PFS HR (methylated vs unmethylated) - 1.67 (95% CI 1.36–2.05)
Wick et al, 2017[16]	Adults with recurrent glioblastoma Randomized to lomustine + bevacizumab vs lomustine (phase III) 437 patients randomized → 270 evaluable samples → methylation status determined in 270 (100% of evaluable samples)	450k array	Methylated: 124/270 patients (45.9%) Unmethylated: 146/270 patients (54.1%) Undetermined: 97 patients	Median PFS - 5.7 months (95% CI 4.4–6.9) Median PFS - 2.8 months (95% CI 2.6– 2.9) Median PFS - 3.0 months (95% CI 2.8–4.2)
Perry et al. 2017 (CE.6 trial)[17]	Adults ≥65 years old with primary glioblastoma Randomized to short-course RT + TMZ vs short-course RT (phase III) 562 patients randomized → 462 evaluable samples → methylation status determined in 354 (77% of evaluable samples)	MS-PCR	Methylated: 165/354 patients (46.6%) Unmethylated: 189/354 patients (53.4%)	OS HR (RT + TMZ vs RT): - 0.53 (95% CI 0.38–0.73) OS HR (RT + TMZ vs RT): - 0.75 (95% CI 0.56–1.01)

EFS: event-free survival; HR: hazard ratio; TTF: time to treatment failure.

or without 1p/19q codeletion, have a methylated *MGMT* promoter.[28]

The *MGMT* Promoter

The promoter of the *MGMT* gene contains a CpG island that is 777 bp long and contains 97 CpG dinucleotides (Fig. 1).

To determine methylation at which specific CpG site(s) influence transcription, Malley et al correlated the *MGMT* mRNA expression level with methylation status of each CpG dinucleotide within the CpG island in both GBM cell lines and xenografts.[29] Ninety-six CpG dinucleotides were tested and the authors demonstrated that the island can be segmented into distinct, nonrandom methylated blocks. Among these, 2 distinct regions (differentially methylated region 1 [DMR1] and DMR2) (Fig. 1) had the greatest impact on mRNA expression. Through selective site-directed mutagenesis, mutation of any CpG dinucleotide within DMR2 altered the expression of *MGMT*. Furthermore, it was shown that DMR2 was always methylated when DMR1 was methylated, making DMR2 the critical region for methylation testing. This finding has been further validated by Bady et al[26]; using results from the patient cohort in Hegi et al for classification of methylation for a training set, the authors showed that DMR1 and DMR2 not only were found to correlate inversely with *MGMT* expression when methylated but also were associated with better OS when exposed to alkylating chemotherapy.[26]

Methylation Detection Assays

A number of assays are currently available to measure *MGMT* promoter methylation status, and categorical variability between institutions and tests exists (Table 2). For all assays, except for the Illumina Infinium MethylationEPIC BeadChip, the bulk of the sample preparation is conducted locally while sequencing is performed in core labs. This difference is reflected in the costs as well. In the USA, the reimbursement for pathologists is governed primarily by CPT code 88363, which outlines reimbursement for facility and non-facility fees. When sample preparation from archival tissue is needed, CPT code 88380 or 88381 is used and bundled with 88363.

There is currently no consensus regarding the best assay.[30] Lassman et al analyzed concordance of MGMT analyses between local and central laboratories from tissue specimens obtained as part of a recently completed randomized phase III trial (Radiation Therapy Oncology Group [RTOG] 3508/AbbVie).[31] The authors compared local versus central biomarker results among patients screened for M12-356 (a prior trial) or RTOG 3508 at Columbia University Medical Center. *MGMT* promoter methylation was analyzed by methylation-specific PCR (MSP). One hundred and nine GBMs were molecularly profiled from 73 patients who underwent 1–4 resection(s) and *MGMT* promoter methylation was observed in 27% of tumors tested locally versus 43% centrally; *MGMT* methylation

interlaboratory concordance was only 61%.[31] Considering the impact of this biomarker on treatment allocation in clinical trials and practice, this underscores the importance of using a validated and quality-controlled assay.

