The Role of Gene Body Cytosine Modifications in MGMT Expression and Sensitivity to Temozolomide

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Abstract

The DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) is known to play a role in sensitivity to temozolomide. Promoter hypermethylation of MGMT is commonly used to predict low expression levels of MGMT in gliomas, despite observed discordance between promoter methylation and protein levels. Here, we investigated the functional role of gene body cytosine modification in regulating levels of MGMT gene expression and sensitivity to temozolomide. In 91 human glioblastoma samples, we observed significant variation in MGMT expression levels in patients with an unmethylated promoter, with higher levels of gene body cytosine modification correlating with higher gene expression levels. Furthermore, inducing hypomethylation across the MGMT gene body with decitabine corresponded with decreased levels of MGMT gene expression in lymphoblastoid and glioblastoma cell lines, indicating an important functional role for gene body cytosine modifications in maintaining gene expression. We reasoned that the decrease in MGMT expression induced by decitabine may render resistant glioblastoma cell lines more sensitive to temozolomide. Consistent with this reasoning, we found that the MGMT-expressing glioblastoma cell lines exhibiting an unmethylated MGMT promoter that were pretreated with decitabine became significantly more sensitive to temozolomide. Overall, our results suggest a functional role for gene body cytosine modification in regulating gene expression of MGMT and indicate that pretreating patients whose tumors have an unmethylated MGMT promoter with decitabine before temozolomide treatment may increase their response to therapy.

Introduction

The O6-methylguanine-DNA methyltransferase (MGMT) gene encodes for a DNA repair protein that repairs O6-alkylguanine-DNA adducts (1). This type of DNA damage can occur after certain environmental exposures, such as tobacco-specific nitrosamines, and from DNA alkylating agents used for chemotherapy (2, 3). Temozolomide is an alkylating agent that was FDA approved to treat brain tumors in 2005, including anaplastic astrocytoma and glioblastoma multiforme, the most aggressive form of brain cancer (4). Only 5% to 10% of the methylation glioblastoma multiforme, the most aggressive form of tumors in 2005, including anaplastic astrocytoma and agents used for chemotherapy (2, 3). Temozolomide is an alkylguanine-DNA adducts (1). This type of DNA damage are repaired (8, 9). Patients with gliomas with an aberrantly hypermethylated MGMT promoter respond better to temozolomide treatment compared with patients with an unmethylated MGMT promoter, because they lack MGMT protein expression (10, 11). Although approximately 40% to 70% of patients with gliomas have a methylated MGMT promoter (9, 12–14), promoter methylation does not always show a strong correlation with MGMT protein levels (13, 15). For example, one study investigating the correlation between MGMT promoter methylation and protein levels showed that 7 of 40 gliomas had an unmethylated promoter yet no detectable MGMT protein, and 16 of 40 gliomas had aberrant methylation at the promoter yet still had detectable MGMT protein (13). Therefore, additional mechanisms of transcriptional and translational regulation are likely affecting expression of MGMT.

The role of gene body cytosine methylation and hydroxymethylation in regulating gene expression levels is not fully understood, but several studies have observed a positive correlation between gene body cytosine modifications and gene expression levels. For MGMT in particular, gene body methylation was first shown to correlate with gene expression levels in 1992 (19). Two
years later, it was demonstrated that glioma cell lines with lower expression of MGMT tend to have a more highly methylated promoter and low levels of methylation along the gene body compared with glioma cell lines showing higher levels of MGMT expression (8, 19). However, these studies have only shown correlation, and the field has been focused primarily on methylation of the promoter region after it was shown to predict survival among patients with glioblastoma. It remains unclear whether modulation of gene body cytosine modifications would be sufficient to disrupt gene expression levels without any change in the status of cytosine modification in the promoter region.

We hypothesized that incorporating gene body cytosine modification levels in models of temozolomide response may lead to better prediction of MGMT expression levels and, more importantly, improved methods of prediction for temozolomide response in patients with glioma. In this study, we used lymphoblastoid cell lines, glioblastoma cell lines, and human glioblastoma tissue data from The Cancer Genome Atlas (TCGA) to investigate the role of gene body cytosine modification in regulating MGMT expression levels and sensitivity to temozolomide.

