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Inhibition of Histone Deacetylation Potentiates the Evolution of Acquired Temozolomide Resistance Linked to MGMT Upregulation in Glioblastoma Xenografts¹

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Abstract

PURPOSE—The therapeutic benefit of temozolomide (TMZ) in glioblastoma (GBM) is limited by resistance. The goal of this study was to elucidate mechanisms of TMZ resistance in GBM.

EXPERIMENTAL DESIGN—We developed an *in vivo* GBM model of TMZ resistance and used paired parental and TMZ 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 demonstrated upregulation of 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 demonstrated an association between MGMT upregulation and elevated acetylation of lysine 9 of histone H3 (H3K9-ac) and decreased di-methylation (H3K9-me2) in GBM12 and GBM14. In contrast, TMZ 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 GBM12 TMZ resistant line, MGMT re-expression 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 TMZ and the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) favored the evolution of TMZ resistance by MGMT over-expression as compared to treatment with TMZ alone.

CONCLUSION—This study demonstrates, for the first time, a unique mechanism of TMZ 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.

Keywords

glioblastoma; MGMT; chromatin; HDAC; temozolomide

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Introduction

Temozolomide (TMZ) is an important component of therapy for glioblastoma multiforme (GBM). Unfortunately, tumor progression while receiving TMZ therapy occurs in over 40% of patients (1-4), and progression during second-line therapy is essentially universal (3, 5, 6). Thus, understanding mechanisms of primary and acquired TMZ resistance is critically important. The DNA repair protein O6-methylguanine-DNA-methyltransferase (MGMT) and the mismatch repair (MMR) pathway are critical mediators of TMZ sensitivity (7). MGMT removes cytotoxic O6-methylguanine (O6-MG) lesions induced by TMZ (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 TMZ 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 GBM patients treated with TMZ (3, 12, 13). Mutations in MMR genes are rare in primary GBM, but acquired MSH6 mutations are associated with development of TMZ 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 TMZ resistance.

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 TMZ 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 TMZ doses. These paired primary and TMZ-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 TMZ resistance linked with chromatin-mediated MGMT upregulation. Further, we show that combined therapy with TMZ and an HDAC inhibitor promotes this epigenetically-driven mechanism of resistance.

Materials and Methods

Establishment of Primary and TMZ-resistant GBM xenograft lines

The Mayo GBM xenograft panel has been previously described (19). TMZ resistance models were developed from mice with established flank tumors treated with either with 20 mg/kg/day \times 3 and then 66 mg/kg/day \times 3 after initial tumor re-growth (GBM12), or treated with 66 mg/kg/day \times 3 (GBM14, 22, 28 and 39). The resulting TMZ-resistant lines were completely resistant to a challenge of 120 mg/kg/day \times 5 days). The efficacy of TMZ 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, Carlsbad, CA).

***In vitro* cytotoxicity assays**

Explant cultures were plated in triplicate on 96-well plates, and treated with graded concentrations of TMZ \pm 10 μ M 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/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 RT-PCR as described (23). The same specimens were processed for western blotting using the antibodies: MGMT (R & D systems, Minneapolis, MN), β -actin (Sigma, St. Louis, MO) and horseradish peroxidase-conjugated to secondary antibodies (Pierce, Rockford, IL) (23). Bisulfite-modified DNA was evaluated by pyrosequencing using a PyroMark MD system (Qiagen, Valencia, CA).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using the EZ-ChIP™ kit (Millipore, Billerica, MA). Tumor samples were minced, and crosslinking was performed with 1% formaldehyde and quenched with 0.1 M 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, Danvers, MA. The anti-SP1, -C-JUN, -NF- κ B and -p300/CBP antibodies were from Santa Cruz Biotech., Santa Cruz, CA). The distal promoter region critical for MGMT silencing by hypermethylation (24) was PCR amplified with human-specific primer sequences: 5'-GCCCCGGATATGCTGGGAC-3' (forward) and 5'-GGGCAACACCTGGGAGGCAC-3' (reverse). A 35 cycle PCR was performed using 60°C annealing temperature. NIH Image J (<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 TMZ resistance