Methylation-Specific Polymerase Chain Reaction

The use of the MSP method for detection of *MGMT* methylation status is supported by evidence from multiple randomized trials.[2,32,33] First described by Herman et al,[34] the technique is dependent on bisulfite conversion (which converts unmethylated cytosines to uracils), followed by use of primers designed to measure multiple CpG dinucleotides within the *MGMT* promoter to specifically amplify alleles with either unconverted or converted cytosines, representing methylated and unmethylated sequences, respectively (Fig. 1).[2,17,35] The amplified sequences are then assessed via gel electrophoresis, and a qualitative interpretation of methylation signal is made. While distinctly positive and negative signals are simple to interpret, faint signals are typically referred to as "equivocal."[36] When samples are run in replicates, performed infrequently, a variation noted may be termed "inconsistently" methylated. Retrospectively assessing 465 GBM samples in which MGMT status was determined using MSP, Xia et al demonstrated an inconsistency rate of 12% among their MSP replicates.[32] The survival of patients with inconsistent results paralleled that of patients with unmethylated samples. The authors noted a "dose-response" trend, wherein the ratio of methylated:total number of replicates correlated with survival and this trended toward significance. Others have argued that even patients with low levels of methylation may benefit from alkylating agents such as TMZ, based on evidence suggesting that these tumors could have glioma-initiating cells that are enriched for methylated *MGMT*.[37]

Quantitative MSP (qMSP) uses quantitative PCR technology and normalizes the copy number of methylated *MGMT* to the copy number of an unmethylated gene, such as beta-actin (*ACTB*), controlled by standard curves. The technical cutoff, where the probability of being methylated/unmethylated is 50%, is usually applied to dichotomize the test result.[14,33] A respective certified commercial qMSP assay has been used for central prospective testing in most recent phase III trials for GBM for patient selection or stratification.[15,38,39] The term "equivocal" or "gray zone" has also been applied to the qMSP method, as the uncertainty in the vicinity of the cutoff is high. Therefore, trials selecting for *MGMT* unmethylated patients and omitting TMZ in the treatments have used the lower bound of the 95% confidence interval as a safety margin in order not to deny patients a potentially effective therapy.[20,21] The technical cutoff has shown to be a good predictor of outcome in GBM trials where patients have been treated with TMZ.[12,14,15] However, for strategies aiming at enriching patient populations with only methylated or unmethylated tumors, respectively, this technical cutoff may need to be further optimized for a "clinical" cutoff.