Materials and Methods

Cell lines and reagents

Lymphoblastoid cell lines were cultured in RPMI supplemented with 15% FBS and 1% L-glutamine at 37°C. Confirmation of cell line identities for the lymphoblastoid cell lines is described previously (18). The glioblastoma cell line U18MG (HTB-15) was purchased from the American Type Culture Collection (ATCC) in March 2013, and was authenticated by ATCC by evaluating the STR profile. U-87MG and A-172 were obtained from Dr. M. Lesniak in February 2013, and T98G was obtained from Dr. B. Yamini in March 2013 at the University of Chicago and no subsequent authentication was performed on these cell lines. SF-188 was obtained from Dr. J. Costello at the Neurosurgery Tissue Bank at University of California, San Francisco, in August 2013. These cells were authenticated by UCSF using the PowerPlex16 System (Promega Corp.). Glioblastoma cells were grown in Dulbecco’s modified Eagle medium (ATCC Catalog No. 30-2002) supplemented with 10% FBS and 1% penicillin/streptomycin.

IDH1/2 genotyping

All 6 glioblastoma cell lines were genotyped for the IDH1 and IDH2 mutations. Primers for IDH1 (R132) are as follows: forward-GCTGCACTTCTTACGAAG, reverse-ATGTTGAGATGGACGCCT. Primers for IDH2 (R140 and R172) are as follows: forward-TTCTGTGTAGAAGATGCGG, reverse-GATGGCTAGGCGAGGAG. Genomic DNA was PCR-amplified using Platinum HiFi Taq polymerase (Life Technologies) under the following conditions: initial denaturation at 94 °C for 2 minutes, 94°C for 30 seconds, 62°C for 30 seconds, and 68°C for 1 minute for 35 cycles, and a final extension at 68°C for 5 minutes.

Drug treatment

Cells in a T25 flask were treated with 200 nmol/L of decitabine or vehicle control (H2O) every 24 hours for 5 days. At the end of the fifth day, cells were plated at 10,000 cells/well (lymphoblastoid cell lines) or 5,000 cells/well (glioblastoma cell lines) in a 96-well plate, and the remainder was spun down into 2 pellets and kept at −80°C until subsequent DNA and RNA isolation. Twenty-four hours after plating, cells were treated with a range of doses of temozolomide (Sigma Aldrich): 0, 50, 100, 200, 500, 750, and 1,000 μmol/L for lymphoblastoid cell lines and 0, 10, 50, 100, 500, 1,000, and 2,000 μmol/L for the glioblastoma cell lines for a total of 72 hours. AlamarBlue was added 24 hours before absorbance reading at wavelengths of 570 and 600 nm (Synergy-HT multidetection plate reader; BioTek). The percentage of cells that survived was quantified relative to vehicle control wells, and at least 2 separate experiments for each glioblastoma cell line were performed in technical triplicates for each concentration. To determine the significance of the effect of decitabine pretreatment on temozolomide cytotoxicity, we fit the following linear model: survival = temozolomide dose × decitabine, where decitabine is 1 for decitabine pretreatment and 0 for vehicle control. This model was compared with a baseline model without the interaction term using ANOVA to determine if the interaction between decitabine and temozolomide dose was significant. The linear models were fit using the lm function in R.

Bisulfite sequencing

DNA was isolated from whole cell lysates using phenol-chloroform, and genomic DNA was treated with sodium bisulfite (20). For bisulfite PCR amplification, primers (Supplementary Table S1) were designed to amplify ~250 base pair fragments within the MGMT promoter and gene body that included CpG sites interrogated by the Illumina Infinium HumanMethylation 450 Beadchip (450K array) as well as adjacent CpGs. Primers for the CpG island in the promoter region were designed and optimized in a previous publication (21). ZymoTaq polymerase (Zymo Research) was used for PCR amplification at the indicated temperatures, according to manufacturer’s instructions. For the glioblastoma cell lines, the PCR product was TA cloned using the TOPO Cloning Kit (Invitrogen), and at least 10 clones per condition were sequenced. Lymphoblastoid cell lines were sequenced by bulk sequencing of the PCR product. DNA sequencing was performed at The University of Chicago Sequencing Core Facility.

