

MSH6 Mutations Arise in Glioblastomas during Temozolomide Therapy and Mediate Temozolomide Resistance

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Abstract Purpose: Over the past few years, the alkylating agent temozolomide has become the standard-of-care therapy for patients with glioblastoma, the most common brain tumor. Recently, large-scale cancer genome sequencing efforts have identified a hypermutation phenotype and inactivating *MSH6* mismatch repair gene mutations in recurrent, post-temozolomide glioblastomas, particularly those growing more rapidly during temozolomide treatment. This study aimed to clarify the timing and role of *MSH6* mutations in mediating glioblastoma temozolomide resistance. **Experimental Design:** *MSH6* sequence and microsatellite instability (MSI) status were determined in matched prechemotherapy and postchemotherapy glioblastomas identified by The Cancer Genome Atlas (TCGA) as having posttreatment *MSH6* mutations. Temozolomide-resistant lines were derived *in vitro* through selective growth under temozolomide, and the *MSH6* gene was sequenced in resistant clones. The role of *MSH6* inactivation in mediating resistance was explored using lentiviral short hairpin RNA knockdown and *MSH6* reconstitution. **Results:** *MSH6* mutations were confirmed in posttreatment TCGA glioblastomas but absent in matched pretreatment tumors. The posttreatment hypermutation phenotype displayed a signature bias toward CpC transitions and was not associated with MSI. *In vitro* modeling through exposure of an *MSH6* wild-type glioblastoma line to temozolomide resulted in resistant clones; one clone showed an *MSH6* mutation, Thr¹²¹⁹Ile, that had been independently noted in two treated TCGA glioblastomas. Knockdown of *MSH6* in the glioblastoma line U251 increased resistance to temozolomide cytotoxicity and reconstitution restored cytotoxicity in *MSH6*-null glioma cells. **Conclusions:** *MSH6* mutations are selected in glioblastomas during temozolomide therapy both *in vitro* and *in vivo* and are causally associated with temozolomide resistance.

The standard of care for newly diagnosed glioblastomas involves surgery and external beam radiation therapy (XRT) in conjunction with the alkylating agent temozolomide (1). The benefit from temozolomide is most noted in patients whose tumors have transcriptional silencing of the *O*⁶-methylguanine methyltransferase (*MGMT*) gene mediated by promoter methylation (2), which occurs in approximately half of the tumors (3). Nonetheless, the prognosis for patients with glioblastoma

remains bleak. Virtually all patients recur after initial therapy, and average survival remains around 12 months (4).

Our knowledge of the genetic changes underlying glioblastoma, although considerable, is largely confined to pretreatment cases (5). Given that all glioblastomas recur and that the recurrent lesions invariably lead to patient death, there is a pressing need to understand the molecular changes that occur during treatment and that characterize the therapeutically resistant recurrences (6). In this regard, we first identified inactivating somatic mutations in the mismatch repair gene *MSH6* in two recurrent glioblastomas treated with temozolomide (7). A survey of the genome in these two tumors revealed large numbers of somatic mutations with mutational signatures consistent with those resulting from defects in DNA mismatch repair. Of note, studies in normal and neoplastic cells had shown that inactivation of *MSH6* results in resistance to cytotoxicity mediated by alkylating agents (8–12). We therefore proposed that *MSH6* inactivation may be one mechanism underlying temozolomide resistance in glioblastomas. In a follow-up study, we examined a larger series of pretreatment and posttreatment glioblastomas for *MSH6* mutations and *MSH6* expression (13). *MSH6* alterations (mutations and/or absent expression) were not found in any pretreatment glioblastomas and not in any posttreatment glioblastomas given only XRT, but were detected in approximately half of the recurrent glioblastomas treated with temozolomide and XRT.

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Translational Relevance

Glioblastomas are highly malignant brain tumors. Current standard therapy uses temozolomide and radiation. Previously, we showed that the mismatch repair gene *MSH6* is mutated in some recurrent, post-temozolomide glioblastomas, and recent data from The Cancer Genome Atlas (TCGA) project has confirmed this observation. We further show mutations of *MSH6* in the posttreatment and not in the pretreatment TCGA tumors and have modeled this situation *in vitro*. Chronic exposure of a glioblastoma line to temozolomide generated multiple resistant clones, with one clone harboring an *MSH6* mutation. Knockdown of *MSH6* expression enhanced survival with cytotoxic doses of temozolomide, and *MSH6* reconstitution restored temozolomide sensitivity in *MSH6*-null glioblastoma cells. These results indicate that *MSH6* is an important mediator of temozolomide cytotoxicity and its inactivation is associated with treatment failure in glioblastomas.

Furthermore, temporal measurements of three-dimensional reconstructed magnetic resonance images showed that *MSH6*-negative glioblastomas showed more rapid radiologic progression while under temozolomide treatment compared with *MSH6*-positive tumors. These data supported a role for *MSH6* inactivation in the emergence of temozolomide resistance in glioblastoma patients.

