Combined analysis of O\textsuperscript{6}-methylguanine-DNA methyltransferase protein expression and promoter methylation provides optimized prognostication of glioblastoma outcome


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Background. Promoter methylation of the DNA repair gene, O-6-methylguanine-DNA methyltransferase (MGMT), is associated with improved treatment outcome for newly diagnosed glioblastoma (GBM) treated with standard chemoradiation. To determine the prognostic significance of MGMT protein expression as assessed by immunohistochemistry (IHC) and its relationship with methylation, we analyzed MGMT expression and promoter methylation with survival in a retrospective patient cohort.

Methods. We identified 418 patients with newly diagnosed GBM at University of California Los Angeles Kaiser Permanente Los Angeles, nearly all of whom received chemoradiation, and determined MGMT expression by IHC, and MGMT promoter methylation by methylation-specific PCR (MSP) and bisulfite sequencing (BiSEQ) of 24 neighboring CpG sites.

Results. With use of the median percentage of cells staining by IHC as the threshold, patients with \(<30\%\) staining had progression-free survival (PFS) of 10.9 months and overall survival (OS) of 20.5 months, compared with PFS of 7.8 months \((P < .0001)\) and OS of 16.7 months \((P < .0001)\) among patients with \(\geq 30\%\) staining. Inter- and intrareader correlation of IHC staining was high. Promoter methylation status by MSP was correlated with IHC staining. However, low IHC staining was frequently observed in the absence of promoter methylation. Increased methylation density determined by BiSEQ correlated with both decreased IHC staining and increased survival, providing a practical semiquantitative alternative to MSP. On the basis of multivariate analysis validated by bootstrap analysis, patients with tandem promoter methylation and low expression demonstrated improved OS and PFS, compared with the other combinations.

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Conclusions. Optimal assessment of MGMT status as a prognostic biomarker for patients with newly diagnosed GBM treated with chemoradiation requires determination of both promoter methylation and IHC protein expression.

Keywords: biomarker, glioblastoma, immunohistochemistry, methylation, MGMT.

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor in adults. Current standard treatment for GBM includes cytoreductive surgery followed by radiation (RT) and chemotherapy with temozolomide (TMZ). CpG methylation of the O-6-methylguanine-DNA methyltransferase (MGMT) gene promoter has emerged as the most robust predictor of TMZ treatment outcome for newly diagnosed GBM with prognostic implications. MGMT encodes for a DNA repair enzyme that provides resistance to alkylating chemotherapies, such as TMZ. Because MGMT transcription can be silenced by promoter methylation in tumor cells, it is widely assumed that MGMT promoter methylation in patient tumors causes decreased MGMT protein expression, thereby abrogating DNA repair activity necessary for TMZ resistance. However, the relationship between MGMT protein expression and promoter methylation in patient tumor samples has remained controversial. Furthermore, studies examining MGMT immunohistochemistry (IHC) and survival have not shown consistent correlation with outcome.

The lack of definitive evidence has been attributed primarily to difficulty with MGMT IHC scoring; however, other possible reasons include small cohort size, nonuniform treatments, and various IHC scoring categories and cutoffs.

Because of the importance of MGMT status for predicting treatment outcome, stratifying clinical trial patients, and guiding treatment decisions, the aims of the current single-arm retrospective study were to evaluate MGMT protein expression as a prognostic marker for patients with newly diagnosed GBM who were receiving standard of care RT/TMZ, to investigate the relationship between MGMT promoter methylation and protein expression, and to optimize the assessment of MGMT status. On the basis of a large cohort of 418 patients with newly diagnosed primary GBM, 410 of whom received chemo-radiation with TMZ, we show that, although MGMT IHC is prognostic of patient survival, the combination of IHC and methylation testing provides optimized assessment of MGMT status.

