Induction of MGMT expression is associated with temozolomide resistance in glioblastoma xenografts

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Temozolomide (TMZ)-based therapy is the standard of care for patients with glioblastoma multiforme (GBM), and resistance to this drug in GBM is modulated by the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT). Expression of MGMT is silenced by promoter methylation in approximately half of GBM tumors, and clinical studies have shown that elevated MGMT protein levels or lack of *MGMT* promoter methylation is associated with TMZ resistance in some, but not all, GBM tumors. In this study, the relationship between MGMT protein expression and tumor response to TMZ was evaluated in four GBM xenograft lines that had been established from patient specimens and maintained by serial subcutaneous passaging in nude mice. Three *MGMT* unmethylated tumors displayed elevated basal MGMT protein expression, but only two of these were resistant to TMZ therapy (tumors GBM43 and GBM44), while the other (GBM14) displayed a level of TMZ sensitivity that was similar in extent to that seen in a single *MGMT* hypermethylated line (GBM12). In tissue culture and animal studies, TMZ treatment resulted in robust and prolonged induction of MGMT expression in the resistant GBM43 and GBM44 xenograft lines, while MGMT induction was blunted and abbreviated in GBM14. Consistent with a functional significance of MGMT induction, treatment of GBM43 with a protracted low-dose TMZ regimen was significantly less effective than a shorter high-dose regimen, while survival for GBM14 was improved with the protracted dosing regimen. In conclusion, MGMT expression is dynamically regulated in some *MGMT* nonmethylated tumors, and in these tumors, protracted dosing regimens may not be effective. *Neuro-Oncology 11, 281–291, 2009 (Posted to Neuro-Oncology [serial online], Doc. D08-00172, October 24, 2008. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2008-090)*

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Glioblastoma multiforme (GBM) tumors are highly malignant brain tumors that remain a major treatment challenge in oncology. Historically, these tumors have been treated with maximal surgical debulking followed by external beam radiation therapy. Survival can be further extended by adding the alkylator temozolomide (TMZ) to the treatment regimen, and concomitant TMZ and radiation followed by adjuvant TMZ is now the standard of care for patients with GBM. Despite this aggressive combined modality approach, the 2-year survival for GBM patients remains only 25%, and few patients survive beyond 5 years.[1] Understanding the molecular mechanisms that contribute to this resistant phenotype will provide important insight for developing novel therapeutic approaches and for identifying those patients most likely to benefit from a specific treatment regimen.

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TMZ resistance in previously untreated GBM patients is mediated predominantly by the O^6-methylguanine-DNA methyltransferase (MGMT) protein.[2–4] Although methylation at the O^6 position of guanine accounts for less than 10% of DNA adducts formed by TMZ, unrepaired O^6-methylguanine lesions are highly cytotoxic.[5,6] O^6-methylguanine lesions are removed in a single-step, irreversible reaction mediated by MGMT. Once the methyl group is transferred from guanine to the active cysteine residue of MGMT, the protein is no longer functional and is degraded.[7,8] Consistent with the importance of this repair activity, tumor cells lacking MGMT activity are significantly more sensitive to the cytotoxic effects of TMZ than are cells expressing functional MGMT.[9–12] In general, high tumor MGMT protein expression in patient samples is associated with TMZ resistance in GBM patients, although this association is not completely predictive of individual tumor response.[13] MGMT expression is commonly suppressed in tumors by CpG methylation within the *MGMT* promoter, and tumor *MGMT* hypomethylation is associated with TMZ resistance in patients with GBM.[3,4,14,15] However, similar to clinical analyses of MGMT protein expression, approximately 15% of patients with *MGMT* hypomethylation derive benefit from TMZ therapy.[4]

The available data suggest that MGMT activity is mechanistically linked to TMZ resistance, and that *MGMT* promoter hypomethylation or MGMT protein expression may be useful prognostic markers associated with clinical TMZ resistance. Unfortunately, neither MGMT protein expression levels nor *MGMT* promoter methylation status is a sufficiently accurate predictor of TMZ responsiveness to decide a priori whether a patient should receive TMZ-based therapies. To better understand the limitations of either assay in identifying TMZ-resistant tumors, we used the Mayo GBM xenograft panel of serially transplantable GBM xenografts established from patient tumor samples to investigate relationships between TMZ sensitivity and MGMT protein. TMZ treatment resulted in variable induction of MGMT repair activity among *MGMT* hypomethylated tumors, and the extent of MGMT induction was associated with TMZ resistance. This observation has important clinical implications relative to the potential for altered TMZ dosing regimens to overcome TMZ resistance.

Materials and Methods

GBM Xenograft Panel

The establishment and maintenance of the human GBM xenograft panel used in this study have been described previously.[16,17] Briefly, patient tumor specimens were implanted into the flank of nude mice and serially passaged as heterotopic tumor to maintain these xenograft lines exclusively in animals.

MGMT *Promoter Methylation Assay*

DNA was extracted from flank GBM xenograft tissues using the Gentra DNA extraction kit (Puregene, Minneapolis, MN, USA). Isolated tumor DNA was treated with bisulfite according to a protocol supplied with the CpGenome bisulfite modification kit (Chemicon Inc., Temecula, CA, USA). The modified DNA was amplified using primers specific for both the methylated and the nonmethylated *MGMT* promoter sequences as described previously.[18,19] The predicted fragment size is 93 bp for methylated PCR and 100 bp for nonmethylated PCR. The PCR conditions were 95°C (30 s), 59°C (30 s), and 72°C (60 s) for 40 cycles, with an initial denaturation step at 95°C for 12 min and final elongation at 72°C for 10 min. All methylation-specific PCR (MS-PCR) products were visualized on 3% ethidium bromide–stained agarose gels.