Two other studies have now reported *MSH6* mutation in glioblastomas following alkylating agent chemotherapy. The Cancer Genome Atlas (TCGA) reported an analysis of 91 glioblastomas with matched peripheral blood, of which 19 cases were recurrent glioblastomas that had received alkylating agent chemotherapy (14). In keeping with our findings, the TCGA reported nonsynonymous *MSH6* mutations in five of the recurrent glioblastomas (26% of recurrent tumors), all with a hypermutation phenotype consistent with mismatch repair defects. Two other tumors had a hypermutation phenotype, one with a mutation in another mismatch repair gene. In addition, Maxwell et al. showed 7 nonsynonymous and therefore putative *MSH6* mutations (2 of them truncating) out of 27 post-temozolomide samples (26%); of note, this series included some malignant gliomas other than glioblastoma, such as oligodendroglial tumors that in our experience to date do not undergo frequent *MSH6* alterations (15). Unfortunately, only two cases with sequence variations had available matched pretreatment samples and both of these had only a common *MSH6* polymorphism rather than nonsynonymous mutations. Thus, although the TCGA and Maxwell et al. reports confirm that *MSH6* alterations are common in those recurrent glioblastomas exposed to alkylating agents, neither had access to matched pretreatment samples to assess the timing of these mutations.

To further pursue this hypothesis, we undertook additional studies to clarify the timing of *MSH6* inactivation in the TCGA clinical cases, as well as to model such inactivation *in vitro* and to directly evaluate the role of *MSH6* in temozolomide resistance *in vitro*. The results of these studies support the hypothesis that *MSH6* inactivation occurs during alkylating

agent chemotherapy and that, at least in the common setting of MGMT inactivation, *MSH6* inactivation is directly related to therapeutic resistance.

Materials and Methods

TCGA tissue samples and DNA stocks. Unstained slides from formalin-fixed, paraffin-embedded, anonymous, matched prechemotherapy and postchemotherapy glioblastomas were obtained from the M.D. Anderson Cancer Center, Houston, TX. The posttreatment samples were the same ones used by TCGA for genomic and epigenomic profiling and were identified as displaying the hypermutation phenotype and somatic *MSH6* mutations (14). Tumor tissue from unstained formalin-fixed, paraffin-embedded glass slides was deparaffinized in xylene followed by immersion in graded alcohols until rehydration, and genomic DNA was extracted using the Genra PureGene Kit (Qiagen). DNA quantitation was done using a Nanodrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies).

Mutation-targeted PCR and sequencing. Targeted PCR or PCR primers were designed to amplify *MSH6* mutations initially identified by the TCGA consortium and posted in the publicly accessible database⁶ (14). PCR was done as described previously (13). Targeted sequencing was done using the standard Sanger method in both forward and reverse directions. Each individual sequencing reaction was repeated for confirmation. Analysis of DNA tracings was carried out using Mutation Surveyor version 3.2 (Softgenetics).

Microsatellite instability testing. PCR was conducted in 20 μ L volumes using 1 \times Platinum Taq PCR buffer, 200 mmol/L deoxynucleotide triphosphates, 2.0 mmol/L $MgCl_2$, 0.4 mmol/L primers, 1.0 unit of Platinum Taq polymerase (all from Invitrogen) with 40 ng of tumor DNA as template. Primer sets comprised the five reference panel markers recommended by the National Cancer Institute, with 5' phosphoramidite fluorescent labeling of forward primers as follows: BAT-25 (NED), BAT-26 (6-FAM), D5S346 (VIC), D17S250 (6-FAM), and D2S123 (VIC). The primer sequences for D2S123 were 5'-AACATTGCTGGAAGTCTGG-3' (forward) and 5'-GTGTCTTGACTTTCACCTATGGGACTG-3' (reverse). Primer sequences for the remaining loci were identical to those previously described (16) except that a 5'-GTGTCTT sequence was added to each reverse primer to facilitate nontemplate adenylation of the 3' end of the forward strand.

PCR was done in a Mastercycler PCR machine (Eppendorf) with an initial denaturing step at 94°C for 5 min; followed by 38 cycles of denaturing at 94°C for 30 s, annealing at either 50°C or 55°C for 30 s, and extension at 72°C for 30 s; and a final elongation step at 72°C for 10 min. PCR products were pooled and fractionated by size using an Applied Biosystems 3130 DNA Analyzer with GeneMapper software. Microsatellite loci at which tumor DNA showed a novel allele profile not present in the corresponding normal DNA were classified as having microsatellite instability (MSI).

Tissue culture. The human glioblastoma cell lines A172 and U251 were originally obtained from the American Tissue Culture Collection. The primary human glioblastoma cell culture Gli60 was established from recurrent tumor xT3162 post-temozolomide and radiotherapy; the tumor had the somatic mutation p.Val⁸⁰⁹X in *MSH6*, which results in premature termination, and had lost the remaining copy of chromosome 2, leading to null expression of the protein (7, 13). Temozolomide was purchased from Sequoia Research Products Limited (Pangbourne), reconstituted to a stock concentration of 100 mmol/L, and stored at -80°C. O⁶-benzylguanine (O⁶-BG) was obtained from Sigma-Aldrich, reconstituted to stock concentration of 320 mmol/L with DMSO, and stored at -80°C. A172, U251, and Gli60 cells were maintained in DMEM supplemented with 10% FCS, 1% L-glutamine (Invitrogen), and grown at 37°C humidified atmosphere containing

⁶ <http://tcga-data.nci.nih.gov/tcga/findArchives.htm>

Table 1. *MSH6* mutations in pretreatment and posttreatment TCGA glioblastoma cases

Case ID	Treatment status	Chemotherapy	<i>MSH6</i> somatic mutations	Amino acid change
TCGA-02-0043	Pretreatment	—	None	None
	Posttreatment	CCNU	c.3656C>T	p.Thr ¹²¹⁹ Ile
TCGA-02-0083	Pretreatment	—	None	None
	Posttreatment	Temozolomide	c.3166G>A	p.Val ¹⁰⁵⁶ Met
TCGA-02-0099	Pretreatment	—	None	None
	Posttreatment	PCV	c.3656C>T	p.Thr ¹²¹⁹ Ile
TCGA-02-0114	Pretreatment	—	None	None
	Posttreatment	PCV	c.1450G>A, c.2294G>A	p.Glu ⁴⁸⁴ Lys, p.Cys ⁷⁶⁵ Tyr

Abbreviations: CCNU, lomustine; Pcv, procarbazine, CCNU, vincristine.