The principle of MSP aims at amplification of fully methylated *MGMT* alleles that are considered to be biologically most relevant for gene silencing. This increases the
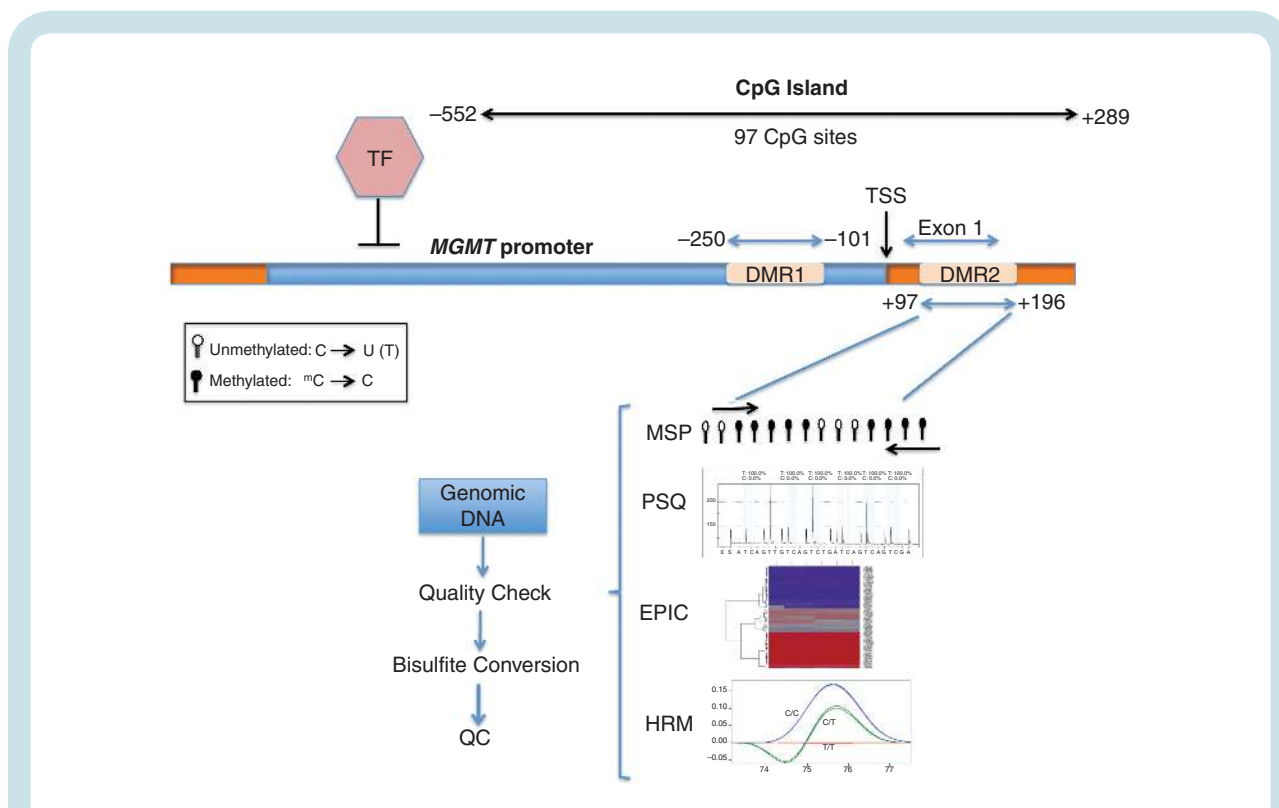
Fig. 1   MGMT promoter region and commonly used methylation assays. The promoter region and exon 1 of the *MGMT* gene contain the CpG island which spans 97 CpG sites. Methylation of CpG sites within 2 regions, DMR1 and DMR2, has been shown to negatively influence transcription. Five commonly methylated CpG sites within DMR2 are shown. The assays require isolation of DNA, followed by quality check (QC) for purity and integrity of the DNA. The efficiency of bisulfite conversion should also be tested to avoid false negative results. PSQ: pyrosequencing; TF: transcription factor; TSS: transcription start site.

specificity but reduces the sensitivity, as heterogeneous methylation may not be detected. This applies for any qualitative or quantitative MSP assay. Despite limitations, the large body of data, including from large clinical trials, supports the clinical utility of qMSP. In our opinion, qMSP provides a good and reproducible balance between reliability, availability, and cost.

Pyrosequencing

Pyrosequencing also relies on bisulfite conversion of DNA and PCR amplification. However, through sequencing DNA by synthesis, this technique also enables quantification of methylated DNA within each CpG site tested, which is displayed as a "pyrogram" (Fig. 1). Pyrosequencing allows for better detection of heterogeneous patterns of methylation than MSP-based technology, as it yields quantitative methylation values for individual CpG dinucleotides. The technology is robust and cutoffs for specific sets of CpGs have been validated in independent, although small, datasets.[40] In a large prospective study, utilizing both MSP and pyrosequencing, Reifenberger et al demonstrated a strong concordance between both assays when a cutoff of <8% vs ≥8% methylated alleles was used for the pyrosequencing method. Furthermore, a significantly better outcome was achieved in response to alkylating chemotherapy when a

cutoff of >25% methylated alleles was used.[41] However, while quantitatively more methylated alleles may provide prognostic value for survival, there is uncertainty regarding the cutoff to define *MGMT* "methylated" versus "unmethylated" status for stratifying patients into treatment groups.[42] Moreover, "partial methylation," wherein not all CpG sites are methylated, is a scenario that arises with pyrosequencing that is difficult to interpret in terms of clinical relevance.[40] As pyrosequencing is significantly more expensive than MSP, it may be reserved for high-volume settings such as clinical trials.[43]

High-Resolution Melt

Quantitative real-time PCR high-resolution melt (PCR-HRM) has been shown to have high reproducibility in assessing methylation.[44] This method also involves bisulfite conversion of DNA followed by PCR amplification and analysis of melting profiles of the PCR products. In one study, PCR-HRM was found to be more accurate than MSP for predicting PFS and OS of high-grade glioma patients treated with radiation and TMZ, and was comparable in detection ability to pyrosequencing.[45] Quillien et al, on the other hand, compared HRM with MSP and pyrosequencing and found that HRM had a weaker predictive value, and a higher percentage of patients with heterogeneous methylation as