Mice with GBM12 tumors were treated with placebo or 50 mg/kg/day TMZ for 5 days every 28 days for 3 cycles. Tumor volume was measured thrice weekly, and mice with tumors exceeding 1500 mm³ were euthanized. Tumors from each mouse were flash frozen and cryopreserved. In a second study, GBM12 xenografts were treated with placebo or TMZ (50 mg/kg days 1-5) with or without SAHA (100 mg/kg on days 1-7 and 15-21 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 TMZ resistant) as the ratio of median survival between TMZ and placebo treated mice and compared across all lines using a two-sample rank sum test. Differences in the *in vitro* cell survival, PCR band intensity and pyrosequencing were analyzed using a two-sample t-test.

Results

Establishment of pair-matched TMZ 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 TMZ in orthotopically-implanted xenografts correlated highly with MGMT promoter methylation and protein expression (20). From this panel, 4 TMZ sensitive tumors (GBM12, 14, 22 and 39) and 1 relatively resistant tumor (GBM28) were subjected to *in vivo* selection for TMZ resistance. These derivative TMZ 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 TMZ in each derivative line was evaluated in an orthotopic survival study and compared to published results for the corresponding parental lines (Figure 1 and supplemental Figure S1). As summarized in the supplemental Table S1, the survival benefit associated with TMZ therapy is significantly greater in each of the parental lines as compared to the derivative TMZ-resistant line (median relative survival for TMZ treatment relative to placebo: parental lines 3.6 versus TMZ resistant line 1.2; $p < 0.01$). Thus, the TMZ selection method generated highly TMZ-resistant tumor lines.

Re-expression of MGMT in TMZ resistant GBM xenografts

MGMT expression is an important mediator of TMZ resistance (3, 13, 18, 25), and therefore, the MGMT protein and mRNA levels were evaluated in the paired lines. Consistent with epigenetic regulation of expression, MGMT protein was undetectable in the 3 parental MGMT methylated lines (GBM12, 22, 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 appears to be a common occurrence in the derivative TMZ-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 TMZ was ineffective in all 3 resistant lines, co-treatment with O6-BG significantly enhanced TMZ sensitivity in GBM12TMZ and GBM14TMZ but not GBM22TMZ (Fig. 2C). Specifically, treatment with 30 μ M TMZ without or with O6-BG was associated with a relative absorbance, compared to 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, treatment with TMZ plus O6-BG resulted in a significant reduction in neurosphere formation (14 ± 4.4) as compared to TMZ alone (98 ± 3.8 ; $p < 0.0001$), while O6-BG alone had no impact on neurosphere formation (Fig. 2D). Together, these data demonstrate that the TMZ resistance observed in GBM12TMZ and GBM14TMZ is mechanistically linked to over-expression of MGMT.

Delineation of epigenetic changes within MGMT promoter region

Since promoter methylation is an important mechanism regulating MGMT expression and TMZ 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 TMZ resistant lines demonstrated 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 performed on GBM12, GBM14, GBM22 and the derivative TMZ lines. As shown in Figure 3B, the average percent 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 demonstrated elevated methylation while no CpGs had decreased methylation (supplementary Table S2). Thus, there were no major changes in MGMT promoter methylation status that can explain the MGMT upregulation observed in GBM12TMZ and GBM14TMZ.

Since post-translational 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, GBM14 and GBM22. Consistent with a closed chromatin structure and low MGMT expression, all 3 parental tumors GBM12, GBM14 and GBM22 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 to parental tumors (Fig. 4A-B), while 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-KB and p300 to the MGMT promoter (Fig. 4D). Collectively, these data suggest that MGMT upregulation linked with the acquisition of TMZ resistance is modulated by histone post-translational modifications that favor transcriptionally active chromatin within the MGMT promoter.

