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, O^6-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 IC_{50} 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 prediction of temozolomide sensitivity ($P < 0.005$). Notably, MGMT alone showed a significant correlation with temozolomide sensitivity (MGMT activity, $P < 0.0001$; *MGMT* expression, $P \leq 0.0001$). 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.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Data Deposition: Gene expression data are available at Gene Expression Omnibus accession no. GSE10916 (http://www.ncbi.nlm.nih.gov/geo/).

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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 O^6-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

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Translational Relevance

With the increasing incidence of melanoma and the historically poor response rates to traditional chemotherapy, it is important 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 O^6-methylguanine-DNA methyltransferase (MGMT). Our results suggest 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 temozolomide and those patients for whom combination therapy or alternative chemotherapeutic reagents might be desirable.

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 signatures, 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 instability, 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 melphalan purchased from Sigma-Aldrich. Stock solutions of temozolomide or melphalan were prepared in 100% DMSO and stored at -20°C until use; when added to cell cultures, the final DMSO concentration did not exceed 1%. WST-1 cell proliferation reagent was purchased from Roche Applied Science. Iscove's modified Dulbecco's medium and 0.05% trypsin-EDTA were purchased from Invitrogen/Life Technologies, Inc., and fetal bovine serum was purchased from Hyclone. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich.

Cell lines and cell culture. Twenty-six human melanoma-derived cell lines were used in these studies. Twenty-four of the cell lines were kindly provided by Dr. Hilliard Seigler (Duke University Medical Center, Durham, NC) and were derived from primary biopsies of metastatic melanoma obtained under a Duke University Institutional Review Board–approved protocol. The other two cell lines, A2058 and SkMel28, were obtained from American Type Tissue Collection. All cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1% penicillin/streptomycin and incubated at 37°C, 5% CO_2.

Cytotoxicity assay. The sensitivity of each cell line to temozolomide or melphalan was measured with a colorimetric assay using WST-1, a tetrazolium salt that is cleaved to formazan by mitochondrial dehydrogenases in metabolically active cells. Two protocols were used to assess temozolomide cytotoxicity: an "acute" assay and a "12-d" assay. For the acute assay, cells were plated at a density of 3,000 to 5,000 in 100 μL culture medium per well of a 96-well plate. After incubation overnight, a stock temozolomide solution was added to achieve the desired final concentrations. After 72-h incubation, 10 μL of WST-1 reagent were added to each well and the absorbance at 450 nm was measured 4 h later (Bio-Rad Benchmark Microplate reader). For the 12-d assay, 1×10^5 cells/mL were incubated in temozolomide for 2.5 h followed by plating into 96-well plates (100-500 cells/100 μL culture medium per well). The culture medium was changed after 6 d, and cell viability measured after 12 d as described above. For melphalan sensitivity, cells were plated (3,000-5,000 cells/100 μL per well of a 96-well plate), incubated overnight, and stock melphalan solution was added to the desired concentration. Cell viability was measured after 48-h incubation, as described above. Cell survival, defined as the absorbance of the treatment group divided by the absorbance of the control group, was plotted as a function of temozolomide or melphalan concentration, and the area under the dose-response curve (AUC_{drug}) was computed using GraphPad Prism v4.0 software over a concentration range of 0 to 1 mmol/L (acute assay) or 0 to 0.5 mmol/L (12-d assay) for temozolomide and 0 to 125 μmol/L for melphalan. The level of resistance to chemotherapy was defined as R (fraction resistant to drug), where

$$R = \mathrm{AUC}_{\mathrm{drug}}/\mathrm{AUC}_{\mathrm{max}}$$

AUC_{max} represents no loss of cell viability at the drug doses tested (AUC_{max} = 1 for temozolomide acute assay, AUC_{max} = 0.5 for temozolomide 12-d assay, and AUC_{max} = 125 for melphalan assay).

