Sensitivity to temozolomide in brain tumor initiating cells

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Molecular alterations in glioblastoma have the potential to guide treatment. Here, we explore the relationship between temozolomide (TMZ) response and O⁶-methylguanine DNA methyltransferase (MGMT) status in brain tumor initiating cells (BTICs). Methylation, expression, and sensitivity were assessed in 20 lines; associations were evaluated by Fisher’s exact test. Some BTICs were sensitive. Sensitivity to TMZ was only associated with protein expression \( (P = .001) \). There were atypical BTICs including TMZ-resistant lines in which the methylation-specific PCR reaction revealed both methylated and unmethylated bands. BTICs are not uniformly resistant to TMZ; some are sensitive. MGMT status does not predict TMZ response with high precision.

Keywords: brain tumor stem cells, glioblastoma, MGMT methylation, temozolomide.

In 2005, silencing of the O⁶-methylguanine DNA methyltransferase (MGMT) gene by promoter methylation emerged as a potentially useful test to identify patients with glioblastoma (GBM) who would benefit from adding temozolomide (TMZ) chemotherapy to radiotherapy.⁵ Despite the compelling rationale to personalize cancer treatment and data supporting the association between MGMT methylation and benefit from TMZ, MGMT testing is not used clinically. Oncologists have been reluctant to adopt such a test because in the study by Hegi et al.,² there were several patients with unmethylated tumors who had long survival times after treatment with TMZ and many patients with methylated tumors who had short survival despite TMZ. The failed promise of a predictive test prompted us to explore the relationship between MGMT status and TMZ sensitivity in brain tumor initiating cell (BTIC) lines, which may be a disease reservoir in GBM.³ We used permanent cell lines that were established using the neurosphere culture method developed at the University of Calgary by Reynolds and Weiss.⁴ These GBM-initiating cell lines, 16 of which express the surface marker CD133, have retained the cardinal features of stem cells, including the ability to self-renew and to differentiate into multiple neural cell lineages.⁵ Furthermore, they also possess the signature characteristics of transformed cells, including continuous proliferation, growth factor-independent replication, and tumor formation in immune-deficient mice.⁵ Additionally, they harbor the hallmark molecular alterations that occur in GBM tissues, including p53, PTEN, and epidermal growth factor receptor mutations⁶ (data not shown). This resource afforded us the opportunity to rigorously evaluate the link between response to TMZ and MGMT methylation in a cell type that may be important to the maintenance of GBM tumors.

Materials and Methods

Cell Culture

All BTIC lines were derived from patients with GBMs. They were established in the laboratory of S.W. (Hotchkiss Brain Institute, University of Calgary) and maintained in basal neural stem cell media plus EGF, FGF, and heparin, as recommended by the manufacturer (Stem Cell Technologies) and described elsewhere.⁵

Methylation-specific PCR

Methylation-specific PCR was performed as described elsewhere² and modified from Herman et al. Brieﬂy, DNA was isolated using the DNeasy kit (Qiagen): 2 μg of DNA in 20 μL water was denatured in NaOH...
performed as described.2,6 Each assay was performed
with NaOH (final concentration of 0.3 M) at
37°C for 15 minutes, followed by ethanol precipitation.
The DNA was resuspended in water, and PCR was
performed as described.2,6 Each assay was performed
with positive and negative controls and repeated to
ensure reproducibility.

**Real-Time PCR**

RNA was isolated from each line, and quantitative RT–
PCR was performed for MGMT gene expression. Pelleted cells were washed in cold PBS, and RNA was extracted using the RNEasy kit (Qiagen) following
the manufacturer’s protocol. Sensiscript Reverse
Transcriptase (Qiagen) was used for reverse transcription. Real-time PCR was performed on the 7900HT
Fast Real-Time PCR system, using an MGMT probe
set and GAPDH (all from Applied Biosystems).

**Western Blotting**

Tumor cell pellets were resuspended in an equal volume
of RIPA lysis buffer (50 mM Tris, 10% SDS, 150 mM
NaCl, 12 mM sodium deoxycholate, 50 mM NaF, 1%
Triton X-100, 0.5% NP40, 1 mM PMSF, 1 mM DTT,
1 μg/mL aprotinin, and 1 μg/mL pepstatin). The
suspension was placed on ice for 15 minutes, vortexed
for 10 seconds, and spun-down for 10 minutes at
12 000 rpm at 4°C. The supernatant was removed and
protein concentration determined using the BioRad
Protein Assay kit (specifications from BioRad). Lysates
were supplemented with 500 mM DTT and protein
sample buffer (Invitrogen) and heated to 70°C for
10 minutes. Samples were loaded and run on precast
4%–12% gradient SDS–PAGE gels (Nupage,
Invitrogen) and transferred to nitrocellulose membranes
in a semi-dry gel transfer module (Nupage, Invitrogen).
Antibody hybridizations were performed, and
signals were visualized with a chemiluminescence kit
(Amersham). MGMT (FL-207) secondary goat-anti-
rabbit (G1305) and anti-actin (C2) antibodies were
from Santa Cruz.

