

Inhibition of Histone Deacetylation Potentiates the Evolution of Acquired Temozolomide Resistance Linked to MGMT Upregulation in Glioblastoma Xenografts

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Abstract

Purpose: The therapeutic benefit of temozolomide in glioblastoma multiforme (GBM) is limited by resistance. The goal of this study was to elucidate mechanisms of temozolomide resistance in GBM.

Experimental Design: We developed an *in vivo* GBM model of temozolomide resistance and used paired parental and temozolomide-resistant tumors to define the mechanisms underlying the development of resistance and the influence of histone deacetylation (HDAC) inhibition.

Results: Analysis of paired parental and resistant lines showed upregulation of O₆-methylguanine-DNA methyltransferase (MGMT) expression in 3 of the 5 resistant xenografts. While no significant change was detected in MGMT promoter methylation between parental and derivative-resistant samples, chromatin immunoprecipitation showed an association between MGMT upregulation and elevated acetylation of lysine 9 of histone H3 (H3K9-ac) and decreased dimethylation (H3K9-me₂) in GBM12 and GBM14. In contrast, temozolomide resistance development in GBM22 was not linked to MGMT expression, and both parental and resistant lines had low H3K9-ac and high H3K9-me₂ within the MGMT promoter. In the GBM12TMZ-resistant line, MGMT reexpression was accompanied by increased recruitment of SP1, C-JUN, NF-κB, and p300 within the MGMT promoter. Interestingly, combined treatment of GBM12 flank xenografts with temozolomide and the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) favored the evolution of temozolomide resistance by MGMT overexpression as compared with treatment with temozolomide alone.

Conclusion: This study shows, for the first time, a unique mechanism of temozolomide resistance development driven by chromatin-mediated MGMT upregulation and highlights the potential for epigenetically directed therapies to influence the mechanisms of resistance development in GBM. *Clin Cancer Res*; 18(15): 4070–9. ©2012 AACR.

Introduction

Temozolomide is an important component of therapy for glioblastoma multiforme (GBM). Unfortunately, tumor progression while receiving temozolomide therapy occurs in more than 40% of patients (1–4), and progression during second-line therapy is essentially universal (3, 5, 6). Thus, understanding mechanisms of primary and acquired temozolomide resistance is critically important. The DNA repair protein O₆-methylguanine-DNA methyltransferase (MGMT) and the mismatch repair (MMR) pathway are

critical mediators of temozolomide sensitivity (7). MGMT removes cytotoxic O₆-methylguanine (O₆-MG) lesions induced by temozolomide (7–9). Suppression of MGMT expression, often mediated by MGMT promoter hypermethylation, leads to persistent O₆-MG lesions that are mispaired with thymidine during replication. This mispairing subsequently is engaged by futile cycles of MMR that lead to collapsed replication forks and death (3, 7). High MGMT expression and disrupted MMR function are mechanistically linked to temozolomide resistance in multiple tumor models (10, 11), and elevated MGMT expression and/or lack of MGMT promoter hypermethylation in patient tumor specimens is associated with a worse outcome in patients with GBM treated with temozolomide (3, 12, 13). Mutations in MMR genes are rare in primary GBM, but acquired MSH6 mutations are associated with development of temozolomide resistance (14, 15). However, this mechanism only accounts for a small proportion of patients, and thus there is significant interest in delineating other mechanisms of temozolomide resistance.

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Translational Relevance

The therapeutic benefit of temozolomide, the only drug known to significantly enhance survival in newly diagnosed patients with glioblastoma multiforme (GBM), is limited by rapid evolution of secondary resistance of which the principal mechanisms are poorly known. In this report, the authors have developed an *in vivo* model of secondary temozolomide resistance and subsequently showed that histone deacetylase (HDAC) inhibition can influence the mechanism of temozolomide resistance emergence, particularly favoring resistance linked with chromatin-mediated O₆-methylguanine-DNA methyltransferase (MGMT) upregulation. With an increasing number of epigenetically targeted therapies being tested in oncology, these results highlight the potential for this class of novel therapeutics to influence resistance emergence of a coadministered cytotoxic agent.

The limited number of clinically relevant tumor models is a major impediment to evaluating mechanisms of chemoresistance in GBM. Traditional glioma cell lines have been cultured for decades and are genetically distinct from primary human tumors (12, 16, 17), and genetically engineered models do not adequately recapitulate potential epigenetic mechanisms of resistance. While collections of paired patient tumor samples from diagnosis and recurrence following temozolomide therapy have been reported (18), most of these samples are paraffin-embedded, and small tissue samples limit the molecular assays that can be applied to these scarce samples. To address the limitations, we subjected primary GBM xenograft lines from the Mayo panel to *in vivo* selection with escalating temozolomide doses. These paired primary and temozolomide-resistant lines provide unlimited amounts of tissues that can be analyzed to define mechanisms of acquired resistance. Herein, we use this model to establish a unique mechanism of acquired temozolomide resistance linked with chromatin-mediated MGMT upregulation. Furthermore, we show that combined therapy with temozolomide and an HDAC inhibitor promotes this epigenetically driven mechanism of resistance.

Materials and Methods

Establishment of primary and temozolomide-resistant GBM xenograft lines

The Mayo GBM xenograft panel has been previously described (19). Temozolomide resistance models were developed from mice with established flank tumors treated either with 20 mg/kg/d for 3 days and then 66 mg/kg/d for 3 days after initial tumor regrowth (GBM12) or treated with 66 mg/kg/d for 3 days (GBM14, 22, 28, and 39). The resulting temozolomide-resistant lines were completely resistant to a challenge of 120 mg/kg/d for 5 days. The

efficacy of temozolomide in resistant models was evaluated using an orthotopic therapy model (20). All animal studies were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Short-term explant cell cultures

Short-term explant cultures grown in serum-containing media were derived from the parental and resistant flank xenografts as described (21). Serum-free explant cultures were established as described by others (22); mechanically disaggregated tumors were plated on laminin-coated flasks overnight in neurobasal serum-free media (StemPro NSC SFM; Invitrogen).