Materials and Methods

Patients

Pretreatment formalin-fixed, paraffin-embedded tumor samples were available for 418 adult patients with newly diagnosed confirmed GBM, 410 of whom were treated with TMZ and RT. A total of 281 patients who received a diagnosis during 2000–2007 were retrospectively identified on the basis of an electronic database query of adult patients with primary GBM receiving upfront TMZ and treated at the University of California Los Angeles (UCLA) or Kaiser Permanente Los Angeles (KPLA). One hundred thirty-seven additional patients (who received a diagnosis during 2007–2010) whose samples were directed to the laboratory in an unselected manner were also included. The collection of human brain tumor samples was approved by the UCLA Institutional Review Board, and informed consent was obtained from all patients. Some MGMT MSP and IDH1 sequencing data have been previously reported. The range of follow-up was 2–137 months. The median follow-up period determined by method of Schmep and Smith was 70 months. We had missing overall survival data on 1 patient and missing progression data on 6 patients.

Results

Patient Characteristics

As described in Methods, we derived a cohort of 418 patients with newly diagnosed primary GBM with available formalin-fixed, paraffin-embedded tumor samples from the initial surgery prior to any treatment. The clinical characteristics of the patients are listed in Table 1. The RT/TMZ treatment protocol varied slightly among the 410 total patients: 235 patients received concurrent daily RT/TMZ followed by TMZ (Stupp), 127 patients received maintenance dose TMZ overlapping with RT (modified Stupp), and 48 patients received TMZ after RT (pre-Stupp). Variation in treatment protocol depended mostly on when patients were diagnosed and reflected evolving treatment patterns over time. In addition, some patients received additional upfront agents, such as isotretinoin or bevacizumab. As determined by Kaplan-Meier survival analysis (data not shown), the overall survival (OS) for the entire cohort was 18.2 months, consistent with recent studies, and the progression-free survival (PFS) was 9.0 months, possibly elevated because of inclusion of patients receiving upfront bevacizumab with RT/TMZ.

MGMT IHC Is Prognostic of Survival

To determine whether MGMT IHC was predictive of survival in our cohort, we assessed MGMT expression levels by IHC in 355 patients. MGMT expression was determined by a trained neuropathologist (W.H.Y.) using a scoring system consistent with published recommendations based on percentage of tumor cells with...
positive immunostaining results, with particular attention placed on excluding staining from nontumor sources, such as endothelial cells and normal brain.11,17 The pathologists were blinded to the results of other MGMT assays or to clinical data. Consistent with other published studies,10,14–17 a histogram of the IHC scores shows that low MGMT expression is a common feature of untreated GBMs (Figure S1).

Using the median percentage of cells staining for MGMT by IHC as the staining threshold, we separated patients into a low expression group defined as $< 30\%$ (183/355, 52\% of patients) and a high expression group defined as $\geq 30\%$ (172/355, 48\% of patients). By Kaplan-Meier analysis, patients with low MGMT protein expression demonstrated a median OS of 20.5 months, whereas patients with high MGMT protein expression demonstrated a median OS of 16.7 months (log-rank, $P < .0001$; Fig. 1A). PFS was also higher among low-expressing patients than among high-expressing patients (10.9 months vs. 7.8 months; $P < .0001$; Fig. 1B). Multivariate analysis including other parameters, such as age, sex, KPS, extent of resection, bevacizumab treatment at any time, and $IDH1R132$ mutation status, showed MGMT IHC as a prognostic biomarker for both OS (hazard ratio [HR] = 1.63; $P < .0001$) and PFS (HR = 1.49; $P = .001$; Table S1). These results were validated by bootstrap analysis.29–31

In the intrarater assessment (W.H.Y.), the mean difference $\pm$ standard deviation (SD) between the 2 readings was $3.5 \pm 15.6$, and the median was 0 (interquartile range [IQR], −5 to 13). The estimated intraclass correlation coefficient was 0.86 (95\% confidence interval [CI], 0.80–0.90). If the IHC value was dichotomized into 2 levels (low and high) with use of median as the cutoff point, 97 (83.6\%) patients were classified into the same level by both readings, and 19 (16.4\%) patients were classified into different levels. The agreement between the 2 readings was significantly high: the estimated $k$ was 0.67 (95\% CI, 0.54–0.81; $P < .0001$). In the interrater assessment (W.H.Y. and N.K.), the mean difference $\pm$ SD between the 2 reviewers was $2.5 \pm 18.9$ (median, 3.0; IQR, −4.5 to 15). The estimated intraclass correlation coefficient was 0.80 (95\% CI, 0.74–0.85). Eighty-three percent of patients were classified into the same level, and 17\% of patients were classified into a different level by the 2 neuropathologists if the IHC value was dichotomized by median. The agreement between these 2 reviewers was significantly high; the estimated $k$ was 0.66 (95\% CI, 0.51–0.80; $P < .0001$). Both assessments indicate that IHC can be reliably scored.