Primary Cell Cultures

Flank glioblastoma tissues were minced and mechanically disaggregated and subsequently cultured on flasks (BD Biosciences, Franklin Lakes, NJ, USA) coated with growth factor–reduced Matrigel (Fisher Scientific, Hampton, NH, USA). Cells were washed 1–5 days after plating to remove debris and then maintained under a low-serum condition (Dulbecco's modified Eagle's medium [DMEM]/1% fetal bovine serum, 1% penicillin/streptomycin) to select against murine fibroblast growth. Using human- and mouse-specific fluorescence in situ hybridization DNA probes, we found that maintenance of cells in low serum for 2 weeks eliminated contaminating murine fibroblasts (data not shown). After 2 weeks, serum concentrations were increased to 10%, and these cells were then used for in vitro analyses.

Western Blot Assay

Proteins were extracted from flank GBM tissues or primary cell cultures in a radioimmunoprecipitaton assay (RIPA) lysis buffer (50 mM Tris HCl [pH 8.0], 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]; Sigma, St. Louis, MO, USA) supplemented with a cocktail of protease inhibitors (Roche, Indianapolis, IN, USA), incubated for 15 min on ice, and then clarified by centrifugation. Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and then electrotransferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). Membranes were blocked for 1 h in 5% skim milk dissolved in phosphate-buffered saline containing 0.1% Tween 20 and incubated overnight at 4°C with a murine anti-MGMT monoclonal antibody (NeoMarkers, Fremont, CA, USA) diluted in the same blocking buffer. After washing, membranes were incubated with goat antimouse (Pierce, Rockford, IL, USA) antibodies conjugated to horseradish peroxidase. Blots were developed with SuperSignal chemiluminescence reagent (Pierce). For loading control, membranes were

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stripped and reprobed with a human-specific antibody against β-actin (Sigma).

In Vivo TMZ Response

The effects of TMZ therapy on survival prolongation in mice with established orthotopic xenografts were used to assess the sensitivity of GBM xenografts to TMZ. Short-term primary cell cultures were injected (3×10^5 cells per mouse, suspended in 10 μl) into the right basal ganglia of anesthetized athymic nude mice (Athymic Ncr-nu/nu: NCI-Frederick, Frederick, MD, USA) using a small-animal stereotactic frame (ASI Instruments, Houston, TX, USA). Just before treatment initiation, animals were randomized to treatment groups of six to eight mice each. Treatment was initiated 2 weeks prior to the time mice were expected to become moribund, as determined from previous studies with each xenograft line. TMZ was purchased from the Mayo Clinic pharmacy and suspended in Ora-plus (Paddock Laboratories, Minneapolis, MN, USA) for oral administration. All doses were administered by oral gavage. Mice were monitored daily and euthanized when they reached a moribund state. Animals were maintained in a pathogen-free environment with a standard rodent diet and water. All experiments were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

In Vitro TMZ Cytotoxicity Assay

Reagent-grade TMZ was obtained from the NCI Developmental Therapeutics Program and dissolved in dimethyl sulfoxide at a concentration of 100 mM. Short-term cell cultures were plated in a 96-well plate (1,500/well) in quadruplicate wells and treated with graded concentrations of TMZ 24 h later. In the indicated experiments, TMZ was added to the culture media a total of three times without changing media. Cells were incubated in standard culture conditions for 96 h, and cell number was then assessed by staining cells with methylene blue and measuring the absorbance (optical density) at 650 nm. Where indicated, cells were treated with 10 μM of the MGMT inhibitor O^6-benzylguanine (O6BG; Sigma) concurrently with the first dose of TMZ.

Effect of TMZ on the Expression of MGMT

To examine the effect of TMZ on the expression of MGMT in vitro, cells were plated in 100-mm petri dishes (BD Biosciences) and allowed to grow to 70%–80% confluence. Cells were washed in phosphate-buffered saline followed by incubation in serum-free medium (DMEM supplemented with 0.1% bovine serum albumin) for 48 h. Cells were then treated with a single dose of TMZ (100 μM) and harvested at the indicated time points. To evaluate the effect of TMZ on the expression of MGMT in vivo, we used flank xenografts. Briefly, GBM14 and GBM43 cells were injected in the flanks of athymic nude mice (NCI-Frederick), and once tumors reached 1 cm in diameter, mice were treated orally with TMZ at 50 mg/kg/day × 5 days and sacrificed on days 1, 4, and 7 after initiation of treatment. Vehicle-treated mice received the vehicle Oral-plus. Immediately after sacrifice, tumor tissues were snap-frozen in liquid nitrogen followed by storage at –80°C until analysis. Two mice were evaluated at each time point.

MGMT Activity Assay

An oligonucleotide-based repair assay (Sigma) was used to monitor the levels of MGMT repair activity in tumor cell lysates. In this assay, lysates are incubated with an 18-bp ^{32}P-labeled oligonucleotide substrate containing a single O^6-methylguanine residue located within a *Pst*I restriction site. *Pst*I will cut only nonmethylated DNA, which allows the extent of the demethylation reaction to be evaluated conveniently using this restriction endonuclease. Following manufacturer instructions, tumor tissues were suspended in MGMT substrate buffer (50 mM Tris-HCl, 1 mM dithiothreitol, 0.5 mM EDTA, 5% glycerol [pH 8.0]), pulse-sonicated on ice five times, and then clarified by centrifugation. Equal amounts of protein were incubated with 0.2 pmol oligonucleotide substrate at 37°C for 2 h. Oligonucleotide DNA then was isolated and digested with *Pst*I (Roche Molecular, Indianapolis, IN, USA). After formamide loading buffer (5 μl) was added to each sample, they were heated at 95°C for 5 min and then resolved by polyacrylamide gel electrophoresis. The gel was directly exposed to film for 3 days at –80°C.

RNA Isolation and Real-Time Reverse Transcriptase PCR

Total cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA). RNA concentration was determined by spectrophotometric absorbance at 260 nm, and the quality was determined by Agilent Bioanalyzer software (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription (RT) PCR was performed with 2 μg of the isolated total RNA and synthesized to cDNA in a 20-μl reaction system using reverse transcriptase (Promega, Madison, WI, USA). RT conditions were 5-min denaturation at 70°C, 60 min at 37°C, and 5 min at 75°C, in a thermocycler. Reverse transcription was carried out with 0.5 μg random primer (Invitrogen), 1 U reverse transcriptase, 1 U RNase inhibitor (Invitrogen), and 2 μM deoxyribonucleoside triphosphates in RT buffer. The resulting RT product was diluted 1:4 in RNase-free double-distilled H_2O, and 1 μl was used for real-time PCR. The primers and probes used for quantitation of MGMT and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) endogenous control were purchased from Applied Biosystems (Foster City, CA; Assay-On-Demand: catalogue nos. Hs.00172470_m1 for MGMT and Hs.99999905_m1 for GAPDH).

