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Minor changes in expression of the mismatch repair protein MSH2 exert a major impact on glioblastoma response to temozolomide

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Abstract

Glioblastoma (GBM) is often treated with the cytotoxic drug temozolomide (TMZ) 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 TMZ sensitivity. RNAi-mediated attenuation of MSH2 and MSH6 showed that such modest decreases provided an unexpectedly strong mechanism of TMZ resistance. In a mouse xenograft model of human GBM, small changes in MSH2 were sufficient to suppress TMZ-induced tumor regression. Using the Cancer Genome Atlas to analyze mRNA expression patterns in tumors from TMZ-treated GBM patients, we found that MSH2 transcripts in primary GBM could predict patient responses to initial TMZ 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 TMZ sensitivity while maintaining microsatellite stability. Overall, our results highlight the

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Conflict of interest statement

The authors declare no conflict of interest.

Author Contributions

J.L.M.F., N.T., J.A.L., M.T.H., F.M.W. and L.D.S. designed research. J.L.M.F., C.J.B, M.S., Z.D.N, P.M., D.S., E.C., K.B., A.V, and Y.C., performed research. J.L.M.F., C.J.B, M.S., Z.D.N, and D.S. analyzed data. C.J.B. and M.S. contributed equally to this work. J.L.M.F., F.M.W. and L.D.S. wrote the article.

powerful effects of MSH2 attenuation as a potent mediator of TMZ resistance, and argue that MMR activity offers a predictive marker for initial therapeutic response to TMZ 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_N1 mono-alkylating agent shown to increase overall survival when administered with radiotherapy (2). Although considered a success, on average TMZ extends survival by only one to two months, with recurrent GBM showing a strong chemoresistant phenotype.

While TMZ induces a variety of DNA base lesions, toxicity is mediated primarily by DNA mismatch repair (MMR) dependent processing at O^6-methylguanine (O^6-meG) base lesions produced by TMZ (3); such processing can be prevented by O^6-methylguanine methyltransferase (MGMT) mediated removal of the methyl group from the O^6 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 TMZ (5,6). In the absence of MGMT mediated O^6-meG repair, the MMR machinery potentiates the toxicity of O^6-meG lesions. During replication, DNA polymerase inserts thymine opposite O^6-meG and the MutSα recognition complex, composed of an MSH2-MSH6 heterodimer, binds the O^6-meG:T mismatch recruiting MutLα (composed of an MLH1 and PMS2 heterodimer) and Exo1. These proteins excise a stretch of single-stranded DNA containing the thymine opposite O^6-meG creating a gap in the DNA. To complete mismatch repair, DNA polymerase fills the gap prior to DNA ligation, only to once again insert thymine opposite O^6-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). TMZ resistance can be achieved either by increased MGMT levels or by mutations in the MMR machinery that prevent futile MMR cycling at unrepaired O^6-meG lesions. Recurrent GBM tumors only occasionally harbor mutations in MMR genes accompanied by microsatellite instability (MSI) (9–12); this has been taken to mean that MMR 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 TMZ resistance to identify changes associated with decreased TMZ sensitivity. As in human tumors, we observed that decreases in certain MMR machinery proteins correlate with TMZ resistance. Strikingly, we show that remarkably small decreases in some MMR components, primarily MSH2, lead to unexpected TMZ resistance *in vitro*. We demonstrate that such modest decreases in MSH2

leads to a significant growth advantage for GBM tumor cells in an *in vivo* mouse model of GBM TMZ chemotherapy. Finally, we show that low *MSH2* and *MSH6* transcript levels in GBM tumors are prognostic for patient survival after TMZ 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 (NYU). Cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum (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 TMZ resistant GBM cell lines U87MG, LN229 and A172 cells were treated with TMZ at the specified concentrations (Fig. 1A) for 3 hr in serum-free media. More details regarding this protocol can be found in the Supplemental Material and Methods section. For acute TMZ and BCNU treatments, GBM cells were treated for 1 hour in serum-free media at the specified concentrations; drug-containing media was 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 (16).

Cell cycle analysis

Cell cycle profiles of GBM cells were obtained by ethanol fixation followed by staining with propidium iodide as described in (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 was performed as described in (17) and Supplemental Materials and Methods.

Host cell reactivation

GG mismatch and O^6-meG:C containing fluorescent plasmids were transfected into parental and TMZ^{R3} GBM cells by electroporation. Fluorescent protein expression was assessed by flow cytometry and compared to 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 regarding this protocol can be found in the Supplemental Material and Methods section and in (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 post-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.

TCGA data analysis

TCGA datasets and clinical patient data were downloaded from the Broad Firehose data portal and the NCI TCGA data matrix, respectively. Data was z-scored and patients treated with TMZ 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 Supplemental Materials Methods section.