**Confirmation of Prognostic Value of MGMT Methylation-Specific PCR**

To determine whether our cohort showed the expected survival stratification by methylation status,4–6,12 we performed standard 2-stage (nested) MSP6 and derived results for 402 (96\%) of 418 patients. By Kaplan-Meier analysis, methylated patients demonstrated a median OS of 24.7 months, whereas unmethylated patients demonstrated a median OS of 16.2 months (log-rank, $P < .0001$; Fig. 1C). PFS was also higher among methylated than among unmethylated patients (13.3 months vs. 7.8 months; log-rank, $P < .0001$; Fig. 1D). Multivariate analysis including other parameters, such as age (continuous variable), sex, KPS, extent of resection, bevacizumab treatment at any time, and $IDH1R132$ mutation status, showed that MGMT promoter methylation status determined by
Fig. 1. Kaplan-Meier analysis of overall survival (OS, A, C, E) and progression-free survival (PFS, B, D, F) comparing (A and B) IHC < 30% (solid) to IHC ≥ 30% (dashed), (C and D) MSP methylated (solid) to MSP unmethylated (dashed), and (E and F) BiSEQ ≥ 3 (hypermethylated, solid) to BiSEQ < 3 (hypomethylated, dashed). A and B, IHC < 30% showed improved outcomes compared to IHC ≥ 30% (OS, log rank $P < .0001$; PFS, log rank $P < .0001$). C and D, MSP methylated (solid) showed improved outcomes compared to MSP unmethylated (dashed) (OS, log rank $P < .0001$; PFS, log rank $P < .0001$). E and F, BiSEQ ≥ 3 (solid) showed improved outcomes compared to BiSEQ < 3 (dashed) (OS, log rank $P < .0001$; PFS, log rank $P = .0001$).
MSP was a prognostic variable for both OS (HR = 0.47; P < .0001) and PFS (HR = 0.53; P < .0001; Table S2). These results were validated by bootstrap analysis.

**MGMT Methylation Can Be Determined by BiSEQ Assessment of CpG Methylation Density**

In contrast to MSP evaluation, BiSEQ using conventional sequencing instruments provides information for individual CpG sites within the entire sequenced region. To investigate whether BiSEQ could provide an alternative to MSP, we sequenced 24 CpG sites in the differentially methylated region 2 (DMR2) region of the MGMT promoter that contains the MSP region (Fig. 2). We obtained BiSEQ data on 312 patients and derived semi-quantitative CpG methylation densities, ranging from 0 to 24 methylated CpG sites of the 24 total sites in this region (Fig. 2). We used the median number of methylated CpG sites as the threshold defining hypomethylated (<3 sites, 160/312, 51%) and hypermethylated (≥3 sites, 152/312, 49%) patients. By Kaplan-Meier analysis, we found that patients with ≥3 methylated CpG sites (hypermethylated) had OS of 23.1 months, whereas patients with <3 methylated CpG sites (hypomethylated) had OS of 15.6 months (log-rank, P < .0001; Fig. 1E). PFS was also higher among hypermethylated patients than among hypomethylated patients (11.5 months vs. 7.9 months; log-rank, P = .0001; Fig. 1F). Multivariate analysis using the same variables as previously described showed that BiSEQ dichotomized at 3 CpG sites was a prognostic variable for both OS (HR = 0.46; P < .0001) and PFS (HR = 0.64; P = .0006, Table S3). These results were validated by bootstrap analysis.