In vitro cytotoxicity assays

Explant cultures were plated in triplicate on 96-well plates and treated with graded concentrations of temozolomide \pm 10 μ mol/L O₆-benzylguanine (O₆-BG). After 6 days, samples were analyzed using a CyQUANT Assay (Invitrogen) according to manufacturer's instructions. For a neurosphere assay, explant cultures in neurobasal media were plated in triplicate at 500 cells per well and treated as above. Intact neurospheres were counted after 14 days.

Analysis of MGMT promoter methylation, mRNA, and protein levels

Tumor samples were analyzed for MGMT promoter methylation by methylation-specific PCR (MS-PCR) and expression by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) as described (23). The same specimens were processed for Western blotting using the antibodies: MGMT (R & D systems), β -actin (Sigma), and horseradish peroxidase-conjugated to secondary antibodies (Pierce; ref. 23). Bisulfite-modified DNA was evaluated by pyrosequencing using a PyroMark MD system (Qiagen).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was conducted using the EZ-ChIP Kit (Millipore). Tumor samples were minced, and cross-linking was conducted with 1% formaldehyde, quenched with 0.1 mol/L glycine, and then processed according to manufacturer's instructions. Antibodies used were anti-acetyl-lysine 9 histone H3 (H3K9-ac) and anti-dimethyl-lysine 9 histone H3 (H3K9-me2) from Millipore, and anti-trimethyl-lysine 27 histone H3 (H3K27-me3) from Cell Signaling Technology. The anti-SP1, -C-JUN, -NF- κ B, and -p300/CBP antibodies were from Santa Cruz Biotechnology, Inc.). The distal promoter region critical for MGMT silencing by hypermethylation (24) was PCR-amplified with human-specific primer sequences: 5'-GCCCGGATATGCTGGGAC-3' (forward) and 5'-GGGCAACACCTGGGAGGAC-3' (reverse). A 35-cycle PCR was conducted using 60°C annealing temperature. NIH ImageJ (<http://rsbweb.nih.gov/ij/>) was used to quantitate PCR bands and relative enrichment was a ratio of the immunoprecipitated signal versus input.

Effect of HDAC inhibitor SAHA on the evolution of temozolomide resistance

Mice with GBM12 tumors were treated with placebo or 50 mg/kg/d temozolomide for 5 days every 28 days for 3 cycles. Tumor volume was measured thrice weekly, and mice with tumors exceeding 1,500 mm³ were euthanized. Tumors from each mouse were flash-frozen and cryopreserved. In a second study, GBM12 xenografts were treated with placebo or temozolomide (50 mg/kg for days 1–5) with or without suberoylanilide hydroxamic acid (SAHA; 100 mg/kg on days 1–7 and 15–21 for every 28 days) for 3 cycles.

Statistical analysis

The log-rank test was used to compare animal survival between placebo- and drug-treated mice. Relative survival was calculated for each tumor line (parental and temozolomide resistant) as the ratio of median survival between temozolomide and placebo-treated mice and compared across all lines using a 2-sample rank-sum test. Differences in the *in vitro* cell survival, PCR band intensity, and pyrosequencing were analyzed using a 2-sample *t* test.

Results

Establishment of pair-matched temozolomide-resistant and parental GBM lines

The Mayo GBM panel was established by implantation of patient samples into mice and maintained by serial heterotopic transplantation. Using this method, xenografts faithfully maintain the histopathologic and molecular characteristics of the primary patient tumor samples. Similar to clinical results, the benefit of temozolomide in orthotopically implanted xenografts correlated highly with MGMT promoter methylation and protein expression (20). From this panel, 4 temozolomide-sensitive tumors (GBM12, 14, 22, and 39) and 1 relatively resistant tumor (GBM28) were subjected to *in vivo* selection for temozolomide resistance. These derivative temozolomide-resistant lines are referred to with a "TMZ" suffix added to the parental tumor designation (i.e., GBM12TMZ is derived from parental GBM12). The efficacy of temozolomide in each derivative line was evaluated in an orthotopic survival study and compared with published results for the corresponding parental lines (Fig. 1 and Supplementary Fig. S1). As summarized in the Supplementary Table S1, the survival benefit associated with temozolomide therapy is significantly greater in each of the parental lines as compared with the derivative temozolomide-resistant line (median relative survival for temozolomide treatment relative to placebo: parental lines 3.6 versus temozolomide resistant line 1.2; $P < 0.01$). Thus, the temozolomide selection method generated highly temozolomide-resistant tumor lines.

Reexpression of MGMT in temozolomide-resistant GBM xenografts

MGMT expression is an important mediator of temozolomide resistance (3, 13, 18, 25), and therefore, the MGMT protein and mRNA levels were evaluated in the paired lines.