Supplemental Materials and Methods

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

Results

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

Results from The Cancer Genome Atlas 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 TMZ resistance in GBM, we generated resistant cell lines by periodic exposure of U87MG GBM cells that were p53-proficient (Control) and p53-deficient (p53kd) (Supp. Fig. 1A and 1B) to increasing doses of TMZ; the selection process is shown in Fig. 1A. The periodic exposures to increasing doses of TMZ emulated standard TMZ chemotherapy regimens currently used for GBM therapy (75 mg/kg/m^2 cycle, followed by a 150 mg/kg/m^2 and finally a high dose of 200 mg/kg/m^2) (2). Pharmacokinetic studies have shown a maximum cerebrospinal fluid (CSF) TMZ concentration of approximately 10 μM after 200 mg/kg/m^2 dosing (19). TMZ displays linear pharmacokinetics up to and past the maximum tolerated single dose of 750–1000 mg/kg/m^2 (20,21). Additionally, GBM tumors display a local breakdown of the blood-brain-barrier (22), resulting in increased intra-tumoral TMZ

levels compared to the CSF. Taken together, these results suggest that the concentrations used here to induce TMZ resistance are indeed clinically relevant.

Previous reports suggest that p53 loss may sensitize GBM cells to TMZ (23,24). However, we find that Control and p53kd cells achieved confluence at similar times following the various TMZ treatment cycles (data not shown). A cell survival assay (16) was used to measure the TMZ sensitivity of parental cells (Control and p53kd) and of cells from the third round of TMZ selection (Control-TMZ^{R3} and p53kd-TMZ^{R3}). Control and p53kd cells exhibited very similar TMZ sensitivity. In contrast, both Control-TMZ^{R3} and p53kd-TMZ^{R3} displayed a strong TMZ-resistant phenotype (Fig. 1B) demonstrating that both p53 proficient and p53 deficient cells are capable of acquiring TMZ resistance. Consistent with the role of p53 in the tetraploid checkpoint (25), p53kd cells rapidly became polyploid in response to TMZ exposure and maintained polyploidy throughout subsequent rounds of TMZ selection (Supp. Fig. 2A). Metaphase chromosome analysis confirmed the polyploid phenotype (Supp. Fig. 2B and 2C). 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 TMZ treatment (Fig. 1C and 1D). This timing corresponds to the time at which MMR-induced processing at O^6-meG leads to double strand break formation at collapsed replication forks (3). In contrast, TMZ^{R3} GBM cells did not activate a cell cycle checkpoint two cell cycle times after drug exposure. Immunoblot analysis of H2AX phosphorylation after TMZ treatment revealed that TMZ^{R3} cells exhibit decreased H2AX phosphorylation compared to parental lines (Fig. 1E and Supp. Fig. 3).

As the toxicity of TMZ is attributed primarily to the formation of O^6-meG lesions in the DNA, we investigated TMZ induced levels of O^6-meG in parental and TMZ^{R3} cells by isotope dilution tandem mass spectrometry. This analysis revealed that parental and TMZ^{R3} cells acquire very similar levels of O^6-meG upon TMZ exposure, eliminating the possibility that cells become resistant by somehow preventing TMZ from reacting with genomic DNA (Supp. Table 1).

The TMZ resistant phenotype is specific for O^6-meG formation, does not confer resistance to ionizing radiation or 1,3-bis-(2-chloroethyl)-1-nitroso-urea (BCNU) and is independent of MGMT

To assess whether TMZ resistance was accompanied by resistance to other types of DNA damaging agents relevant to GBM therapy, cells were exposed to MNNG (an S_N1 alkylating agent), BCNU (a DNA crosslinking bifunctional alkylating agent) and ionizing radiation (an agent that induces DSB's and various oxidized DNA bases). TMZ^{R3} cells displayed strong resistance to MNNG, demonstrating that resistance extends to S_N1 alkylating agents that induce O^6-meG and is not specific to the structure of TMZ (Fig. 1F). Parental and TMZ^{R3} cells did not display significant differences in their sensitivity to ionizing radiation, suggesting that TMZ resistance was not due to increased double strand break repair (Fig. 1G). Prior to the adoption of TMZ as the frontline chemotherapeutic agent for GBM patients, BCNU was the major chemotherapeutic agent used to treat GBM. It is well documented that MGMT expression greatly reduces the sensitivity of cells to BCNU, a DNA crosslinking agent whose mechanism of action initially involves formation of O^6-

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 TMZ^{R3} cells were equally sensitive to BCNU treatment (Fig. 1H), suggesting that TMZ^{R3} cells are unlikely to have reactivated MGMT expression. The TMZ resistant phenotype of GBM cells obtained after selection appears to be specific for monofunctional S_N1 alkylating agents and likely independent of MGMT-mediated enhanced O^6-meG repair. Immunoblot analysis of parental and TMZ^{R3} cells confirmed that MGMT was not expressed in any of the TMZ^{R3} 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 O^6-meG in an MGMT independent manner, we employed an in-cell Host Cell Reactivation (HCR) assay for O^6-meG repair (Supp. Fig. 4A). Parental and TMZ^{R3} cells displayed equally low O^6-meG repair activity confirming that increased MGMT activity is not responsible for the TMZ resistant phenotype of TMZ^{R3} 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 TMZ resistance.