As shown in Fig. 3A, the majority of BiSEQ hypermethylated patients were also determined to be MSP methylated (open triangles), and the majority of BiSEQ hypomethylated patients were found to be MSP unmethylated (filled squares). Both the nondichotomized (point biserial correlation coefficient = 0.71, P < .0001) and dichotomized (κ = 0.58; 95% CI, 0.50–0.67; P < .0001) BiSEQ results were highly concordant with MSP status. There were 21 patients methylated on MSP but had <3 methylated CpG sites on BiSEQ (filled triangles). Survival (OS and PFS) among these 21 patients was similar to that among MSP unmethylated patients (data not shown), suggesting that these discordant results represent clinically silent methylation detectable by MSP but not BiSEQ. In addition, there were 43 patients who were MSP unmethylated who had evidence of ≥3 methylated CpG sites (open squares). The occurrence of these cases can be explained by the inability of MSP to detect methylation in regions outside the primer regions or incomplete methylation within the specific MSP primer location.2 Of interest, BiSEQ density (binned into 4 groups) was prognostic of patient survival, with increasing methylation density correlating with increased OS and PFS (trend test, P < .0001 for both; Fig. 3B and C). These results suggest that BiSEQ may provide added value, compared with MSP, by providing clinically relevant methylation density information.
MGMT Promoter Methylation Correlates with Reduced Expression

To determine the relationship between MGMT expression level (IHC) and MGMT methylation status as determined by either MSP or BiSEQ, we plotted expression levels for MSP methylated and MSP unmethylated patients (Fig. 4A) and BiSEQ hypermethylated and hypomethylated patients (Figure S2A). We found that MGMT MSP was correlated with nondichotomized MGMT IHC scores (point biserial correlation coefficient = –0.41; \( P < .0001 \)) and with IHC dichotomized at 30% (\( \kappa = –0.39; 95\% \ CI, –0.29 \) to –0.48; \( P < .0001 \)). Seventy-eight percent of MSP methylated patients had reduced MGMT expression (100/129), whereas 37% of unmethylated patients (79/215) also had reduced MGMT expression (Fig. 4A; \( \chi^2 \) test, \( P < .0001 \)). When BiSEQ data were binned into 4 groups of increasing CpG density, we observed that increasing number of methylated CpG sites was also correlated with decreasing IHC score in our samples (Fig. 4B; Spearman correlation coefficient = –0.48; \( P < .0001 \)). These observations are consistent with prior studies that showed that increasing MGMT promoter methylation density in DMR2 is correlated with decreasing MGMT protein expression in vitro. The 29 MSP methylated patient samples that demonstrated high MGMT expression could result from low-level methylation or persistent expression that escapes methylation silencing (Fig. 4A). Overall, these results clearly demonstrate the relationship between promoter methylation and reduced expression in patient samples and also had reduced MGMT expression (Fig. 4A; \( \chi^2 \) test, \( P < .0001 \)).
Fig. 4. Correlation of methylation and IHC. (A) Scatterplot (open squares (MSP unmethylated, IHC ≥30%; n = 136), filled squares (MSP unmethylated, IHC <30%; n = 79), open triangles (MSP methylated, IHC ≥30%; n = 29), and filled triangles (MSP methylated, IHC <30%; n = 100) showing correlation between MSP and IHC (non-dichotomized IHC, point biserial correlation coefficient = -0.41, P < .0001, and dichotomized IHC, Kappa = -0.39, P < .0001). (B) Scatterplot showing correlation between CpG density and decreased protein expression (Spearman correlation coefficient = -0.48, P < .0001). Open squares (0 CpG sites methylated), triangles (1–7 CpG sites methylated), circles (8–18 CpG sites methylated), and diamonds (19–24 CpG sites methylated) indicate IHC ≥30%. Filled squares (0 CpG sites methylated), triangles (1–7 CpG sites methylated), circles (8–18 CpG sites methylated), and diamonds (19–24 CpG sites methylated) indicate IHC <30%. Kaplan-Meier analysis of (C) OS and (D) PFS showing survival curves for groups with different MSP/IHC combinations (MSP methylated, IHC <30% = solid line; MSP methylated, IHC ≥30% = alternating dashed and dotted line; MSP unmethylated, IHC ≥30% = dotted line). Patients with the combination of MSP methylated and IHC <30% demonstrated improved OS and PFS compared to all other groups (P < .0001 for both). No significant difference in OS or PFS was observed among the other three groups.
confirm the importance of this CpG island region in down-regulating protein expression. However, our data also suggest that reduced expression can occur via other mechanisms, such as methylation outside the DMR2 region\(^{32,34}\) or non–methylation-dependent transcriptional silencing.\(^{35–37}\)