5% CO₂. Cells were confirmed to be free of *Mycoplasma* using the Lonza MycoAlert detection kit.

Generation of temozolomide-resistant glioblastoma subclones. The glioblastoma cell line A172 was treated with temozolomide at 100 μmol/L or DMSO solvent control at a final concentration of 0.1% for 3 wk. To generate temozolomide-resistant glioma sublines, A172 cells were cultured in six-well plates and allowed to adhere overnight at a 37°C incubator. Control groups were treated with 0.1% DMSO alone. Cell treatment was repeated every 24 h for 5 consecutive days and then exposure to the fresh temozolomide was done every 3 d to a total of 3 wk. Each single clone was grown to derive stable resistant cell lines for subsequent study. *MSH6* and MGMT expression was measured by Western blot using the cell lysates treated with temozolomide.

Reconstitution of *MSH6* expression in Gli60 using lentiviral approach. The vector backbone for lentiviral reconstitution of Gli60 and the viral packaging procedure have been described previously (17, 18). Briefly, cDNA for *MSH6* was cloned into the construct under the control of the cytomegalovirus promoter, and cDNA for the fluorescence marker GFP was under the control of internal ribosomal entry sequence. We used empty constructs expressing only GFP as an infection control. Gli60 cells were infected with lentiviral particles in the presence of protamine sulfate (Sigma) at a multiplicity of infection of 10:1. Successful infection was confirmed by the appearance of green fluorescing cells and Western blot evaluation for *MSH6* protein.

Generation of lentiviral constructs for shRNA-mediated *MSH6* knockdown. Bacterial plasmid constructs containing shRNA sequence candidates against *MSH6* mRNA were obtained from the Massachusetts General Hospital shRNA core (Dr. Toshi Shioda, Massachusetts General Hospital). Subsequent steps in the production of lentiviral particles and infection of glioblastoma cells were done according to protocols listed in the RNA Consortium/Broad Institute of MIT and Harvard (TRC/BROAD) website.⁷ Lentiviral supernatants were removed from U251 cells after overnight incubation and the cells were allowed to grow in DMEM with 10% FCS for 48 h before selection with increasing doses of puromycin from 0.5 to 2.0 μg/mL (Sigma-Aldrich). *MSH6* and MGMT protein levels in U251 cells stably infected with the five candidate shRNA constructs were assessed by Western blot analysis.

Cell viability assay. To compare cell viability between parental glioblastoma cell lines and temozolomide-treated, resistant, or *MSH6* knockdown cell lines, we used the Promega CellTiter96 Aqueous cell proliferation assay cytotoxicity assay measuring absorbance at 490 nm (Promega). Briefly, 1,000 cells were seeded per well in 96-well tissue culture plates before the drug treatment. Cells were treated daily with fresh temozolomide (100 μmol/L) for 7 d. U251 and U251 Sh1 cells were additionally treated with temozolomide in the presence of O⁶-BG (40 μmol/L). Gli60 cells reconstituted with wild-type *MSH6* and with empty control vector were incubated with various concentrations of temozolomide for 5 d and cell growth was evaluated by the MTS assay.

⁷ <http://www.broad.mit.edu/genome.bio/trc/publicProtocols.html>

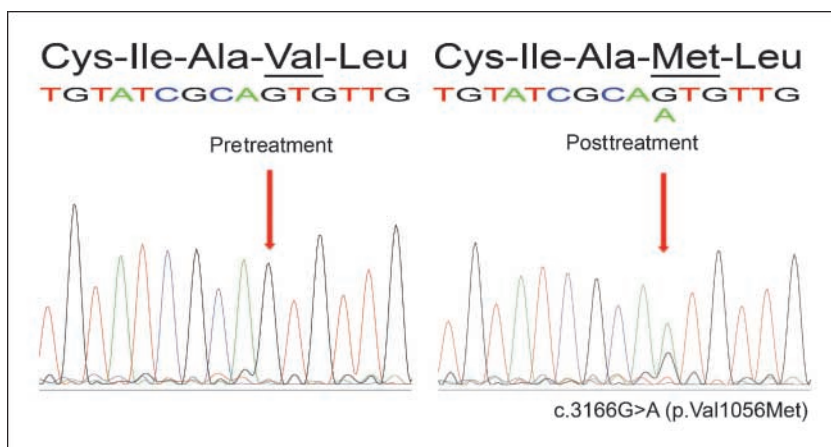
Western blot analysis. Antibodies were obtained from the following sources: *MSH6* monoclonal antibody from BD Biosciences, MGMT from Lab Vision Co., and β-actin monoclonal antibody from Santa Cruz Biotechnology. Cells were lysed in radioimmunoprecipitation assay protein extraction buffer (Sigma-Aldrich) together with protease and phosphatase inhibitors at 1:100 dilutions (Sigma-Aldrich) and then centrifuged 10 min at 4°C to harvest the supernatant. The concentration of the extracted protein was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories). Twenty micrograms per lane of the extracted protein were loaded onto 4% to 12% Tris-glycine gels (Invitrogen) for electrophoresis, and electro-transferred to Pure Nitrocellulose Membrane (Bio-Rad Laboratories). The membrane was blocked with 5% nonfat dry milk in Tris-buffered Saline-Tween buffer [10 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.05% Tween 20] for 1 h at room temperature, followed by incubation with different antibodies in the blocking buffer overnight at 4°C. After washing with TBS-T buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (Promega Co.) at a dilution of 1:2,000 in blocking buffer for 2 h at room temperature. The membrane was developed using the enhanced chemiluminescence system (Perkin-Elmer, Inc.) and exposed to Biomax XAR film (Kodak).