Table 2   Summary of assays used for analysis of *MGMT* promoter methylation

Assay	Turnaround Time	Benefits	Disadvantages	Estimated cost per Sample (USD)
Simple MSP	2 days	- Shown to have predictive and prognostic value - Relatively inexpensive	- Unreliable results - Poor reliability in FFPE tissues	$5/sample
Quantitative MSP	2 days	- Cutoff point validated in clinical trials	- Unreliable results with mosaic methylation patterns - Poor reliability in FFPE tissues	$10–20/sample
Pyrosequencing	5 days	- Quantitative	- High cost - Longer time to results - Cutoff threshold not validated in clinical trials - High-throughput core facility required	$10–30/sample
High resolution melt	2 days	- Quantitative	- Cutoff not validated in clinical trials	$20–30 /sample
EPIC	5 days	- Offers testing of other biomarkers (1p/19q, G-CIMP) - Compatible with different sample preparations	- Longer time to results - High cost - High-throughput core facility required - Cutoff not validated in clinical trials	$500–700/sample
IHC	2 days	- Low cost	- High interobserver variability - Inconsistent correlation with clinical outcomes	$10–30/sample
MLPA	2 days	- No need for bisulfite conversion of samples - Low cost	- Predictive utility is not validated	$10–30/sample

'Turnaround time' is estimated from DNA isolation point. The timelines presented are the minimum amount of time required for sample processing and may increase depending on availability of equipment and core facility.

observed by pyrosequencing were defined as "unmethylated" using HRM.[46] Therefore, the current evidence for HRM is not robust.

Multiplex Ligation-Dependent Probe Amplification

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) is a semi-quantitative method that does not require the DNA-modifying bisulfite treatment step and, therefore, avoids additional damage to the DNA.[35] MS-MLPA can detect changes in both CpG methylation and copy number of approximately 40 chromosomal regions per reaction.[47] In MS-MLPA, the ligation of probes is combined with digestion of template DNA-probe complex with the methylation-sensitive endonuclease HhaI. Probe–DNA complex is first treated with HhaI and then subjected to PCR. The limitation is that the methylation-specific probes can only be designed for sequences that contain the HhaI restriction site GCGC. If the CpG locus is methylated, the HhaI restriction site is protected from restriction enzyme digestion and the PCR product is not generated. An additional limitation of the MS-MLPA method is the need for dedicated equipment and costly reagents.

Compared with other well-established assays, the utility of MS-MLPA in detection of methylation status has been assessed in a limited number of studies.[48,49] Although the semi-quantitative aspect of MS-MLPA may prove to be of great value, additional studies will need to be conducted to evaluate the prognostic ability and clinical utility of this method.

Immunohistochemistry

Assessment of *MGMT* status at the protein level through immunohistochemistry (IHC) has also demonstrated variable accuracy across studies.[46,50,51] The advantage of IHC is that it enables focused interpretation of signal in regions of high tumor purity, avoiding a potentially erroneous "signal" from samples mixed with nontumor tissue. However, conventional IHC is also associated with poor reproducibility and high interobserver variability, along with variations in the detection capabilities of different antibodies used across laboratories.[52] Furthermore, individual MGMT proteins are sacrificed upon interaction with the appropriate substrate and, therefore, the amount of protein detected may not necessarily be a reliable indicator of expression.[53]

The accuracy of IHC may be increased when expression levels are assessed in combination with assessment of *MGMT* promoter methylation.[54,55] Quantitative methods may overcome some of the major shortcomings of conventional IHC. However, IHC is substantially limited by false positives, MGMT expression by nontumor cells, or false negatives as expression of MGMT is induced upon

treatment in unmethylated tumors.[56] These and other considerations are discussed comprehensively elsewhere.[57] In conclusion, despite its simplicity, MGMT determination by IHC is not sufficiently reliable for basing management decisions upon.