The real-time PCR was performed on the Applied Biosystems Prism 7900 PCR and detection instrument. The real-time PCR reactions were performed on plates using adhesive seals as covers. A master mix was pre-

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pared for the target (MGMT) and endogenous control (GAPDH) in a 50-μl reaction, using TaqMan Universal PCR Master Mix (Applied Biosystems). The real-time PCR was programmed as follows: 95°C for 10 min, followed by 95°C for 15 s and 60°C for 1 s cycled 40 times. Each sample was amplified in triplicate. A no-template control for each master mix was always included in the experiments. The relative MGMT mRNA expression was determined using SDS RQ 1.2 software (Applied Biosystems). For this analysis, GAPDH was used as the endogenous control and the MGMT mRNA expression level in untreated cells or vehicle-treated mice was used as a calibrator.

Statistical Analysis

Cumulative survival probabilities were estimated using the Kaplan-Meier method. The log-rank test was used to compare survival across groups. Different treatment regimens were compared across experiments by pooling the survival data from individual experiments and then using the log-rank test to compare survival across treatment experiments. Differences in cell survival for the in vitro studies were calculated using a two-sample *t*-test or rank sum test, as appropriate. In all cases, *p*-values < 0.05 were considered statistically significant.

Results

MGMT *Promoter Methylation Status in Glioblastoma Xenografts*

The Mayo GBM xenograft panel was established by direct implantation of patient samples and subsequent serial subcutaneous propagation in nude mice, and these xenograft lines then can be used to establish intracranial xenografts for therapy evaluations. *MGMT* promoter methylation status was evaluated for all members of the xenograft panel, and four xenograft lines were selected for further study: tumor GBM12 with methylated *MGMT* and tumors GBM14, GBM43, and GBM44 with nonmethylated *MGMT* (Fig. 1A). In parallel with *MGMT* methylation analyses, we examined the expression of MGMT protein in the same tumor specimens (Fig. 1B). The xenograft with *MGMT* promoter methylation (GBM12) expressed low levels of MGMT protein, while the three xenografts with *MGMT* promoter hypomethylation (GBM14, -43, and -44) expressed higher levels of this protein.

MGMT *Promoter Methylation and In Vivo TMZ Response*

The influence of TMZ treatment on survival was evaluated in mice with intracranial tumors established from the four tumor cell lines (Fig. 2). Similar to the dosing regimen used for adjuvant therapy in patients, TMZ was administered daily for 5 days by oral gavage. The *MGMT* methylated GBM12 xenograft was highly sensitive to TMZ with a 51-day prolongation in median survival ($p < 0.0001$). In contrast, two of the nonmethylated tumors were relatively resistant to TMZ therapy, with a 25-day prolongation and a 6-day reduction in median survival for GBM43 ($p = 0.003$) and GBM44 ($p = 0.81$), respectively. Interestingly, GBM14 displayed high sensitivity to TMZ therapy (70-day prolongation in median survival, $p < 0.0001$; Table 1) despite the hypomethylated status of the *MGMT* promoter and moderate basal levels of MGMT protein.

In Vitro Sensitivity to TMZ

In parallel with the in vivo survival studies, the sensitivity of the xenograft lines was evaluated in cell culture. In distinct contrast to the animal studies, the GBM14-derived cells showed a similar level of TMZ resistance as the other *MGMT* nonmethylated tumor lines 96 h after a single exposure to TMZ, while only the methylated GBM12 cell line was significantly sensitive to TMZ (Fig. 3). Specifically, cell survival after treatment with 100 μM TMZ was 39% for GBM12 versus 80%, 83%, and 88% for GBM14, GBM43, and GBM44, respectively (significance vs. GBM14: $p < 0.001$ for GBM12, $p = 0.76$ for GBM43, and $p = 0.20$ for GBM44). To address the possibility that differences in dosing schedule between the in vivo studies (daily × 5 days) and the in vitro study (single dose) might influence TMZ responsiveness, the in vitro experiments were repeated with GBM14 and GBM43 using three doses of TMZ administered either every 6 h (12 h total) or every 24 h (48 h total). Similar to the results obtained with a single TMZ exposure, both

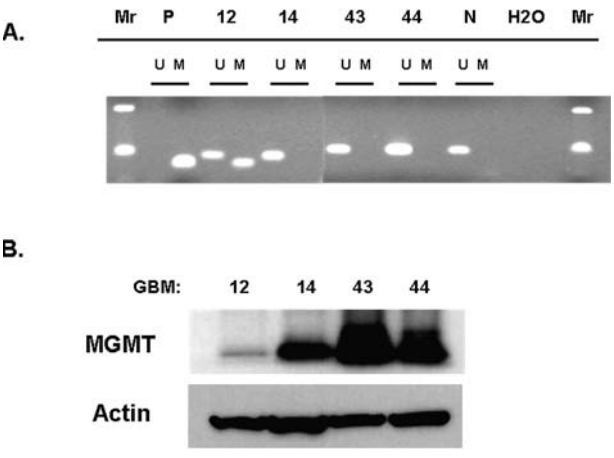

Fig. 1. O^6-Methylguanine-DNA methyltransferase (*MGMT*) promoter methylation and protein expression in glioblastoma multiforme (GBM) xenografts. Flank tumor specimens for the indicated xenograft lines were processed for assessment of *MGMT* promoter methylation by methylation-specific PCR (MS-PCR) (A) and for MGMT protein expression by Western blotting (B). For the MS-PCR reaction, universally methylated DNA and normal brain DNA were used as positive (P) and negative (N) controls, respectively. The MS-PCR amplification products were resolved by electrophoresis, and the corresponding ethidium bromide–stained gel is shown. Abbreviations: Mr, relative molecular weight markers; U, unmethylated; M, methylated. GBM12 is the only *MGMT* methylated tumor line.