Quantitative real-time PCR

Total RNA was extracted from 1 million cells using TRIzol (Life Technologies) following the manufacturer’s protocol. RNA quality assessment and quantification were conducted using the optical spectrometry 260 to 280 nm ratio. Subsequently, mRNA was reverse transcribed to cDNA using Applied Biosystems High Capacity Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) was performed for...
MGMT, and *huB2M* (beta-2-microglobulin) and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) were included as endogenous controls for lymphoblastoid and glioblastoma cell lines, respectively, using TaqMan Gene Expression Assays on the Applied Biosystems Viia7 RT-PCR system.

**Statistical analyses of 450K array data**

Initial analyses of the 450K array cytosine modification data (GSE39672) in the CEU and YRI lymphoblastoid cell lines are explained in detail previously (18). Level 3 450K array, exon array, and survival data were downloaded from TCGA Data Matrix. All 3 datasets were available for 91 patients with glioblastoma. All of our analyses of patient with TCGA glioblastoma data were performed using the R statistical software.

*K*-means clustering was performed using the “kmeans” function in R. *K*-means clustering aims to divide *n* observations into *k* clusters in which each observation belongs to the cluster with the nearest mean, so as to minimize the within-cluster sum of squares. A plot of the within cluster sum of squares by number of clusters helped us to determine that 3 groups was the appropriate number of clusters to minimize the within-cluster sum of squares for clustering our data. After 3 groups, the within-group sum of squares plateaus, so increasing the number of groups would not significantly decrease the within-group sum of squares (Supplementary Fig. S1).

**Results**

**A temozolomide-associated SNP is a modified cytosine quantitative trait locus for MGMT gene body cytosine modification levels**

Our group has generated 450K array data for 60 Caucasian (CEU) and 73 African (YRI) lymphoblastoid cell lines (18). This array requires bisulfite-modified DNA, and modified cytosines, both 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC), are distinguished from unmodified cytosines using this technique (22). Our analysis of cytosine modification and gene expression levels revealed that the averaged levels of cytosine modification within the gene body of *MGMT* were strongly positively correlated at genome-wide significance with gene expression levels (*P* < 5.3 × 10^{-12}; Fig. 1A). Thus, we aimed to investigate the biological function of gene body cytosine modification in regulating *MGMT* transcription and how it may affect sensitivity to temozolomide. Interestingly, a recent genome-wide association study of temozolomide sensitivity in a cohort of Caucasian lymphoblastoid cell lines identified several SNPs in high linkage disequilibrium within the gene body of *MGMT* to be significantly associated with cellular sensitivity to temozolomide (23).

The most significant SNP they identified was an expression quantitative trait locus (eQTL) for *MGMT* expression, but it was not correlated with promoter methylation levels in their lymphoblastoid cell line cohort. We hypothesized that the most significant temozolomide-associated SNP and *MGMT* expression quantitative trait locus (rs477692) identified in that study would be associated with gene body cytosine modification levels in our cohort of Caucasian (CEU) lymphoblastoid cell lines.