**MGMT Methylation and Low Expression by IHC in Combination Are Additively Prognostic of Outcome**

By Kaplan-Meier analyses, combined testing of MSP and IHC enabled identification of a long-term survival group when methylation and low expression were observed in tandem (Fig. 4C; OS: \(P < .0001\), compared with other 3 combinations; PFS: \(P < .0001\); Fig. 4D). Further inspection of the Kaplan-Meier curves shows that patients with high protein expression have poor outcomes despite the presence of methylation and that patients with low expression without methylation also have poor outcomes. These results are summarized in Table 2.

To confirm this, we performed multivariate analysis by including the 4 (2 × 2 table) combinations of methylation and IHC into the Cox regression model adjusting for the same clinical variables and setting the combination of methylation and low expression (<30) as the reference. We found that combined methylation by MSP and low expression by IHC prognosticated improved outcome for OS and PFS, compared with the other combinations (Table 3). Using bootstrap analysis to provide unbiased estimates of HR and \(P\) values, we found similar results.

These data indicate that both MSP and IHC were individually inferior in prognosticating survival for a significant portion of patients, compared with when they were used in combination, and suggest that low IHC expression is only prognostic of improved outcome when associated with methylation (Table 3).

### Table 2. Combined MGMT Analysis with IHC and Methylation

<table>
<thead>
<tr>
<th>IHC Methylation</th>
<th>Percentage of patients</th>
<th>Overall Survival Months (Median, 95% CI)</th>
<th>Progression-Free Survival Months (Median, 95% CI)</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;30% MSP Methylated</td>
<td>29%</td>
<td>27.8 (21.0, 36.2)^*</td>
<td>15.2 (11.5, 22.4)^*</td>
<td>Best survival group</td>
</tr>
<tr>
<td>≥30% MSP Methylated</td>
<td>8%</td>
<td>18.5 (11.7, 22.9)^</td>
<td>9.8 (4.7, 11.6)^</td>
<td>Methylated alone inadequate for good outcome</td>
</tr>
<tr>
<td>&lt;30% MSP Unmethylated</td>
<td>23%</td>
<td>16.4 (13.6, 20.0)^^</td>
<td>8.2 (6.6, 9.9)^</td>
<td>Low expression alone inadequate for good outcome</td>
</tr>
<tr>
<td>≥30% MSP Unmethylated</td>
<td>40%</td>
<td>16.3 (14.5, 18.2)^</td>
<td>7.6 (6.5, 8.7)^</td>
<td>Unmethylated or high expression either together or alone adequate for poor outcome</td>
</tr>
<tr>
<td>&lt;30% BISEQ ≥3 sites methylated</td>
<td>38%</td>
<td>26.3 (20.5, 29.2)^*</td>
<td>13.3 (10.4, 17.6)^*</td>
<td>Best survival group</td>
</tr>
<tr>
<td>≥30% BISEQ ≥3 sites methylated</td>
<td>11%</td>
<td>19.3 (13.6, 22.4)^</td>
<td>9.6 (6.3, 11.2)^</td>
<td>Low methylation alone inadequate for good outcome</td>
</tr>
<tr>
<td>&lt;30% BISEQ &lt;3 sites methylated</td>
<td>20%</td>
<td>15.3 (12.3, 20.0)^</td>
<td>8.3 (6.6, 10.9)^</td>
<td>Low expression alone inadequate for good outcome</td>
</tr>
<tr>
<td>≥30% BISEQ &lt;3 sites methylated</td>
<td>31%</td>
<td>16.3 (11.9, 18.3)^</td>
<td>7.7 (5.9, 8.1)^</td>
<td>Low methylation or high expression either together or alone adequate for poor outcome</td>
</tr>
</tbody>
</table>