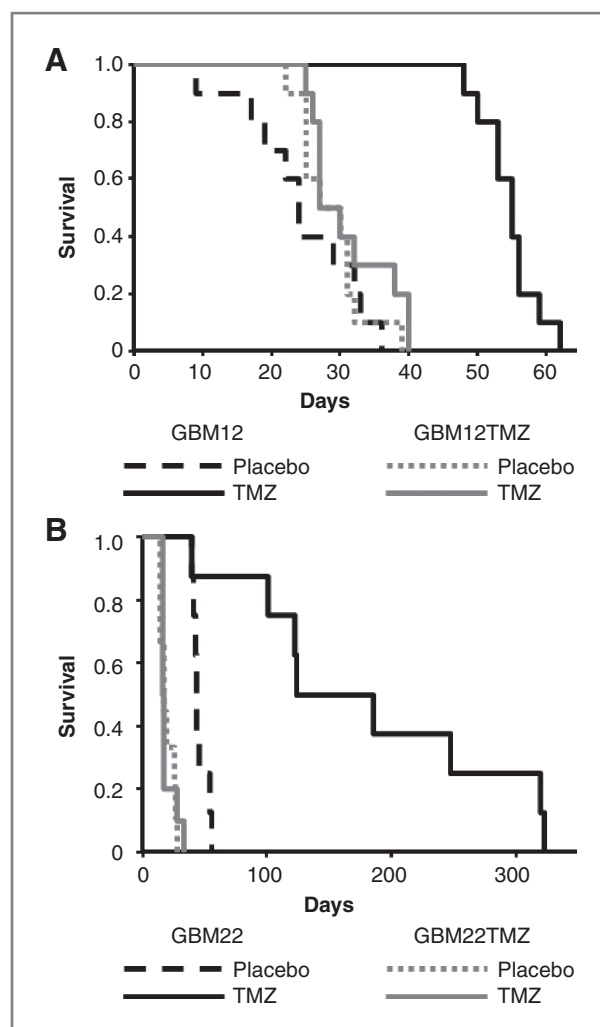


Figure 1. Evaluation of temozolomide (TMZ) response in parental compared with the derived temozolomide-resistant xenografts. The efficacy of 66 mg/kg temozolomide daily for 5 days was compared with placebo treatment in parental and derivative temozolomide-resistant tumor lines. Shown are the representative Kaplan-Meier survival plots for (A) GBM12/GBM12TMZ and (B) GBM22/GBM22TMZ.

Consistent with epigenetic regulation of expression, MGMT protein was undetectable in the 3 parental MGMT-methylated lines (GBM12, 22, and 39) and in 1 partially methylated line (GBM14) but readily detectable in the unmethylated line (GBM28; Fig. 2A). In contrast, MGMT protein was significantly elevated in 2 derivative lines (GBM12TMZ and GBM14TMZ) and further elevated in the GBM28TMZ line. Similarly, MGMT mRNA was increased only in GBM12TMZ, GBM14TMZ, and GBM28TMZ (Fig. 2B). Thus, MGMT upregulation seems to be a common occurrence in the derivative temozolomide-resistant xenografts.

The contribution of MGMT to resistance was evaluated *in vitro* in GBM12TMZ, GBM14TMZ, and GBM22TMZ in a cytotoxicity assay using O6-BG, a selective MGMT inhibitor. While temozolomide was ineffective in all 3 resistant lines, cotreatment with O6-BG significantly enhanced

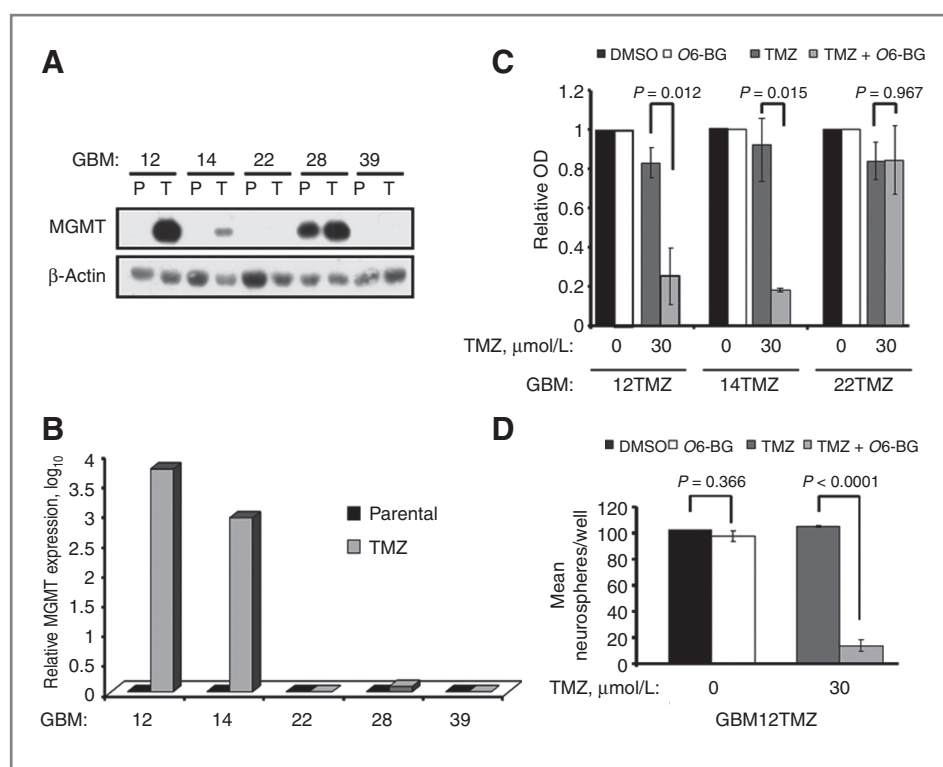


Figure 2. Expression of MGMT in temozolomide-resistant xenografts. A, pooled triplicate tumor samples derived from parental (P) or derivative temozolomide-resistant (T) xenografts were processed for Western blotting of MGMT and β -actin. B, total RNA from the same triplicate tumor samples was pooled and analyzed for MGMT expression by qRT-PCR in parental and temozolomide-resistant xenografts. For each line, the relative level of MGMT expression in temozolomide lines is plotted relative to parental lines. C, short-term explant cultures from GBM12TMZ, GBM14TMZ, and GBM22TMZ were treated with graded concentrations of temozolomide with or without 10 μ mol/L O_6 -BG and analyzed for survival with a CyQUANT assay 7 days later. OD, optical density. D, short-term GBM12TMZ explant stemcell-like cultures were plated in 96-well plates and analyzed for neurosphere formation 14 days later. Results for C and D are the mean relative absorbance or mean neurosphere number (\pm SEM), respectively, from 3 independent experiments. DMSO, dimethyl sulfoxide.

