Minor Changes in Expression of the Mismatch Repair Protein MSH2 Exert a Major Impact on Glioblastoma Response to Temozolomide

José L. McFaline-Figueroa1,2,3, Christian J. Braun1,2, Monica Stanciu1,2, Zachary D. Nagel3,4, Patrizia Mazzucato3,4, Dewaker Sangaraju5, Edvinas Černiauskas3,4, Kelly Barford3,4, Amanda Vargas3,4, Yimin Chen3,4, Natalia Tret'yakova5, Jacqueline A. Lees1,2, Michael T. Hemann1,2, Forest M. White2,3,4, and Leona D. Samson1,2,3,4

Abstract

Glioblastoma (GBM) is often treated with the cytotoxic drug temozolomide, but the disease inevitably recurs in a drug-resistant form after initial treatment. Here, we report that in GBM cells, even a modest decrease in the mismatch repair (MMR) components MSH2 and MSH6 have profound effects on temozolomide sensitivity. RNAi-mediated attenuation of MSH2 and MSH6 showed that such modest decreases provided an unexpectedly strong mechanism of temozolomide resistance. In a mouse xenograft model of human GBM, small changes in MSH2 were sufficient to suppress temozolomide-induced tumor regression. Using The Cancer Genome Atlas to analyze mRNA expression patterns in tumors from temozolomide-treated GBM patients, we found that MSH2 transcripts in primary GBM could predict patient responses to initial temozolomide therapy. In recurrent disease, the absence of microsatellite instability (the standard marker for MMR deficiency) suggests a lack of involvement of MMR in the resistant phenotype of recurrent disease. However, more recent studies reveal that decreased MMR protein levels occur often in recurrent GBM. In accordance with our findings, these reported decreases may constitute a mechanism by which GBM evades temozolomide sensitivity while maintaining microsatellite stability. Overall, our results highlight the powerful effects of MSH2 attenuation as a potent mediator of temozolomide resistance and argue that MMR activity offers a predictive marker for initial therapeutic response to temozolomide treatment.

Introduction

Glioblastoma (GBM), or WHO grade IV glioma, is the most common and aggressive type of brain cancer with a median survival of 9.7 months after patient diagnosis (1). GBM treatment consists of surgical resection of the main tumor mass followed by radiotherapy and concomitant chemotherapy. Frontline chemotherapy in the treatment of GBM consists of temozolomide (TMZ), an oral S-1 mono-alkylating agent shown to increase overall survival when administered with radiotherapy (2). Although considered a success, on average temozolomide extends survival by only 1 to 2 months, with recurrent GBM showing a strong chemoresistant phenotype.

Although temozolomide induces a variety of DNA base lesions, toxicity is mediated primarily by DNA mismatch repair (MMR)-dependent processing at O6-methylguanine (O6-meG) base lesions produced by temozolomide (3); such processing can be prevented by O6-methylguanine methyltransferase (MGMT)-mediated removal of the methyl group from the O6 position of guanine (4). In approximately half of all GBM, MGMT is epigenetically silenced by promoter methylation at the MGMT locus, and MGMT levels are inversely correlated to the response of GBM patients to temozolomide (5, 6). In the absence of MGMT-mediated O6-meG repair, the MMR machinery potentiates the toxicity of O6-meG lesions. During replication, DNA polymerase inserts thymine opposite O6-meG and the MutS recognition complex, composed of an MSH2-MSH6 heterodimer, binds the O6-meG:T mismatch recruiting MutLg (composed of an MLH1 and PMS2 heterodimer) and Exo1. These proteins excise a stretch of single-stranded DNA containing the thymine opposite O6-meG creating a gap in the DNA. To complete mismatch repair, DNA polymerase fills the gap before DNA ligation, only to once again insert thymine opposite O6-meG, stimulating another round of MMR. This futile MMR cycling and accumulation of ssDNA gaps generate double-strand breaks upon subsequent rounds of replication, resulting in cell-cycle arrest and/or cell death (3, 7, 8). Temozolomide resistance can be achieved either by increased MGMT levels or by mutations in the MMR machinery that prevent futile MMR cycling at unreppaired O6-meG lesions. Recurrent GBM...
tumors only occasionally harbor mutations in MMR genes accompanied by microsatellite instability (MSI; refs. 9–12); this has been taken to mean that MSI infrequently plays a role in the resistant phenotype of recurrent disease. However, a recent study by the German Glioma network found frequent decreases in MMR protein levels in recurrent GBM relative to their initial tumors, suggesting that MMR deficiencies are more common than currently appreciated (13). Additional studies have also identified subsets of GBM patients that present with decreased MMR protein levels at recurrence (14).