MMR protein levels and activity are deregulated in TMZ^{R3} cells

MMR is known to mediate toxic processing of O^6-meG (30). Immunoblot analysis of parental and TMZ^{R3} cells revealed decreases in the MutSα MMR recognition complex components, MSH6 and MSH2 (Fig. 2C) with virtually no change in the MutLα components, MLH1 and PMS2 (data not shown). However, these decreases were modest with 50% MSH6 and 70% MSH2 protein remaining (Fig. 2D and 2E). An in-cell HCR assay was employed to determine whether these modest decreases in MSH2 and MSH6 correlated with diminished MMR capacity in TMZ^{R3} cells (Supp. Fig. 4B). TMZ^{R3} cells displayed roughly 50% decreased MMR capacity compared to their respective parental cells (Fig. 2F). We infer that diminished MMR capacity likely contributes to TMZ resistance in TMZ^{R3} cells, and sought to confer whether modest MMR decreases could account for the resistance of TMZ^{R3} cells.

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

Using a panel of lentiviral vectors encoding short hairpin RNAs targeting *MSH2* or *MSH6* transcripts, we created a library of U87MG GBM cells with varying degrees of MSH2 or MSH6 knockdown (Fig. 3A, 3B and Supp. Fig. 5). A sharp threshold for TMZ sensitivity was observed as a function of MSH6 knockdown with a transition to TMZ resistance in cells with 35% or less residual MSH6 protein (Fig. 3A, 3C and 4C). TMZ resistance correlated with decreased late-S/G2-M accumulation after TMZ treatment (Supp. Fig. 6A and 6B). Strikingly, the TMZ sensitivity of MSH2 knockdown cells revealed that a modest 20% decrease in MSH2 protein levels was sufficient to yield robust TMZ resistance compared to cells expressing a non-silencing hairpin control (Fig. 3B, 3D and 4D). Again, the TMZ resistant phenotype correlated with decreased late-S/G2-M accumulation after TMZ treatment (Supp. Fig. 7A and 7B). It is important to note that, like TMZ^{R3} cells, none of the MSH2 and MSH6 knockdown cells showed any resistance to BCNU compared to control

(Fig. 3E and 3F). Therefore BCNU treatment could be an effective alternative for GBM patients with recurrent disease.

As MSH2 appears to be such a potent mediator of TMZ resistance we investigated whether decreased MSH2 levels are selected for in other GBM backgrounds. Selecting for TMZ 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 TMZ and decreased MMR activity compared to parental cells (Supp. Fig. 8A-B/E-F). Analysis of MSH2 protein levels revealed that minor decreases in MSH2 levels are selected for in both GBM backgrounds (Supp. Fig. 8C-D/G-H). It should be noted, however, that in a separate experiment we obtained TMZ resistant LN229 cells that did not display decreased MSH2 protein levels despite decreased sensitivity to TMZ and decreased MMR activity (data not shown).

To investigate how TMZ resistance correlated to MMR activity in MSH knockdown cells, MMR-HCR was used to measure MMR activity in MSH knockdown cell lines that displayed sensitivity to TMZ (MSH6 kd #2 with 51% residual MSH6 protein) and resistance to TMZ (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 TMZ sensitive MSH6 kd #2 cell line was statistically indistinguishable from cells expressing a non-silencing hairpin control. In contrast, decreased MMR activity was observed for the three MSH knockdowns that displayed TMZ resistance (Fig. 4A). These observations are consistent with MSH6 monomer levels found in excess compared to free MSH2, making MSH2, the limiting factor in MutSα formation (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 MutSα and MutSβ heterodimers, respectively. In contrast, MSH6 only dimerizes with MSH2 (32) which recognize and bind O^6-meG:T mismatches (3). Given that MSH2 has two binding partners, we investigated MSH2 stability in the MSH6 knockdown GBM cells; resistance to TMZ was only seen when MSH6 loss began to destabilize MSH2 as reflected by decreased MSH2 protein levels (Fig. 4C/E and Supp. Fig. 9A). Analysis of MSH6 stability in the MSH2 knockdown cells revealed that MSH6 protein levels decreased linearly with decreasing MSH2 protein levels (Fig. 4D/F and Supp. Fig. 9B). Therefore, it appears that resistance to TMZ was observed at MSH6 or MSH2 knockdown levels where destabilization of the binding partner becomes apparent, which presumably accompanies decreased MutSα dimer levels and decreased binding to O^6-meG-T mispairs.