*Abbreviations: IHC, immunohistochemistry; MSP, methylation specific PCR; BISEQ, bisulfite sequencing.*

\(^{*P < .001\) vs. all other groups. \(^{\wedge}P > .01\) among these 3 groups.*

### Discussion

Although CpG island promoter methylation represents an established mechanism of transcriptional silencing and resultant reduced protein expression, the prognostic significance of MGMT protein expression as assessed by IHC and its relationship with methylation remain unclear in patients with newly diagnosed GBM treated with standard radiation and TMZ.\(^3,4,8–11,15,17,19,32,34\) On the basis of concurrent analyses of MGMT IHC and promoter methylation by MSP and BiSEQ in a 418-patient cohort, our results demonstrate that optimal assessment of MGMT status as a prognostic biomarker for newly diagnosed GBM treated with RT and TMZ should take into consideration both protein expression and methylation status. Using both evaluations, we observed substantially improved outcomes for patients with GBM that demonstrated simultaneous methylation and low expression. In addition, we showed that methylation was correlated with reduced protein expression, although low expression occurred frequently in the absence of methylation (79/215, 37% of unmethylated patients; Table 3). In this 2 institution study, it is important to note that most but not all cases were subjected to central review of pathology (85% of cases) and central review of progression (88% of cases).

Aside from challenges in scoring IHC staining,\(^9,11,17\) possible reasons for the lack of agreement between previous studies attempting to correlate IHC with survival include small cohort size,\(^3,4,5,10,13,18\) various IHC scoring categories and cutoffs,\(^4,9,10,21\) small tumor sample size with tissue arrays,\(^9\) multiple glioma subtypes,\(^15\) and nonuniform treatment protocols.\(^5,9,10,13\)

In contrast, our study investigated a large number of newly diagnosed GBM treated with TMZ and RT. Furthermore, we scored IHC staining as a percentage of positive tumor cells, whereas many other studies...
Table 3. Cox Proportional Hazard Analysis Including MSP and IHC Combinations with Bootstrap Validation