RNA isolation and gene expression profiling. RNA was isolated using the RNeasy Mini Kit from Qiagen according to the manufacturer's instructions. The RNA concentration was measured and an initial quality assessment obtained (A_{260}/A_{280} ratio). Before labeling and hybridization, the RNA quality was further assessed using the Agilent 2100 Bioanalyzer at the Duke University Microarray Core Facility where reverse transcription, labeling, and hybridization of the RNA samples were also done. Biotinylated cRNA targets were generated using a one-cycle eukaryotic target labeling assay, purified, and fragmented. The fragmented cRNA was hybridized to the Affymetrix Human Genome U133plus2 (HU133+2) GeneChip, which contains ~47,000 25-mer oligonucleotide probe sets for 38,000 characterized human genes. Each probe set contains 11 to 20 pairs of perfect match and mismatch oligonucleotides that differ by a single nucleotide. GeneChips were scanned and gene expression data preprocessed using the Affymetrix GeneChip Operating Software. After background adjustment and normalization, an expression value for each gene was calculated as the difference between the perfect match and mismatch oligonucleotides in each probe set.

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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 O^6-[^{3}H]methylguanine from a [^{3}H]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 (N^7 and O^6) 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 O^6-methylguanine released from the DNA substrate (femtomoles of O^6 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 ± SE.

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. See Supplementary Table S1 for primer sequences.

Analysis of MMR status by microsatellite instability. Mismatch repair status of 16 melanoma cell lines was determined by assessing the level of microsatellite instability (MSI). Briefly, multiplex PCR was carried out with genomic DNA to determine MSI at five mononucleotide markers, namely, BAT-26 (hMSH2; 120 bp), BAT-25 (c-kit; 124 bp), NR-21 (SLC7A8; 103 bp), NR-22 (transmembrane precursor protein B5; 142 bp), and NR-24 (ZNF-2; 132 bp). The PCR products were then separated by capillary electrophoresis using ABI Prism 3100 Genetic Analyzer (Applied Biosystems), and the data were analyzed with the GeneScan Analysis software to determine the MSI status. MSI in three or more markers was defined as MMR deficient, and MSI in two or fewer markers was considered MMR proficient.

Expression of MMR- and BER-associated genes. The expression of genes encoding proteins involved in MMR (*hMSH2*, *hMSH3*, *hMSH6*, *hMLH1*, and *hPMS2*) and BER (*POLB*, *XRCC1*, *APEX*, *APC*, *PARP1*, *PCNA*, *LIG1*, and *MPG*) were obtained from background-adjusted and normalized GeneChip gene expression data (see above).

Statistical analysis. Measured temozolomide resistance was plotted against MGMT activity, MGMT expression, or predicted temozolomide resistance. Correlation and linear regression and one-way ANOVA analyses were done using GraphPad Prism software (v4.0) to evaluate the relationship between measured parameters and the differences across cell lines, respectively.

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* ranged from very sensitive (DM738) to very resistant (DM443; ref. 20). 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 statistically insignificant differences between the cell lines with respect to their sensitivity to temozolomide (one-way ANOVA using GraphPad Prism v4.0: P = 0.37, r^2 = 0.39). In contrast, the *in vitro* sensitivity of these cell lines determined with the 12-day assay showed a broader spectrum of response ranging from very sensitive ($R \leq 0.3$; DM440) to very resistant ($R \geq 0.9$; DM443 and DM6; one-way ANOVA analysis comparing cell lines: P < 0.0001, r^2 = 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 ($R \leq 0.3$) to very resistant ($R \geq 0.9$; 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 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, r^2 = 0.6) between measured temozolomide resistance and MGMT activity.