Infinium Methylation EPIC BeadChip Array (27k, 450k, or 850k)

Genome-wide analysis of DNA methylation patterns has improved our understanding of glioma biology and has contributed to the advancement of tumor classification.[58–60] The analysis of DNA methylation using BeadChip arrays allows interrogation of 850 000 CpG sites in its most recent version (EPIC, 850k). The platform shows good performance also for DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue samples and is therefore suitable for analysis of clinical samples,[61] which lends itself well to the possibility of application for centralized testing. Furthermore, the array technology also enables inference of genome-wide copy number changes[26,59,61] and assessment sample purity.[62]

Of interest for the present review, the *MGMT* gene is also well covered. A BeadChip-based classifier for *MGMT* methylation status has been developed and validated using 2 CpG probes located in the *MGMT* promoter (one in DMR1, the other in DMR2), whose methylations were identified to be most influential for *MGMT* expression, as well as for outcome in TMZ-treated GBM patients (MGMT-STP27).[26] The assay was shown to be also valid for predicting the MGMT methylation status in other tumor types and demonstrated similarity in methylation score regardless of DNA sources (frozen versus FFPE).[5,26] The usefulness of the MGMT-STP27 classifier for outcome prediction was shown in several clinical trials.[16,63,64]

A binary classification of methylated versus unmethylated carries the risk of erroneous misclassification of patients close to the technical cutoff, where uncertainty is high. In the original model, Bady et al established upper and lower confidence intervals around the cutoff, which defines a "gray zone" to minimize the margin of error in identifying "unmethylated" and "methylated" patients.[16] It is also of note that there may be a difference between the technical cutoff (50% probability to be methylated) and the clinically relevant cutoff.

The array technology has yet to be accredited by the College of American Pathologists (CAP) and certified by Clinical Laboratory Improvement Amendments (CLIA). Though this is on the horizon, it will likely increase cost. Furthermore, the technology is not available at all sites and a minimum number of samples are necessary to conduct the assay.[24] However, DNA methylation-based diagnosis of tumors is likely the future of pathology, redefining histological classifications in up to 12% of cases.[60] The added costs of the array technology will therefore likely be offset in the long run by the increase in diagnostic accuracy and the ability to assess a broad range of molecular prognostic markers. Hence, we trust that there is great utility for this assay in both clinical and research settings, particularly for centralized assessment of equivocal cases.

The Need for Quality Control and Establishing Standards

The caveat for the evaluation of MGMT tests is that the large body of publications that interrogate different sets of CpGs in heterogeneous patient populations rarely validate their assays for technical reproducibility and most often lack validation in an independent dataset. Rigorous quality control necessitates the collaborative effort of neuro-oncological surgeons, oncologists, neuropathologists, molecular pathologists, and other team members involved in the processing and assessment of data. The adequacy of the sample provided, including amount of tumor tissue and sampling from geographically independent regions of the tumor tissue, would contribute to decreasing heterogeneity. Sampling of nontumor cells (eg, macrophages and neighboring brain parenchyma) and nonviable tumor regions is a major source of inconsistencies. During histological determination of tumor subtype, selection of regions of high tumor cell purity within a hematoxylin and eosin–stained slide for processing and methylation assessment is critical. This emphasizes the importance of input from an expert neuropathologist.[35]

Aside from MLPA, all of these assays are limited by the efficiency of bisulfite reaction[32] resulting in false positive results.[35] This should be addressed in the quality control of the assays. Based on these and other factors, the debate regarding the cutoffs to be applied—hence the amount of methylation that should be considered significant—persists for quantitative methods. This certainly also depends on the clinical question (eg, patient stratification to balance trials versus selection for therapy excluding TMZ).[19,20,65] Everhard et al reported that a minimum of at least 9% mean methylation, as determined by pyrosequencing, over 52 CpGs associated with expression was associated with low MGMT RNA expression.[66] Brigliadori et al, on the other hand, suggested that a cutoff of >30% of 10 CpG dinucleotides within DMR2 through the pyrosequencing method was most predictive of patient survival.[6] Simple qualitative methods (simple MSP) are likely inferior to quantitative methods, as they preclude setting safety margins or evaluating the extent of methylation. Furthermore, the dichotomous classification of methylated versus unmethylated may need to be reevaluated. To this end, we believe that the consideration of 95% confidence intervals around a signal threshold status may be prudent.