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

To determine whether the effect of minor decreases in MSH2 protein levels on the response of cultured GBM cells to TMZ are relevant when treating brain tumors, we employed 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. 5A, Supp. Fig. 10). The infection was carried out such that 20–40% of GL261 cells expressed shRNA and a marker GFP. Assessment of the fraction of GFP positive cells post-TMZ 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 TMZ treatment *in vitro* or *in vivo* (Fig. 5C and D). In contrast, the Msh2 hairpin 2 expressing cells conferred a large growth advantage upon TMZ treatment *in vitro* (Fig. 5C). Msh2 hairpin 1 expressing cells displayed a trend towards a growth advantage but this was only significant for the 45 μM dose (Fig. 5C). Similarly to MSH2 knockdown in U87MG cells, decreased Msh2 did not confer a growth advantage to GL261 cells post BCNU exposure (Supp. Fig. 11). More importantly, *in vivo*, significant enrichment upon TMZ 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 5E). 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 TMZ treatment.

MSH2 transcript levels are predictive for the overall survival of TMZ treated primary GBM patients

Our results suggest that moderate decreases in MSH2 levels alter the response of GBM tumors to TMZ 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 TMZ chemotherapy. To test our hypothesis, we ranked *MSH2* and *MSH6* transcript levels of resected primary tumors among GBM patients who had been treated with TMZ. Transcript levels were derived from The Cancer Genome Atlas (TCGA) data and ranked by a z-score of +/− 0.5 as described in Material and Methods (10). We observed a trend for low *MSH6* transcript levels being associated with decreased survival in TMZ treated TCGA patients, but the difference did not reach significance for the overall survival of TMZ 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 TMZ treatment (top 5^{th} percentile), low *MSH6* levels did significantly correlate with decreased GBM patient survival after TMZ treatment ($p<0.05$, Fig. 6D and Supp. Fig. 12A). Strikingly, low *MSH2* transcript levels showed a highly significant correlation with decreased overall survival of TMZ treated GBM patients ($p<0.05$) and the correlation was stronger ($p<0.001$) for TMZ treated patients minus the top 5^{th} percentile for patient survival after TMZ treatment (Fig. 6B and 6E). Moreover, for this subset there was a significant correlation between *MSH2* transcript levels and survival down to +/− 0.25 z-score (Supp. Fig. 12B). *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 data set, 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 6F). Taken together, it appears *MSH2* levels are a strong predictor of GBM patient response to initial TMZ 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 decreases in MMR protein levels in recurrent GBM (13) can be responsible for the observed TMZ resistance at recurrence. These decreases in isolation are unlikely to induce MSI accounting for the underreporting of MMR alterations in recurrent disease. Furthermore, our results suggest that the variation of MMR protein levels in patients with newly diagnosed glioma, a population essentially devoid of MMR mutations, may mediate the innate resistance to TMZ of numerous patients in this population.

The study presented here indicates that *MSH2* transcript and protein levels strongly predict the response of GBM tumors to TMZ treatment, presumably reflecting decreased MutSα activity. Characterization of MSH2 deficiency in whole animals revealed that in terms of mutation spectrum and whole body sensitivity to MNNG, a TMZ analog, MSH2 heterozygotes were analogous to MSH2 wild type mice. As expected, MSH2 null mice were resistant to alkylation damage and had a mutational spectrum consistent with MutSα loss (37,38). These results suggest that for a majority of tissues loss of one copy of *MSH2* does not lead to haploinsufficiency or resistance to O^6-meG producing compounds. More recent work by Marra and colleagues found that lymphoblastoid cells from HNPCC patients that were carriers for an *MSH2* mutation, and therefore heterozygous for wild type *MSH2*, were resistant to TMZ-induced damage (39). Therefore, it appears that for some cell types haploinsufficiency of *MSH2* can result in resistance to alkylation damage. It should be noted, however, that this haploinsufficiency resulted in an average of 40% of residual MSH2 protein, a much larger decrease than what is necessary to decrease TMZ sensitivity in GBM cells. This heterogeneous response to moderate decreases in *MSH2* levels and resistance to S_N1 alkylating agents may be a reflection of the total MutSα levels or the ratio of total MSH2 to MSH6 in different cell types.