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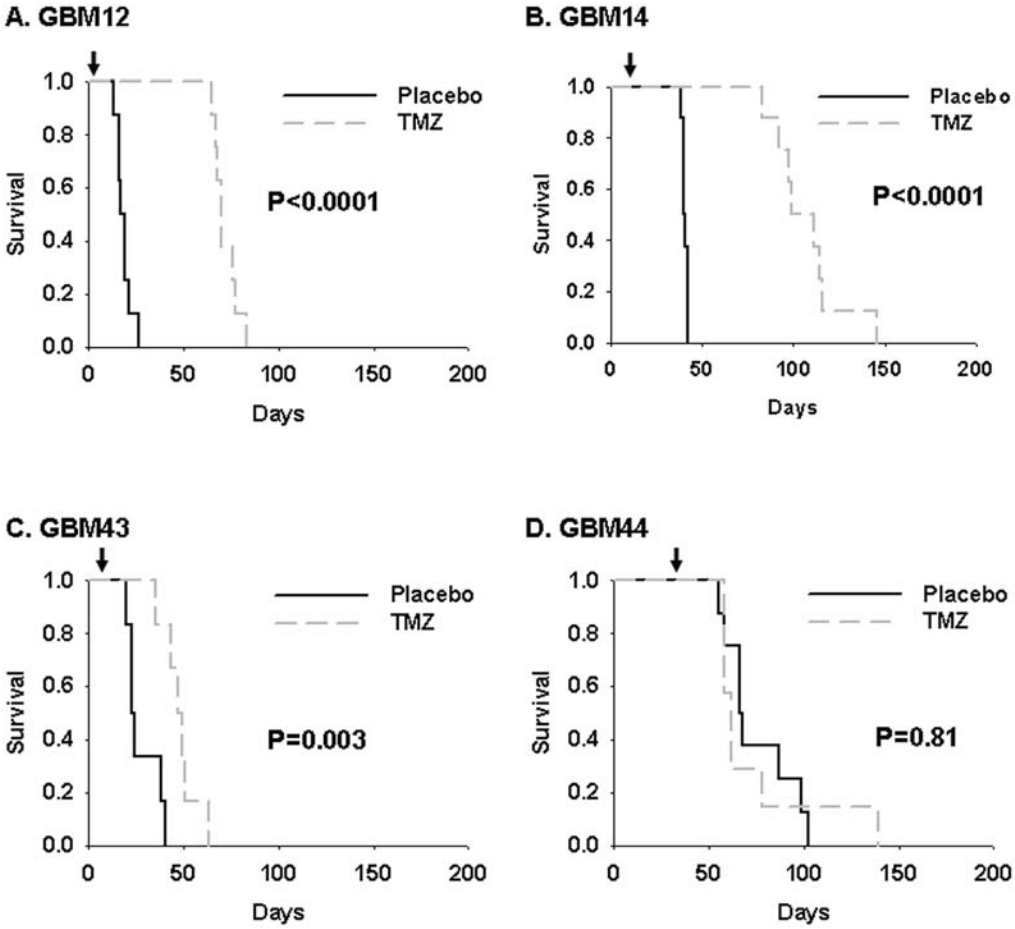

Fig. 2. Effects of temozolomide (TMZ) therapy on survival. Mice with established orthotopic xenografts for the indicated glioblastoma multiforme (GBM) tumor lines were randomized to treatment with vehicle or 120 mg/kg TMZ daily for 5 days. Mice were euthanized once they became moribund. Arrows denote when treatment was initiated. (A) GBM12 (methylated; $p < 0.0001$). (B) GBM14 (unmethylated; $p < 0.0001$). (C) GBM43 (unmethylated; $p = 0.003$). (D) GBM44 (unmethylated; $p = 0.81$).

tumor lines were resistant to three doses of TMZ delivered over 12 h (Fig. 4A), with 83% and 84% survival on day 5 in GBM14 and GBM43, respectively ($p = 0.67$ comparing survival for GBM14 vs. GBM43). To determine whether MGMT activity was an important contributor to TMZ resistance in these xenograft lines, cells were treated concurrently with TMZ and the MGMT inhibitor O6BG. O6BG significantly enhanced TMZ cytotoxic effects in cell cultures of each tumor treated with the short, 12-h regimen: survival with TMZ + O6BG was 56% for GBM14 ($p = 0.005$ relative to TMZ only) and 63% for GBM43 ($p = 0.0004$ relative to TMZ only). Thus, with either a single dose or repeated doses delivered over 12 h, GBM14 was relatively resistant to TMZ therapy in vitro, while GBM14 was highly sensitive to TMZ delivered over 5 days in vivo.

Table 1. Survival prolongation for temozolomide (TMZ)-treated relative to vehicle-treated glioblastoma multiforme (GBM) xenografts

GBM Tumor Line	Median Survival (Days)		Relative Survival Prolongation[a]	*p*-Value
	Vehicle Treated	TMZ Treated		
GBM12	18	70	3.9	$p < 0.0001$
GBM14	41	105	2.6	$p < 0.0001$
GBM43	23.5	48	2.0	$p = 0.003$
GBM44	67	62	0.9	$p = 0.903$

[a]Determined by a ratio of median survival in TMZ-treated versus vehicle-treated tumors.