<table>
<thead>
<tr>
<th>Factor</th>
<th>Overall Survival (95% CI), P-value</th>
<th>Overall Survival (Bootstrap) Estimate HR (95% CI), P-value</th>
<th>Progression Free Survival (95% CI), P-value</th>
<th>Progression Free Survival (Bootstrap) Estimate HR (95% CI), P-value</th>
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</thead>
<tbody>
<tr>
<td>MGMT MSP and IHC</td>
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</tr>
<tr>
<td>1. MSP = M, IHC &lt; 30 (n = 100)</td>
<td>1.92 (1.18, 3.14), P = .0087</td>
<td>1.92 (1.18, 3.14), P = .0087</td>
<td>1.92 (1.18, 3.14), P = .0087</td>
<td>1.92 (1.18, 3.14), P = .0087</td>
</tr>
<tr>
<td>2. MSP = M, IHC ≥ 30 (n = 29)</td>
<td>1.92 (1.18, 3.14), P = .0087</td>
<td>1.91 (1.16, 3.16), P = .0114</td>
<td>1.78 (1.10, 2.88), P = .0186</td>
<td>1.82 (1.13, 2.93), P = .0312</td>
</tr>
<tr>
<td>3. MSP = U, IHC &lt; 30 (n = 29)</td>
<td>2.20 (1.54, 3.12), P &lt; .0001</td>
<td>2.14 (1.47, 3.12), P &lt; .0001</td>
<td>1.98 (1.41, 2.79), P &lt; .0001</td>
<td>2.02 (1.41, 2.90), P &lt; .0001</td>
</tr>
<tr>
<td>4. MSP = U, IHC ≥ 30 (n = 136)</td>
<td>2.62 (1.91, 3.60), P &lt; .0001</td>
<td>2.57 (1.84, 3.60), P &lt; .0001</td>
<td>2.15 (1.59, 2.92), P &lt; .0001</td>
<td>2.23 (1.60, 3.10), P &lt; .0001</td>
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<td>Gender</td>
<td>Male vs. Female</td>
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<tr>
<td></td>
<td>1.48 (1.15, 1.91), P = .0021</td>
<td>1.47 (1.10, 1.97), P = .0091</td>
<td>1.30 (1.02, 1.67), P = .0371</td>
<td>1.28 (0.98, 1.68), P = .0691</td>
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<td>Age</td>
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<td></td>
<td>1.02 (1.01, 1.04), P &lt; .0001</td>
<td>1.02 (1.01, 1.04), P &lt; .0001</td>
<td>1.02 (1.01, 1.03), P &lt; .0001</td>
<td>1.02 (1.01, 1.03), P &lt; .0001</td>
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<tr>
<td>KPS</td>
<td>100</td>
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<tr>
<td></td>
<td>1.48 (1.15, 1.91), P = .0021</td>
<td>1.48 (1.15, 1.91), P = .0021</td>
<td>1.48 (1.15, 1.91), P = .0021</td>
<td>1.48 (1.15, 1.91), P = .0021</td>
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<tr>
<td></td>
<td>1.21 (0.83, 1.75), P = 0.3246</td>
<td>1.24 (0.87, 1.78), P = 0.2310</td>
<td>1.05 (0.73, 1.52), P = 0.7908</td>
<td>1.04 (0.74, 1.46), P = 0.8306</td>
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<td></td>
<td>1.24 (0.82, 1.88), P = 0.2989</td>
<td>1.27 (0.85, 1.88), P = 0.2384</td>
<td>0.91 (0.60, 1.38), P = 0.6595</td>
<td>0.90 (0.63, 1.28), P = 0.5544</td>
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<td></td>
<td>1.91 (1.19, 3.05), P = 0.070</td>
<td>2.03 (1.08, 3.83), P = 0.0279</td>
<td>1.68 (1.05, 2.68), P = 0.0309</td>
<td>1.72 (1.04, 2.84), P = 0.0336</td>
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<td>Resection</td>
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<td>GTR vs. STR and Bx</td>
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<td></td>
<td>0.68 (0.53, 0.88), P = 0.028</td>
<td>0.66 (0.50, 0.87), P = 0.0028</td>
<td>0.65 (0.51, 0.84), P = 0.0008</td>
<td>0.64 (0.49, 0.83), P = 0.0006</td>
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<td>Bevacizumab Yes vs. No</td>
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<td></td>
<td>0.78 (0.60, 0.99), P = 0.044</td>
<td>0.75 (0.57, 0.99), P = 0.014</td>
<td>1.07 (0.84, 1.37), P = 0.5778</td>
<td>1.08 (0.84, 1.38), P = 0.5464</td>
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<td>WT vs. MUT</td>
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<tr>
<td></td>
<td>0.52 (0.26, 1.01), P = 0.0528</td>
<td>0.50 (0.25, 0.97), P = 0.0416</td>
<td>0.59 (0.32, 1.08), P = 0.0867</td>
<td>0.59 (0.31, 1.11), P = 1.015</td>
</tr>
</tbody>
</table>

Abbreviations: Bx, biopsy; GTR, gross-total resection; IHC, immunohistochemistry; KPS, Karnofsky performance status; MGMT, O6-methylguanine DNA methyltransferase; MSP, methylation specific PCR; M, methylated; MUT, mutant; STR, sub-total resection; U, unmethylated; WT, wildtype.

divided IHC staining into categories. Studies suggest that it is critical to ensure that only staining from tumor cells are included with specific consideration to exclude staining from normal brain tissue, endothelium, or macrophages. From the median percentage of cells staining for MGMT by IHC, we used 30% staining as the threshold separating percentage IHC staining into 2 groups. This threshold is similar to the 10%–20% thresholds used in several smaller studies showing positive correlation between MGMT IHC staining and outcome in malignant gliomas. In addition, stratification of our IHC data using other thresholds of 10%–50% also yielded significant prognostive value, as evidenced by Kaplan-Meier analysis (data not shown). On the basis of our results demonstrating that IHC and methylation additively prognosticate outcome, we recommend that evaluation of MGMT methylation and IHC be incorporated into standard molecular testing for newly diagnosed GBM. Because of the relative ease and low expense, many diagnostic histopathology laboratories have the ability to establish and offer MGMT IHC evaluation on resected tumor tissue. The most significant benefit in tandem evaluation of IHC and methylation is to identify patients with both low protein expression and promoter methylation, because our results show that outcome for this group is most favorably separated from the 3 other possible combinations (Tables 2 and 3).