In conclusion, we identify minor MMR deficiencies as potent mediators of TMZ resistance in GBM. Further, this work identifies MMR activity as a modulator of initial therapeutic response to TMZ. Future work identifying mechanisms by which to increase MMR activity, possibly by increasing MSH2 levels, offer a point of intervention to potentiate TMZ efficacy in GBM patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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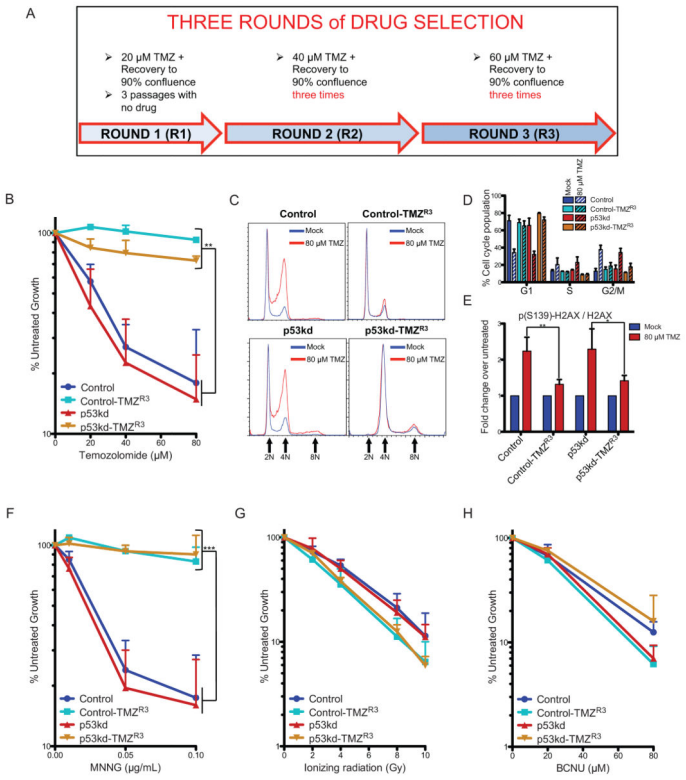

Figure 1. Periodic exposure of GBM cells to TMZ produces a chemoresistant phenotype

(A) Treatment scheme for the *in vitro* selection of TMZ resistant GBM cells.

(B) Sensitivity of p53 proficient and p53 deficient GBM cells prior to and after TMZ selection (n=3, ± s.d., p< 0.01** ANOVA).

(C) Cell cycle profiles of parental and TMZ^{R3} GBM cells two cell cycle times post-TMZ exposure.

(D) Quantitation of cell cycle changes in parental and TMZ ^{R3} GBM cells two cell cycle times post-TMZ exposure.

(E) H2AX serine 139 phosphorylation in parental and TMZ^{R3} GBM cells two cell cycle times post-TMZ exposure (n=3, ± s.d., p< 0.01** Student's t-test).

(F–H) Sensitivity of parental and TMZ^{R3} GBM cells to MNNG (F), BCNU (G) and ionizing radiation (H) (n=3, ± s.d., p< 0.01** ANOVA).

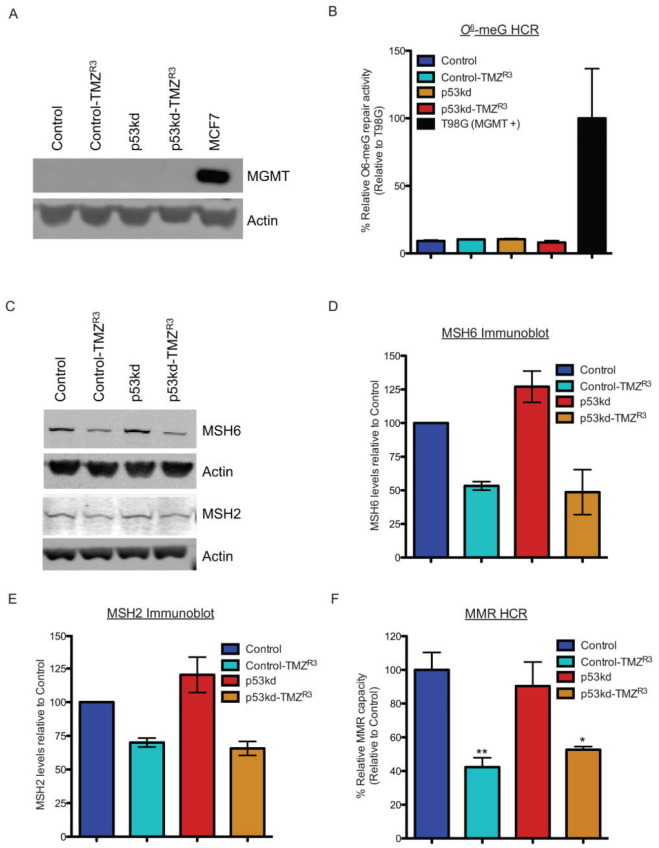

Figure 2. The TMZ resistant phenotype in TMZ^{R3} GBM cells is not due to increased repair of *O*^{6}-methylguanine lesions but is correlated to decreased MMR activity

(A) Immunoblot of MGMT levels in parental and TMZ^{R3} GBM cells.

(B) *O*^{6}-meG repair capacity of parental and TMZ^{R3} GBM cells (n=3, ± s.d.).

(C) Immunoblot of MSH6 and MSH2 levels in parental and TMZ^{R3} GBM cells.

(D–E) Quantitation of MSH6 (D) and MSH2 (E) immunoblots. Protein levels were normalized to MSH6 and MSH2 levels in Control cells (n=3, ± s.d., p< 0.01** Student's t-test).