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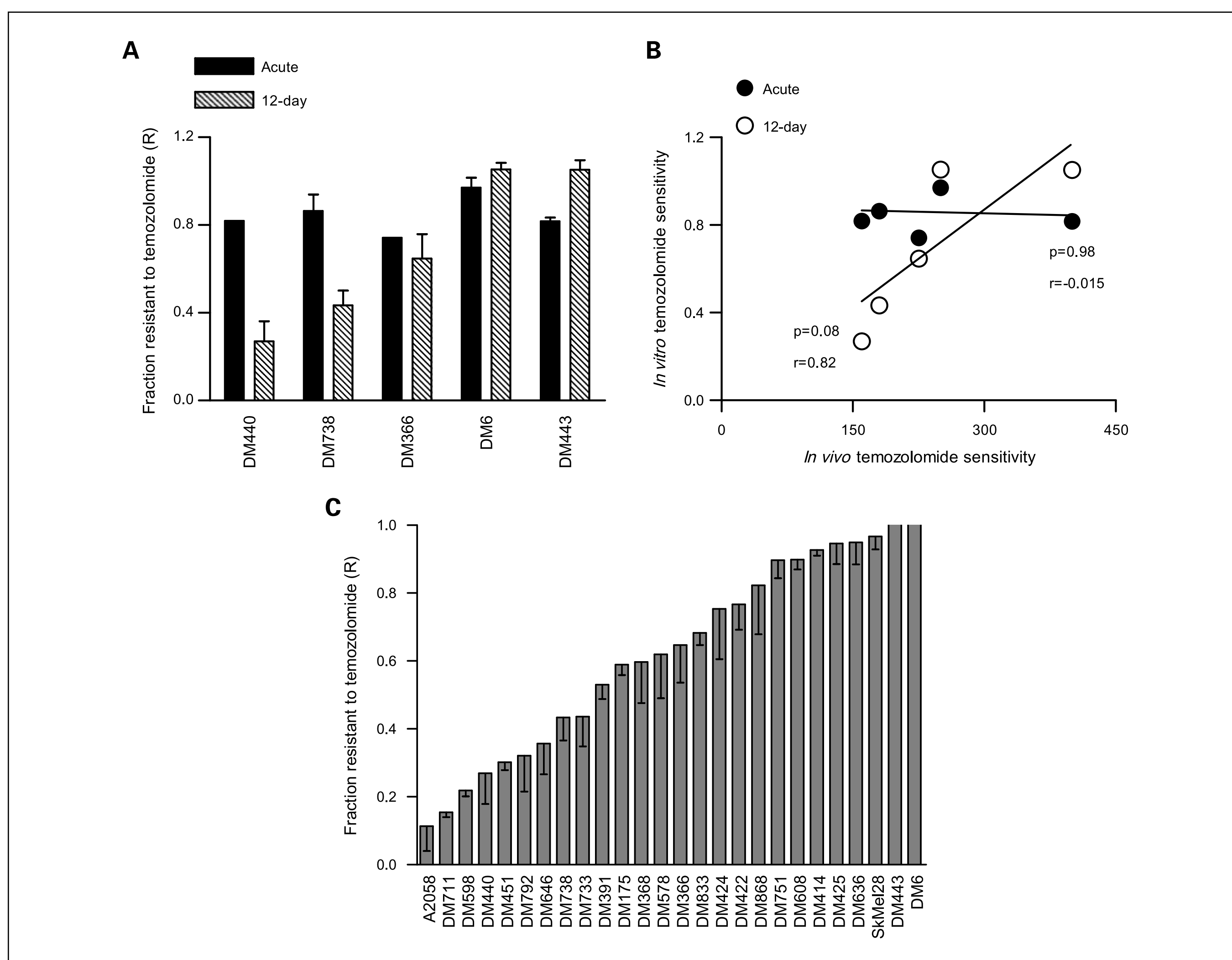

Fig. 1. *A,* temozolomide resistance in five melanoma-derived cell lines in response to a 72-h acute (*solid columns*) or 12-d (*hatched columns*) *in vitro* cytotoxicity assay. AUC was measured up to 1.0 mmol/L (acute) or 0.5 mmol/L (12-d) temozolomide and normalized (see Materials and Methods). Columns, mean of three experiments for each cell line; bars, SE. *B,* sensitivity to isolated limb infusion with temozolomide was measured in an animal model of extremity melanoma. Response to temozolomide was measured as percent change in tumor volume at day 30 (*in vivo* sensitivity). This is plotted against *in vitro* sensitivity (fraction resistant to temozolomide, *R*) measured either acutely (*filled symbols*) or using the 12-d assay (*open symbols*). *In vitro* sensitivity correlated strongly correlated with *in vivo* sensitivity measured with the 12-d assay, but not with the acute assay. The lines are from a linear regression analysis of the data. *C,* temozolomide resistance measured using the 12-d assay in 26 melanoma-derived cell lines. Temozolomide resistance was measured as described above. Columns, mean of at least three experiments for each cell line; bars, SE.