First, we validated that the SNP was an expression quantitative trait locus for *MGMT* in our Caucasian lymphoblastoid cell lines (*P* = 0.004; Fig. 1B). We did not observe this SNP to be an expression quantitative trait locus for *MGMT* expression in the African (YRI) lymphoblastoid cell lines (*P* = 0.6), suggesting different genetic predictors of *MGMT* expression between the 2 populations. Furthermore, we found that this SNP is a modified cytosine QTL (mQTL) for the averaged levels of *MGMT* gene body cytosine modification (Fig. 1C). We also analyzed the SNP against each CpG within *MGMT* individually (n = 94) using a linear model in the Caucasian lymphoblastoid cell lines, and identified 5 CpGs that were significantly associated with expression at *P* < 0.05 after correcting for multiple testing. We performed bisulfite sequencing around 3 of these CpGs to demonstrate technical validation of the association using a separate methodology (Supplementary Fig. S2). From these results, we hypothesized that *MGMT* gene body cytosine modification levels are functionally important in regulating *MGMT* expression, thereby effecting cellular sensitivity to temozolomide.

**Cytosine modification levels are correlated across the gene body of MGMT**

We were interested in determining whether a certain pattern or region of cytosine modification across the *MGMT* gene would be able to predict gene expression levels more precisely than promoter methylation levels alone. In the Caucasian lymphoblastoid cell lines, a correlation matrix including all CpGs across the promoter region and gene body showed that the gene body cytosine modifications were positively correlated with each other, and they were slightly negatively correlated with CpGs in the promoter region (Fig. 1D). Across the gene body, there are several regions that show higher association with expression than others. Using a backward elimination regression model (24), we were able to identify 4 CpGs, which were all located within the second intron, that could explain 69% of the variance in expression levels in the Caucasian lymphoblastoid cell lines (Fig. 1D marked by asterisks and Table 1). When we performed a backwards elimination regression model using the African lymphoblastoid cell lines, we were able to identify 3 CpGs, 1 in the promoter and 2 in the second intron, that could explain 29% of the variance in expression levels (Table 1). Therefore, in Caucasian and African lymphoblastoid cell lines, the second intron, which spans more than 171 kb, contains several CpGs whose cytosine modification levels are significantly associated with expression levels of *MGMT*.

**Gene body cytosine modification levels predict MGMT expression levels in patients with glioblastoma with an unmodified MGMT promoter**

We next investigated whether *MGMT* gene body cytosine modification levels were correlated with gene
expression levels in human glioblastoma samples. We obtained the 450K array, expression, and survival data for 91 glioblastoma samples from TCGA. To determine whether a certain pattern of cytosine modification across the entire gene was predictive of MGMT expression or patient survival, we performed k-means clustering to group the patients into 3 groups based on similar cytosine modification patterns across the MGMT promoter and gene body (Fig. 2A). Our rationale for clustering the patients into 3 groups is described in Methods and in Supplementary Fig. S1. Group 1 patients (black circles) had the highest levels of cytosine modification across the gene body, with the mean β value for all CpGs being 0.803 (80.3% modified). This group has the highest levels of gene expression compared with the other 2 groups (Fig. 2B). Group 2 (gray circles) had moderate levels of cytosine modification across the gene body, with the mean β value for all CpGs being 0.702 (70.2% modified; Fig. 2A). Group 3 (white circles) had the lowest levels of cytosine modification across the gene body, with the mean β value for all

Figure 1. MGMT gene body cytosine modification is positively correlated with gene expression levels in lymphoblastoid cell lines. A, MGMT expression is positively correlated with the averaged levels of cytosine modification across the gene body. B, a temozolomide-associated SNP (rs477692) is an expression quantitative trait locus for MGMT gene expression levels in the CEU HapMap lymphoblastoid cell lines. C, a temozolomide-associated SNP (rs477692) is a mQTL for MGMT gene body cytosine modification levels in CEU HapMap lymphoblastoid cell lines. D, cytosine modifications across the gene body are positively correlated with each other and negatively correlated with the promoter region. Gray bars, the –log10 P value of the association of each CpG with expression. Starred bars indicate the CpGs that, together, explain 69% of the variance of MGMT expression in CEU lymphoblastoid cell lines.
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Table 1. Certain gene body CpGs explain a high proportion of variance of MGMT expression levels