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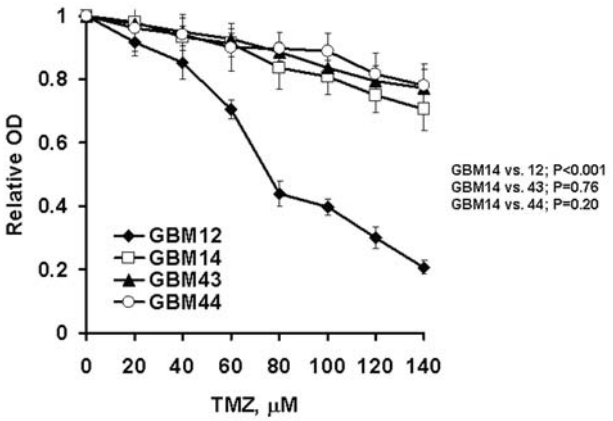

Fig. 3. In vitro temozolomide (TMZ) cytotoxicity. Short-term cell cultures from the indicated glioblastoma multiforme (GBM) xenograft lines were treated with a single dose of TMZ, and the effects on cell survival were evaluated 96 h later using a methylene blue staining assay. Mean ± SD for relative optical density (OD) versus TMZ concentrations are plotted from three independent experiments. Significance compared with GBM14: GBM12, $p < 0.001$; GBM43, $p < 0.76$; GBM44, $p < 0.20$.

In contrast to the short in vitro TMZ regimen, GBM14 was significantly more sensitive to three doses of TMZ delivered in a protracted regimen over 48 h (60% survival) compared with GBM43 (85% survival, $p < 0.001$ comparing survival for GBM14 vs. GBM43; Fig. 4B). Interestingly, inhibition of MGMT with O6BG in GBM14 did not further sensitize cells to the prolonged 48-h TMZ regimen (58% survival for TMZ + O6BG, 60% for TMZ only; $p = 0.71$), while O6BG did enhance the efficacy of this regimen in GBM43 (63% survival for TMZ + O6BG, 85% for TMZ only; $p = 0.0002$). Thus, dosing schedule was an important determinant of TMZ sensitivity related to MGMT activity in GBM14.

MGMT Induction after TMZ Treatment

The sensitizing effects of O6BG in GBM43 but not GBM14, when combined with protracted TMZ dosing, suggested that MGMT activity might be differentially suppressed in GBM14 relative to the other xenograft lines. To test this hypothesis, short-term cell cultures from each of the four xenograft lines were treated with 100 μM TMZ and then processed for Western blot analysis of MGMT at various time points after treatment. Interestingly, distinct MGMT expression responses were observed among the four tumor cell cultures: graded increases in MGMT expression were observed in GBM43 and GBM44, whereas MGMT protein levels were suppressed in GBM14 (Fig. 5A). As expected, there was no significant MGMT induction in GBM12, which has silenced MGMT expression due to *MGMT* promoter hypermethylation (data not shown). The increased MGMT protein corresponded with a 2.0 ± 0.1-fold increase in MGMT mRNA levels in TMZ-treated GBM43 cells over the course of 48 h ($p = 0.005$; Fig. 5B).

Increased MGMT Activity after In Vivo TMZ Treatment

On the basis of these in vitro studies, the in vivo effects of TMZ on MGMT activity was evaluated in GBM14 and GBM43 flank xenografts using an MGMT repair

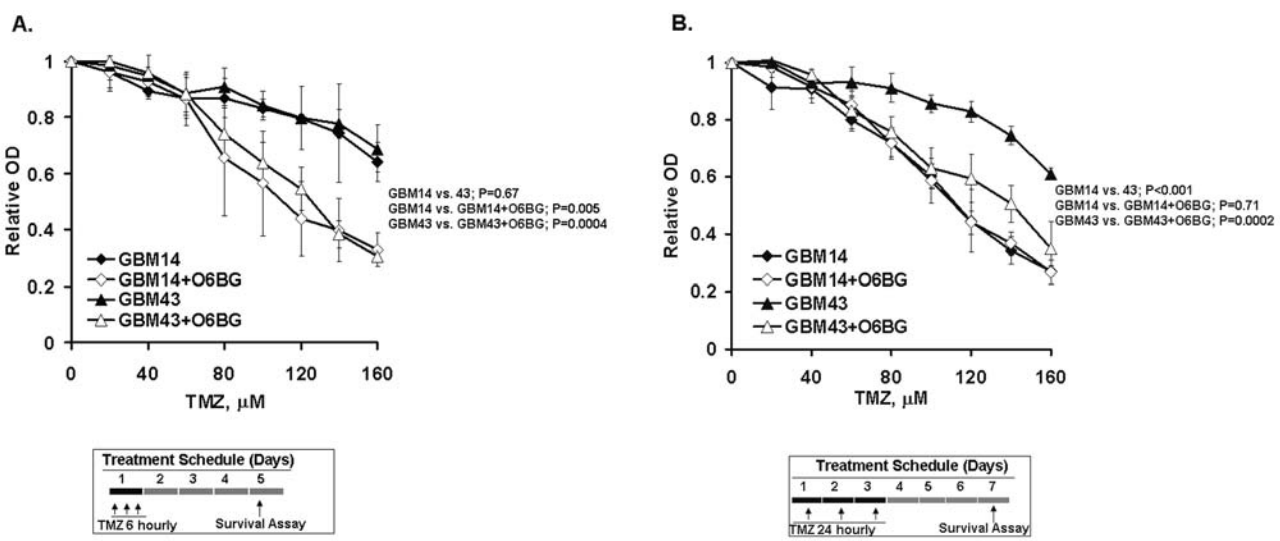

Fig. 4. Short versus protracted dosing regimens. Short-term cell cultures from glioblastoma multiforme (GBM) tumors GBM14 and GBM43 were treated with three doses of 100 μM temozolomide (TMZ) delivered once every 6 h (A) or once every 24 h (B). Where indicated, cultures were also dosed once with 10 μM O^6-benzylguanine (O6BG) concurrently with the first dose of TMZ. Survival was evaluated with a methylene blue survival assay 96 h after the last dose of TMZ. Mean ± SD for relative optical density (OD) versus TMZ concentrations are plotted from two independent experiments. Survival analyses: (A) GBM14 versus GBM43, $p = 0.67$; GBM14 TMZ + O6BG versus TMZ only, $p = 0.005$; GBM43 TMZ + O6BG versus TMZ only, $p = 0.0004$. (B) GBM14 versus GBM43, $p = 0.001$; GBM14 TMZ + O6BG versus TMZ only, $p = 0.71$; GBM43 TMZ + O6BG versus TMZ only, $p = 0.0002$.