Analysis methods. GraphPad Prism 3.0 was used to determine statistically significant differences between cytotoxicity curves. Best-fit curves for the growth of A172/A172TR3 lines, U251 line, and U251-Sh1 were calculated using the program-derived "exponential growth" nonlinear regression equation with the starting points of all curves held constant at the average absorbance value for all day 1 points. Second-order polynomial nonlinear regression was used for the analysis of survival fractions at the end of the 5-d incubation with temozolomide in Gli60 cells reconstituted with *MSH6* and control vector. To determine significance, only the mean Y value was considered for each replicate point and rate constants of the calculated best-fit curves were compared using the *t* test. Due to multiple testing within each cytotoxicity experiment, statistical significance was defined as *P* < 0.01.

Results and Discussion

Genetic analysis of matched TCGA glioblastoma samples: *MSH6* mutations, hypermutation phenotype, and MSI status. We previously reported somatic *MSH6* mutations in 3 of 11 recurrent glioblastomas treated with alkylating agents and XRT, but in no pretreatment tumors or in posttreatment tumors treated with XRT only, suggesting that *MSH6* mutations arise specifically after alkylating agent chemotherapy (13). Given that the TCGA has now found somatic *MSH6* mutations in 5 of 19 recurrent glioblastomas, we sought to determine the timing of these genetic changes. We obtained matched pretreatment and posttreatment unstained formalin-fixed, paraffin-embedded tumor tissue sections from four of the five cases identified

Fig. 1. *MSH6* mutation c.3166G>A (p.Val¹⁰⁵⁶Met) is present in the posttreatment TCGA-02-0083 glioblastoma (right) but not in the matched pretreatment sample (left).



by TCGA as having somatic *MSH6* mutations (TCGA-02-0043, -02-0083, -02-0099, and -02-0114). To show that sequencing from formalin-fixed, paraffin-embedded tissues was of adequate sensitivity and specificity to detect mutations, we sequenced the posttreatment samples and confirmed all five *MSH6* mutations reported by TCGA in the four cases (the TCGA-02-0114 posttreatment specimen had two distinct *MSH6* somatic mutations). Notably, however, sequencing of the matched pretreatment glioblastomas (including two prechemotherapy specimens from TCGA-02-0114) showed no mutations (Table 1; Fig. 1). The absence of germline and of somatic pretreatment *MSH6* mutations strongly suggests that *MSH6* mutations do not contribute to the development of glioblastoma. Rather, the presence of *MSH6* mutations in the posttreatment samples is consistent with *de novo* alterations in the tumor cell genome in association with treatment.

The TCGA report noted a hypermutation phenotype in all four of these *MSH6*-mutant glioblastomas (14). However, the published data did not clarify the sequence context of the mutations beyond classifying them as CpG or non-CpG. We therefore undertook a detailed analysis of the somatic mutation data of these four cases, and confirmed our prior findings of a hypermutation phenotype with a preponderance of C:G>T:A transitions at CpC dinucleotides that is striking when compared

with recurrent, post-temozolomide glioblastomas without *MSH6* mutations (Table 2; ref. 7). For example, TCGA-02-0083 contains 94 C>T somatic mutations, of which 60 are within the context of CpC dinucleotides. Interestingly, this recurrent tumor also harbors somatic mutations in two other mismatch repair genes, *MSH2* and *MLH1*, which could account for the larger number of such mutations compared with the other hypermutant cases. The other three posttreatment TCGA cases in this series (TCGA-02-0043, -0099, and -0114) also contained markedly higher numbers of somatic mutations and a preponderance of mutations in the context of CpC dinucleotides. On the other hand, these cases did not show high MSI; we did not detect MSI-high (>3/5 unstable loci) in any cases and detected only minor shifts in <2 loci in two cases (TCGA-02-0043 and -02-0083, data not shown). These findings are in keeping with a recent evaluation of the role of MSI as a surrogate marker for *MSH6* inactivation in recurrent malignant gliomas; Maxwell et al. (15) showed no correlation between *MSH6* mutations and MSI, as assessed by a panel of five mononucleotide loci. That study, however, did not determine whether the recurrent tumors with *MSH6* mutations had a hypermutation phenotype. In our study, the overwhelming number of somatic mutations in these tumors, in conjunction with *MSH6* mutations, are wholly consistent with *MSH6* inactivation

Table 2. Mutational burden and sequence context of C:G>T:A mutations in the four posttreatment TCGA glioblastomas with hypermutation phenotype (top), compared with four nonhypermutation posttreatment glioblastomas (bottom)