Influence of HDAC inhibition on MGMT upregulation

TMZ resistance selection was repeated in GBM12 using multiple mice with established heterotopic xenografts and a clinically relevant dosing schedule. Twenty mice with established tumors were treated with placebo or TMZ for 5 days every 28 days for 3 cycles and then allowed to regrow. Once tumors exceeded 1500 mm^3 , mice were euthanized and tumors were used to derive neurosphere cultures and archived for molecular analysis. As seen in Figure 5A, only 2 of 10 TMZ-treated xenografts exhibited marked MGMT upregulation, while none of the placebo-treated tumors had elevated MGMT. Significant TMZ resistance was confirmed using an *in vitro* neurosphere assay in all 10 TMZ-treated tumors as compared to placebo-treated tumors (data not shown). Consistent with the importance of MGMT over-expression for TMZ resistance in a subset of tumors, TMZ sensitivity was restored with O6-BG co-treatment in the MGMT expressing GBM12TMZ tumor #3080 (relative neurosphere formation was 0.11 ± 0.03 as compared to 1.31 ± 0.2 for TMZ alone, $p<0.01$, Fig. 5B), while no such effect was observed in the non-MGMT expressing GBM12TMZ tumor #5920 (relative neurospheres/well was 0.83 ± 0.16 with TMZ alone and 0.89 ± 0.09 with TMZ/O6-BG; ($p<0.440$; Fig. 5B). Consistent with the initial findings in GBM12TMZ, only those lines with MGMT elevation demonstrated increased H3K9-ac occupancy within the MGMT promoter compared to 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 TMZ and resistance was not rescued by O6-BG (Fig. 5D, lower panel). Consistent with persistent resistance in this sub-line, subsequent *in vivo* TMZ re-challenge failed to delay the growth (data not shown) and resulted in partial restoration of MGMT expression. Thus, while the mechanism(s) of TMZ resistance in the non-MGMT expressing tumors is being evaluated, these data demonstrate the potential for heterogenous mechanisms of TMZ resistance development even within the same tumor.

Since the above findings suggest that MGMT-mediated TMZ resistance is associated with increased histone acetylation, the influence of HDAC inhibition on resistance emergence was tested in a second GBM12 experiment. Similar to before, 40 mice with established xenografts were randomized to therapy with placebo, monotherapy with SAHA or TMZ, 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 Figure 6A, global acetylation of H3K9 was markedly elevated in brain, liver and flank tumor following drug therapy, compared to placebo-treated mice. In the tumor growth assay, there was no difference in the time for flank tumors to reach 1000 mm³ in mice treated with placebo or SAHA (median survival 26 days versus 31 days, respectively; $p=0.16$), while both TMZ treatment (median survival 75 days) and SAHA + TMZ (median survival 92.5 days) significantly extended time for tumor growth beyond 1000 mm³ compared to placebo ($p<0.01$) (Fig. 6B and Supplementary Fig. S3A). Interestingly, despite no difference in treatment efficacy between TMZ vs. TMZ + SAHA ($p=0.12$), SAHA co-treatment promoted MGMT upregulation. In this experiment, none of the tumors treated with TMZ alone or SAHA alone demonstrated MGMT upregulation at recurrence, while 5 of the 8 evaluable tumors from TMZ + SAHA treatment group had high level MGMT expression (Fig. 6C). TMZ sensitivity was restored with O6-BG co-treatment in the MGMT expressing GBM12TMZ+SAHA tumor #5500 (relative neurosphere/well was 0.14 ± 0.02 as compared to 0.93 ± 0.01 for TMZ alone, $p<0.01$, Fig. 6D), while 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 TMZ resistant GBM12 xenografts (Fig. 6E), and ChIP assay revealed increased recruitment of SP1, C-JUN, NF-kB 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 TMZ resistance in GBM12 xenografts.

Discussion

The efficacy of TMZ 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 TMZ 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 subjected to multiple serial tumor passages. Moreover, response to TMZ in this panel was significantly associated with MGMT promoter methylation status, and in a subset of tumors derived from patients ultimately treated with TMZ, there was a good correlation between clinical response to TMZ therapy and sensitivity of the corresponding xenografts to TMZ (20, 23). Finally,

reminiscent of patients with an initial response but subsequent progression on TMZ therapy, the survival prolongation observed with a single cycle of TMZ 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 TMZ therapy. Collectively, these data suggest that these xenograft GBM models are highly relevant for analyzing mechanisms of TMZ resistance.