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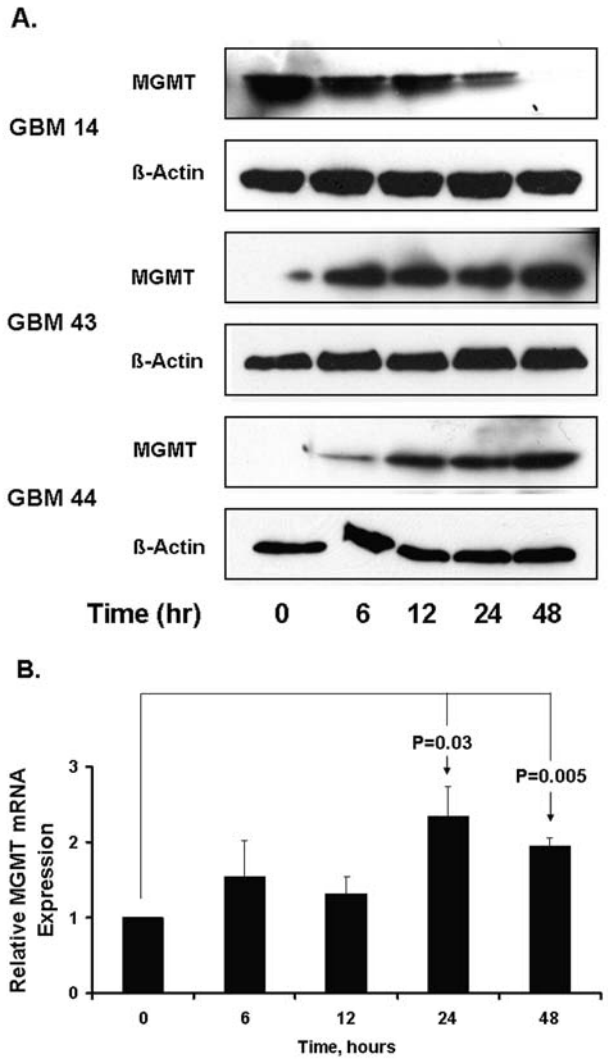

Fig. 5. Effects of temozolomide (TMZ) treatment on O^6-methylguanine-DNA methyltransferase (MGMT) expression in vitro. Short-term cell cultures for glioblastoma multiforme (GBM) tumors GBM12, -14, -43, and -44 were treated with 100 μM TMZ and harvested at the indicated time points. (A) Samples were processed for Western blotting for MGMT and then β-actin. (B) Total RNA from GBM43 was reverse transcribed, and the effect of TMZ treatment on MGMT mRNA was evaluated using real-time PCR. The data from three independent experiments performed in triplicate are presented as mean ± SD.

assay. Consistent with results of the in vitro studies, MGMT repair activity increased 13-fold in TMZ-treated GBM43 compared with vehicle-treated control tumors. In contrast, TMZ-treated GBM14 tumors showed no induction of MGMT activity (Fig. 6A). These same flank tumor samples for GBM43 showed a corresponding increase in MGMT mRNA expression that reached a peak of 2.4-fold 7 days after TMZ treatment, compared with vehicle (Fig. 6B). Thus, induction of MGMT activity after TMZ treatment is associated with increased levels of MGMT mRNA transcripts.

Influence of Treatment Schedule on Response in Orthotopic Xenografts

We hypothesized that dose-dense TMZ treatment regimens may be ineffective in tumors with robust MGMT induction. To test this hypothesis, two treatment regimens were selected: 50 mg/kg 5 days per week for 2 weeks (500 mg/kg total) or 120 mg/kg once per week for 2 weeks (240 mg/kg total). Both treatment regimens conferred significant prolongation in survival for mice with intracranial GBM14 compared with vehicle-treated mice: median survival was 35 days for vehicle compared with 82 days for 120 mg/kg TMZ × 2 days and 128 days for 50 mg/kg TMZ × 10 days (both $p < 0.0001$ vs. vehicle; Fig. 7A). The protracted TMZ dosing regimen of 50 mg/kg/day was associated with a significant prolongation in survival compared with the once-weekly 120 mg/kg treatment regimen ($p < 0.0001$). In GBM43, both treatment regimens resulted in a marginal but statistically significant prolongation in survival relative to vehicle: median survival was 24 days for vehicle, 31 days for 120 mg/kg TMZ × 2 days, and 31 days for 50 mg/kg TMZ × 10 days (both $p = 0.03$ vs. vehicle; Fig. 7B). In contrast to the GBM14 survival results, the protracted daily dosing regimen was not superior to the weekly treatment regimen ($p = 0.93$) in GBM43 tumors.

To investigate whether regimen variation results in differential survival benefit in tumors with *MGMT* promoter methylation, the survival experiment was repeated for mice with intracranial GBM12 tumors. Similar to the results for GBM14, the more dose-intensive treatment regimen was associated with superior survival in GBM12 tumors ($p < 0.001$) compared with the once-weekly dosing regimen: median survival was 18 days for vehicle, 60 days for 120 mg/kg TMZ × 2 days, and 71 days for 50 mg/kg TMZ × 10 days (both $p < 0.0001$ vs. vehicle; Fig. 7C). Our initial survival studies (Fig. 2) were performed using a TMZ dosing regimen of 120 mg/kg × 5, and thus, to compare the relative efficacy of the three TMZ treatment regimens tested (120 mg/kg × 5 days, 50 mg/kg × 10 days, and 120 mg/kg × 2 days), the results from both experiments for each xenograft line were pooled and then analyzed by the log-rank test (Table 2). In this analysis, the protracted 10-day treatment regimen was marginally more effective in mice with intracranial GBM14 tumors, the more dose-intensive treatment of 120 mg/kg × 5 days was most effective in treating GBM43 tumors, and both regimens were equally effective in treating GBM12 tumors.