Here, we used an in vitro model of acquired temozolomide resistance to identify changes associated with decreased temozolomide sensitivity. As in human tumors, we observed that decreases in certain MMR machinery proteins correlate with temozolomide resistance. Strikingly, we show that remarkably small decreases in some MMR components, primarily MSH2, lead to unexpected temozolomide resistance in vitro. We demonstrate that such modest decreases in MSH2 lead to a significant growth advantage for GBM tumor cells in an in vivo mouse model of GBM temozolomide chemotherapy. Finally, we show that low MSH2 and MSH6 transcript levels in GBM tumors are prognostic for patient survival after temozolomide treatment.

Materials and Methods

Cell culture

U87MG, LN229, and A172 GBM cells were purchased from ATCC, expanded and used within 10 passages. Mouse GL261 GBM cells lines, previously described (15), were a gift from Dr. David Zagzag (New York University, New York, NY). Cell lines were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin (pen-strep) under standard incubation conditions.

Generation of p53, MSH2, and MSH6 knockdown cells

Lentiviral shRNA constructs and packaging plasmids (psPAX2 and pMD2.G) were transfected into 293T cells to produce lentiviral particles. U87MG cells were infected with lentivirus and shRNA-expressing cells selected in puromycin.

Drug treatments and cell survival measurements

For the generation of temozolomide-resistant GBM cell lines, U87MG, LN229, and A172 cells were treated with temozolomide at the specified concentrations (Fig. 1A) for 3 hours in serum-free media. More details about this protocol can be found in the Supplementary Materials and Methods. For acute temozolomide and BCNU treatments, GBM cells were treated for 1 hour in serum-free media at the specified concentrations; drug-containing media were then replaced with complete media. For ionizing radiation treatment, cells were irradiated in complete media using a gamma cell irradiator for the time period necessary to achieve the specified exposure. For MNNG treatment, cells were treated in complete media and exposure time determined by its rapid decay. Sensitivity to treatment was measured using a flow cytometry-based proliferation assay as described in ref. 16.

Cell-cycle analysis

Cell-cycle profiles of GBM cells were obtained by ethanol fixation followed by staining with propidium iodide as described in ref. 17.

Immunoblotting

Cells were harvested at the appropriate conditions by scraping into ice cold PBS, centrifuged, washed, lysed and protein was quantified. Gel electrophoresis, membrane transfer, and blotting for p53, MSH2, MSH6, MLH1, PMS2, phosphoserine H2AX, and total H2AX levels were performed as described in ref. 17 and Supplementary Materials and Methods.

Host cell reactivation

GG mismatch and O6-meG:C containing fluorescent plasmids were transfected into parental and TMZ23 GBM cells by electroporation. Fluorescent protein expression was assessed by flow cytometry and compared with the expression levels of a transfection using plasmids containing no lesion. Each transfection also contained a transfection control to calculate DNA mismatch and MGMT repair capacities of GBM cells. More details about this protocol can be found in the Supplementary Materials and Methods section and in ref. 18.