MGMT repairs cytotoxic O6-MG DNA lesions induced by TMZ. Multiple studies employing molecular or pharmacologic manipulation of MGMT have demonstrated a mechanistic link between MGMT activity and TMZ 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 TMZ resistance is not well established. In this study, a comparison of 5 primary xenograft lines with corresponding derived models of TMZ resistance demonstrated a marked upregulation of MGMT expression in a subset of resistant GBM xenograft lines. In these lines, MGMT is mechanistically linked to TMZ resistance emergence since the relatively specific MGMT inhibitor O6-BG restored TMZ sensitivity only in lines that overexpressed MGMT (GBM12TMZ and GBM14TMZ). Ongoing studies are focused on understanding the underlying mechanisms of acquired TMZ resistance in non-MGMT expressing tumors. Although previous studies have linked TMZ 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, since 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 TMZ-resistant GBM xenografts.

Promoter CpG hypermethylation can suppress MGMT expression and is associated with greater clinical benefit from TMZ in patients with newly diagnosed GBM (2-4, 28). Importantly in the current study, MGMT mRNA and protein expression levels were markedly increased following selection for TMZ resistance in methylated lines without a marked change in MGMT promoter methylation. Although the biological 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 TMZ 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 demonstrated role of histone modifications (decreased H3K9-ac and increased H3K9-me2) in transcriptional silencing of MGMT (30), the mechanisms by which TMZ exposure reverses these chromatin marks to upregulate MGMT expression are unknown and the subject of ongoing studies.

The most intriguing and clinically relevant finding of this study is the demonstration of heterogeneous mechanisms of acquired TMZ resistance in a single xenograft tumor. We observed a 100% re-growth of GBM12 xenografts after treatment with a clinically relevant TMZ regimen. Interestingly, even though these tumors were generated from a single short-term explant culture, MGMT re-expression was only detected in 2 of the 10 tumors that

recurred after TMZ therapy, and increased H3K9-ac was observed only in tumors with MGMT upregulation. The reversal of TMZ resistance by co-treatment with the MGMT inhibitor O6-BG demonstrates 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 co-emerge in the same tumor, MGMT expression was lost in the absence of TMZ selective pressure in GBM12TMZ#0461 despite this tumor remaining highly resistant to TMZ. The restoration of MGMT expression following re-treatment with TMZ 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 over-expression 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 TMZ-SAHA treatment (Fig. 6E). This finding contradicts recent publications, in which SAHA alone induced H3K9-ac mediated re-activation 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 to 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 to relatively steady concentrations of SAHA encountered *in vitro*. Moreover, since TMZ treatment alone can induce heterochromatin re-organization (32), we speculate that TMZ-induced epigenetic events may contribute to heterochromatin remodeling within the MGMT promoter associated with co-treatment with SAHA.

Because there is an ongoing clinical trial evaluating the integration of SAHA with TMZ and radiation in newly diagnosed GBM patients, the results in this study are reassuring that concurrent SAHA did not accelerate the development of TMZ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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STATEMENT OF TRANSLATIONAL RELEVANCE

The therapeutic benefit of temozolomide (TMZ), the only drug known to significantly enhance survival in newly diagnosed glioblastoma (GBM) patients, 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 TMZ resistance and subsequently demonstrated that HDAC inhibition can influence the mechanism of TMZ resistance emergence, particularly favoring resistance linked with chromatin-mediated 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 co-administered cytotoxic agent.

