Genomic and Molecular Profiling Predicts Response to Temozolomide in Melanoma

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Abstract

Purpose: Despite objective response rates of only ~13%, temozolomide remains one of the most effective single chemotherapy agents against metastatic melanoma, second only to dacarbazine, the current standard of care for systemic treatment of melanoma. The goal of this study was to identify molecular and/or genetic markers that correlate with, and could be used to predict, response to temozolomide-based treatment regimens and that reflect the intrinsic properties of a patient’s tumor.

Experimental Design: Using a panel of 26 human melanoma-derived cell lines, we determined in vitro temozolomide sensitivity, O6-methylguanine-DNA methyltransferase (MGMT) activity, MGMT expression and promoter methylation status, and mismatch repair proficiency, as well as the expression profile of 38,000 genes using an oligonucleotide-based microarray platform.

Results: The results showed a broad spectrum of temozolomide sensitivity across the panel of cell lines, with IC50 values ranging from 100 μmol/L to 1 mmol/L. There was a significant correlation between measured temozolomide sensitivity and a gene expression signature derived from expression of 38,000 genes using an oligonucleotide-based microarray platform. Notably, MGMT alone showed a significant correlation with temozolomide sensitivity (MGMT activity, P < 0.0001; MGMT expression, P ≤ 0.0001). The promoter methylation status of the MGMT gene, however, was not consistent with MGMT gene expression or temozolomide sensitivity.

Conclusions: These results show that melanoma resistance to temozolomide is conferred predominantly by MGMT activity and suggest that MGMT expression could potentially be a useful tool for predicting the response of melanoma patients to temozolomide therapy.

Malignant melanoma is increasing at a rate faster than any other cancer, with an expected 62,000 new cases this year (1). Despite advances in our understanding of melanoma biology and the development of several targeted therapeutics, the overall response rates of malignant melanomas to therapy continue to be low.

Currently, the drug of choice for the treatment of systemic melanoma is dacarbazine (DTIC). Although DTIC as a single agent has yielded response rates of ~15% against melanoma, most of these are incomplete and last only a few months. Temozolomide is a second-generation alkylating agent with a mechanism of action similar to DTIC through the active metabolite 5-(3-methyltriazen-1-yl)imidazole-4-carboximide (MTIC; ref. 2). A randomized phase III trial comparing temozolomide with DTIC showed that temozolomide improved health-related quality of life, had greater systemic exposure to both the parent drug and active metabolite, and was associated with longer progression-free survival (3).

It is well established that failure to respond to temozolomide is largely due to both inherent and acquired tumor resistance (4, 5). Drugs that target specific resistance pathways when used in combination with primary chemotherapeutic agents have been shown to improve the tumor response rates to chemotherapy (6–9). Among the factors that contribute to temozolomide resistance are elevated O6-methylguanine-DNA methyltransferase (MGMT), a protein that removes drug-induced alkylguanine adducts from DNA; base excision repair [BER; in particular poly(ADP-ribose) polymerase 1 (PARP1)]; and mismatch repair (MMR; ref. 10). Similar to MGMT, BER plays an important role in repairing the cytotoxic methyl DNA adducts created by temozolomide, and high BER activity can confer tumor resistance to temozolomide. On the other hand, a deficiency in the MMR pathway can lead...
to tolerance of temozolomide-generated DNA adducts, a
continuation of DNA replication, and a loss of the cytotoxic
effects of temozolomide.

Gene expression profiling provides a powerful means of
classifying tumors based on their underlying biology. It has
been used to identify genetic markers that are predictive of
disease recurrence in breast cancer (11), to identify previously
undetected subtypes of cutaneous melanoma (12), to identify
a gene expression pattern that correlates with BRAF mutation
status in melanoma cell lines (13), and to characterize the
progression of melanoma (14). Similarly, expression profiling
has enabled the identification of genetic markers, or signature,
that are predictive of response to primary chemotherapy
in ovarian cancer (15). More recently, this approach has been
used to identify gene expression patterns that are predictive of
oncogenic signaling (16) and sensitivity to chemotherapy
(17). Whereas most of these studies have been done retrospectively, the potential clinical utility of this technology lies in the ability to prospectively characterize the underlying
drug resistance of a patient’s tumor and, ultimately, predict
response to therapy.