temozolomide sensitivity in GBM12TMZ and GBM14TMZ but not GBM22TMZ (Fig. 2C). Specifically, treatment with 30 μ mol/L TMZ without or with O_6 -BG was associated with a relative absorbance, compared with control treatment, of 0.83 ± 0.08 and 0.25 ± 0.14 , respectively ($P = 0.012$), for GBM12TMZ, 0.93 ± 0.16 and 0.18 ± 0.01 , respectively ($P = 0.016$), for GBM14TMZ, and 0.84 ± 0.1 and 0.84 ± 0.17 , respectively ($P = 0.494$), for GBM22TMZ. A neurosphere formation assay with GBM12TMZ and treatment with temozolomide plus O_6 -BG resulted in a significant reduction in neurosphere formation (14 ± 4.4) as compared with temozolomide alone (98 ± 3.8 ; $P < 0.0001$), whereas O_6 -BG alone had no impact on neurosphere formation (Fig. 2D). Together, these data show that the temozolomide resistance observed in GBM12TMZ and GBM14TMZ is mechanistically linked to overexpression of MGMT.

Delineation of epigenetic changes within MGMT promoter region

Because promoter methylation is an important mechanism regulating MGMT expression and temozolomide response in tumors (3), the paired lines were analyzed for methylation by MS-PCR. Despite marked upregulation of

MGMT in GBM12TMZ and GBM14TMZ, MS-PCR comparison of parental and temozolomide-resistant lines showed no change in promoter methylation status for any tumor pair (Fig. 3A). To evaluate the methylation of the MGMT promoter in greater detail, pyrosequencing of the CpG sites (CpG 75–96; ref. 23) encompassing a region covered by the MS-PCR assay was conducted on GBM12, 14, 22, and the derivative temozolomide lines. As shown in Fig. 3B, the average percentage of methylation of all sites was $71.1\% \pm 15.2\%$ for GBM12 versus $74.0 \pm 19.6\%$ for GBM12TMZ ($P = 0.641$), $28.4\% \pm 1.4\%$ for GBM14 versus $24.0\% \pm 1.9\%$ for GBM14TMZ ($P = 0.139$), and $73.2\% \pm 19.3\%$ for GBM22 versus $80.8\% \pm 16.3\%$ for GBM22TMZ ($P = 0.211$). In an analysis of methylation of each individual CpG, relatively subtle changes in the extent of CpG methylation were noted in a comparison between GBM12/12TMZ lines with statistically significantly increased methylation seen in 11 CpGs and decreased methylation seen in 4 CpGs. Similar comparison between GBM14/14TMZ lines revealed statistically significantly increased methylation in 2 CpGs and decreased methylation in 3 CpGs. In GBM22, only 2 CpGs showed elevated methylation, whereas no CpGs had decreased methylation (Supplementary Table S2).

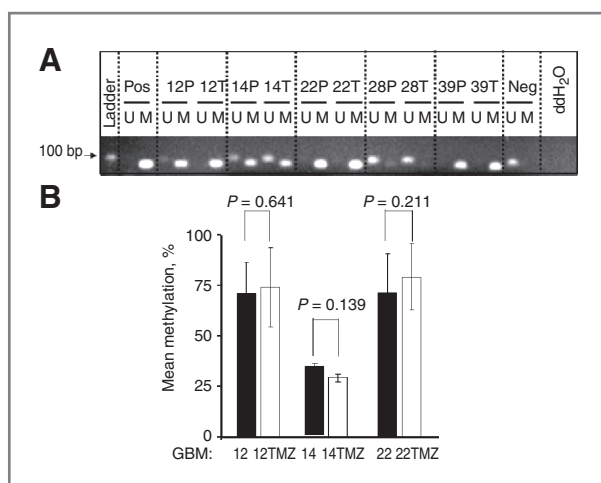


Figure 3. Evaluation of MGMT promoter methylation status in the parental and derived temozolomide-resistant xenografts. **A**, MGMT promoter methylation by MS-PCR. For each parental (P) and temozolomide-resistant (T) line, unmethylated (U) and methylated (M)-specific PCR reactions are shown, along with positive (Pos) and negative (Neg) controls. **B**, validation of MGMT promoter methylation by pyrosequencing in paired parental and temozolomide-resistant GBM12, 14, and 22. Shown is the average methylation across 22 CpG dinucleotides (CpG75–96) encompassing a region interrogated by the MS-PCR assay.

Thus, there were no major changes in MGMT promoter methylation status that can explain the MGMT upregulation observed in GBM12TMZ and GBM14TMZ.

Because posttranslational modifications of histones can modulate chromatin structure and control gene expression (26), acetylation and methylation of a key lysine residue, H3K9, and methylation of H3K27 was assessed by ChIP in paired flank tumor specimens from GBM12, 14, and 22. Consistent with a closed chromatin structure and low MGMT expression, all 3 parental tumors GBM12, 14, and 22 had low H3K9-ac and high H3K9-me2 occupancy within the MGMT promoter (Fig. 4A–C). In contrast, elevated MGMT expression in GBM12TMZ and GBM14TMZ was associated with a significant increase in H3K9-ac and concomitant decrease in H3K9-me2 promoter occupancy compared with parental tumors (Fig. 4A and B), whereas these changes were not seen in GBM22TMZ (Fig. 4C). No significant change in H3K27-me3 status was observed in any of the lines (Fig. 4A–C). The increased MGMT expression, mediated by chromatin changes in GBM12TMZ, was associated with recruitment of the transcription factors Sp1, C-JUN, NF-κB, and p300 to the MGMT promoter (Fig. 4D). Collectively, these data suggest that MGMT upregulation linked with the acquisition of temozolomide resistance is modulated by histone posttranslational modifications that favor transcriptionally active chromatin within the MGMT promoter.