**BTIC Viability**

The sensitivity of BTICs to TMZ was assessed using the
alamarBlue viability assay (Medicorp). Briefly, single-cell suspensions of BTICs were plated in a 96-well plate,
5000 cells/well in a final volume of 200 μL/well.
 Cultures were treated with vehicle (DMSO) or increasing
concentrations of TMZ chemotherapy (1–10 μg/mL;
Schering Plough). Every second day, alamamarBlue was
added to the culture media. After 24 hours, absorbance
was measured at 560 and 600 nm, and cell viability calcu-
lated as directed in the manufacturer’s protocol. Final
measurements were made 12 days after TMZ treatment
began. Each treatment group was repeated in duplicate
and each experiment in triplicate. Sensitivity was assessed
in 2 ways: relative viability was determined, first, by
comparing the absorbance values of TMZ-treated cells
with vehicle-treated cells, and second, by the absolute
absorbance values for each treatment group. Lines were
classified as sensitive if their relative viability decreased
compared with controls and their absolute absorbance
decreased or remained unchanged. In addition, there
was an extended period of observation after treatment
to confirm that sensitive lines stopped proliferating
(data not shown).

**Statistical Considerations**

The relationship between TMZ sensitivity and MGMT
methylation was analyzed in 2 ways: first, grouping
hemi-methylated lines with methylated lines as
described in Hegi et al.,2 and then pooling hemi-
methylated lines with unmethylated lines. The relations-
ships between promoter methylation, transcript
eexpression, and protein expression were analyzed after
excluding hemi-methylated cases; all other comparisons
included hemi-methylated lines. Fisher’s exact test was
used to evaluate the significance of associations
(Graphpad Prism Software).

**Results and Discussion**

**TMZ Sensitivity**

There is a commonly held view that BTICs are resistant
to chemotherapy even though the experimental data on
this point are conflicting. Some have found that BTICs
are resistant to chemotherapy,9 whereas others have
reported their sensitivity.7 Here, we performed a com-
prehensive analysis of the response of BTICs to TMZ
chemotherapy using 20 newly established lines from
patients with GBM. We assessed sensitivity with a well-
characterized cell viability assay (alamarBlue) and chose
dose range (1–10 μg/mL) that spanned the clinically
relevant concentrations of TMZ. This range was specifi-
cally selected to reflect drug concentrations that have
been achieved in the cerebrospinal fluid of patients
with GBM undergoing treatment with TMZ.10
Response data for representative sensitive and resistant
lines are shown (Fig. 1). Lines varied in their response
to TMZ: 9 were sensitive and 11 resistant (Table 1).
Some lines were highly sensitive to TMZ and others
highly resistant. Response to TMZ was difficult to cat-
egorize in 2 lines, BT067 and BT085; both continued
to proliferate after TMZ exposure, but more slowly
than vehicle-treated cells. For the purpose of subsequent
analysis, these lines were scored resistant. There was no
association between CD133 expression and response to
TMZ (data not shown).
These results derived from a careful survey of 20 BTIC lines from GBMs are noteworthy because they immediately call into question the popular view that BTICs are inherently and invariably resistant to chemotherapy. Perhaps, conflicting data in the literature reflect the fact that conclusions about the behavior of BTICs have been based on small numbers of lines.\textsuperscript{7,8}

**MGMT Status**

Three patterns of MGMT promoter methylation were observed in BTIC lines—methylated, unmethylated, and hemi-methylated (Fig. 2). Five BTIC lines were methylated, 12 were unmethylated, and 3 displayed a hemi-methylated configuration (Table 1). The MGMT transcript was not detected in methylated lines and was found in only 7 of 12 unmethylated lines; similarly, protein was not detected in methylated lines and was found in only 6 of 12 unmethylated lines (Table 1). When detected, the levels of expression of the MGMT transcript and the MGMT protein were highly variable between the lines (data not shown). A very strong correlation was observed between the transcript and the protein expression ($P < .0001$). There was a weak association between MGMT promoter methylation and the expression of MGMT transcript ($P = .04$) and no association between methylation and protein expression ($P = .1$). These observations are similar to the results of comparable analyses performed in GBM tissues and traditional glioma cell lines derived from GBMs.\textsuperscript{11}