In this study, we used a panel of 26 human melanoma-
derived cell lines in an effort to identify molecular and/or
genetic markers with potential for use as predictors of response
to temozolomide in melanoma patients. We evaluated the
sensitivity of these cell lines to temozolomide as well as DNA
repair pathway efficiency, specifically MGMT activity and
promoter methylation status, as well as microsatellite instabili-
ity, as a measure of DNA MMR status. The gene expression
profile of each cell line was obtained using a high-density
oligonucleotide chip. The relationship between temozolomide
sensitivity, DNA MMR efficiency, and gene expression patterns
was evaluated to define a set of biomarkers that could be
predictive of patient response to temozolomide.

Materials and Methods

Drugs, chemicals, and other reagents. Temozolomide was provided by Schering-Plough, and melphan purchased from Sigma-Aldrich. Stock solutions of temozolomide or melphan were prepared in 100% DMSO and stored at -20°C until use; when added to cell cultures, the

Translational Relevance

With the increasing incidence of melanoma and the histori-
cally poor response rates to traditional chemotherapy, it is im-
portant to develop tools that can be used prospectively to
characterize a patient’s tumor with regard to chemoresistance
pathways. The findings in this study show striking differences
in terms of DNA repair pathway efficiency and temozolomide
response across a broad sampling of melanoma cell lines and
that, in melanoma, resistance to temozolomide is conferred
largely by the activity of the DNA repair enzyme O6-methyl-
guanine-DNA methyltransferase (MGMT). Our results sug-
gest that MGMT expression could potentially be a useful tool
for personalizing treatment strategies in melanoma patients
by identifying those patients most likely to respond to temo-
zolomide and those patients for whom combination therapy
or alternative chemotherapeutic reagents might be desirable.
Gene expression analysis. Analysis of gene expression data for predicted drug resistance was done as previously described (17). In brief, from the NCI-60 database, a panel of cell lines was selected based on their response to temozolomide. A binary regression analysis was done wherein the training set was the NCI-60 cell lines, defined as sensitive (0) or resistant (1), and the validation set was our panel of 26 melanoma cell lines, defined as unknown sensitivity (2): see Supplementary data for additional details.

**MGMT activity.** MGMT activity was measured as the removal of [3H]methyguanine from a [3H]methylated DNA substrate as previously described (18). Briefly, pelleted cells were homogenized, sonicated, and centrifuged for 30 min. For each sample, a known amount of protein was incubated in the reaction mixture at 37°C for 30 min. DNA was precipitated by adding ice-cold perchloric acid (250 mmol/L), hydrolyzed by the addition of 0.1 N HCl, and incubated at 70°C for 30 min. Following filtration, bases (N7 and O6) were separated by reverse-phase high-performance liquid chromatography with 9% methanol in 0.5 mol/L ammonium formate. MGMT activity was calculated as the amount of O6-methylguanine released from the DNA substrate (femtomoles of O6 lost) divided by the amount of sample protein added to the reaction mixture. Values are expressed as the mean of at least three separate experiments ± SD.

**Quantitative PCR for MGMT gene expression.** MGMT gene expression level was measured using SYBR Green Real-Time PCR. Total RNA was isolated from cell lines as described above. First-strand cDNA synthesis was carried out using Roche Transcriptor First Strand cDNA Synthesis Kit. PCR was done in a 20-µL volume containing 2 µL sample cDNA, 6 µL distilled water, 10 µL ABI SYBR Green Master Mix, and 1 µL each of the forward and reverse primers. Quantitative PCR and data analysis were done using the ABI 7900 sequence detector system. See Supplementary Table S1 for primer sequences. MGMT expression values were normalized to β-actin expression values and expression in HT29 cells. Data are presented as the mean of at least three separate experiments ± SD.