Influence of HDAC inhibition on MGMT upregulation

Temozolomide resistance selection was repeated in GBM12 using multiple mice with established heterotopic xenografts and a clinically relevant dosing schedule. Twenty

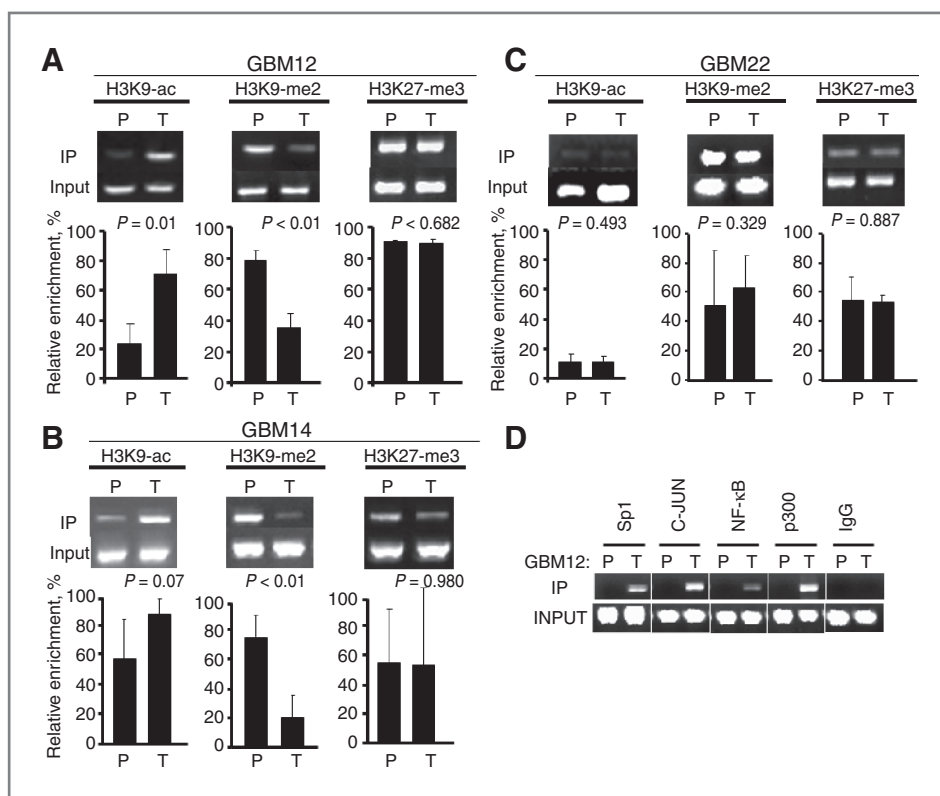


Figure 4. Evaluation of the chromatin marks within MGMT promoter region comparing pair-matched parental and temozolomide-resistant GBM12, 14, and 22. Shown are the acetylation of lysine 9 of histone H3 (H3K9-ac), dimethylation of lysine 9 of histone H3 (H3K9-me2), and trimethylation of lysine 27 of histone H3 (H3K27-me3) for (A) GBM12, (B) GBM14, and (C) GBM22 from a single tumor (above) and densitometric analysis of mean \pm SD from 3 independent tumor samples (graphs). **D**, ChIP assay showing a differential recruitment of transcription factors to the MGMT promoter region in parental (P) and temozolomide-resistant (T) GBM12 cells. IP, immunoprecipitation.

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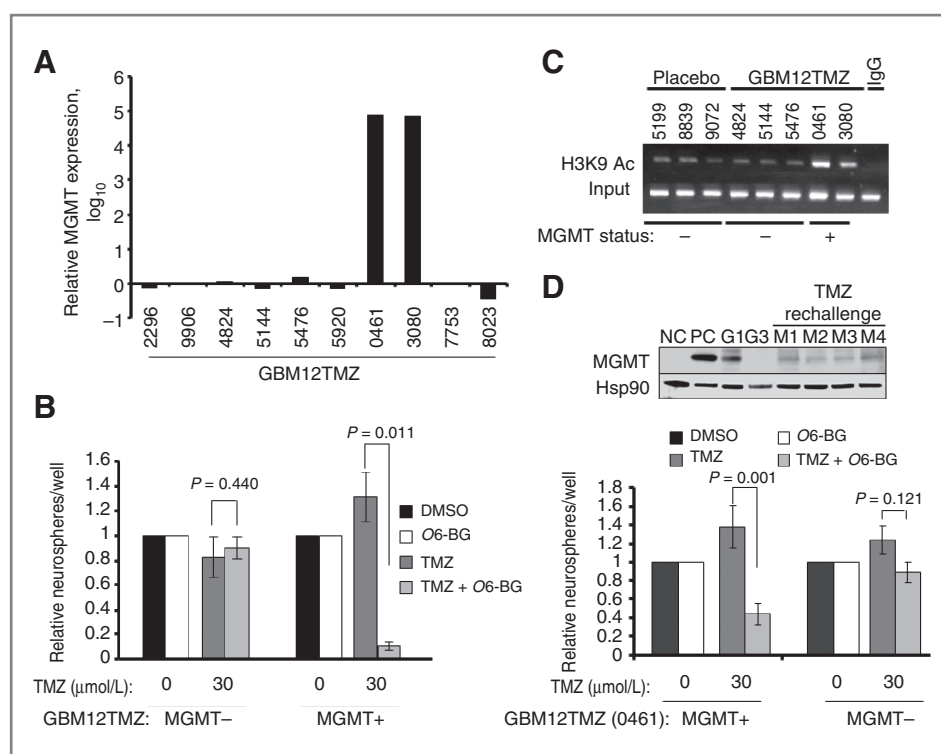