**Sensitivity vs MGMT**

The most widely studied and best characterized mechanism of resistance to TMZ in GBM is the expression of the DNA repair protein, MGMT. MGMT removes methyl adducts from O\textsuperscript{6}-guanine, a site of lethal DNA damage by TMZ.\textsuperscript{12} GBM tumors that actively express MGMT are more resistant to TMZ than identical looking tumors in which the MGMT gene has been silenced.\textsuperscript{13} In GBM, the expression of the MGMT is silenced epigenetically via methylation of the MGMT gene promoter. For unknown reasons, methylation of the promoter occurs in up to 50\% of GBMs.\textsuperscript{2} By silencing the MGMT, methylation of the gene promoter renders the tumor more sensitive to TMZ. Given its critical role in governing the response to TMZ, we assessed MGMT promoter methylation in GBM-derived BTICs that had different sensitivities to TMZ. Unlike in Hegi et al.,\textsuperscript{2} where the benefit from TMZ in GBM was associated with methylation of the MGMT gene promoter, we were unable to demonstrate a statistically significant association between sensitivity to TMZ and MGMT methylation in BTIC lines ($P = .3$). However, when the hemi-methylated lines were pooled with the
unmethylated BTICs instead of methylated BTICs, as occurs clinically,\(^2\)  we found a significant association between sensitivity to TMZ and methylation status \((P = .008; \text{Table 1})\).  Overall, our findings in BTIC lines support the prevailing viewpoint that MGMT methylation status, by itself, should not be used to predict response to TMZ, unmethylated lines sometimes had anomalous responses to TMZ.  There were 2 groups of atypical patterns of MGMT expression or unpredictable behavior.\(^3\)  Whereas all methylated lines in this series were sensitive to TMZ, unmethylated lines sometimes had anomalous responses to TMZ.  Such obstacles to reliable measurement may not be insurmountable, however.  Similar challenges were successfully addressed by the breast cancer translational research community, who developed consensus criteria for therapeutic decision-making based on her2-neu expression.\(^4\)  Her2-neu, like the MGMT protein, is detected by immunohistochemical analysis of tumor tissues that may contain a mixture of normal and neoplastic cells.  Of course, no criteria are perfect or apply to all possible situations.  Extrapolating from this BTIC study, it would be difficult to personalize the use of TMZ for patients with tumors that behaved like BT042, BT067, and BT085.  These lines defy simple interpretation; they express neither the MGMT transcript nor protein, yet are resistant to TMZ.

**Hemi-Methylated and Atypical BTICs**

One of the unexpected findings in this study was the existence of hemi-methylated lines.  The phenomenon of hemi-methylation has been seen before in the analyses of GBM tissues but attributed to the inadvertent contamination of the test sample by normal brain tissue.  Normal tissue contamination is clearly an untenable explanation for a hemi-methylated pattern in BTICs, but whether hemi-methylation implies that only 1 of the 2 MGMT alleles is methylated in each cell within a single line, as suggested by Piccirillo et al.,\(^5\)  is unknown at this time.  In either case, the finding of hemi-methylation in some BTICs raises the intriguing possibility that hemi-methylation might also be a characteristic of some GBM tumors and is not due to normal tissue contamination in all instances.  This possibility could have implications for the interpretation of MGMT test results and may have additional significance when coupled with our finding that TMZ sensitivity is significantly associated with MGMT methylation when hemi-methylated lines are assigned to the unmethylated group.  Indeed, in a clinical trial, the pooling of TMZ-resistant hemi-methylated cases with sensitive methylated cases could undermine a positive study by shifting poor prognosis patients to a good prognosis subgroup.  At the very least, miss-assignment of hemi-methylated lines are assigned to the unmethylated group.  The phenomenon of hemi-methylation has been seen before in the analyses of GBM tissues but attributed to the inadvertent contamination of the test sample by normal brain tissue.  Normal tissue contamination is clearly an untenable explanation for a hemi-methylated pattern in BTICs, but whether hemi-methylation implies that only 1 of the 2 MGMT alleles is methylated in each cell within a single line, as suggested by Piccirillo et al.,\(^5\)  is unknown at this time.  In either case, the finding of hemi-methylation in some BTICs raises the intriguing possibility that hemi-methylation might also be a characteristic of some GBM tumors and is not due to normal tissue contamination in all instances.  This possibility could have implications for the interpretation of MGMT test results and may have additional significance when coupled with our finding that TMZ sensitivity is significantly associated with MGMT methylation when hemi-methylated lines are assigned to the unmethylated group.  Indeed, in a clinical trial, the pooling of TMZ-resistant hemi-methylated cases with sensitive methylated cases could undermine a positive study by shifting poor prognosis patients to a good prognosis subgroup.  At the very least, miss-assignment of hemi-methylated lines could explain short survivors in a series of methylated cases.