Case ID	CpA				CpC				CpG				CpT				Total C:G>T:A mutations	Mutations in CpC context
	ACA	CCA	GCA	TCA	ACC	CCC	GCC	TCC	ACG	CCG	GCG	TCG	ACT	CCT	GCT	TCT		
MSH6 mutated posttreatment cases with hypermutation																		
TCGA-02-0043	0	0	0	0	8	9	6	4	0	0	3	2	2	2	2	0	38	27
TCGA-02-0083	1	2	3	0	10	19	15	16	2	3	2	1	4	9	4	3	94	60
TCGA-02-0099	0	1	0	0	2	3	0	7	1	2	1	1	3	2	3	3	29	12
TCGA-02-0114	1	2	5	0	10	11	12	15	0	2	3	1	6	3	8	6	85	48
MSH6 wild-type posttreatment cases without hypermutation																		
TCGA-02-0021	0	1	0	0	0	1	0	0	1	1	1	0	1	0	0	0	6	1
TCGA-02-0024	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	2	5	1
TCGA-02-0058	0	0	0	0	1	0	1	0	0	1	1	1	0	0	0	0	5	2
TCGA-02-0116	0	0	0	0	1	0	0	0	0	0	0	3	2	1	0	2	9	1

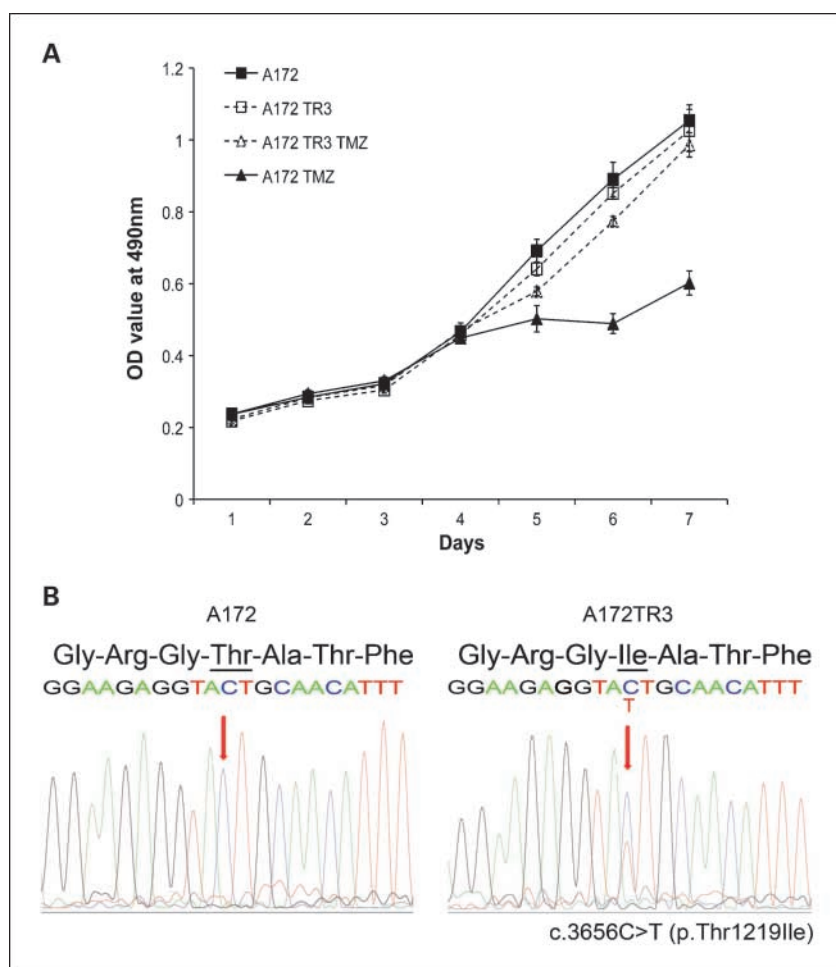


Fig. 2. *A*, the parental A172 and the temozolomide-resistant subclone A172TR3 exhibit similar growth in the absence of temozolomide (solid and dashed lines, square markers). Parental A172 (solid line, triangle marker) is sensitive to 100 $\mu\text{mol/L}$ temozolomide for 7 d, whereas A172TR3 growth (dashed line, triangle marker) is minimally affected at 100 $\mu\text{mol/L}$ temozolomide ($P < 0.01$). *B*, the temozolomide-resistant subclone TR3 (left) has a c.3656C>T *MSH6* mutation, resulting in a Thr to Ile amino acid change at codon 1219, in comparison with the temozolomide-sensitive, parental glioblastoma A172 line (right).

causing the hypermutation phenotype, and the absence of MSI is consistent with known *MSH6* function (7, 13, 19).

Molecular characterization of *in vitro* derived temozolomide-resistant glioblastoma cell line. We next sought to model the phenomenon of *MSH6* inactivation *in vitro*. To do so, we exposed the human glioblastoma cell line A172 to temozolomide to generate drug-resistant clones and found reduced *MSH6* protein level in a highly resistant line, A172TR3. There was no difference in growth rate between parental A172 and A172TR3; however, the resistant clone showed significantly enhanced survival in the presence of 100 $\mu\text{mol/L}$ temozolomide compared with the parental A172 ($P < 0.01$; Fig. 2A). We further reasoned that, similar to the situation in human glioblastomas, the development of a temozolomide-resistant clone with reduced *MSH6* protein could result from *in vitro* somatic *MSH6* mutation. In fact, sequencing of *MSH6* in the resistant line identified a novel *MSH6* alteration that was not present in the parental A172 cells. This clone, A172TR3, had a c.3656C>T *MSH6* somatic mutation, altering threonine to isoleucine at amino acid position 1219 (Fig. 2B). This same mutation has been identified in two TCGA recurrent glioblastomas with the hypermutation phenotype (TCGA-02-0043 and -02-0099) in the malignant melanoma cell line MZ7-mel derived from a postchemotherapy splenic metastasis (Wellcome Trust Sanger Institute, COSMIC database, The Cancer Genome Project⁸), and as a germline mutation in a colorectal cancer and

a case of complex nonatypical endometrial hyperplasia (20). Notably, the MZ7-mel cell line also contains a significant number of somatic mutations consistent with that of the hypermutation phenotype. The Thr¹²¹⁹Ile mutation is therefore likely important to *MSH6* function and suggests that the *in vitro* induced mutation of Thr¹²¹⁹Ile in the temozolomide-resistant A172TR3 cells is biologically and functionally significant.