GL261 in vitro and in vivo competition assay

The effects of decreased MSH2 levels on the sensitivity of murine GL261 GBM cells were assessed when cultured in vitro as well as when injected into mouse brains to recapitulate GBM tumors in vivo, using a competition assay. For both competition assays, GL261 cells were infected with shRNA vectors such that 20% to 40% of cells express the shRNA and are marked by GFP. For the in vitro competition assay, 96 hours after exposure, single cell suspensions were prepared and the percentage of GFP-positive cells quantified by flow cytometry. For the in vivo competition assay, mice were euthanized when appropriate criteria presented, brains were removed, tumors localized, excised, and dissociated. Suspensions of GL261 cells were analyzed by flow cytometry to assess the percentage of GFP-positive cells.

The Cancer Genome Atlas data analysis

The Cancer Genome Atlas (TCGA) datasets and clinical patient data were downloaded from the Broad Firehose data portal and the NCI TCGA data matrix, respectively. Data were z-scored and patients treated with temozolomide were chosen for analysis on the effects of MSH2, MSH3, MSH6, PMS2, MLH1, and MGMT expression levels on survival. More details can be found in the Supplementary Materials and Methods section.

Supplementary materials and methods

Supplementary discussion, figures, tables, and in depth description of the experimental procedures and reagents used are available in a separate document.

Results

Generation of temozolomide-resistant p53-proficient and p53-deficient GBM cells by periodic exposure to escalating doses of temozolomide

Results from TCGA Research Network identified mutations in TP53, a major node in the cellular response to DNA damage, in almost 40% of tumor samples obtained mostly from primary GBM patients (10). To identify changes associated with acquired temozolomide resistance in GBM, we generated resistant cell lines by periodic exposure of U87MG GBM cells that were p53-proficient (control) and p53-deficient (p53kd; Supplementary Fig. S1A and S1B) to increasing concentrations (Fig. 1A) for 3 hours in media. More details about this protocol can be found in the Supplementary Materials and Methods section and in ref. 18.

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Three rounds of drug selection

- Round 1 (R1): 20 μmol/L TMZ + recovery to 90% confluence
- Round 2 (R2): 40 μmol/L TMZ + recovery to 90% confluence
- Round 3 (R3): 40 μmol/L TMZ + recovery to 90% confluence

Figure 1.
Periodic exposure of GBM cells to temozolomide produces a chemoresistant phenotype. A, treatment scheme for the in vitro selection of temozolomide-resistant GBM cells. B, sensitivity of p53-proficient and p53-deficient GBM cells before and after temozolomide selection (n = 3; ± SD; **, P < 0.01 ANOVA). C, cell-cycle profiles of parental and TMZR3 GBM cells two cell-cycle times after temozolomide exposure. D, quantitation of cell-cycle changes in parental and TMZR3 GBM cells two cell-cycle times after temozolomide exposure. E, H2AX serine 139 phosphorylation in parental and TMZR3 GBM cells two cell-cycle times after temozolomide exposure (n = 3; ± SD; **, P < 0.01 Student t test). F-H, sensitivity of parental and TMZR3 GBM cells to MNNG (F), BCNU (G), and ionizing radiation (H; n = 3; ± SD; **, P < 0.01 ANOVA).
Figure 2.
The temozolomide-resistant phenotype in TMZR3 GBM cells is not due to increased repair of O6-methylguanine lesions but is correlated to decreased MMR activity. A, immunoblot analysis of MGMT levels in parental and TMZR3 GBM cells. B, O6-meG repair capacity of parental and TMZR3 GBM cells (n = 3; ± SD). C, immunoblot analysis of MSH6 and MSH2 levels in parental and TMZR3 GBM cells. D and E, quantitation of MSH6 (D) and MSH2 (E) immunoblot analyses. Protein levels were normalized to MSH6 and MSH2 levels in control cells (n = 3; ± SD; **, P < 0.01 Student t test). F, mismatch repair capacity against a G:G mismatch in parental and TMZR3 GBM cells (n = 3; ± SD; *, P < 0.05; **, P < 0.01 Student t test).
doses of temozolomide emulated standard temozolomide chemotherapy regimens currently used for GBM therapy (75 mg/kg/m² cycle, followed by a 150 mg/kg/m² and finally a high dose of 200 mg/kg/m²; ref. 2). Pharmacokinetic studies have shown a maximum cerebrospinal fluid (CSF) temozolomide concentration of approximately 10 μmol/L after 200 mg/kg/m² dosing (19). Temozolomide displays linear pharmacokinetics up to and past the maximum tolerated single dose of 750 to 1,000 mg/kg/m² (20, 21). In addition, GBM tumors display a local breakdown of the blood-brain-barrier (22), resulting in increased intratumoral temozolomide levels compared with the CSF. Taken together, these results suggest that the concentrations used here to induce temozolomide resistance are indeed clinically relevant.