**MGMT promoter methylation.** DNA was isolated using Qiagen DNeasy Kit, and sodium bisulfite modification was done on 2 µg DNA using EZ DNA methylation gold kit (Zymo Research Corporation). Following bisulfite modification of DNA, all unmethylated cytosine residues were converted to uracil whereas all methylated cytosine residues remained as cytosine. PCR amplification of the promoter region of MGMT spanning nucleotides 1064 to 1156 (19) was done using HotStar Taq (Qiagen) in a final volume of 25 µL containing 2 µg DNA and 0.3 mmol/L ammonium formate. MGMT activity was calculated as the amount of O6-methylguanine released from the DNA substrate (femtomoles of O6 lost) divided by the amount of sample protein added to the reaction mixture. Values are expressed as the mean of at least three separate experiments ± SD.

Results

**Temozolomide resistance of melanoma cell lines.** The results of the analysis of temozolomide sensitivity across 26 melanoma cell lines are summarized in Fig. 1. Initially, five cell lines were examined for their response to temozolomide therapy in vivo in a xenograft animal model of extremity melanoma. The temozolomide response of these five cell lines in vivo is shown in Table 1. The in vitro temozolomide sensitivity of these five cell lines was determined using both the acute and 12-day assays. The results of the acute assay (Fig. 1A) showed a broad spectrum of response ranging from very sensitive (R ≤ 0.3; DM440) to very resistant (R ≥ 0.9; DM443 and DM6; one-way ANOVA analysis comparing cell lines: P = 0.0001, r2 = 0.90; Fig. 1A). The correlation (Fig. 1B) between in vivo and 12-day in vitro sensitivities was higher (P = 0.086) than between in vivo and acute in vitro sensitivities (P = 0.98). We used the 12-day in vitro cytotoxicity assay to measure temozolomide sensitivity across all 26 cell lines and observed a broad spectrum of response ranging from very sensitive (R ≤ 0.3) to very resistant (R ≥ 0.9; Fig. 1C).

We evaluated three DNA repair pathways important in temozolomide resistance by examining the activity and expression of MGMT as well as the expression of genes involved in DNA MMR and BER.

**Relationship between MMR and BER status and temozolomide resistance.** MMR activity was measured as a function of microsatellite stability using five mononucleotide markers (for details, see Materials and Methods) to classify the cell lines as having high microsatellite instability (MSI-H) or as being microsatellite stable (MSS). The results showed that, based on the presence of a polymorphism or somatic deletion of 3-6 bp (4 bp for BAT-26) in three or more markers as MSI-H and MMR deficient and a deletion in two or fewer markers as MSS and MMR proficient, none of the 16 melanoma cell lines tested were found to be MSI-H across the 16 markers tested (see Table 1), indicating that all the cell lines are MMR proficient. The observed microsatellite stability correlated with high expression of several genes in the MMR pathway (MSH2, MSH3, MSH6, MLH1, and PMS2) across all 26 cell lines (Supplementary Table S2).

The expression of several genes directly and indirectly involved in BER, including PARP1, DNA polymerase β (POLB), APEX nuclease 1 (APEX1), ATP-dependent DNA ligase 1 (LIG1), and N-methylpurine-DNA glycosylase (MPG), showed no correlation with sensitivity to temozolomide (Supplementary Table S3), suggesting that BER does not significantly contribute to temozolomide resistance in melanoma.

**MGMT activity and temozolomide resistance.** Figure 2A summarizes the results of MGMT activity across the panel of 26 melanoma cell lines and shows a spectrum ranging from no detectable activity (DM711, DM733, DM738, and DM792) up to >800 fmol/mg protein for DM425. This parallels the marked differences observed in temozolomide resistance. As shown in Fig. 2B, there is a significant correlation (P < 0.0001, r2 = 0.6) between measured temozolomide resistance and MGMT activity.
To examine the relationship between MGMT activity and MGMT gene expression, the latter was measured in the panel of 26 cell lines using two platforms: standard quantitative PCR and microarray-based technology. MGMT gene expression across the panel of cell lines was heterogeneous, and measurements obtained from both quantitative PCR and microarray were significantly correlated with each other ($P < 0.0001$; Supplementary Fig. S1) and with MGMT activity ($P < 0.0001$; Supplementary Fig. S2), as well as with measured temozolomide resistance ($P < 0.0001$, $r^2 = 0.46$ (quantitative PCR) or $r^2 = 0.72$ (microarray); Fig. 2C and D).