In terms of how IHC can enhance methylation testing by MSP, our results show that IHC status can stratify outcome in the methylated group. In other words, 22% of methylated patients (29/129) would be predicted to have poor outcomes on the basis of having high protein expression. This methylated with high IHC subgroup is relatively small, making it less clear whether this delineates a biologically relevant set of tumors showing MGMT expression that can escape methylation-dependent downregulation or whether this is attributable to detection of clinically silent levels of methylation. Despite this uncertainty, the addition of IHC to MSP.
will optimize prognosis for methylated patients with high IHC staining whom we observed to have poor outcome despite the presence of methylation.

In situations in which MSP testing is unavailable, our results indicate that IHC has clinical use for identifying patients with high expression, because OS among these patients is independent of *MGMT* methylation. High-expressing (≥30%) patients had poor outcome regardless of methylation status, whereas low-expressing patients with associated promoter methylation (56% of low IHC) have clinically significant improved outcomes, indicating that patients with low IHC staining should undergo methylation testing. A possible explanation for the poor outcomes of low-expressing tumors without associated methylation could be that these tumors have inducible *MGMT* protein expression. Alternatively, *MGMT* promoter methylation may be associated with additional survival/treatment benefits unrelated to *MGMT* expression.

Another reason for the perceived lack of utility of IHC in prognosticating treatment outcome for GBM has been the apparent lack of correlation with promoter methylation. In our cohort, we found that promoter methylation by MSP is correlated with protein expression, as evidenced by low expression in the majority of patients with methylation. This observation is consistent with several previous studies that showed correlation between *MGMT* promoter methylation and protein expression. This is also consistent with in vitro studies in which methylation of the *MGMT* promoter caused protein downregulation. However, our data and those from other studies show that nearly half (44%) of the patients with low IHC staining do not have promoter methylation. It is possible that the presence of this population obscured the correlation between methylation and IHC in other studies, although factors, such as mixed tumor subtypes in the study cohort and nonuniform treatment protocols, are also possible limitations.

The correlation between methylation and protein expression is further supported by data from BiSEQ, which we performed as an alternative evaluation of promoter CpG sites. BiSEQ is straightforward and can be performed on conventional DNA sequencing instruments without any specialized procedures beyond bisulfite conversion, which is also necessary for MSP. The 24 CpG sites evaluated coincide with 1 of the 2 differentially methylated regions in the *MGMT* promoter, DMR2, found to be particularly important for *MGMT* downregulation. By showing that expression level correlates with CpG methylation density in this region, we confirm the importance of DMR2 methylation for transcriptional regulation of *MGMT*. Consistent with the study by Dunn et al., we also found that methylation density was correlated with survival. Overall, BiSEQ results were highly correlated with MSP, and it provides an attractive semiquantitative alternative to MSP. We did not determine the contribution of CpG sites outside this region or of individual CpG sites towards expression and prognosis, such as previously described.

On the basis of our results, we recommend that both IHC and methylation determination be obtained and considered in combination for prognostication of GBM treatment outcome. In institutions where only IHC is available, high expression alone is prognostic of poor outcome. However, low expression is less informative, because these patients can have significantly different outcomes depending on methylation status. Our results highlight the possibility that low expression can occur for likely non–methylation-dependent reasons. Further investigation is necessary to understand why low-expressing patients with associated methylation outperform those without methylation and whether our results are applicable to low-grade and anaplastic gliomas. Our findings need to be validated in an independent dataset, preferably in the context of controlled clinical trials.

**Supplementary Material**

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/).

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**References**