(F) Mismatch repair capacity against a G:G mismatch in parental and TMZ^{R3} GBM cells (n=3, ± s.d., p< 0.05*; 0.01** Student's t-test).

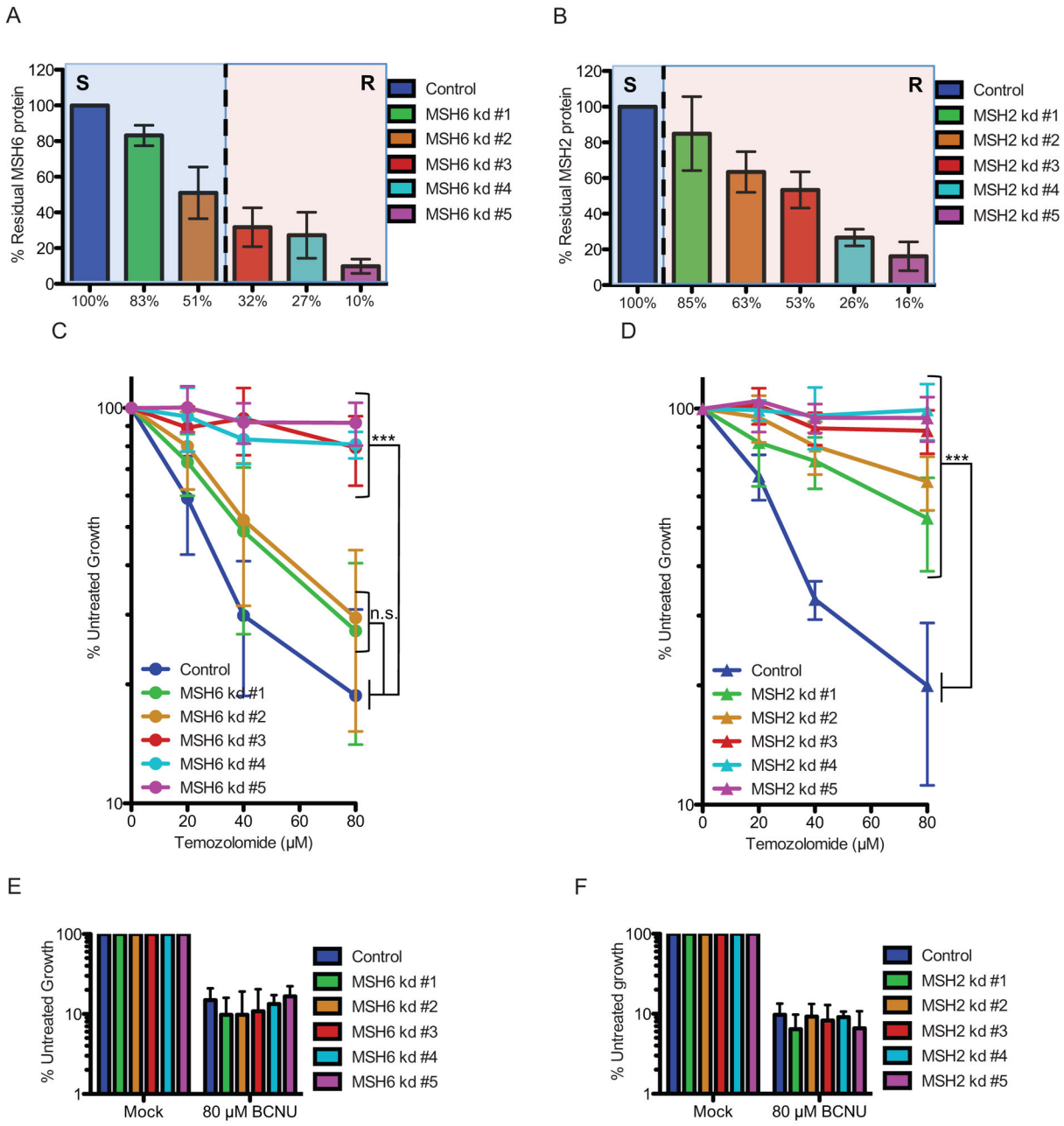

Figure 3. Small decreases in MSH2 protein levels drastically alter the sensitivity of GBM cells to TMZ

(A–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 red shaded regions denote MSH6 and MSH2 knockdown cells where sensitivity (**S**) or resistance (**R**) to TMZ was observed (n=3, ± s.e.m.).

(C–D) Sensitivity of MSH6 (C) and MSH2 (D) knockdown cells to TMZ. (n=5, ± s.d., $p < 0.001$*** Two-way ANOVA).

(E–F) Sensitivity of MSH6 (C) and MSH2 (D) knockdown cells to BCNU (n=3, ± s.d.).