MGMT promoter methylation, MGMT expression and activity, and temozolomide resistance. To determine the relationship between MGMT gene expression and promoter methylation and whether MGMT promoter methylation status can be used to predict response to temozolomide in melanoma, we measured promoter methylation across the panel of 26 melanoma cell lines. PCR amplification of the promoter region, spanning nucleotides 1064 to 1156, was done using matched primer pairs. The results (Fig. 3A; Table 1) showed 11 of the 26 cell lines to harbor the unmethylated allele and 15 to have both methylated and unmethylated alleles. Notably, no correlation was observed between MGMT promoter methylation status at the target regions evaluated and response to temozolomide (Fig. 3B; unpaired $t$ test comparing mean $R$ of methylated samples to mean $R$ of unmethylated sample: $P = 0.399$).

Gene expression signature of predicted temozolomide resistance. A signature of gene expression that correlated with resistance to temozolomide was derived from the NCI-60 panel of cancer cell lines (ref. 17; see also Supplementary data). From this panel of 60 cell lines, a smaller subset of 15 was selected that represented two extremes of sensitivity to temozolomide; nine of these cell lines were classified as “resistant” and six as...
“sensitive.” Using the gene expression profiles of these cell lines, we identified 45 genes that showed significantly different expression patterns between the “resistant” and “sensitive” cell lines and thus provided a “temozolomide sensitivity gene signature” (see Supplementary Table S4). The color-coded heatmap of expression of the 45 “temozolomide” genes across these 15 cell lines (Fig. 4A) shows 8 genes (red) that were more highly expressed in the resistant than in the sensitive cell lines, whereas 37 genes (blue) were more highly expressed in the sensitive than in the resistant cell lines. The robustness of the signature was evaluated by a “leave-one-out” cross validation analysis. The results showed the six “sensitive” cell lines, with metagene scores between 2 and 5, to be distinct from the nine “resistant” cell lines, with metagene scores between -1 and -4 (Supplementary Fig. S3).

To validate the capacity of the NCI-60 derived temozolomide sensitivity gene signature to predict response, we performed a regression analysis of the gene expression profiles obtained for each of the 26 melanoma cell lines and determined a predicted probability of temozolomide resistance on a scale of 0 to 1 (1, resistant; 0, sensitive). The cell lines displayed a broad spectrum of predicted resistance to temozolomide, as shown in Fig. 4B. Furthermore, the predicted temozolomide resistance based on the NCI-60 temozolomide sensitivity gene signature correlated significantly \((P < 0.005)\) with the measured resistance obtained using the 12-day cytotoxicity assay described above (Fig. 4C). As a further test of the specificity of the gene expression signature for temozolomide resistance, we analyzed our panel of melanoma cell lines for resistance to melphalan and found no correlation between predicted temozolomide resistance and measured melphalan resistance, as shown in Fig. 4D.

**Discussion**

Melanoma is an increasing health problem with dismal prognosis for stage IV patients who have metastatic disease (21). As with other cancers, the ability to prospectively predict patient response to treatment using molecular or genetic markers that reflect the underlying biology of a patient’s disease will facilitate the design of more effective and less toxic treatment strategies for melanoma patients. In this study, we evaluated the molecular, genetic, and sensitivity profiles of a panel of 26 human melanoma-derived cell lines to identify a marker(s) that could be used clinically to predict the response of melanoma patients to temozolomide-based therapy. A unique aspect of this study is the diverse array of melanoma cell lines used that we believe better captures the molecular and genetic heterogeneity of clinical disease.