Atypically behaving lines also deserve comment.  Whereas all methylated lines in this series were sensitive to TMZ, unmethylated lines sometimes had anomalous patterns of MGMT expression or unpredictable responses to TMZ.  There were 2 groups of atypical lines.  BT041, BT050, BT060, and BT069 had unmethylated promoters, yet were sensitive to TMZ; alternative sites of promoter methylation, not examined in this study, might explain such cases.\(^6\)  BT042 and

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**Table 1.** BTIC lines \((n = 20)\) were characterized for MGMT methylation status using MS-PCR and classified as methylated \((M)\), unmethylated \((U)\), or hemi-methylated \((U/M)\).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MGMT methylation</th>
<th>MGMT transcript</th>
<th>MGMT protein</th>
<th>TMZ response</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT048</td>
<td>M</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>BT053</td>
<td>M</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>BT084</td>
<td>M</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>BT089</td>
<td>M</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>BT094</td>
<td>M</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>BT041</td>
<td>U</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>BT050</td>
<td>U</td>
<td>Present</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>BT060</td>
<td>U</td>
<td>Present</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>BT069</td>
<td>U</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
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<td>U</td>
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<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>BT085</td>
<td>U</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>BT012</td>
<td>U</td>
<td>Present</td>
<td>Present</td>
<td>S</td>
</tr>
<tr>
<td>BT030</td>
<td>U</td>
<td>Present</td>
<td>Present</td>
<td>S</td>
</tr>
<tr>
<td>BT073</td>
<td>U</td>
<td>Present</td>
<td>Present</td>
<td>S</td>
</tr>
<tr>
<td>BT074</td>
<td>U</td>
<td>Present</td>
<td>Present</td>
<td>S</td>
</tr>
<tr>
<td>BT075</td>
<td>U</td>
<td>Present</td>
<td>Present</td>
<td>S</td>
</tr>
<tr>
<td>BT090</td>
<td>U</td>
<td>Present</td>
<td>Present</td>
<td>S</td>
</tr>
<tr>
<td>BT025</td>
<td>U/M</td>
<td>Present</td>
<td>Present</td>
<td>S</td>
</tr>
<tr>
<td>BT067</td>
<td>U/M</td>
<td>ND</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>BT068</td>
<td>U/M</td>
<td>Present</td>
<td>Present</td>
<td>S</td>
</tr>
</tbody>
</table>

Real-time PCR was used to detect MGMT gene transcript (ND, no transcript; Present, transcript detected).  Protein expression was examined by Western blot analysis (ND, no protein; Present, protein detected).  TMZ response was characterized over time by alamarBlue viability after exposure to increasing concentrations of TMZ.  Resistant lines \((R)\) demonstrated minimal or no change in viability in response to clinically relevant doses of TMZ.  Sensitive lines \((S)\) demonstrated a significant decrease in viability after drug treatment.

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**Fig. 2.** Representative methylation-specific PCR (MS-PCR) data.  DNA was isolated from each BTIC line and MS-PCR performed for the MGMT promoter.  Three methylation patterns were seen: unmethylated (BT012), hemi-methylated (BT025), and methylated (BT048).
BT085 did not express MGMT protein, yet were resistant to TMZ. Neither BT042 nor BT085 had a mutation of MSH6; presumably other mechanisms of TMZ resistance were operative in these cases. Further characterization of anomalous lines may contribute to our understanding of atypically responding patients and assist in the development of robust predictive tests for TMZ use.

Our data demonstrate that BTICs have varying sensitivities to TMZ. In this study, methylation status did not predict response to TMZ precisely, although the reassignment of hemi-methylated cases to the unmethylated group improved our accuracy. All methylated lines were sensitive to TMZ and lines that expressed MGMT protein were resistant. Although our findings have not yet been confirmed in vivo, it would appear from this and other data that predicting response to TMZ with “clinical grade” precision will require more complex profiling of BTICs and, by inference, GBMs.

Conflict of interest statement. None declared.

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References