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 [$P \leq 0.0001$, $r^2 = 0.46$ (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

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"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

Cell line	Measured temozolomide resistance	MGMT activity (fmol/mg protein)	MGMT expression (qPCR)	MGMT expression (204880_at*)	Predicted temozolomide resistance	MGMT methylation status	MSI status
A2058	0.113	14.77	0	12.6	0.137	M/U	ND
DM711	0.155	0	0	5.7	0.267	M/U	MSS
DM598	0.218	6.32	0	62.1	0.132	U	ND
DM440	0.270	35.69	0.083	35.5	0.405	M/U	MSS
DM451	0.302	28.67	0.038	271.2	0.288	U	ND
DM792	0.321	0	0	5.4	0.855	M/U	MSS
DM646	0.357	9.04	0	8.5	0.458	M/U	MSS
DM738	0.434	0.000	0	29.7	0.609	M/U	MSS
DM733	0.437	0.0	0	57.7	0.181	M/U	MSS
DM391	0.530	139.6	0.080	440.3	0.197	U	MSS
DM175	0.590	486.18	0.311	582.4	0.248	M/U	MSS
DM368	0.597	275.57	0.225	407.9	0.194	U	ND
DM578	0.620	228.88	0.200	445.3	0.749	M/U	MSS
DM366	0.647	373.2	0.233	328.0	0.396	U	MSS
DM833	0.683	268.71	0.165	375.3	0.652	M/U	MSS
DM424	0.754	721.27	0.490	675.6	0.268	U	ND
DM422	0.767	92.21	0.065	211.3	0.228	U	MSS
DM868	0.823	692.72	0.700	617.8	0.806	U	ND
DM751	0.897	292.34	0.300	415.4	0.860	M/U	MSS
DM608	0.899	333.75	0.329	425.6	0.726	M/U	MSS
DM414	0.927	980.25	1.155	939.7	0.866	U	ND
DM425	0.946	1033.3	0.770	877.6	0.611	U	ND
DM636	0.949	489.17	0.480	663.4	0.582	M/U	MSS
SkMel28	0.967	272.26	0.160	500.1	0.276	U	ND
DM443	1.000	636.35	0.195	713.5	0.813	M/U	ND
DM6	1.000	668.25	0.459	725.8	0.831	M/U	MSS

Abbreviations: qPCR, quantitative PCR; M, methylated; U, unmethylated; ND, not determined.
*Affymetrix GeneChip Hu133 plus 2 Probe ID number.

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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