Recent clinical trials have shown that for systemic treatment of metastatic melanoma, temozolomide is as effective as DTIC, the only widely used drug that is Federal Drug Administration approved for melanoma, for which objective response rates of only 13% to 20% are achieved (22, 23). Temozolomide has the added advantage of oral delivery as well as the ability to

### Table 1. Summary of melanoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Measured temozolomide resistance</th>
<th>MGMT activity (fmol/mg protein)</th>
<th>MGMT expression (qPCR)</th>
<th>MGMT expression (204880_at*)</th>
<th>Predicted temozolomide resistance</th>
<th>MGMT methylation status</th>
<th>MSI status</th>
</tr>
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<tbody>
<tr>
<td>A2058</td>
<td>0.113</td>
<td>14.77</td>
<td>0</td>
<td>12.6</td>
<td>0.137</td>
<td>M/U</td>
<td>ND</td>
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<tr>
<td>DM711</td>
<td>0.155</td>
<td>6.32</td>
<td>0</td>
<td>5.7</td>
<td>0.267</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM598</td>
<td>0.218</td>
<td>35.69</td>
<td>0.083</td>
<td>35.5</td>
<td>0.405</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM440</td>
<td>0.302</td>
<td>28.67</td>
<td>0.038</td>
<td>271.2</td>
<td>0.288</td>
<td>U</td>
<td>ND</td>
</tr>
<tr>
<td>DM792</td>
<td>0.321</td>
<td>0</td>
<td>0</td>
<td>5.4</td>
<td>0.855</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM646</td>
<td>0.357</td>
<td>9.04</td>
<td>0</td>
<td>8.5</td>
<td>0.458</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM738</td>
<td>0.434</td>
<td>0.000</td>
<td>0</td>
<td>29.7</td>
<td>0.609</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM733</td>
<td>0.437</td>
<td>0</td>
<td>0</td>
<td>57.7</td>
<td>0.181</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM391</td>
<td>0.530</td>
<td>139.6</td>
<td>0.080</td>
<td>440.3</td>
<td>0.197</td>
<td>U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM175</td>
<td>0.590</td>
<td>486.18</td>
<td>0.311</td>
<td>582.4</td>
<td>0.248</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM368</td>
<td>0.597</td>
<td>275.57</td>
<td>0.225</td>
<td>407.9</td>
<td>0.194</td>
<td>U</td>
<td>ND</td>
</tr>
<tr>
<td>DM578</td>
<td>0.620</td>
<td>228.88</td>
<td>0.200</td>
<td>445.3</td>
<td>0.749</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM366</td>
<td>0.647</td>
<td>373.2</td>
<td>0.233</td>
<td>328.0</td>
<td>0.396</td>
<td>U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM833</td>
<td>0.683</td>
<td>268.71</td>
<td>0.165</td>
<td>375.3</td>
<td>0.652</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM424</td>
<td>0.754</td>
<td>721.27</td>
<td>0.490</td>
<td>675.6</td>
<td>0.268</td>
<td>U</td>
<td>ND</td>
</tr>
<tr>
<td>DM422</td>
<td>0.767</td>
<td>92.21</td>
<td>0.065</td>
<td>211.3</td>
<td>0.228</td>
<td>U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM586</td>
<td>0.823</td>
<td>692.72</td>
<td>0.700</td>
<td>617.8</td>
<td>0.806</td>
<td>U</td>
<td>ND</td>
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<tr>
<td>DM751</td>
<td>0.897</td>
<td>292.34</td>
<td>0.300</td>
<td>415.4</td>
<td>0.860</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM608</td>
<td>0.899</td>
<td>333.75</td>
<td>0.329</td>
<td>425.6</td>
<td>0.726</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>DM414</td>
<td>0.927</td>
<td>980.25</td>
<td>1.155</td>
<td>939.7</td>
<td>0.866</td>
<td>U</td>
<td>ND</td>
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<tr>
<td>DM425</td>
<td>0.946</td>
<td>1033.3</td>
<td>0.770</td>
<td>877.6</td>
<td>0.611</td>
<td>U</td>
<td>ND</td>
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<tr>
<td>DM636</td>
<td>0.949</td>
<td>489.17</td>
<td>0.480</td>
<td>663.4</td>
<td>0.582</td>
<td>M/U</td>
<td>MSS</td>
</tr>
<tr>
<td>SKMel28</td>
<td>0.967</td>
<td>272.26</td>
<td>0.160</td>
<td>500.1</td>
<td>0.276</td>
<td>U</td>
<td>ND</td>
</tr>
<tr>
<td>DM443</td>
<td>1.000</td>
<td>636.35</td>
<td>0.195</td>
<td>713.5</td>
<td>0.813</td>
<td>M/U</td>
<td>ND</td>
</tr>
<tr>
<td>DM6</td>
<td>1.000</td>
<td>668.25</td>
<td>0.459</td>
<td>725.8</td>
<td>0.831</td>
<td>M/U</td>
<td>MSS</td>
</tr>
</tbody>
</table>