<table>
<thead>
<tr>
<th>Population</th>
<th>CpGs</th>
<th>$r^2$</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>CEU</td>
<td>cg11309842</td>
<td>0.69</td>
<td>$1.13 \times 10^{-13}$</td>
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<tr>
<td></td>
<td>cg188502933</td>
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<td></td>
<td>cg0617303</td>
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<td></td>
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<tr>
<td></td>
<td>cg14485787</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YRI</td>
<td>cg14194475</td>
<td>0.29</td>
<td>$9.73 \times 10^{-5}$</td>
</tr>
<tr>
<td></td>
<td>cg18651291</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cg19680672</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients with glioblastoma with an unmodified promoter</td>
<td>cg25394042</td>
<td>0.18</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>cg14485787</td>
<td></td>
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</tr>
<tr>
<td></td>
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<td></td>
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NOTE: Backward elimination regression identifies 4 CpGs that explain 69% of variation and 3 CpGs that explain 29% of variation of MGMT expression levels in the Caucasian (CEU) and African (YRI) populations, respectively. In patients with glioblastoma with an unmodified promoter, 5 CpGs were identified that explain 18% of the variance in expression levels, calculated using Spearman correlation backward elimination.

CpGs being 0.460 (46.0% modified; Fig. 2A). These groups were not predictive of survival, which may be a result of the significant number of the patients with glioblastoma who were lost to follow-up (Supplementary Fig. S3).

As expected, cytosine modification within the gene promoter was negatively associated with gene expression (Fig. 2C). However, we observed a significant amount of variation in expression levels among patients with glioblastoma with an unmodified promoter (Fig. 2C). When analyzing only patients with an unmodified promoter (averaged $\beta$ values between 0 and 0.2), we found that levels of gene body cytosine modification were linearly correlated with expression levels of $MGMT$ (Fig. 2D, right). Hence, among the patients with an unmodified promoter, patients who had the lowest levels of gene body modification (Group 3) expressed significantly lower levels of $MGMT$ compared with patients with an unmodified promoter and high levels of gene body cytosine modification (Group 1). The patients whose cytosine modification levels within the promoter averaged to be at least 20% modified (average $\beta$ value > 0.2) did not show a correlation between gene body cytosine modification levels and expression, and, as expected, all groups had very low levels of expression (Fig. 2D, left). This suggests that disruption of cytosine modification levels across the gene body may introduce variability in expression levels in patients who have an unmodified promoter. To identify which CpGs along the gene body contributed most to variation in expression within patients with an unmodified promoter, we performed backward elimination to identify 5 CpGs, 1 in the first intron and 4 in the second intron, that explained 18% of the variation in expression (Table 1).

Decitabine decreases $MGMT$ expression in cell lines with an unmethylated $MGMT$ promoter

We next investigated whether hypomethylating the $MGMT$ gene body correlated with reduced gene expression levels. To do so, we treated lymphoblastoid cell lines that have an unmodified promoter and high levels of $MGMT$ expression with decitabine, a hypomethylating agent. After a 5-day treatment with a low dose of decitabine (200 nmol/L), we examined changes in gene expression and gene body cytosine modification levels. We included 6 lymphoblastoid cell lines that all had relatively equal baseline levels of $MGMT$ expression and gene body cytosine modification: GM7000, GM7357, GM12005, GM12043, GM12810, and GM12812. After decitabine treatment, 3 lymphoblastoid cell lines (GM7357, GM12005, and GM12810) showed a decrease in $MGMT$ expression, which corresponded to a decrease in cytosine modification levels in the examined regions along the gene body, measured by bisulfite sequencing (Fig. 3A–C, black bars, left). The other 3 lymphoblastoid cell lines (GM7000, GM12043, and GM12812) showed no change in expression and experienced less hypomethylation across the gene (Fig. 3A–C, white bars, right) after decitabine treatment. These phenotypes were reproducible over 2 biologic replicates, indicating that decitabine was consistently more effective in the same 3 cell lines. We noted that the growth rates of the 3 lymphoblastoid cell lines that showed hypomethylation of the $MGMT$ gene body were faster than the other 3 lymphoblastoid cell lines. It is thought that decitabine is dependent on cell division (25), and the cells that divide more frequently will incorporate more of the drug into the nascent DNA strand. Thus, the difference in $MGMT$ gene body hypomethylation and expression observed between these 2 groups is likely because of the differences in cellular growth rate. The unmethylated $MGMT$ promoter status remained completely unchanged among the lymphoblastoid cell lines that showed a decrease in $MGMT$ expression, confirming that the change in gene expression was not because of changes within the promoter (Supplementary Fig. S4).