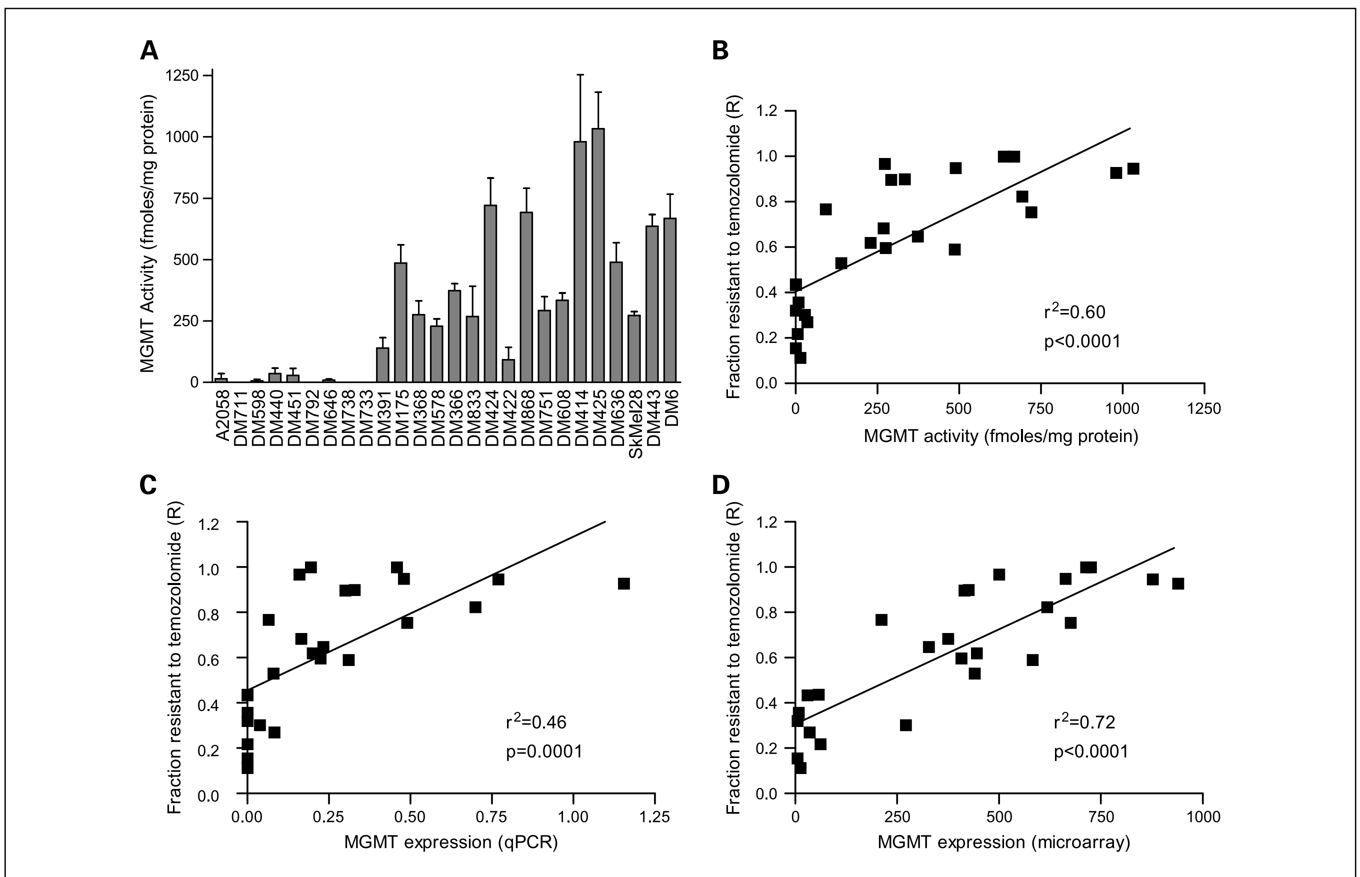

Fig. 2. *A,* MGMT activity in the panel of 26 melanoma cell lines, measured as described in Materials and Methods. Columns, mean of at least three measurements; bars, SE. Cell lines are plotted in order of increasing measured resistance to temozolomide. *B,* MGMT activity plotted as a function of measured temozolomide resistance (fraction resistant to temozolomide, *R*). The line is from a linear regression analysis of all the points (r^2 = 0.60 and 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 and P = 0.0001 for quantitative PCR and r^2 = 0.72 and P < 0.0001 for microarray).

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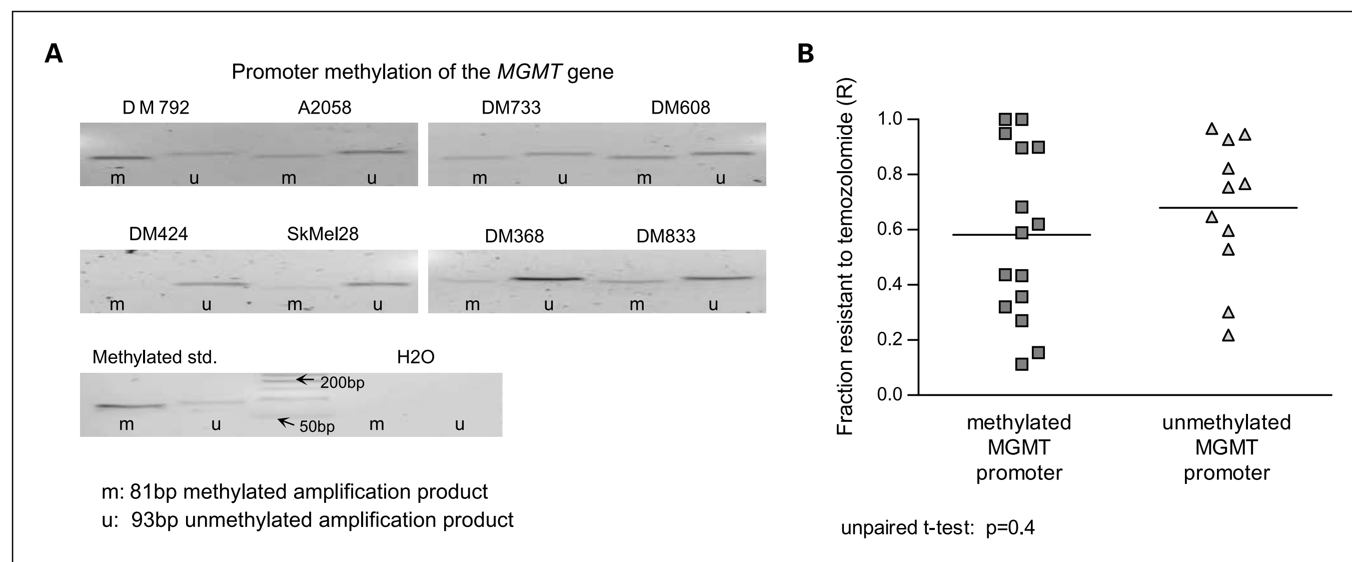