Figure 3.
Small decreases in MSH2 protein levels drastically alter the sensitivity of GBM cells to temozolomide. A and B, MSH6 (A) and MSH2 (B) protein levels in panels of MSH6 and MSH2 knockdown GBM cells measured by quantitative immunoblotting. Residual protein levels after MSH6 or MSH2 knockdown can be found at the bottom of each bar. Blue and pink shaded regions denote MSH6 and MSH2 knockdown cells where sensitivity (S) or resistance (R) to temozolomide was observed (n = 3; ± SEM). C and D, sensitivity of MSH6 (C) and MSH2 (D) knockdown cells to temozolomide (n = 5; ± SD; *** P < 0.001 two-way ANOVA). E and F, sensitivity of MSH6 (C) and MSH2 (D) knockdown cells to BCNU (n = 3; ± SD).

www.aacrjournals.org Cancer Res; 75(15) August 1, 2015 3131

Published OnlineFirst May 29, 2015; DOI: 10.1158/0008-5472.CAN-14-3616
Figure 4.
Small decreases in MSH2 protein alter MSH6 protein levels and lead to decreased mismatch repair activity. A, mismatch repair capacity against a GG mismatch substrate in select MSH6 and MSH2 knockdown GBM cells. n = 3; ± SD; *P < 0.05; **P < 0.01; ***P < 0.001, Student t test. B, model of the effects of moderate MSH2 decreases on cellular MutSα activity. Ovals and squares represent MSH2 and MSH6 in monomer or dimer form, respectively. In a setting where MSH2 is rate limiting for the formation of MutSα dimers, small decreases in MSH6 lead to a drop in MSH6 monomer levels but do not appreciably alter MutSα dimer levels. (Continued on the following page.)
Previous reports suggest that p53 loss may sensitize GBM cells to temozolomide (23, 24). However, we find that control and p53kd cells achieved confluence at similar times following the various temozolomide treatment cycles (data not shown). A cell survival assay (16) was used to measure the temozolomide sensitivity of parental cells (control and p53kd) and of cells from the third round of temozolomide selection (control-TMZR3 and p53kd-TMZR3). Control and p53kd cells exhibited very similar temozolomide sensitivity. In contrast, both control-TMZR3 and p53kd-TMZR3 displayed a strong temozolomide-resistant phenotype (Fig. 1B) demonstrating that both p53-proficient and p53-deficient cells are capable of acquiring temozolomide resistance. Consistent with the role of p53 in the tetraploid checkpoint (25), p53kd cells rapidly became polyploid in response to temozolomide exposure and maintained polyploidy throughout subsequent rounds of temozolomide selection (Supplementary Fig. S2A). Metaphase chromosome analysis confirmed the polyploid phenotype (Supplementary Fig. S2B and S2C). Control and p53kd GBM cells underwent a robust cell-cycle arrest at the late S/G2–M boundary two cell-cycle times after a single temozolomide treatment (Fig. 1C and D). This timing corresponds to the time at which MMR-induced processing at O6-methylguanine leads to double-strand break formation at collapsed replication forks (3). In contrast, TMZR3 GBM cells did not activate a cell-cycle checkpoint two cell-cycle times after drug exposure. Immunoblot analysis of H2AX phosphorylation after temozolomide treatment revealed that TMZR3 cells exhibit decreased H2AX phosphorylation compared with parental lines (Fig. 1E and Supplementary Fig. S3). As the toxicity of temozolomide is attributed primarily to the formation of O6-methylguanine lesions in the DNA, we investigated temozolomide-induced levels of O6-methylguanine in parental and TMZR3 cells by isotope dilution tandem mass spectrometry. This analysis revealed that parental and TMZR3 cells acquire very similar levels of O6-methylguanine upon temozolomide exposure, eliminating the possibility that cells become resistant by somehow preventing temozolomide from reacting with genomic DNA (Supplementary Table S1).