Figure 5. Generation of temozolomide-resistant GBM12 xenografts using a clinically relevant temozolomide regimen. Twenty mice with GBM12 flank tumors were treated with 3 cycles of temozolomide (50mg/kg/d for 5 days every 28 days) or placebo. **A**, Total RNA from the tumor samples was analyzed for MGMT expression by qRT-PCR in placebo and temozolomide-resistant xenografts and shown is the relative level of MGMT expression in the resistant lines plotted relative to mean expression in placebo. The numbers shown on the x-axis correspond with the animal/tumor number. **B**, short-term explant stemcell-like cultures from MGMT-expressing GBM12-resistant tumor (#3080; MGMT+) and non-MGMT-expressing tumor (#5920; MGMT-) were plated in 96-well plates, treated with temozolomide alone or with O₆-BG and analyzed for neurosphere formation 14 days later. Shown is the mean of 3 independent experiments ± SEM. **C**, evaluation of the H3K9-ac histone mark within MGMT promoter region comparing placebo and temozolomide-treated non-MGMT- and MGMT-expressing samples (MGMT status - or +, respectively). **D**, MGMT expression by Western blotting (top) of GBM12TMZ #0461 serially transplanted xenografts after initial isolation at generation 1 (G1) and generation 3 (G3) without temozolomide treatment and then 4 individual mice (M1-M4) at generation 4 that were rechallenged *in vivo* with temozolomide (50 mg/kg for 5 days; NC, negative control; PC, positive control). The initial G1 (MGMT+) and G3 (MGMT-) tumors were used to generate neurospheres and evaluated as in (B) for *in vitro* temozolomide sensitivity (bottom). DMSO, dimethyl sulfoxide.

mice with established tumors were treated with placebo or temozolomide for 5 days every 28 days for 3 cycles and then allowed to regrow. Once tumors exceeded 1,500 mm³, mice were euthanized and tumors were used to derive neurosphere cultures and archived for molecular analysis. As seen in Fig. 5A, only 2 of 10 temozolomide-treated xenografts exhibited marked MGMT upregulation, whereas none of the placebo-treated tumors had elevated MGMT. Significant temozolomide resistance was confirmed using an *in vitro* neurosphere assay in all 10 temozolomide-treated tumors as compared with placebo-treated tumors (data not shown). Consistent with the importance of MGMT overexpression for temozolomide resistance in a subset of tumors, temozolomide sensitivity was restored with O6-BG cotreatment in the MGMT-expressing GBM12TMZ tumor #3080 (relative neurosphere formation was 0.11 ± 0.03 as compared with 1.31 ± 0.2 for temozolomide alone, $P < 0.01$, Fig. 5B), whereas no such effect was observed in the non-MGMT-expressing GBM12TMZ tumor #5920 (relative neurospheres/well was 0.83 ± 0.16 with temozolomide alone and 0.89 ± 0.09 with temozolomide/O6-BG; $P <$

0.440 ; Fig. 5B). Consistent with the initial findings in GBM12TMZ, only those lines with MGMT elevation showed increased H3K9-ac occupancy within the MGMT promoter compared with placebo-treated tumors (Fig. 5C). One of the MGMT-expressing GBM12TMZ tumors (#0461) lost MGMT expression with subsequent passages in mice without treatment, but even after losing MGMT expression, the tumor remained resistant to temozolomide and resistance was not rescued by O6-BG (Fig. 5D, bottom). Consistent with persistent resistance in this subline, subsequent *in vivo* temozolomide rechallenge failed to delay the growth (data not shown) and resulted in partial restoration of MGMT expression. Thus, while the mechanism(s) of temozolomide resistance in the non-MGMT-expressing tumors is being evaluated, these data show the potential for heterogeneous mechanisms of temozolomide resistance development even within the same tumor.

Because the above findings suggest that MGMT-mediated temozolomide resistance is associated with increased histone acetylation, the influence of HDAC inhibition on resistance emergence was tested in a second GBM12