It is also noteworthy that we were able to derive the *MSH6* Thr¹²¹⁹Ile mutation after temozolomide exposure because the same mutation has been reported independently in glioblastomas that failed therapy with other alkylating agents. TCGA-02-0043 was treated with the alkylating agent CCNU (lomustine) and TCGA-02-0099 was treated with the PCV combination therapy (procarbazine, lomustine, and vincristine). In this regard, it is interesting to note that temozolomide is a S_N1-type methylating agent that mediates guanine modification, resulting in base pair mismatch during replication, whereas CCNU causes interstrand cross-linking (12, 21, 22). Moreover, whereas the role of the mismatch repair pathway in facilitating temozolomide-mediated cell death is well understood (12), the role of mismatch repair proteins in mediating CCNU cytotoxicity is less clear. These results may suggest a separable

⁸ <http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=sample;id=753596>

convergence in downstream signaling function from DNA damage recognition, as has been hypothesized (19, 23).

MSH6 inactivation may be heterozygous and is expectedly not associated with high MSI. Some of the recurrent TCGA glioblastomas with a hypermutation phenotype contain heterozygous somatic *MSH6* mutations rather than biallelic inactivation. In addition, A172TR3 showed a heterozygous Thr¹²¹⁹Ile *MSH6* mutation that was clearly associated with temozolomide resistance *in vitro*. In addition, patients with colorectal cancers and *MSH6* missense mutations often have preserved *MSH6* immunoreactivity; in fact, colorectal cancer cells and cells from endometrial complex nonatypical hyperplasia with the germline Thr¹²¹⁹Ile missense mutation have been reported to have preserved *MSH6* immunoreactivity (20). It would therefore seem that partial *MSH6* inactivations, both *in vivo* and *in vitro*, may be associated with functional effects.

MMR proteins function in multimeric complexes; *MSH6* dimerizes with *MSH2* to form the eukaryotic equivalent of the bacterial mutS α complex (19). The *MSH6*:*MSH2* dimer functions to detect single nucleotide mismatches, and subsequent corrective actions are undertaken by complexes of other MMR family members. In this regard, it has been reported that compromise of MMR function can occur following the mutation of one allele. This may occur through a dominant-negative effect through "soaking up" of the normal binding partner—*MSH2* in the case of *MSH6* (24). Alternatively, inactivation of one copy of *MSH6*, as seen in the TCGA recurrent tumors and by us, could contribute to compromised MMR function through a "gene-dosage" effect, which could be exaggerated in an environment with strong selective pressures, as in the tumor microenvironment in the presence of

temozolomide. Notably, analyses of changes in *MSH6* protein tertiary structure secondary to the missense mutations in the four recurrent TCGA glioblastoma show significant alterations of protein folding in functional domains—in the case of Thr¹²¹⁹Ile, a putative protein-protein interaction domain.⁹ These data suggest that the elucidation of the differential effects of homozygous versus heterozygous *MSH6* inactivation in glioblastoma therapeutic resistance will be an important subject for further inquiry.

As noted, inactivation of *MSH6* is typically associated with the MSI-L phenotype and not with high MSI (25, 26). Moreover, for *MSH6*, there exists functional dichotomy between mismatch repair (or surveillance) function and apoptotic signaling secondary to cytotoxic agents (24). This might further explain the lack of correlation between missense *MSH6* mutations and level of MSI in recurrent glioblastomas. There also exists a substantial body of literature on the role of *MSH6* in mediating somatic hypermutation in the generation of antibody gene diversity (27, 28). This is not surprising because *MSH6* functions solely in the survey of the genome for single base-pair mismatches and does not participate in their subsequent repair (29). These observations are in keeping with our findings and argue that the role of *MSH6* in temozolomide response is not dependent on MSI.

Genetic knockdown of *MSH6* in U251 glioblastoma cell line and correlation with temozolomide resistance. To investigate the specific functional role of *MSH6* in glioblastomas, particularly in response to temozolomide treatment, we knocked down the expression of wild-type *MSH6* protein in the human glioblastoma cell line U251 through a lentiviral-mediated shRNA approach. We obtained clones of shRNA against the human *MSH6* gene from the TRC/BROAD consortium and generated lentiviral constructs of the five candidate clones (Table 2, Supplemental Data). Infection of U251 cells by lentiviral constructs expressing the five *MSH6*