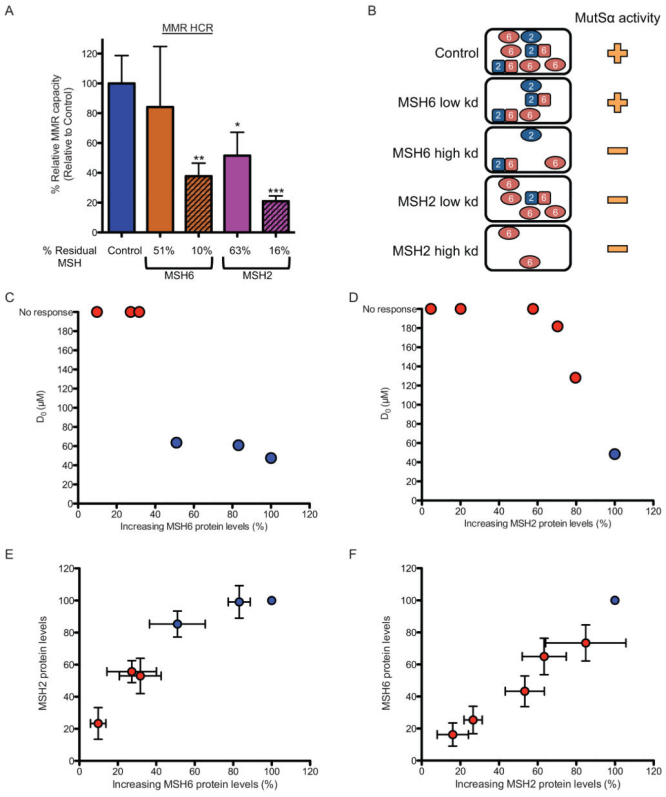

Figure 4. Small decreases in MSH2 protein alter MSH6 protein levels and lead to decreased mismatch repair activity

(A) Mismatch repair capacity against a GG mismatch substrate in select MSH6 and MSH2 knockdown GBM cells. Blue and red shaded regions denote areas of MSH6 and MSH2 knockdown where sensitivity (**S**) or resistance (**R**) to TMZ was observed (n=3, ± s.d. p< 0.05*; 0.01**; 0.001*** Student's 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. Low decreases in MSH2 however, immediately deplete MSH2 from its dimer form resulting in decreased MutSα activity.

(C/D) Relationship between MSH6 (A) and MSH2 (C) protein levels and sensitivity to TMZ in MSH2 and MSH6 knockdown cells. Blue and red shaded regions denote areas of MSH6 and MSH2 knockdown where sensitivity (**S**) or resistance (**R**) to TMZ was observed (n=3, ± s.e.m.).

(E/F) Effects of MSH6 (B) and MSH2 (D) knockdown on the stability of its dimerization partner. Immunoblot analysis was used to assess MSH2 and MSH6 protein levels in MSH6 and MSH2 knockdown cells, respectively. Blue and red shaded regions denote areas of MSH6 and MSH2 knockdown where sensitivity (**S**) or resistance (**R**) to TMZ was observed (n=3, ± s.e.m.).