We also tested this observation in 5 glioblastoma cell lines. We obtained 2 glioblastoma cell lines that had high expression levels of $MGMT$ (T98G and SF188), 1 glioblastoma cell line with low expression of $MGMT$ (U118MG), and 2 glioblastoma cell lines with no $MGMT$ expression because of promoter hypermethylation (U87MG and A172). It has been reported that IDH1/2 mutation status is associated with distinct methylation and hydroxymethylation patterns (26–28), so we genotyped all 6 cell lines for $IDH1$ and $IDH2$ mutations and found that they were all wild-type for both. Therefore, $IDH1/2$ mutations could not explain changes in cytosine modifications between these cell lines.
We observed that a 5-day treatment of low-dose decitabine (200 nmol/L) resulted in a significant decrease in MGMT expression in T98G, SF188, and U118MG (Fig. 4A). This correlated with loss of modified cytosines across regions of the gene body of MGMT, measured by bisulfite sequencing (Fig. 4B). Conversely, U87MG and A172 have
a methylated promoter, and decitabine treatment could therefore hypomethylate the promoter and increase MGMT expression. In U87 and A172, we observed some hypomethylation of the promoter region after decitabine treatment ($P < 0.05$; Supplementary Fig. S5). U87 experienced a very small increase in MGMT expression, yet A172 experienced no increase in MGMT expression (Fig. 4C). This suggests that increased hypomethylation and/or other factors may be required for reactivation of MGMT expression in these cell lines.

Pretreatment with decitabine sensitizes MGMT-expressing glioblastoma cell lines to temozolomide

A significant clinical problem is that patients with glioblastoma whose tumors express MGMT, typically with an unmethylated MGMT promoter, respond poorly to temozolomide, which is the drug of choice in this disease. To determine whether the decrease in MGMT expression observed in the decitabine-treated glioblastoma cell lines is sufficient to sensitize the cells to temozolomide, we pretreated the glioblastoma cell lines with decitabine to determine the effect on cellular sensitivity to temozolomide. The cells were treated with 200 nmol/L of decitabine every 24 hours for 5 days and then plated for a temozolomide dose-curve cytotoxicity assay. T98G, SF188, and U118MG, each of which experienced a decrease in MGMT expression following the decitabine pretreatment, were significantly more sensitive to temozolomide compared with vehicle-treated controls (Fig. 5A). Interestingly, decitabine did not affect the sensitivity to temozolomide in either of the cell lines that had a methylated MGMT promoter (Fig. 5B). This suggests that the effectiveness of decitabine pretreatment is more pronounced in glioblastoma cell lines that have an unmethylated MGMT promoter and are initially resistant to temozolomide.

We also tested the effect of decitabine pretreatment to cellular sensitivity to temozolomide in lymphoblastoid cell lines that showed both no change (GM7000 and GM12812) and a decrease in MGMT expression (GM7357) following decitabine treatment. We observed that none of the lymphoblastoid cell lines tested were sensitized to temozolomide (Supplementary Fig. S6). Lymphoblastoid cell lines are noncancerous cells with high levels of MGMT, which makes them highly resistant to temozolomide compared with cancer cell lines, even when the levels of MGMT in lymphoblastoid cell lines are reduced. Thus, decitabine pretreatment may increase sensitivity of cancer cells to temozolomide with no increase in sensitivity in normal cells, which would potentially reduce unwanted toxicities during combination treatment.