⁹ <http://awabi.cbio.mskcc.org/oma/Report.jsp?mutanId=79>

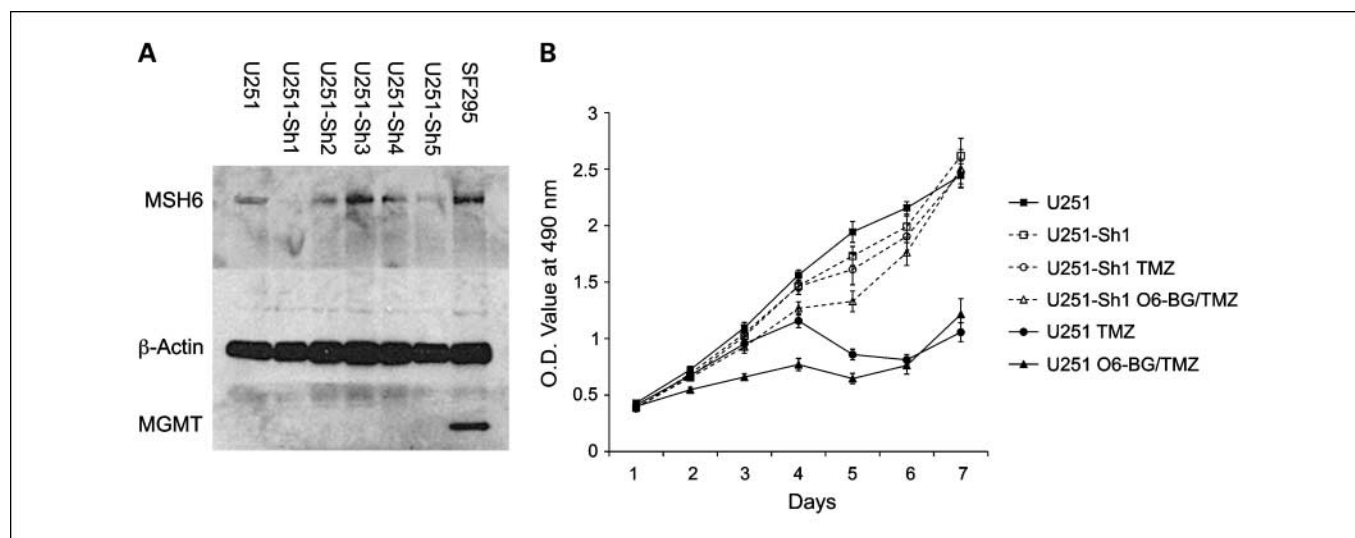


Fig. 3. A, Western blot for *MSH6* protein in U251 cells stably infected with five candidate shRNA lentiviral constructs show variable reduction of *MSH6* expression, with Sh1 showing maximal inhibition. *MGMT* is not expressed by any of the knockdown clones or the parental U251 cells (positive glioblastoma control SF295). B, parental U251 and *MSH6*-knockdown U251-Sh1 cells exhibit similar growth in the absence of temozolomide (solid and dashed lines, square markers). Whereas the growth of parental U251 is significantly reduced in the presence of 100 μ mol/L temozolomide for 7 d (solid line, closed circle), U251-Sh1 cells seem resistant to 100 μ mol/L temozolomide (dashed line, open circle; $P < 0.01$). The addition of 40 μ mol/L of O⁶-BG had no effect on temozolomide cytotoxicity in both parental U251 and *MSH6*-knockdown U251-Sh1 cells (solid and dashed lines, triangle markers).

shRNA candidates was followed by puromycin selection of successfully infected cells. We then carried out Western blot analyses of the MSH6 protein to identify the shRNA candidates with most efficiently down-regulated MSH6 protein (Fig. 3A). The shRNA construct 1 (U251-Sh1) showed 90% knockdown of MSH6 compared with 50% in construct 5 (U251-Sh5). We proceeded to examine the response of these cells to temozolomide in *in vitro* cytotoxicity assays. Both U251-Sh1 and U251-Sh5 are significantly more resistant to temozolomide compared with parental controls when exposed to 100 $\mu\text{mol/L}$ temozolomide for 7 days (Fig. 3A; data for U251-Sh5 not shown; $P < 0.01$). Nonetheless, U251-Sh1 and U251-Sh5 exhibited similar proliferation indices to parental U251 cells, showing that enhanced temozolomide resistance in the MSH6-knockdown cells is not due to alteration in cell growth kinetics. These results confirm a role for MSH6 in mediating temozolomide cytotoxicity.

Restoration of temozolomide cytotoxicity in Gli60 with reconstitution of MSH6 expression. We had previously derived and characterized the primary glioblastoma cell culture Gli60 from a recurrent glioblastoma in a patient who had recurred quickly during treatment with temozolomide and radiotherapy. Gli60 has the somatic *MSH6* mutation delG2425, which results in premature protein termination, and loss of the remaining wild-type *MSH6* gene, with resulting null expression of MSH6 on Western blotting; Gli60 also exhibits the characteristic hypermutation phenotype of C \rightarrow T transitions preferentially at CpC dinucleotides (7, 13). Gli60 thus represents a unique resource for the study of post-temozolomide recurrent glioblastoma. To support our hypothesis that somatic inactivating *MSH6* mutations result in a survival advantage to these cells, we restored MSH6 expression in Gli60 and examined the subsequent response to temozolomide. Gli60 cells were infected with a lentiviral construct expressing MSH6 under the control of the cytomegalovirus promoter (Gli60-MSH6) or with an empty control vector (Gli60-Con vector). We then incubated the cells with different concentrations of temozolomide and examined the cytotoxicity using the MTS assay. Gli60-MSH6 showed restored temozolomide sensitivity compared with Gli60-CON ($P < 0.01$; Fig. 4). This confirms that restoration of MSH6 expression in glioma cells from a patient who failed temozolomide treatment conferred temozolomide sensitivity *in vitro*, and functionally links MSH6 function to temozolomide sensitivity in a glioblastoma that harbors the characteristic hypermutation phenotype.