The temozolomide-resistant phenotype is specific for O6-methylguanine formation, does not confer resistance to ionizing radiation or 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU), and is independent of MGMT

To assess whether temozolomide resistance was accompanied by resistance to other types of DNA-damaging agents relevant to GBM therapy, cells were exposed to MNNNG (an S3′,1′ alkylating agent), BCNU (a DNA cross-linking bifunctional alkylating agent), and ionizing radiation (an agent that induces DSBs and various oxidized DNA bases). TMZR3 cells displayed strong resistance to MNNNG, demonstrating that resistance extends to S3′,1′ alkylating agents that induce O6-methylguanine and is not specific to the structure of temozolomide (Fig. 1F). Parental and TMZR3 cells did not display significant differences in their sensitivity to ionizing radiation, suggesting that temozolomide resistance was not due to increased double-strand break repair (Fig. 1G). Before the adoption of temozolomide as the frontline chemotherapeutic agent for GBM patients, BCNU was the major chemotherapeutic agent used to treat GBM. It is well documented that MGMT repression greatly reduces the sensitivity of cells to BCNU, a DNA cross-linking agent whose mechanism of action initially involves formation of O6-chloroethyl lesions that are efficiently removed by MGMT (26, 27). The U87MG cell line, from which control and p53kd cells are derived, does not express MGMT, due to epigenetic silencing of the MGMT locus by promoter methylation (28), making it feasible that resistance could be achieved by MGMT derepression. Parental and TMZR3 cells were equally sensitive to BCNU treatment (Fig. 1H), suggesting that TMZR3 cells are unlikely to have reactivated MGMT expression. The temozolomide-resistant phenotype of GBM cells obtained after selection appears to be specific for monofunctional S3′,1′ alkylating agents and likely independent of MGMT-mediated enhanced O6-methylguanine repair. Immunoblot analysis of parental and TMZR3 cells confirmed that MGMT was not expressed in any of the TMZR3 cells obtained after selection (Fig. 2A). To rule out the possibility that MGMT protein levels fell below the limit of detection, or that cells repaired O6-methylguanine in an MGMT-independent manner, we used an in-cell host cell reactivation (HCR) assay for O6-methylguanine repair (Supplementary Fig. S4A). Parental and TMZR3 cells displayed equally low O6-methylguanine repair activity confirming that increased MGMT activity is not responsible for the temozolomide-resistant phenotype of TMZR3 cells. T98G cells, a GBM cell line known to expresses MGMT (29), serves as a positive control for MGMT activity (Fig. 2B). These data show that MGMT does not play a role in our system of acquired temozolomide resistance.

MMR protein levels and activity are deregulated in TMZR3 cells

MMR is known to mediate toxic processing of O6-methylguanine (30). Immunoblot analysis of parental and TMZR3 cells revealed decreases in the MutSα MMR recognition complex components, MSH6 and MSH2 (Fig. 2C) with virtually no change in the MutLα complex components, MLH1 and PMS2 (data not shown). However, these decreases were modest with 50% MSH6 and 70% MSH2 protein remaining (Fig. 2D and E). An in-cell HCR assay was used to determine whether these modest decreases in MSH2 and MSH6 correlated with diminished MMR capacity in TMZR3 cells (Supplementary Fig. S4B). TMZR3 cells displayed roughly 50% decreased MMR capacity compared with their respective parental cells (Fig. 2F). We infer that diminished MMR capacity likely contributes to temozolomide resistance in TMZR3 cells, and sought to confer whether modest MMR decreases could account for the resistance of TMZR3 cells.