Discussion

TMZ is a key component of standard therapy for GBM patients. As with radiation treatment, however, sensitivity of GBM tumors to TMZ is variable. Understanding the mechanistic basis of individual tumor responses to TMZ is important for developing accurate predictive markers that could be used to identify those patients most likely to benefit from receiving this agent, as well as those who should receive alternative therapies. In clinical studies, low basal expression of tumor MGMT protein

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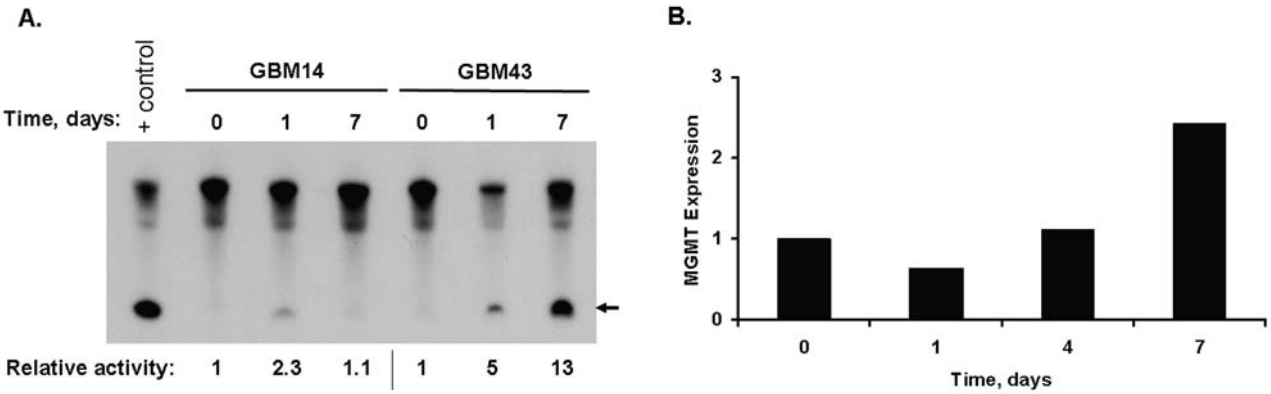

Fig. 6. Effects of temozolomide (TMZ) treatment on O^6-methylguanine-DNA methyltransferase (MGMT) activity and RNA expression in vivo. Mice with established flank glioblastoma multiforme (GBM) tumors were treated with TMZ at 50 mg/kg per day × 5 days, euthanized at the indicated time points, and processed for an analysis of MGMT repair activity (A) or MGMT mRNA levels by reverse transcription PCR (B). (A) For the activity assay, the MGMT activity within tumor samples is measured by incubating cell lysates with a ^{32}P-labeled oligonucleotide containing an O^6-methylguanine residue within a *Pst*I restriction site. Oligonucleotide is then isolated, cut with *Pst*I, and resolved by electrophoresis. MGMT activity in this assay correlates with the level of the cut oligonucleotide (lower band, arrow) and was determined as a ratio of radioactivity in the uncut and cut oligonucleotide bands as determined by film densitometry. The relative change in repair activity for each tumor sample is shown. (B) Pooled total RNA from two tumor samples at each time point was reverse transcribed, followed by real-time PCR amplification. Bars represent MGMT mRNA relative to the vehicle-treated tumors.

and *MGMT* promoter hypermethylation are each associated with prolonged survival for GBM patients treated with TMZ, although a significant subset of patients without these favorable prognostic characteristics also benefit from TMZ therapy.[3,4,14,15] In the present study, we evaluated relationships between MGMT expression and TMZ responsiveness in tumors with nonmethylated *MGMT* promoter in an orthotopic animal model using a series of GBM xenograft lines established directly from patient specimens. Consistent with clinical observations,[4] the three nonmethylated tumor lines examined showed a range in survival benefit after TMZ treatment: GBM44 was highly resistant, GBM43 was modestly resistant, and GBM14 was highly sensitive to TMZ therapy. The extent of survival benefit in this latter xenograft line was similar to that observed in the *MGMT* hypermethylated tumor line GBM12. In response to TMZ treatment, MGMT levels were suppressed in the highly sensitive but *MGMT* nonmethylated tumor line GBM14, while MGMT expression was markedly induced in response to TMZ treatment in the TMZ-resistant GBM43 and GBM44 tumor lines. In cell culture models, restoration of MGMT expression in *MGMT*-null cells confers significant resistance to TMZ, and conversely, treatment with a highly selective MGMT inhibitor markedly sensitizes only MGMT-expressing tumors to TMZ.[20–23] Given the importance of MGMT for recovery after methylation damage, the observed robust induction of MGMT activity in response to TMZ is likely mechanistically linked to TMZ resistance in at least a subset of GBM tumors. Moreover, the observation that MGMT expression changes in response to TMZ treatment and that this effect may influence sensitivity highlights the limitations of using baseline MGMT expression levels as a predictor of TMZ responsiveness.

Studies in tumor cells and human peripheral blood mononuclear cells have shown that MGMT levels are suppressed within hours of TMZ treatment and remain low for more than 24 h.[2,24,25] We have observed similar effects in vitro using the established T98 glioma cell line (G. Kitange, unpublished data) and in this study with GBM14. In contrast to these observations, MGMT protein expression was markedly upregulated in the TMZ-resistant GBM xenograft lines GBM43 and GBM44. Our in vitro and in vivo results are similar to those of a recent study of MGMT mRNA induction in MCF7 breast cancer cells after TMZ exposure[26] and other studies in normal tissue models. In previous studies of rodent or human hepatoma cell lines, significant upregulation of MGMT mRNA and protein was induced by alkylator exposure, ionizing or ultraviolet radiation, cisplatin, mitomycin C, restriction endonucleases, and other DNA-damaging agents.[27–36] Moreover, *MGMT* transcription is known to be induced in multiple rodent organs, including liver and brain, after whole-animal exposure to methylating agents, ionizing radiation, or bleomycin.[32,37–39] Consistent with a predominantly transcriptional mechanism of regulation, pretreatment of cells with the transcription inhibitor actinomycin D blocks DNA-damage–inducible MGMT upregulation.[34,40–43] Although the signal transduction pathways responsible for this induction are not clearly delineated, the cumulative available data suggest that TMZ-induced upregulation of MGMT expression is a specific response to DNA damage.