A Decision-Making Framework Based on MGMT Promoter Methylation Testing

The utilization of *MGMT* methylation testing outside of trial settings may be limited. Assessing the National Cancer Database, Lee et al observed that in ~87% of patients with a diagnosis of GBM, the methylation status was unknown or not coded.[67] The survival of patients with unknown methylation status was similar to unmethylated patients.

With the era of precision medicine upon us, ignoring the strongest prognostic and predictive factor is unacceptable. Standardized algorithms for assessment of methylation status in a much higher proportion of patients will not only lead to better care for methylated patients but also enable the possibility of new therapeutics in unmethylated patients.

Given the universally poor prognosis of high-grade gliomas (histological diagnosis of World Health Organization [WHO] grades III/IV glioma, IDH wildtype, 1p/19q retained), we cannot afford to miss the opportunity to identify the subgroups of patients who may benefit from adjuvant therapy while also minimizing toxicity and developing alternative treatment strategies for others.

Through distilling the evidence on the available assays, we propose a decision-making algorithm outlined in Fig. 2. In this algorithm, the surgical neuro-oncologist samples multiple regions of the tumor for viable tumor cells. In addition to establishing a histological and molecular diagnosis, the neuropathologist is tasked with selecting slides with the highest tumor purity. qMSP is a good initial method to assess *MGMT* methylation status, based on its wide availability, ease of interpretation, and low cost (Table 2). The 2 distinctly methylated regions (DMR1 and DMR2) identified by Malley et al and validated by Bady et al should be the area of focus for probe-based assays.[26,29] We recommend that patients with methylated status based on MSP testing undergo RT/TMZ followed by TMZ therapy if patients meet clinically appropriate criteria.

Equivocal cases based on MSP testing would need further refinement. In high-throughput settings (such as clinical trials or batch testing of clinical samples), either pyrosequencing or Infinium MethylationEPIC BeadChip array can be of great value. The array method has the added value of providing methylation-based tumor classification, including assessing copy number variations and tumor purity. Emerging computer algorithms have the potential to establish the DNA methylation profile of tumors with high fidelity. Head-to-head comparisons, providing correlations with patient outcomes and analysis of cost-effectiveness, would be necessary to select the ideal assay moving forward.

In patients categorized as "unmethylated," particularly those >65 years old, while strong consideration should be given to withholding TMZ, the results of trials such as the CE.6 study must also be taken into account. An open conversation regarding the risk-benefit profile of adjuvant therapy is also necessary with the patient and/or caregivers. Ideally, recently published prognostic categorization of patients based on clinical and molecular parameters would be implemented, which would add objectivity and help facilitate the discussion between the physician and the patient.[55,60] In current practice, the assessment of the various parameters is based on the overall impression of the clinical team and patient preference, which is not captured objectively in clinical trials. Not all elderly patients are homogeneously in a poor clinical condition and thus we suggest the age variable as only part of the decision-making paradigm. Consideration of comorbidities and
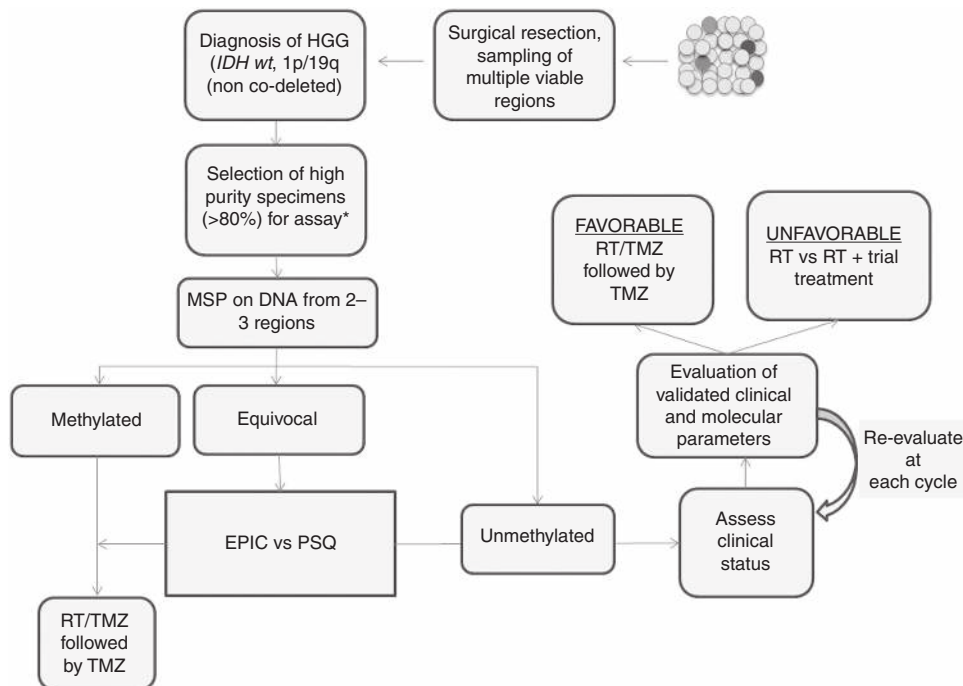