Very limited knockdown of MSH2 protein levels leads to extensive temozolomide chemoresistance in GBM cells in vitro

Using a panel of lentiviral vectors encoding shRNAs targeting MSH2 or MSH6 transcripts, we created a library of U87MG GBM cells with varying degrees of MSH2 or MSH6 knockdown (Fig. 3A and B and Supplementary Fig. S5). A sharp threshold for
Figure 5.
Small decreases in Msh2 confer a growth advantage to GBM tumors after temozolomide challenge. A, Msh2 transcript (top) and protein (bottom) levels in GL261 GBM cells expressing a vector control or one of two hairpins targeting Msh2 transcripts and a marker GFP. FACS was used to obtain a pure population of control or hairpin expressing cells (n = 3; ± SEM). B, a competition assay to assess the effects of decreased Msh2 levels on the response of GL261 GBM tumors to temozolomide. Green, GL261 cells expressing GFP as a marker of hairpin expression. C, temozolomide induced changes in the proportion of GFP-expressing cells in GL261 GBM cells expressing a vector control or one of two hairpins targeting Msh2 transcript as measured in vitro. (Continued on the following page.)
temozolomide sensitivity was observed as a function of MSH6 knockdown with a transition to temozolomide resistance in cells with 32% or less residual MSH6 protein (Figs. 3A and C and 4C). Temozolomide resistance correlated with decreased late $S$-$G_2$-$M$ accumulation after temozolomide treatment (Supplementary Fig. S6A and S6B). Strikingly, the temozolomide sensitivity of MSH2 knockdown cells revealed that a modest 15% decrease in MSH2 protein levels was sufficient to yield robust temozolomide resistance compared with cells expressing a nonsilencing hairpin control (Figs. 3B and D and 4D). Again, the temozolomide-resistant phenotype correlated with decreased late $S$-$G_2$-$M$ accumulation after temozolomide treatment (Supplementary Fig. S7A and S7B). It is important to note that, like TMZR3 cells, none of the MSH2 and MSH6 knockdown cells showed any resistance to BCNU compared with control (Fig. 3E and F). Therefore BCNU treatment could be an effective alternative for GBM patients with recurrent disease.

As MSH2 appears to be such a potent mediator of temozolomide resistance, we investigated whether decreased MSH2 levels are selected for in other GBM backgrounds. Selecting for temozolomide-resistant cells in A172 and LN229 GBM cells using our previously described protocol (Fig. 1A) resulted in the generation of cells with a significant decrease in sensitivity to temozolomide and decreased MMR activity compared with parental cells (Supplementary Fig. S8A, S8B, S8E, and S8F). Analysis of MSH2 protein levels revealed that minor decreases in MSH2 levels are selected for in both GBM backgrounds (Supplementary Fig. S8C, S8D, S8G, and S8H). It should be noted, however, that in a separate experiment we obtained temozolomide-resistant LN229 cells that did not display decreased MSH2 protein levels despite decreased sensitivity to temozolomide and decreased MMR activity (data not shown).

To investigate how temozolomide resistance correlated to MMR activity in MSH2 knockdown cells, MMR-HCR was used to measure MMR activity in MSH1 knockdown cell lines that displayed sensitivity to temozolomide (MSH6 kd #2 with 51% residual MSH6 protein) and resistance to temozolomide (MSH6 kd #5 with 10% residual MSH6 protein; MSH2 kd #2 with 63% residual MSH2 protein and MSH2 kd #5 with 16% residual MSH2 protein). MMR activity in the temozolomide-sensitive MSH6 kd #2 cell line was statistically indistinguishable from cells expressing a nonsilencing hairpin control. In contrast, decreased MMR activity was observed for the three MSH1 knockdowns that displayed temozolomide resistance (Fig. 4A). These observations are consistent with MSH6 monomer levels found in excess compared with free MSH2, making MSH2, the limiting factor in MutS $\alpha$ (Fig. 4B).