Relationship of MSH6 alterations to MGMT status. Temozolomide acts by adding methyl groups to the O⁶ position of guanine nucleotides. The first line of response to repair this chemotherapeutic event is mediated by MGMT, which removes these methyl groups (30). However, a substantial proportion of glioblastomas have transcriptional silencing of the *MGMT* gene through promoter hypermethylation, and cases with intact MGMT expression can have MGMT down-regulated by the MGMT inhibitor O⁶-BG (31, 32). In such cases, the mismatch repair pathway becomes a primary mediator of O⁶-methylguanine cytotoxicity, and defects in the mismatch repair pathway can therefore serve as an alternate resistance mechanism for cancer cells (12, 33). As a result, to develop resistance after temozolomide exposure, cancer cells that cannot up-regulate MGMT expression would be expected to inactivate this mismatch repair pathway.

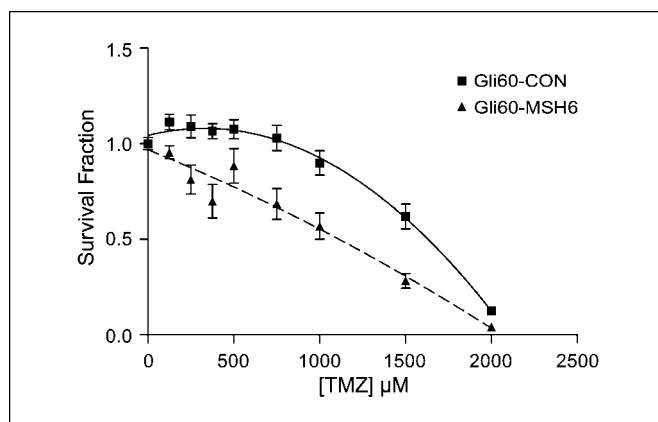


Fig. 4. The Gli60 primary glioblastoma, which is MSH6-null secondary to nonsense mutation and loss of the remaining chromosome 2, infected with control empty lentiviral vector (*Gli60-CON*) and with MSH6-vector (*Gli60-MSH6*), showed differential sensitivity to temozolomide after 5 d of drug exposure. Gli60 cells with restored MSH6 expression are significantly more sensitive to temozolomide (TMZ) than cells infected with control vectors ($P < 0.01$).

In the present experiments, in keeping with what has been found in primary human glioblastomas, both the TCGA cases and the studied cell lines had inactivation of MGMT. All four of the TCGA cases had methylation of the MGMT promoter (14), which has been correlated with decreased expression of MGMT and improved response to temozolomide in initially treated primary tumors (2, 34). The A172 cell line does not express MGMT, nor does the temozolomide-treated resistant clone A172TR3 (data not shown). The U251 cell line does not express MGMT, and MGMT expression is not induced in the knockdown cells (Fig. 3A). Nonetheless, to confirm that MGMT did not play a role in the U251 knockdown experiments, we also added the irreversible MGMT inhibitor O⁶-BG to both parental U251 and the MSH6-knockdown U251-Sh1 and U251-Sh5 clones, and this did not alter drug sensitivity in both the parental and the two MSH6-knockdown clones ($P < 0.01$; Fig. 3B). Thus, the difference in sensitivity to temozolomide between the parental and the genetically modified clones was due principally to MSH6. At the same time, O⁶-BG is being considered as an adjuvant in temozolomide therapy due to its ability to deplete cellular MGMT (31, 35). In this regard, pharmacologic inhibition of MGMT by O⁶-BG *in vivo* or escalation of temozolomide dosing through "dose dense" treatment scheduling could result in accelerated selection pressure to develop alternate escape mechanisms to temozolomide-mediated cytotoxicity (33), one of which would be somatic mutations of a mismatch repair gene such as *MSH6*. Future adjuvant therapies aimed at overcoming MGMT activity could therefore potentially increase the frequency by which we observe alternate escape pathways such as mismatch repair inactivation (36).

In summary, *MSH6* mutations are frequent in recurrent glioblastomas that have been treated with alkylating agents, but have not been found in any prechemotherapy glioblastomas. Combining our data (3 of 11, 27%) with those of the TCGA (5 of 19, 26%) and Maxwell et al. (7 of 27, 26%), *MSH6* mutations have now been found in 15 of 57 (26%) glioblastomas after alkylating agent chemotherapy, with similar incidences found in the three series (13–15). We have also shown that *in vitro* derivation of temozolomide-resistant cells

can be associated with *MSH6* inactivation and mutation. Moreover, *in vitro* inactivation of wild-type *MSH6* protein in glioblastoma cells can result in increased temozolomide resistance, and *in vitro* reconstitution of *MSH6* expression can restore temozolomide sensitivity in glioblastomas lacking *MSH6*. These multiple approaches support an integral role for *MSH6* inactivation in mediating temozolomide resistance in glioblastomas. It is also likely that defects in other mismatch repair proteins could play similar roles: As mentioned above, one case from the TCGA data (TCGA-02-0083) had somatic mutations in two other mismatch repair genes, *MSH2* and

MLH1, and in another large-scale genome-wide study of glioblastomas, one recurrent tumor post-temozolomide/XRT had the hypermutation phenotype, but *MSH6* mutations were not found in this tumor (37). Thus, mismatch repair defects may be a common resistance pathway for treated glioblastomas that have already inactivated MGMT.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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