Fig. 2   Proposed management algorithm for patients with glioblastomas, based on methylation status and other clinical parameters. PSQ: pyrosequencing; KPS: Karnofsky performance status; ECOG: Eastern Cooperative Oncology Group.

performance status must be made in allocating patients with "unmethylated" status to different treatment regimens. The efficacy of novel agents should be assessed in the setting of clinical trials. Assignment of patients to a particular intervention is certainly not rigid and we recommend patient reevaluation at each adjuvant treatment cycle in order to maximize safety and efficacy.

Future Directions

Current studies assessing the predictive value of the various assays are likely underpowered.[43,51] The performance of the most promising assays can potentially be compared head-to-head prospectively in observational or randomized studies. The yield, feasibility, and cost-effectiveness of "double testing" to refine equivocal cases has to be explored. The objective of this approach would be either to randomize patients with equivocal MSP results to MGMT determination using different assays or to simply compare single testing versus double testing, using a consensus assay for the latter. Focusing on equivocal cases can reduce the required sample size; however, the overall low prevalence of these cases may prolong the study. Still, ultimately correlating the test result with OS will be required.

The array method of assessing methylation status would likely impose not only upfront capital cost but also ongoing material and maintenance costs as well. However, considering that the average per-patient cost in a phase III clinical trial can be as high as $42 000, the added cost of this diagnostic assay would only be marginal, considering its many benefits.[68]

In non-GBM tumors, the clinical impact of *MGMT* methylation on benefit from TMZ or other alkylating agents remains to be established. GBMs generally have only one *MGMT* allele, suggesting that methylation of the second allele completely blocks MGMT mediated DNA repair—conferring sensitivity to TMZ treatment. In contrast, other tumor types, including *IDH* mutant low-grade gliomas, retain both alleles. Hence, a higher extent of methylation may be indicative of inactivation of both alleles, as suggested by the predictive value of a high *MGMT* methylation score in IDH mutant low-grade glioma patients treated with TMZ in EORTC 22033.[62] The assessment of methylation status based on Bady et al's algorithm using a higher cutoff may be helpful in predicting benefit from TMZ providing a tool for stratified therapy.[62] Therefore, relevant cutoffs for IDH *mutant* tumors need to be established and validated for quantitative assays.

The selection of a valid endpoint would be critical for study design and sample size calculations. Assessment of the accuracy of each assay in the traditional sense (eg, sensitivity and specificity) may not be feasible for MGMT, particularly given that there is currently no gold standard confirmatory test.[69] Correlation with response to TMZ therapy and survival is likely more clinically relevant. Furthermore, such an endeavor would require standardization of methods across laboratories. Given the increasing constraints of health care resources, health economic evaluations of the utility of these proposed strategies are necessary as well.

While in the setting of research trials stratification of patients based purely on methylation status would be helpful in choosing therapeutic options, implementation of such important biomarkers in clinical practice has its own challenges, not the least being the lack of a better alternative for patients with an unmethylated *MGMT* promoter. Within the limitations of the evidence to date, the clinical management of patients based on the *MGMT* promoter methylation status should be considered together with other molecular and clinical factors that contribute to patient outcome. The merits of this approach need to be examined systematically.

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