Fig. 3. *A,* methylation status of eight melanoma cell lines as determined using methylation-specific PCR. Following sodium bisulfite conversion of the DNA, methylated and unmethylated sequences were amplified by PCR, and the products separated on a 4% agarose gel. u, unmethylated (93 bp); m, methylated (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.

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 O^6-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 O^6-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.

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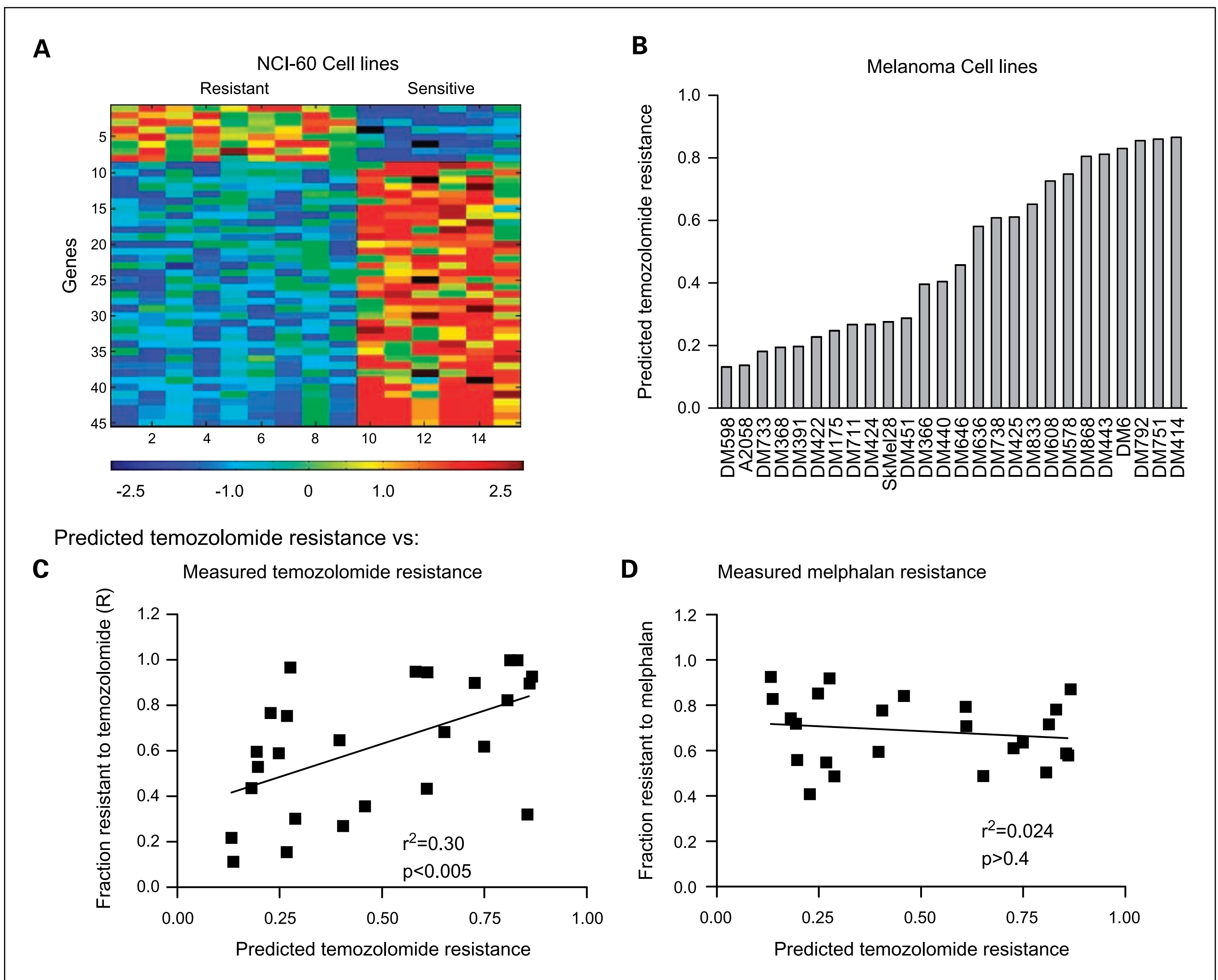