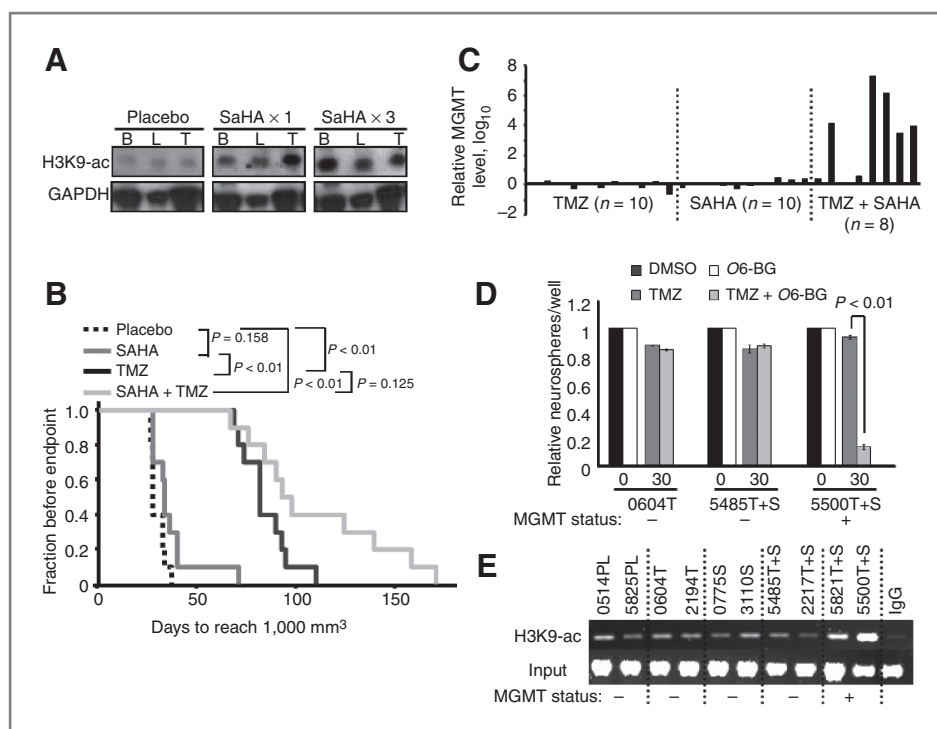


Figure 6. Effect of SAHA on the evolution of acquired resistance to temozolomide. **A**, effect of SAHA on the H3K9 acetylation in pooled tissue samples from brain (B), liver (L), and the tumor (T) obtained from the correlative mice after 1 and 3 doses. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **B**, the time to exceed 1,000 mm³ after treatment with placebo, temozolomide plus placebo, SAHA alone, or temozolomide plus SAHA. **C**, evaluation of MGMT mRNA expression in recurrent xenografts following treatment with temozolomide alone ($n = 10$), SAHA alone ($n = 10$), and SAHA + temozolomide ($n = 8$) by qRT-PCR. The y-axis represents MGMT levels relative to the level in the placebo-treated tumors. **D**, short-term GBM12TMZ explant cultures derived from mice treated with temozolomide alone (0604T), temozolomide + SAHA (5485T + S and 5500T + S) were treated with graded concentrations of temozolomide with or without 10 $\mu\text{mol/L}$ O₆-BG and analyzed for neurosphere formation 14 days later. Results are the mean relative neurosphere number (\pm SEM), respectively, from 3 independent experiments. **E**, ChIP analysis of MGMT H3K9-ac among different treatment groups (PL, placebo; T, temozolomide; S, SAHA; T + S, temozolomide + SAHA). The MGMT expression level (MGMT status) for individual tumors is noted below the figure.

experiment. Similar as before, 40 mice with established xenografts were randomized to therapy with placebo, monotherapy with SAHA or temozolomide, or combination therapy. Additional mice were treated with SAHA alone for 1 or 3 doses and then euthanized to evaluate HDAC activity. As seen in Fig. 6A, global acetylation of H3K9 was markedly elevated in brain, liver, and flank tumor following drug therapy, compared with placebo-treated mice. In the tumor growth assay, there was no difference in the time for flank tumors to reach 1,000 mm³ in mice treated with placebo or SAHA (median survival 26 days vs. 31 days, respectively; $P = 0.16$), whereas both temozolomide treatment (median survival 75 days) and SAHA + temozolomide (median survival 92.5 days) significantly extended time for tumor growth beyond 1,000 mm³ compared with placebo ($P < 0.01$; Fig. 6B and Supplementary Fig. S3A). Interestingly, despite no difference in treatment efficacy between temozolomide vs. temozolomide + SAHA ($P = 0.12$), SAHA cotreatment promoted MGMT upregulation. In this experiment, none of the tumors treated with temozolomide alone or SAHA alone showed MGMT upregulation at recurrence, whereas 5 of the 8 evaluable tumors from temozolomide + SAHA treatment group had high-level

MGMT expression (Fig. 6C). Temozolomide sensitivity was restored with O₆-BG cotreatment in the MGMT-expressing GBM12TMZ + SAHA tumor #5500 (relative neurosphere/well was 0.14 ± 0.02 as compared with 0.93 ± 0.01 for temozolomide alone, $P < 0.01$; Fig. 6D), whereas no such effect was observed in the non-MGMT-expressing GBM12TMZ tumor #0604 and non-MGMT-expressing GBM12TMZ + SAHA tumor #5485 [relative neurosphere/well was 0.88 ± 0.05 vs. 0.84 ± 0.03 , ($P > 0.05$) and 0.85 ± 0.01 vs. 0.87 ± 0.03 ($P > 0.05$), respectively]. There were no significant differences in neurosphere formation efficiency among these resistant lines (Supplementary Fig. S3B). Consistent with a link between histone acetylation and MGMT upregulation, increased H3K9-ac was specifically observed in MGMT-expressing temozolomide-resistant GBM12 xenografts (Fig. 6E), and ChIP assay revealed increased recruitment of SP1, C-JUN, NF- κ B, and p300 to the MGMT promoter only in the MGMT-expressing GBM12TMZ + SAHA tumor #5500 (Supplementary Fig. S3C). Thus, consistent with a link between histone acetylation and MGMT upregulation, HDAC inhibitor therapy promoted an MGMT-mediated mechanism of temozolomide resistance in GBM12 xenografts.

Discussion

The efficacy of temozolomide is limited by rapid emergence of resistance, and yet little is known about the underlying mechanisms of acquired resistance in the majority of GBM. Progress in this area has been slow due to the limited number of clinically relevant models for studying mechanisms of temozolomide resistance. The Mayo GBM xenograft panel is maintained exclusively by serial heterotopic transplantation in nude mice, and the MGMT promoter methylation status of primary patient tumor specimens is preserved in most xenografts that are subjected to multiple serial tumor passages. Moreover, response to temozolomide in this panel was significantly associated with MGMT promoter methylation status, and in a subset of tumors derived from patients ultimately treated with temozolomide, there was a good correlation between clinical response to temozolomide therapy and sensitivity of the corresponding xenografts to temozolomide (20, 23). Finally, reminiscent of patients with an initial response but subsequent progression on temozolomide therapy, the survival prolongation observed with a single cycle of temozolomide therapy is significantly greater than the additional benefit of subsequent cycles in several xenografts analyzed (27). In this study, we relied exclusively on heterotopic xenograft studies to facilitate cryopreservation of tumor samples. While, the brain microenvironment can influence the response to some agents, we have observed similar results between orthotopic and heterotopic xenograft studies with temozolomide therapy. Collectively, these data suggest that these xenograft GBM models are highly relevant for analyzing mechanisms of temozolomide resistance.