The CpG island within the *MGMT* promoter contains nearly 100 CpG dinucleotide sites, and differential methylation of these CpG sites can significantly affect transcriptional activity of the *MGMT* promoter.[18,44,45] Hypermethylation near the transcription start site is especially important for suppressing promoter activity, and the MS-PCR method commonly used to assess

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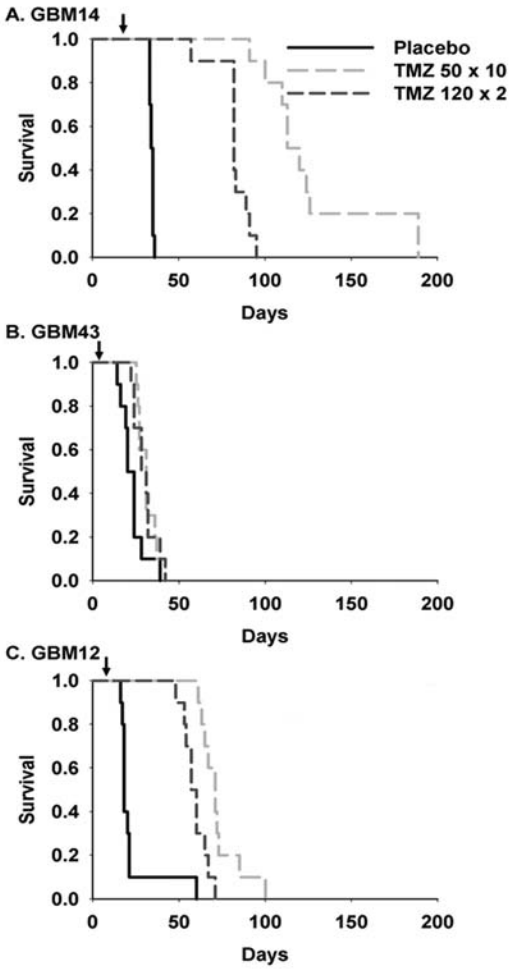

Fig. 7. Effects of temozolomide (TMZ) treatment schedule on survival. Mice with established orthotopic xenografts for glioblastoma multiforme (GBM) tumor lines were randomized to treatment with vehicle, 120 mg/kg TMZ × 2 days, or 50 mg/kg TMZ × 10 days. Mice were euthanized once they became moribund. Arrows denote when treatment was initiated. Both TMZ treatments significantly prolonged median survival in all GBM tumor lines: GBM14, $p < 0.0001$; GBM 43, $p < 0.03$; GBM12, $p < 0.0001$. For GBM14 and GBM12, 50 mg/kg × 10 days was superior to 120 mg/kg × 2 days (GBM14, $p < 0.0001$; GBM12, $p < 0.001$).

MGMT methylation specifically queries the methylation status of several CpG sites near this region.[18] In a recent randomized trial, tumor *MGMT* hypermethylation was associated with significantly greater survival in GBM patients treated with TMZ and radiation; 2-year survival was 46% in patients with tumor *MGMT* hypermethylation versus 14% in patients with *MGMT* hypomethylation.[4] In conjunction with our current understanding of TMZ-mediated toxicity, these clinical observations are consistent with the idea that MGMT repair activity is critical for cellular resistance to TMZ. The present study now provides a more detailed understanding of the link between *MGMT* promoter methylation and TMZ resistance. Although further mechanistic studies are necessary, we propose that TMZ-induced DNA damage leads to activation of *MGMT* transcription in at least some tumors with hypomethylation of the *MGMT* promoter and that this upregulation of MGMT expression may be an important factor contributing to TMZ resistance. In contrast, a damage-specific response may be blunted in other *MGMT* hypomethylated tumors and in *MGMT* hypermethylated tumors. The current focus of our laboratory is to confirm this hypothesis and to identify the relevant signaling pathways that modulate MGMT induction.

The results of this study also address another clinical question of significant interest: whether modulation of TMZ dose intensity can improve survival in patients with GBM. Based on previous observations that MGMT expression is suppressed in peripheral blood mononuclear cells and tumors,[2,25] protracted low-dose TMZ regimens have been promoted as a potential method for enhancing tumor cytotoxicity of TMZ. However, if MGMT expression is induced in response to TMZ exposure, then one might expect protracted dosing regimens to be less effective than more dose-intensive treatments in the subset of resistant tumors. Consistent with this idea, GBM43 demonstrates robust MGMT induction, and in this tumor line, the more protracted dosing regimens (50 mg/kg × 5 days/week × 2 weeks [500 mg/kg total dose] or 120 mg/kg × 1 day/week × 2 weeks [240 mg/kg total dose]) were significantly less effective than a more intensive regimen (120 mg/kg × 5 days/week × 1 week [600 mg/kg total dose]). Only in the GBM14 line, with a blunted MGMT induction response, did protracted treatment regimens provide greater benefit, con-

Table 2. Median survival for temozolomide treatment regimens relative to vehicle-treated tumors

	Relative Survival, by Treatment Schedule		
Cell Line	**120 mg/kg × 5 Days**	**50 mg/kg × 10 Days**	**120 mg/kg × 2 Days**
GBM12	3.9	3.9	3.3[a]
GBM14	3.0	3.3[c]	2.3[b]
GBM43	2.0[a]	1.3	1.3

[a] $p < 0.01$ relative to the other two treatments.

[b] $p < 0.001$ relative to the other two treatments.

[c] $p = 0.14$ relative to 120 mg/kg × 5 days.

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sistent with the suppressive effect of TMZ on MGMT expression in GBM14. Interestingly, both 120 mg/kg × 5 days and 50 mg/kg × 10 days were equally effective in the hypermethylated GBM12 xenograft line. Extrapolating these results to the clinical setting, we anticipate that protracted TMZ dosing regimens will not overcome TMZ resistance in tumors with a robust MGMT induction. More important, our results indicate that information beyond that provided by current standards for *MGMT* methylation and immunohistochemical analysis is needed for improved accuracy of predicting individual patient benefit from TMZ therapy and for optimizing TMZ administration schedules for patients anticipated to benefit from TMZ therapy.

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