Fig. 4. *A,* gene expression signature of temozolomide resistance derived from 15 cell lines in the NCI-60 panel, showing a heatmap representation of genes that are differentially expressed across temozolomide-sensitive and temozolomide-resistant cell lines. Red and blue, genes that are expressed at 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.

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^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^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).

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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.

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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.

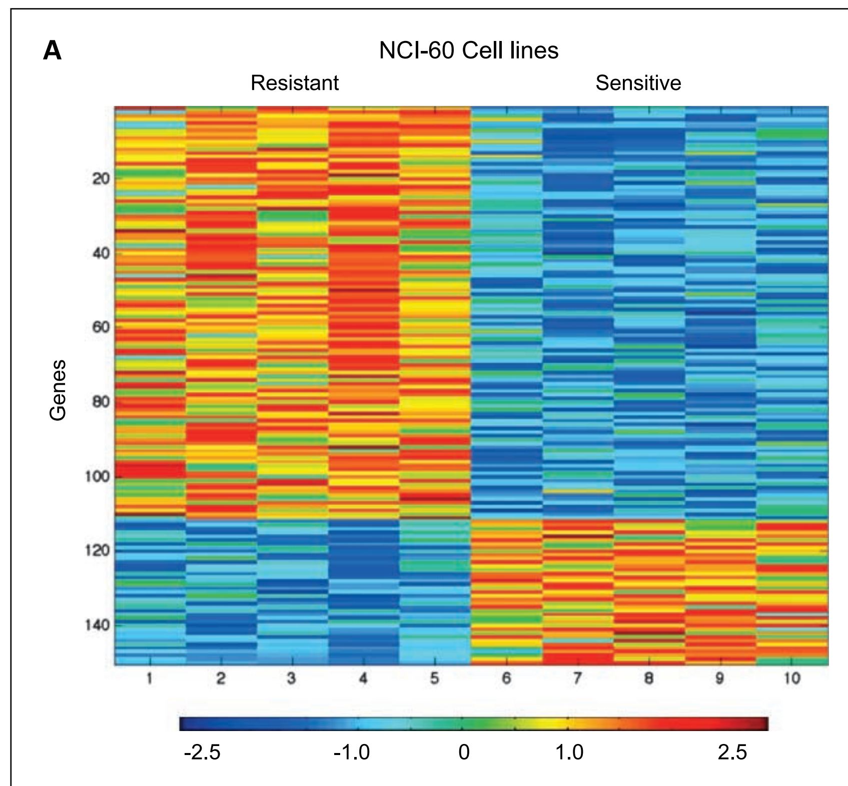

Fig. 4. *A,* gene expression signature of temozolomide resistance derived from 10 cell lines in the NCI-60 panel, showing a heatmap representation of genes that are differentially expressed across temozolomide-sensitive and temozolomide-resistant cell lines. Red and blue, genes that are expressed at high and low levels, respectively. The scale below shows the fold change difference in expression across the color spectrum.

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.

Augustine CK, Yoo JS, Potti A, Yoshimoto Y, Zipfel PA, Friedman HS, Nevins JR, Ali-Osman F, Tyler DS. Genomic and molecular profiling predicts response to temozolomide in melanoma. Clin Cancer Res 2009;15:502 – 10.

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Genomic and Molecular Profiling Predicts Response to Temozolomide in Melanoma

Christina K. Augustine, Jin Soo Yoo, Anil Potti, et al.

Clin Cancer Res 2009;15:502-510.

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