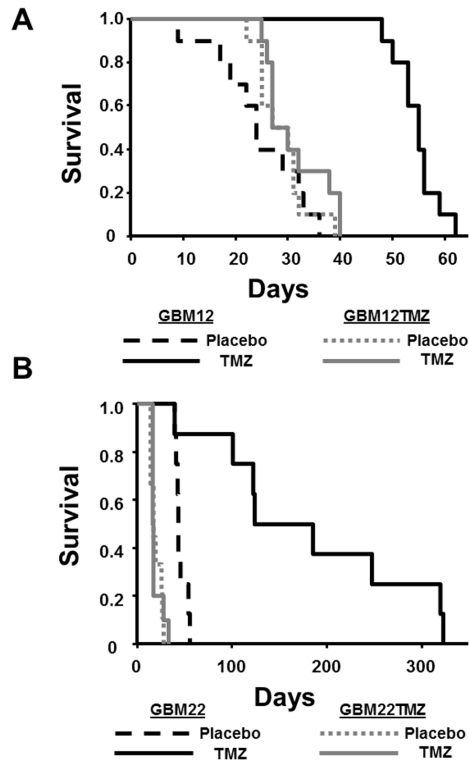


Figure 1. Evaluation of TMZ response in parental compared with the derived TMZ resistant xenografts. The efficacy of 66 mg/kg TMZ daily \times 5 days was compared to placebo treatment in parental and derivative TMZ resistant tumor lines. Shown are the representative Kaplan-Meier survival plots for A)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

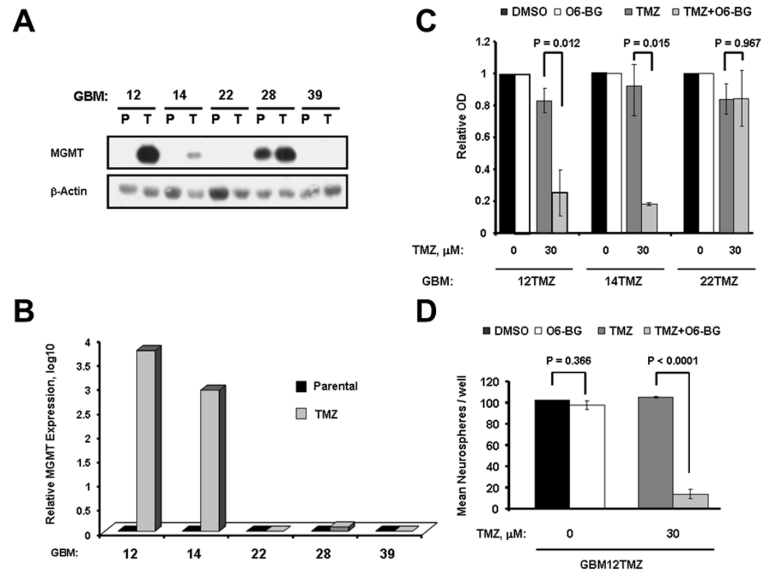


Figure 2. Expression of MGMT in TMZ resistant xenografts. A) Pooled triplicate tumor samples derived from parental (P) or derivative TMZ resistant (T) xenografts were processed for Western blotting of MGMT and β -actin. B) Total RNA from the same triplicate tumor samples was pooled and analyzed for MGMT expression by qRT-PCR in parental and TMZ resistant xenografts. For each line, the relative level of MGMT expression in TMZ lines is plotted relative to parental lines. C) Short-term explant cultures from GBM12TMZ, GBM14TMZ and GBM22TMZ were treated with graded concentrations of TMZ with or without 10 μ M O6-BG and analyzed for survival with a CyQuant assay 7 days later. D) Short-term GBM12TMZ explant stem-cell 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.