Discussion

In this study, we investigated the role of MGMT gene body cytosine modification in regulating MGMT expression levels. Using primary human glioblastoma data from TCGA, we demonstrated that levels of cytosine
Cytosine Modifications in MGMT and Temozolomide Sensitivity

Figure 4. Glioblastoma cell lines that express MGMT show a decrease in expression and gene body cytosine modification levels following decitabine treatment. A and C, qRT-PCR of MGMT expression levels in MGMT-expressing glioblastoma cell lines (A) and MGMT-silenced glioblastoma cell lines (C) treated with either 200 nmol/L decitabine (DAC) for 5 days or vehicle controls. Each bar represents the mean of 3 independent experiments, and error bars denote the standard deviation of the mean. P values were calculated using the Student t test. *, P < 0.05. B, bisulfite sequencing of 2 regions within the gene body of MGMT in MGMT-expression glioblastoma cell lines treated with decitabine or vehicle control. Each pie chart represents the proportion of modified cytosines at a particular CpG, calculated from at least 10 clones. P values were calculated using a χ² statistical test. N.S., not significant.

Modification across the MGMT gene body are associated with expression levels specifically in patients with an unmethylated promoter, and those patients with the lowest levels of gene body cytosine modification have similar levels of MGMT expression as those patients with a methylated promoter. Our results may shed light on previous studies that show discordance between MGMT promoter methylation and MGMT protein levels (13, 15), at least in the cases where the promoter is unmethylated yet there is little or no detectable MGMT protein. An unmethylated promoter leaves the MGMT gene open for transcription, but additional transcription factors and chromatin states, including histone marks and DNA methylation and hydroxymethylation, are likely required for transcriptional initiation and elongation. This interplay between epigenetic modifications and transcription factors may contribute to the variation in expression levels seen in patients with glioblastoma with an unmethylated promoter.

The importance of gene body cytosine modifications in regulating gene transcription is not completely understood. In fact, the interaction between gene body cytosine modification and expression may be bi-directional. It has been shown that transcriptional elongation may promote DNA methylation along gene bodies (29, 30), whereas the interaction of 5-mC with histone marks, such as H3K36me3, may be important for transcriptional elongation (29). It is also thought that gene body cytosine modifications may be important in defining exon–intron boundaries and alternative splicing (31). Furthermore, levels of 5-mC and 5-hmC located within gene bodies may have distinct effects on gene expression. A recent study demonstrated that, in the brain, the ratio of 5-hmC:5-mC within the gene body is a better predictor of gene expression than either epigenetic mark on their own (32). We propose that gene body cytosine modifications may be one of the factors influencing expression levels of MGMT, such that when the gene body becomes hypomethylated, the cell may not be able to successfully transcribe the gene even when the transcriptional machinery has access to the promoter. This implies that, at least in the case of MGMT, maintaining high levels of cytosine modification along the gene body may be required for the cell to continue to transcribe high levels of the gene. Gaining a better understanding of the functional importance of cytosine modifications along the gene body will help us to predict gene expression patterns better, which could have implications on downstream phenotypes such as response to chemotherapeutic agents.

In addition, previous work by our group investigating population-specific gene expression patterns has shown that MGMT is differentially expressed at baseline between Caucasian and African lymphoblastoid cell lines, with Caucasians expressing lower levels of MGMT compared with Africans (33). However, the overall poor prognosis for all patients has made it difficult to discern if health
disparities exist between different ethnic populations (34). Overall, our results indicate that measuring cytosine modification levels in the gene body in addition to those in the promoter will more accurately predict MGMT expression. Importantly, inclusion of gene body cytosine modification levels may allow for better prediction of temozolomide response and long-term survival in patients with glioblastoma. A study of long-term survival of patients with glioblastoma showed that 8 of 36 of glioblastoma long-term survivors had an unmethylated MGMT promoter (35). Our data would suggest that these patients would have low MGMT gene body cytosine modification levels.