It is well documented that MSH2 and MSH6 stability is influenced by their dimerization (31). MSH2 has two dimerization partners, namely MSH6 and MSH3, generating the MutS $\alpha$ heterodimers, respectively. In contrast, MSH6 only dimerizes with MSH2 (32), which recognizes and binds $O^6$-methylguanine (3). Given that MSH2 has two binding partners, we investigated MSH2 stability in the MSH6 knockdown GBM cells; resistance to temozolomide was only seen when MSH2 loss began to destabilize MSH1 as reflected by decreased MSH2 protein levels (Fig. 4C and E and Supplementary Fig. S9A). Analysis of MSH6 stability in the MSH2 knockdown cells revealed that MSH6 protein levels decreased linearly with decreasing MSH2 protein levels (Fig. 4D and F and Supplementary Fig. S9B). Therefore, it appears that resistance to temozolomide was observed at MSH6 or MSH2 knockdown levels where destabilization of the binding partner becomes apparent, which presumably accompanies decreased MutS $\alpha$ dimer levels and decreased binding to $O^6$-methylguanine.

Small reductions in Msh2 decrease the in vivo response of GBM tumors to temozolomide therapy

To determine whether the effect of minor decreases in MSH2 protein levels on the response of cultured GBM cells to temozolomide are relevant when treating brain tumors, we used the GL261 syngeneic mouse model of GBM. GL261 glioma cells form robust tumors that have characteristics consistent with human GBM when injected into the brain of syngeneic C57B6/J mice (15). GL261 GBM cells were infected with retroviral particles containing vectors expressing either a vector control, Msh2 hairpin 1 or Msh2 hairpin 2, leading to 0%, 10%, or 40% Msh2 mRNA knockdown, respectively, and 0%, 25%, or 50% Msh2 protein knockdown, respectively (Fig. S9A and Supplementary Fig. S10). The infection was carried out such that 20% to 40% of GL261 cells expressed shRNA and a marker GFP. Assessment of the fraction of GFP-positive cells after temozolomide treatment establishes whether the Msh2 hairpins confer a selective advantage (yielding enrichment of GFP cells), a selective disadvantage (yielding depletion) or have no effect on the GBM cells (Fig. 5B). These competition experiments were conducted in both cell culture (in vitro), or after transplant into the mouse brain (in vivo). As expected, cells expressing the vector control were neither enriched nor depleted in response to temozolomide treatment in vitro or in vivo (Fig. 5C and D). In contrast, the Msh2 hairpin 2 expressing cells conferred a large growth advantage upon temozolomide treatment in vitro (Fig. 5C). Msh2 hairpin 1 expressing cells displayed a trend toward a growth advantage but this was only significant for the 45 $\mu$mol/L dose (Fig. 5C). Similarly to Msh2 knockdown in U87MG cells, decreased Msh2 did not confer a growth advantage to GL261 cells after BCNU exposure (Supplementary Fig. S11). More importantly, in vivo, significant enrichment upon temozolomide treatment was observed for GFP cells expressing either Msh2 hairpin, with hairpin 2 conferring a stronger growth advantage than hairpin 1 consistent with the higher degree of Msh2 knockdown (Fig. 5D and E). Thus, in two distinct cell lines, and more importantly in the in vivo tumor context, very modest decreases in MSH2 protein levels endow GBM cells with a significant growth advantage during temozolomide treatment.
MSH2 transcript levels are predictive for the overall survival of temozolomide-treated primary GBM patients

