**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, Thr^{1219}Ile, 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.

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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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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 oligodendrogial 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 Gentra 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 µL volumes using 1× Platinum Taq PCR buffer, 200 µmol/L deoxynucleotide triphosphates, 2.0 µmol/L MgCl2, 0.4 µmol/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’-AACATTGCTGGAAGTTCTGG-3’ (forward) and 5’-GTGTCCTGACCTGACCTGACCTGC-3’ (reverse). Primer sequences for the remaining loci were identical to those previously described (16) except that a 5’-GTGTCCTGACCTGACCTGACCTGC-3’ sequence was added to each reverse primer to facilitate nontemplated 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 mM/mL, and stored at −80°C. O6-benzylguanine (O6-BG) was obtained from Sigma-Aldrich, reconstituted to stock concentration of 320 mM/mL 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 

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6 http://tcga-data.nci.nih.gov/tcga/findArchives.htm
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 glioblastoma 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 1:10. Successful infection was confirmed by the appearance of green fluorescent cells and Western blot evaluation for MSH6 protein.

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 cytototoxicity 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 cytototoxic 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 discussed.

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

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Treatment status</th>
<th>Chemotherapy</th>
<th>MSH6 somatic mutations</th>
<th>Amino acid change</th>
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Abbreviations: CCNU, lomustine; PCV, procarbazine, CCNU, vincristine.

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, MSI2 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>
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<th>TcA</th>
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<th>GCC</th>
<th>TCC</th>
<th>ACG</th>
<th>CGG</th>
<th>GCG</th>
<th>TCg</th>
<th>ACT</th>
<th>TCT</th>
<th>ACT</th>
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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 μmol/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 (Welcome 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 SN1-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

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8 http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=sample;id=763596
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\(\alpha\) 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

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![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 \(\mu\)mol/L temozolomide for 7 d (solid line, closed circle), U251-Sh1 cells seem resistant to 100 \(\mu\)mol/L temozolomide (dashed line, open circle; \(P < 0.01\)). The addition of 40 \(\mu\)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).](http://www.aacjournals.org/content/15/14/4627/F3.large.jpg)
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 μmol/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→T transitions preferentially at CpG 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 O<sup>6</sup> position of guanine nucleotides. The first line of response to repair this chemotherapeutic event is mediated by MGMT, which removes O<sup>6</sup>-methylguanine from DNA, 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.

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 O<sup>6</sup>-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, O<sup>6</sup>-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 O<sup>6</sup>-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 glioblastoma. 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.

**References**

Correction: *MSH6* Mutations Arise in Glioblastomas during Temozolomide Therapy and Mediate Temozolomide Resistance

In this article (Clin Cancer Res 2009;15:4622–9), which was published in the July 15, 2009, issue of *Clinical Cancer Research* (1), the parental origin of the TR3 cell line had been mistakenly identified as A172. On the basis of the analysis of more than 60 markers across 17 autosomal short-tandem repeat (STR) and amelogenin loci, it was determined that TR3 was only 34.92% identical to A172. Upon testing of other established cell lines from the laboratory, it was determined that the parental origin of the TR3 cell line was U251 (88.52% identical). Cell Line Authentication Tests were conducted by Genetica DNA Laboratories, Inc. The authors regret this error.

Because of the use of the wrong parental cell line, the growth rate experiments reported in Fig. 2A were repeated using U251 in place of A172 to determine if the conclusions of the original article remained true. In the initial experiments, growth curves were used to compare the effect of temozolomide treatment in A172 and TR3. To confirm the results of the original experiments, growth curves were generated for U251 and TR3 in the absence or presence of temozolomide. In the corrected version of Fig. 2A below, U251 cells were seeded at 1,000 cells per well and TR3 cells at 2,000 cells per well to generate similar nontreated growth curves for comparison. Under these conditions, TR3 was more resistant to temozolomide than the parental U251 cell line, mirroring the results obtained in the original experiments. Because the growth curve experiments required the use of different seeding densities for comparison, survival curves were also generated at multiple seeding densities for confirmation of the findings. At all seeding densities tested, TR3 was more resistant to temozolomide than the parental U251 cell line (corrected Fig. 2B). The U251 cell line was confirmed to have wild-type *MSH6*, and the TR3 cell line was confirmed to have the c.3656C>T *MSH6* somatic mutation as reported previously (1). These analyses with U251 cells substantiate the original conclusion that *MSH6* mutations are causally associated with temozolomide resistance.

![Figure 2](image-url)

*Figure 2.* A, the parental U251 cell line and the temozolomide (TMZ)-resistant subclone TR3 exhibited similar growth in the absence of temozolomide. In contrast, U251 cells were more sensitive to 100 μmol/L temozolomide than TR3 cells. U251 cells were seeded at 1,000 cells per well, and TR3 cells were seeded at 2,000 cells per well. Treated cells were administered with 100 μmol/L temozolomide daily, and cell viability was measured using the MTS assay as described in the original article. B, survival curves for U251 and TR3 cells after 7 days of daily temozolomide treatment. U251 cells were plated at 500 or 1,000 cells per well, and TR3 cells were plated at 500, 1,000, or 2,000 cells per well. At all plating densities, U251 cells were more sensitive to temozolomide than TR3 cells. For all experiments, error bars represent the SD of triplicates, and each individual experiment was replicated for a minimum of two times.
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Reference


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

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