The primary model for the mechanism of action of hypomethylating agents decitabine and azacitidine is that they incorporate into DNA to induce global hypomethylation by covalently trapping the maintenance methyltransferase, DNMT1, to the DNA strand (36). Thus, over the course of cell divisions, the genome becomes hypomethylated. Several studies have investigated decitabine-induced hypomethylation of promoter regions and have shown subsequent upregulation of gene expression (37–39). However, decitabine has been shown to induce both upregulation and downregulation of genes (38, 40). One study investigated genome-wide changes in methylation induced by decitabine and azacitidine and showed that hypomethylation had a preference for CpGs that were not in CpG islands (41). Gene body CpGs may be frequently affected by decitabine, but the effects on gene expression may be more subtle than changes in the promoter region, which acts more like an on/off switch. After treatment with decitabine, DNMT1 may not be able to keep levels of methylation high across the MGMT gene body after several replication events, leading to a decrease in gene expression. Although this gene may be more dependent on gene body cytosine modification levels compared with other genes, this phenomenon could occur in other genes as well, and may explain why certain genes become downregulated following decitabine treatment. However, because decitabine causes global hypomethylation, we cannot rule out the possibility that treatment with decitabine is affecting the expression levels of transcription factors involved in MGMT expression, leading to changes in MGMT expression that are independent of the observed changes in gene body methylation levels.

A recent avenue in current strategies for cancer treatment is the combination of hypomethylating agents with cytotoxic chemotherapy to increase response to the chemotherapeutic agent (42–45). The goal of precision medicine is to use patient-specific biomarkers to help determine their individualized treatment strategy. In this work, we have demonstrated that decitabine can be used to sensitize resistant glioblastoma cell lines with an unmethylated MGMT promoter status to temozolomide. Importantly, the effects of decitabine seem to be dependent on the changes to the MGMT expression levels. Cells that express MGMT become hypomethylated across the gene body after decitabine treatment, corresponding with a decrease in
expression levels and an increase in temozolomide sensitivity. However, cells with a hypermethylated MGMT promoter are not sensitized further to temozolomide following decitabine pretreatment. Although MGMT promoter methylation status is one of the most requested molecular assays in clinical neuro-oncology, there are few therapeutic alternatives to temozolomide for patients with an unmethylated MGMT promoter even though it has been shown that those patients are less likely to respond to treatment (46). In fact, many of the other agents used to treat brain tumors such as carmustine (BCNU), streptozotocin, and dacarbazine also form O6-alkylguanine DNA adducts that are repaired by MGMT (5). Therefore, our strategy of combining decitabine would likely be beneficial with these other agents. In addition, MGMT promoter methylation status and low MGMT expression both were identified as positive prognosticators in patients with newly diagnosed glioblastoma who underwent surgical resection and received Gliadel wafer implants followed by adjuvant radiotherapy and concomitant oral TMZ chemotherapy (47). Finally, we observed that lymphoblastoid cell lines were not sensitized to temozolomide by pretreatment with decitabine, suggesting that this combination may not significantly increase toxicities in noncancerous tissues.

Decitabine crosses the blood–brain barrier, but the dose of decitabine needed to induce hypomethylation in the brain in human patients is not yet known (48). Decitabine is thought to require cell division to induce hypomethylation, and therefore may only be effective on tumors that are actively proliferating. Thus, more work is needed to determine the appropriate combination of decitabine and temozolomide in human patients. For example, to ensure the cancer cells are exposed to decitabine during cellular divisions, a longer duration of a lower dose may be required. In addition, this drug combination may be beneficial to the survival of patients with other malignancies. Diffuse large B-cell lymphoma is another disease in which the promoter of MGMT is hypermethylated and is predictive of survival among patients treated with alkylating chemotherapy (49). Thus, it will be worthwhile to study this drug combination in a lymphoma model as well. In conclusion, our work argues that pretreating patients with glioblastoma with an unmethylated MGMT promoter with decitabine before the start of temozolomide treatment may sensitize their tumors to the chemotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Writing, review, and/or revision of the manuscript: E.L. Moen, A.L. Stark, W. Zhang, M.E. Dolan, L.A. Godley
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