Our results suggest that moderate decreases in MSH2 levels alter the response of GBM tumors to temozolomide therapy. This led us to hypothesize that if there were a range of MSH2 and MSH6 expression levels in primary GBM tumors, patients with low expression would be less responsive to temozolomide chemotherapy. To test our hypothesis, we ranked MSH2 and MSH6 transcript levels of resected primary tumors among GBM patients who had been treated with temozolomide. Transcript levels were derived from TCGA data and ranked by a z-score of ±0.5 as described in Materials and Methods (10). We observed a trend for low MSH6 transcript levels being associated with decreased survival in temozolomide-treated TCGA patients, but the difference did not reach significance for the overall survival of temozolomide-treated GBM patients (Fig. 6A). However, when we exclude patients whose survival falls on the tail end, beyond the normal distribution for patient survival after temozolomide treatment (top 5th percentile), low MSH6 levels did significantly correlate with decreased GBM patient survival after temozolomide treatment ($P < 0.05$; Fig. 6D and Supplementary Fig. S12A). Strikingly, low MSH2 transcript levels showed a highly significant correlation with decreased overall survival of temozolomide-treated GBM patients ($P < 0.05$) and the correlation was stronger ($P < 0.001$) for temozolomide-treated patients minus the top 5th percentile for patient survival after temozolomide treatment (Fig. 6B and E). Moreover, for this subset there was a significant correlation between MSH2 transcript levels and survival down to ±0.25 z-score (Supplementary Fig. S12B). MGMT methylation status and transcript levels are currently the most accepted molecular biomarkers for the survival of GBM patients (5, 33). In this particular TCGA dataset, low MGMT transcript levels were indeed significantly correlated with patient survival ($P < 0.05$), but only when we exclude patients whose survival falls on the tail end of the normal distribution (Fig. 6C and F). Taken together, it appears that MSH2 levels are a strong predictor of GBM patient response to initial temozolomide therapy.

Discussion

It has been assumed that the involvement of MMR in the resistant phenotype of GBM is low due to studies investigating markers of complete MMR deficiency, namely MSI and a mutator phenotype (12). However, several studies have demonstrated that, unlike complete loss of MSH2 or MLH1 function, even severe decreases in MSH2 or MLH1 levels do not effectively induce MSI (34–36). Our results suggest that the recently observed
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by which to increase MMR activity, possibly by increasing MSH2 levels, offers a point of intervention to potentiate temozolomide efficacy in GBM patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J.L. McFalone-Figueroa, J.A. Lees, M.T. Hermann, F.M. White, L.D. Samson
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.L. McFalone-Figueroa, C.J. Braun, M. Stanciu, P. Mazuccato, D. Sangaraju, E. Cermakias, K. Barford, A. Vargas, Y. Chen, N. Tretyakova, L.D. Samson
 Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L. McFalone-Figueroa, C.J. Braun, M. Stanciu, D. Sangaraju, Y. Chen, N. Tretyakova, J.A. Lees, L.D. Samson
 Writing, review, and/or revision of the manuscript: J.L. McFalone-Figueroa, C.J. Braun, J.A. Lees, F.M. White, L.D. Samson

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.L. McFalone-Figueroa, L.D. Samson


Other (LC-MS/MS analysis): D. Sangaraju

Grant Support

Funding support was provided by the Integrative Cancer Biology Program (ICBP) at MIT, U54-CA112967, and U.S. NIH Grants, R01-ES022872, P30-CA05103, P30-ES02109, T32-GM007287, T32-GM061081, and DP1-ES022576. C.J. Braun is the recipient of a Mildred-Schoel fellowship of the German Cancer Foundation. J.L. McFalone-Figueroa is the recipient of a Ruth L. Kirschstein National Research Service Award SF31CA165735 of the National Cancer Institute. L.D. Samson is an American Cancer Society Professor.

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Received December 11, 2014; revised April 13, 2015; accepted May 7, 2015; published OnlineFirst May 29, 2015.

References


Minor Changes in Expression of the Mismatch Repair Protein MSH2 Exert a Major Impact on Glioblastoma Response to Temozolomide

José L. McFalone-Figueroa, Christian J. Braun, Monica Stanciu, et al.

Cancer Res 2015;75:3127-3138. Published OnlineFirst May 29, 2015.

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