Abbreviations: qPCR, quantitative PCR; M, methylated; U, unmethylated; ND, not determined.

*Affymetrix GeneChip Hu133 plus 2 Probe ID number.
penetrate the blood-brain barrier (23, 24), a distinct advantage over DTIC given the high rate of central nervous system metastasis associated with melanoma (25, 26). Furthermore, in an animal model of extremity melanoma, isolated limb infusion with temozolomide was more effective than with melphalan, the drug of choice for regional treatment of melanoma (27).

Despite the shown potential of temozolomide in the treatment of melanoma, there continues to be high rates of progressive disease and recurrences (28). Failure to respond to chemotherapy in general is largely due to tumor drug resistance mechanisms. In melanoma chemotherapy, resistance is, at least in part, inherent to the biology of the tumor (5). The mechanism by which temozolomide exerts its cytotoxic action involves the generation of the active intermediary MTIC, which, in turn, methylates purines and pyrimidines in DNA at several nucleophilic sites including \(N^7\)-guanine (accounting for nearly 70% of the methylated lesions), \(N^3\)-adenine (10% of lesions), and \(O^6\)-guanine (7% of lesions; ref. 29). Resistance to temozolomide arises when the methylated DNA bases are repaired by MGMT (30) or BER (31) or when the MMR pathway is deficient (32) and the damaged DNA is tolerated.

Methylation of the \(O^6\)-guanine base is the most cytotoxic lesion generated by both temozolomide and DTIC (29). Because of the highly cytotoxic nature of \(O^6\)-methylguanine compared with \(N^7\)-methylguanine and \(N^3\)-methyladenine, both of which are very rapidly repaired, elevated MGMT is thought to be the primary mechanism by which resistance to temozolomide and DTIC is conferred. Clinical trials, however, have yielded conflicting results about the relationship between response to temozolomide or DTIC and MGMT levels. Glioblastoma patients with MGMT expression in fewer than 20% of cells (33) or with promoter methylation of the MGMT gene that silences its expression (34) have been shown to benefit from temozolomide therapy. Likewise, response to first-line treatment with temozolomide in patients with primary glioma significantly correlated with hypermethylation of the MGMT gene promoter (35). In contrast to glioblastoma, the relationship between melanoma response to alkylating agents and MGMT levels is more complex, with some reports indicating that MGMT activity (36, 37), expression as measured by immunohistochemistry (38), and promoter methylation (39) did not correlate significantly with response to temozolomide or DTIC. Likewise, in a recent clinical trial, inactivation of MGMT did not enhance the cytotoxic activity of temozolomide in melanoma (40).