MGMT repairs cytotoxic O6-MG DNA lesions induced by temozolomide. Multiple studies using molecular or pharmacologic manipulation of MGMT have shown a mechanistic link between MGMT activity and temozolomide resistance, with suppression of MGMT activity resulting in increased cytotoxicity and MGMT overexpression resulting in resistance (7). However, the role of MGMT in the evolution of acquired temozolomide resistance is not well established. In this study, a comparison of 5 primary xenograft lines with corresponding derived models of temozolomide resistance showed a marked upregulation of MGMT expression in a subset of resistant GBM xenograft lines. In these lines, MGMT is mechanistically linked to temozolomide resistance emergence as the relatively specific MGMT inhibitor O6-BG restored temozolomide sensitivity only in lines that overexpressed MGMT (GBM12TMZ and GBM14TMZ). Ongoing studies are focused on understanding the underlying mechanisms of acquired temozolomide resistance in non-MGMT-expressing tumors. Although previous studies have linked temozolomide resistance to emergence of mutational inactivation of MMR genes in a minority of GBM tumors (14, 15), sequencing of MSH2, MSH6, MLH1, and PMS2 failed to identify any mutations in either GBM12TMZ or GBM14TMZ (unpublished data). Thus, as both of these resistance mechanisms operate within the same DNA repair pathway, we hypothesize that MGMT upregulation or MMR mutation may be mutually exclusive

within any given tumor cell. The unique MSH6 mutation observed in GBM22TMZ (Supplementary Fig. S2) partially support this view, and we are testing this hypothesis in an expanded panel of temozolomide-resistant GBM xenografts.

Promoter CpG hypermethylation can suppress MGMT expression and is associated with greater clinical benefit from temozolomide in newly diagnosed patients with GBM (2–4, 28). Importantly, in the current study, MGMT mRNA and protein expression levels were markedly increased following selection for temozolomide resistance in methylated lines without a marked change in MGMT promoter methylation. Although the biologic relevance of the subtle changes in methylation of discrete CpGs in GBM12TMZ and GBM14TMZ remain to be determined, these changes are unlikely to explain sufficiently the significant changes in MGMT transcript levels associated with temozolomide resistance development. There are multiple levels of epigenetic regulation of gene transcription, and while most attention for MGMT has focused on CpG methylation (reviewed in ref. 29), MGMT expression also is regulated by chromatin remodeling. Accordingly, the increased MGMT expression in GBM12TMZ and GBM14TMZ coincided with increased H3K9-ac, decreased H3K9-me2, and elevated binding of transcription factors within the MGMT promoter region. While these findings are consistent with a previously shown role of histone modifications (decreased H3K9-ac and increased H3K9-me2) in transcriptional silencing of MGMT (30), the mechanisms by which temozolomide exposure reverses these chromatin marks to upregulate MGMT expression are unknown and are the subject of ongoing studies.

The most intriguing and clinically relevant finding of this study is the demonstration of heterogeneous mechanisms of acquired temozolomide resistance in a single xenograft tumor. We observed a 100% regrowth of GBM12 xenografts after treatment with a clinically relevant temozolomide regimen. Interestingly, even though these tumors were generated from a single short-term explant culture, MGMT reexpression was only detected in 2 of the 10 tumors that recurred after temozolomide therapy, and increased H3K9-ac was observed only in tumors with MGMT upregulation. The reversal of temozolomide resistance by cotreatment with the MGMT inhibitor O6-BG shows that the dominant mechanism of resistance is driven by MGMT only in those lines with high-level MGMT expression. However, consistent with the idea that multiple mechanisms of resistance may coemerge in the same tumor, MGMT expression was lost in the absence of temozolomide-selective pressure in GBM12TMZ#0461 despite this tumor remaining highly resistant to temozolomide. The restoration of MGMT expression following retreatment with temozolomide supports the idea that recurrent tumors can be composed of mixed populations of cells with different mechanisms of resistance. Subcloning of the derivative tumors and application of next-generation sequence analysis will be important to further dissect the heterogeneous mechanisms of resistance in these model systems and patient tumors.

MGMT overexpression likely is an important mechanism of resistance in both MGMT promoter hypermethylated and unmethylated GBM tumors. In this study, MGMT overexpression was regulated epigenetically and was specifically potentiated by concurrent HDAC inhibition by SAHA (Fig. 6). While H3K9 acetylation was globally increased by SAHA treatment (Fig. 6A), this mark was only enriched within MGMT promoter of the MGMT-expressing GBM12TMZ lines derived from the combined temozolomide–SAHA treatment (Fig. 6E). This finding contradicts recent publications, in which SAHA alone induced H3K9-ac mediated reactivation of MGMT expression in promoter hypermethylated leukemic and breast cancer cells *in vitro* (31). This difference in results may stem from the use of primary xenograft models compared with established cell lines and the exclusive analysis of SAHA effects *in vivo* in which metabolism of the drug will lead to fluctuating exposure over time compared with relatively steady concentrations of SAHA encountered *in vitro*. Moreover, as temozolomide treatment alone can induce heterochromatin reorganization (32), we speculate that temozolomide-induced epigenetic events may contribute to heterochromatin remodeling within the MGMT promoter associated with cotreatment with SAHA.

Because there is an ongoing clinical trial evaluating the integration of SAHA with temozolomide and radiation in newly diagnosed patients with GBM, the results in this study are reassuring that concurrent SAHA did not accelerate the development of temozolomide resistance, although concurrent therapy resulted in a distinct shift favoring MGMT upregulation. To our knowledge, this is the first ever demonstration that HDAC inhibition can influence the mechanism of resistance emergence when combined with a conventional cytotoxic chemotherapy

agent. With an increasing number of epigenetically targeted therapies being tested in oncology, these results highlight the potential for these novel therapies to affect resistance emergence.

Disclosure of Potential Conflicts of Interest

J.N. Sarkaria has a commercial research grant from Merck, Basilea, and Genentech. No potential conflicts of interest were disclosed by other authors.

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