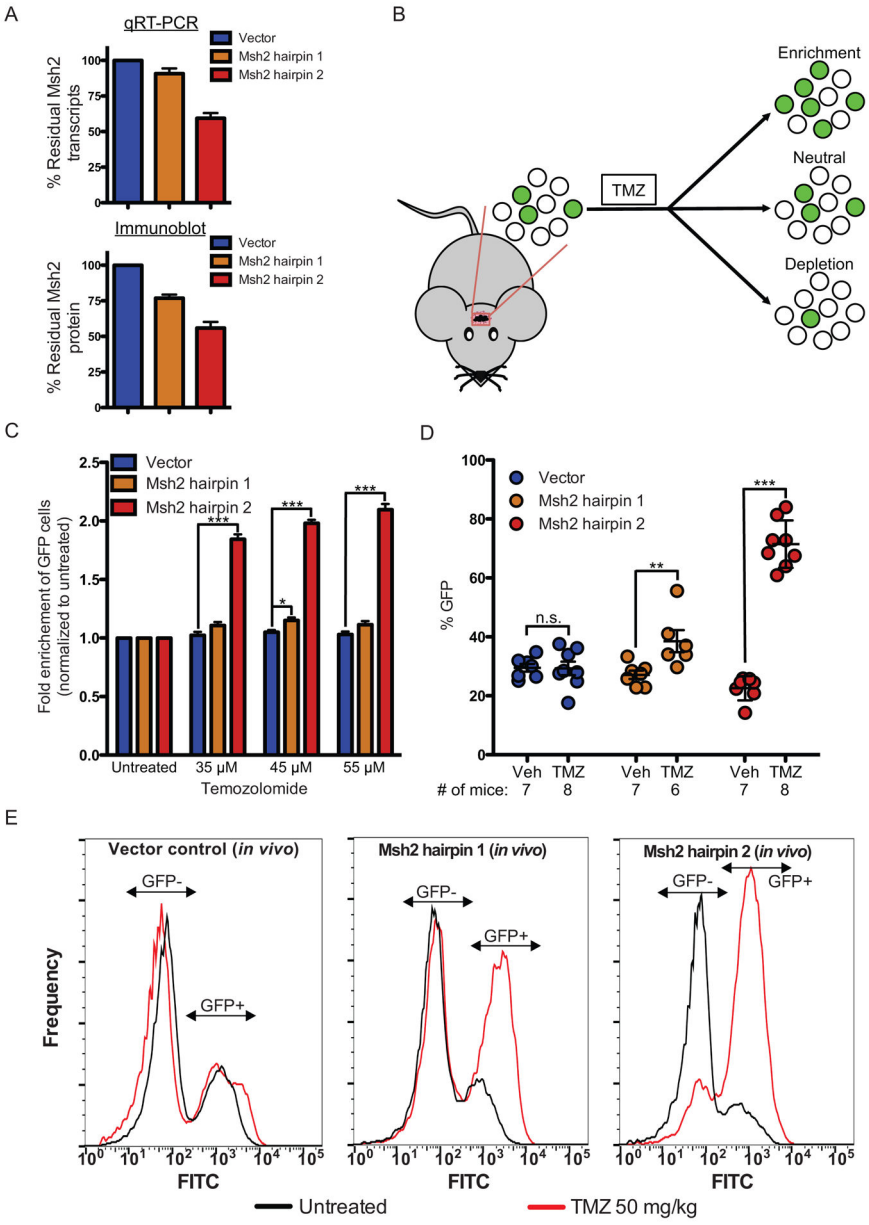

Figure 5. Small decreases in Msh2 confer a growth advantage to GBM tumors after TMZ challenge

(A) Msh2 transcript (top panel) and protein (bottom panel) levels in GL261 GBM cells expressing a vector control or one of two hairpins targeting *Msh2* transcripts and a marker GFP. Fluorescently activated cell sorting was employed to obtain a pure population of control or hairpin expressing cells (n=3, ± s.e.m.).

(B) A competition assay to assess the effects of decreased Msh2 levels on the response of GL261 GBM tumors to TMZ. GL261 cells expressing GFP as a marker of hairpin expression are labeled green.

(C) TMZ-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. Flow cytometry was used to assess changes in the percentage of GFP positive cells 96 hours post-TMZ treatment (n=3, ± s.e.m., p< 0.05*; 0.001*** Student's t test).
(D) *In vivo* enrichment of Msh2 knockdown cells in a TMZ treated GBM tumor model. C56BL6/J mice harboring GL261-derived GBM tumors were treated with TMZ 8 days post-tumor initiation. Changes in the percentage of GFP positive cells was assessed by flow cytometry of dissociated tumors from mice euthanized after euthanasia criteria were observed (± s.d., p< 0.05*; 0.001*** Mann-Whitney test).
(E) Representative histogram obtained from dissociated GL261 tumors expressing a vector control or one of two hairpins targeting *Msh2* transcripts.

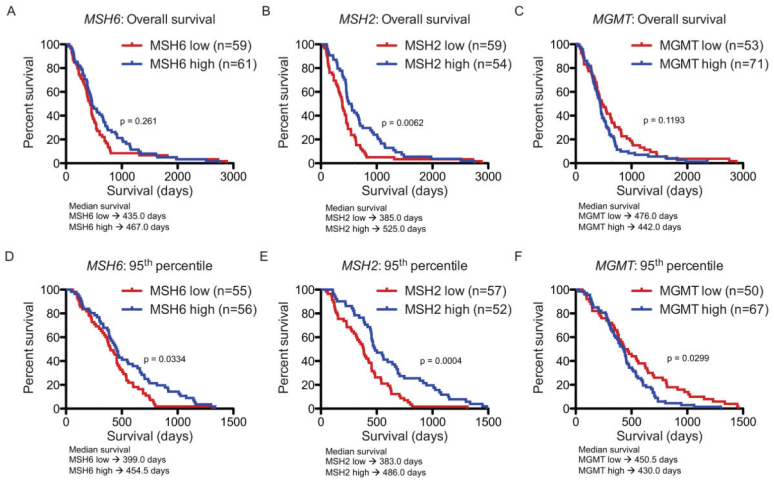

Figure 6. *MSH2* transcript levels are predictive for the survival of TMZ treated GBM patients

(A–C) Effects of *MSH2*, *MSH6* and *MGMT* transcript levels on the overall survival of TMZ treated GBM patients. Patients were stratified as high or low expressers by a z-score cutoff of 0.5. The log rank test was employed to assess significance between the median survivals of both populations.

(D–F) Effects of *MSH2*, *MSH6* and *MGMT* transcript levels on the survival of TMZ treated GBM patients excluding long-term survivors that fall into upper 5th percentile for patient survival after TMZ treatment. Patients were stratified as high or low expressers by a z-score cutoff of 0.5. The log rank test was employed to assess significance between the median survivals of both populations.