Given the multiple cellular events that can contribute to temozolomide resistance, we hypothesized that a larger-scale gene expression analysis, rather than an analysis of single

![Graph A](https://example.com/graphA.png)

**Fig. 2.** A, MGMT activity in the panel of 26 melanoma cell lines, measured as described in Materials and Methods. Columns, mean of at least three measurements; bars, SE. Cell lines are plotted in order of increasing measured resistance to temozolomide. B, MGMT activity plotted as a function of measured temozolomide resistance (fraction resistant to temozolomide, \(R\)). The line is from a linear regression analysis of all the points \((r^2 = 0.60, P < 0.0001)\). MGMT expression measured by quantitative PCR (C) or microarray (D) is plotted as a function of measured temozolomide resistance. The lines are from a linear regression analysis of the data points \((r^2 = 0.46, P = 0.0001, r^2 = 0.72, P < 0.0001)\) for quantitative PCR and \(r^2 = 0.72\) and \(P < 0.0001\) for microarray.)
genes or pathways, would yield a more robust predictor of melanoma response to temozolomide because it would involve analysis of genes associated with multiple mechanisms of resistance. Gene expression profiling has been used successfully to identify gene expression markers, or signatures, that are predictive of in vitro and in vivo sensitivity to several chemotherapy drugs (17). In the present study, we observed a significant correlation between measured temozolomide resistance and a gene expression signature predictive of temozolomide response in an independent panel of cell lines derived from multiple cancer types. Notably, however, our results show that in melanoma cell lines MGMT is a more robust marker of temozolomide response. Evaluation of MGMT expression using both standard quantitative PCR and microarray methods showed similar results. Whereas standard quantitative PCR can be easily adapted to small quantities of tissue, microarray technology has the potential to yield information on the expression of thousands of genes at one time and can be used not only to identify tumors likely to be resistant to temozolomide but also to provide other information about the underlying tumor biology that can be used to guide therapeutic strategies (17).

Our results corroborate those of other studies suggesting that in melanoma the MMR system is largely intact and contributes little to temozolomide resistance (41, 42). The high proficiency of the MMR pathway in this panel of cell lines highlights the importance of MGMT-dependent repair of O⁶-guanine lesions, which, when not repaired by MGMT, trigger MMR leading to apoptosis. Although BER can repair N-methylated lesions generated by alkylating agents such as temozolomide (43), our data suggest that this pathway is likely to have minimal effect on temozolomide resistance in melanoma.

We have previously shown in an animal model of extremity melanoma that inhibiting MGMT can lead to marked enhancement of temozolomide sensitivity (9) and, further, that in vivo sensitivity to temozolomide strongly correlated with activity of the DNA repair protein MGMT (ref. 20; \( P = 0.10 \)) across a panel of five melanoma xenografts. MGMT has been shown to be expressed at a higher level in melanoma metastases after chemotherapy (4), and previous studies have indicated a critical role for MGMT in temozolomide resistance (42). Our results are consistent with these reports and suggest that MGMT-dependent repair of O⁶-methyl groups of guanine is the dominant mechanism conferring resistance to temozolomide in melanoma.

MGMT gene expression is regulated by several mechanisms including activation by SP1 transcription factors and methylation of CpG islands in the promoter region of the MGMT gene, which can lead to transcriptional silencing of the MGMT gene (29, 44, 45). In light of recent reports showing that MGMT promoter methylation is associated with favorable outcome following treatment with temozolomide in glioblastoma (34), we explored the predictive potential of MGMT methylation in melanoma. In contrast to previous reports indicating that methylation of the MGMT gene is an infrequent event in melanoma, occurring in ~11% of tumors (46), in this study we observed methylation of the MGMT promoter to be present in nearly half of the 26 melanoma cell lines that we analyzed. Furthermore, in a larger panel of 40 human melanoma-derived cell lines, we observed 35% to have very low levels of MGMT activity (see Supplementary Fig. S4). Interestingly, the level and pattern of MGMT promoter methylation observed in the melanoma cell lines in this study did not correspond with the observed MGMT expression, activity, or temozolomide resistance of the cell lines. Together these results suggest that the methylation targets analyzed may not be critical regulators of MGMT gene expression and/or that other mechanisms may be more important in the regulation of MGMT expression in melanoma.