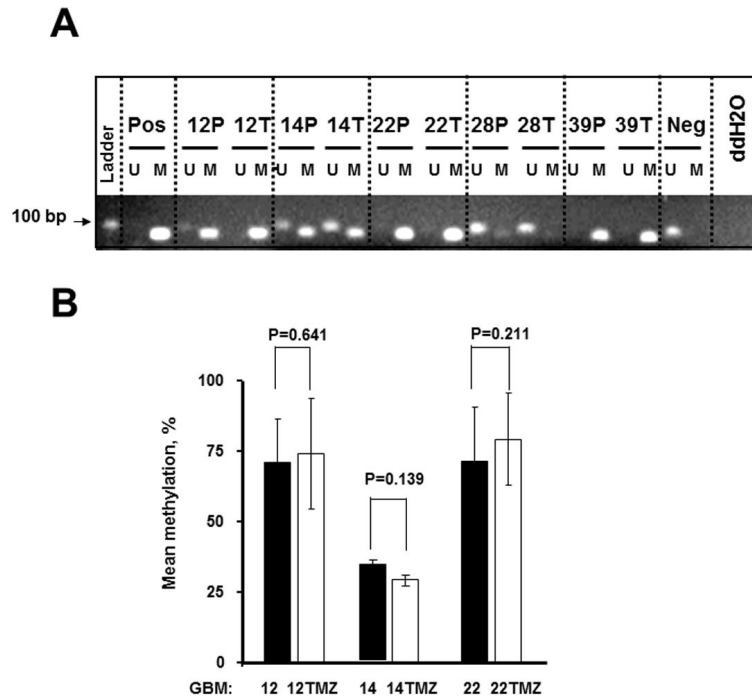
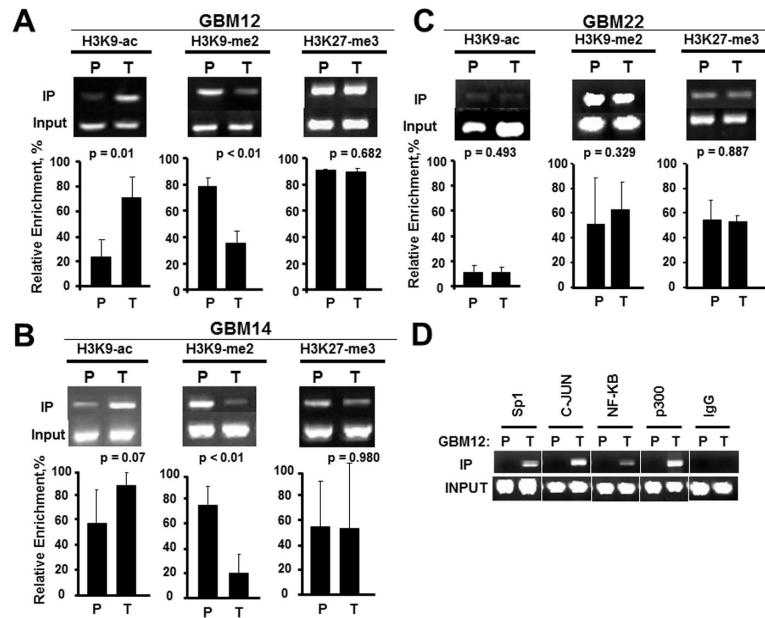
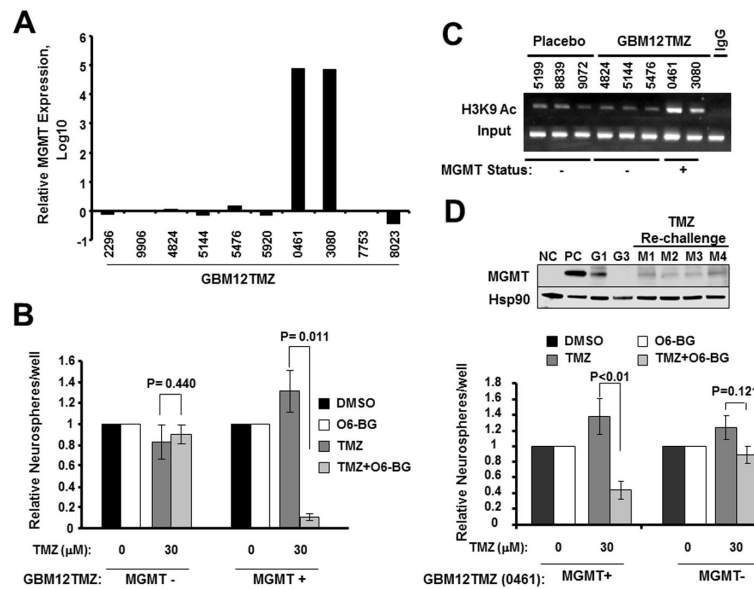


Figure 3. Evaluation of MGMT promoter methylation status in the parental and derived TMZ resistant xenografts. A) MGMT promoter methylation by MS-PCR. For each parental (P) and TMZ-resistant (T) line, an unmethylated (U) and methylated (M) specific PCR reaction are shown, along with positive (Pos) and negative (Neg) controls. B) Validation of MGMT promoter methylation by pyrosequencing in paired parental and TMZ resistant GBM12, GBM14 and GBM22. Shown is the average methylation across 22 CpG dinucleotides (CpG75-96) encompassing a region interrogated by the MS-PCR assay.