Fig. 3. A, methylation status of eight melanoma cell lines as determined using methylation-specific PCR. Following sodium bisulfite conversion of the DNA, methylated and unmethylated sequences were amplified by PCR, and the products separated on a 4% agarose gel. unmethylated (93 bp); m, methylated (81 bp). B, fraction resistant to temozolomide (R) is plotted as a function of methylation status. Mean R for methylated samples (those showing both methylated and unmethylated products) is 0.58, whereas mean R for unmethylated samples is 0.68. \( P = 0.399, \) unpaired t test.
The progress that is being made in our understanding of the mechanisms that limit tumor response to chemotherapy treatment has become critically important in the development of strategies to overcome this resistance clinically. Efforts to improve patient response to temozolomide by inhibiting the activity of MGMT with the substrate analogue O\textsuperscript{6}-benzylguanine have yielded encouraging results (9) and show the therapeutic potential of chemotherapy in combination with a resistance modulator for the treatment of melanoma. However, the role of current MGMT inhibitors, such as O\textsuperscript{6}-benzylguanine, in modulating temozolomide activity clinically will be limited by poor tumor selectivity of these modulators, which results in significant normal tissue toxicity due to inhibition of DNA repair mechanisms and enhancement of temozolomide effects (47). Several reports suggest that the inherent resistance of melanoma to chemotherapy is a consequence of genetic lesions that interfere with the normal apoptotic processes (5). Targeted inhibition of cellular signaling pathways important for the processes of proliferation, apoptosis, and survival could therefore be of potential utility in improving responses to chemotherapy. Many novel drugs are being developed that target specific molecular or genetic lesions, and although targeted therapies alone often show only modest antitumor activity (48), it is likely that when combined with standard chemotherapies, the responses will be higher and more durable (28). Our laboratory has recently shown that targeted disruption of N-cadherin signaling with the small molecule ADH-1 can significantly improve the antimelanoma activity of temozolomide in a murine xenograft model (49).

**Fig. 4.** A, gene expression signature of temozolomide resistance derived from 15 cell lines in the NCI-60 panel, showing a heatmap representation of genes that are differentially expressed across temozolomide-sensitive and temozolomide-resistant cell lines. Red and blue, genes that are expressed at high and low levels, respectively. The scale below shows the fold change difference in expression across the color spectrum. B, the panel of cell lines, each from a different tumor type, illustrated in Fig. 2A, was used to define a predicted probability of temozolomide resistance in our panel of 26 melanoma-derived cell lines. The graph shows the predicted probability of temozolomide resistance for each cell line. The probability of temozolomide resistance predicted from the gene expression signature shown in Fig. 2A is plotted against the fraction of cell lines resistant to temozolomide (C) or melphalan (D), measured as described in Materials and Methods. The lines are from a linear regression analysis of the data.
As shown here, MGMT expression is a sensitive measure that can potentially be used to tailor current temozolomide-based treatment strategies to those patients most likely to respond to this alkylating agent. Furthermore, by identifying those individuals who are likely to be resistant to temozolomide, alternative therapeutic strategies can be developed to include novel combination treatments that incorporate additional reagents directed at overcoming resistance to temozolomide and/or apoptosis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
Correction: Article on Genomic and Molecular Profiling to Predict Response to Temozolomide

The article on genomic and molecular profiling to predict response to temozolomide in the January 15, 2009, issue of *Clinical Cancer Research* presented an incorrect Fig. 4A. The corrected Fig. 4A, Fig. 4A legend and text description are as follows.

**Results**

*Gene expression signature of predicted temozolomide resistance.* A subset of 10 cell lines was selected from the NCI-60 panel of cancer cell lines that represented two extremes of sensitivity to temozolomide; five of these cell lines were classified as “resistant” and five as “sensitive”. Using the gene expression profiles of these cell lines, we identified 150 genes that showed significantly different expression patterns between the “resistant” and “sensitive” cell lines—93 genes (red) were more highly expressed in the resistant than in the sensitive cell lines, whereas 57 genes (blue) were more highly expressed in the sensitive than in the resistant cell lines. A revised companion graph (Fig. S3) and a revised table of genes that make up the gene signature of sensitivity to temozolomide (Supplementary Table S4) are also provided.

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