**Figure 4.**

Evaluation of the chromatin marks within MGMT promoter region comparing pair-matched parental and TMZ resistant GBM12, GBM14 and GBM22. Shown is the acetylation of lysine 9 of histone H3 (H3K9-ac), di-methylation of lysine 9 of histone H3 (H3K9-me2) and tri-methylation 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 S.D. 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 TMZ resistant (T) GBM12 cells.

**Figure 5.**

Generation of TMZ resistant GBM12 xenografts using a clinically relevant TMZ regimen. Twenty mice with GBM12 flank tumors were treated with 3-cycles of TMZ (50mg/kg/day \times 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 TMZ 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 stem-cell 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 TMZ alone or with O6-BG and analyzed for neurosphere formation 14 days later. Shown is the mean of 3 independent experiments \pm SEM. C) Evaluation of the H3K9-ac histone mark within MGMT promoter region comparing placebo and TMZ-treated non-MGMT- and MGMT-expressing samples (MGMT status - or +, respectively). D) MGMT expression by Western (upper panel) of GBM12TMZ #0461 serially-transplanted xenografts after initial isolation at generation 1 (G1) and generation 3 (G3) without TMZ treatment and then 4 individual mice (M1-M4) at generation 4 that were re-challenged *in vivo* with TMZ (50 mg/kg \times 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* TMZ sensitivity (lower panel).

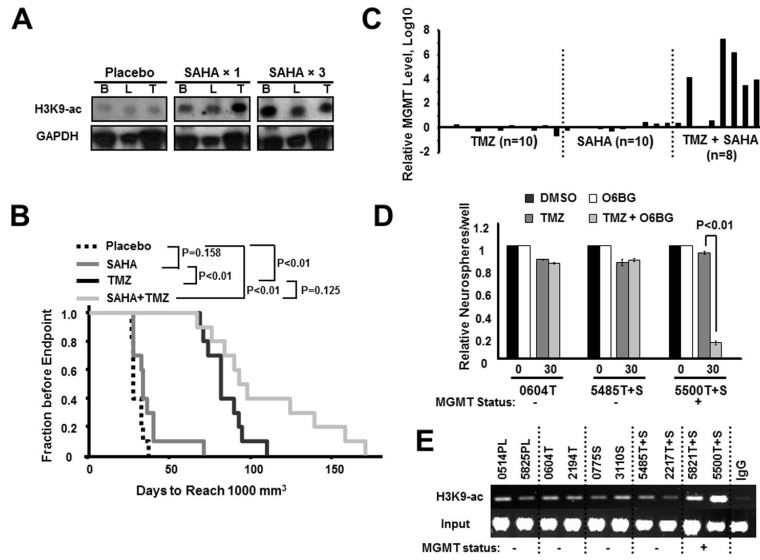


Figure 6. Effect of SAHA on the evolution of acquired resistance to TMZ. A) Effect of SAHA on the H3K9 acetylation in pooled tissue samples from brain (B), liver (L), and the tumor (T) obtained from the correlative mice after 1 and 3 doses. B) The time to exceed 1000 mm³ after treatment with placebo, TMZ alone, SAHA alone or TMZ plus SAHA. C) Evaluation of MGMT mRNA expression in recurrent xenografts following treatment with TMZ alone (n=10), SAHA alone (n=10) and SAHA plus TMZ (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 TMZ alone (0604T), TMZ + SAHA (5485T+S and 5500T+S) were treated with graded concentrations of TMZ with or without 10 μ M 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=TMZ, S=SAHA. T+S=TMZ+SAHA). The MGMT expression level (MGMT status) for individual tumors is noted below the figure.