Novel MSH6 Mutations in Treatment-Naïve Glioblastoma and Anaplastic Oligodendroglioma Contribute to Temozolomide Resistance Independently of MGMT Promoter Methylation

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Abstract

Purpose: The current standard of care for glioblastoma (GBM) involves a combination of surgery, radiotherapy, and temozolomide chemotherapy, but this regimen fails to achieve long-term tumor control. Resistance to temozolomide is largely mediated by expression of the DNA repair enzyme MGMT; however, emerging evidence suggests that inactivation of MSH6 and other mismatch repair proteins plays an important role in temozolomide resistance. Here, we investigate endogenous MSH6 mutations in GBM, anaplastic oligodendroglial tumor tissue, and corresponding brain tumor-initiating cell lines (BTIC).

Experimental Design: MSH6 sequence and MGMT promoter methylation were determined in human tumor samples and BTICs. Sensitivity to temozolomide was evaluated in vitro using BTICs in the absence and presence of O6-benzylguanine to deplete MGMT. The influence of MGMT and MSH6 status on in vivo sensitivity to temozolomide was evaluated using intracranial BTIC xenografts.

Results: We identified 11 previously unreported mutations in MSH6 in nine different glioma samples and six paired BTIC lines from adult patients. In addition, MSH6 mutations were documented in three oligodendrogliomas and two treatment-naive gliomas, both previously unreported findings. These mutations were found to influence the sensitivity of BTICs to temozolomide both in vitro and in vivo, independent of MGMT promoter methylation status.

Conclusions: These data demonstrate that endogenous MSH6 mutations may be present before alkylator therapy and occur in at least two histologic subtypes of adult glial neoplasms, with this report serving as the first to note these mutations in oligodendroglioma. These findings broaden our understanding of the clinical response to temozolomide in gliomas. Clin Cancer Res; 20(18); 1–10. ©2014 AACR.

Introduction

Malignant glial neoplasms are the most frequent primary intra-axial brain tumors in adults. The current standard of care for glioblastoma (GBM), the most aggressive of gliomas, involves a combination of surgery, radiotherapy, and temozolomide chemotherapy (reviewed in ref. 1). Despite aggressive treatment, long-term tumor stabilization of GBM is uncommon and the median survival time of good performance patients of ages 70 years or less is only 14.6 months (2). The most complete and most durable responses to temozolomide and the longest overall survival times are seen in the subgroup of tumors with epigenetic silencing of the MGMT enzyme that normally repairs the cytotoxic O6-methylguanine (O6-MeG) DNA adducts induced by temozolomide (3). However, MGMT methylation status does not correlate with temozolomide sensitivity in all patients, and thus MGMT expression is insufficient to explain temozolomide resistance in all tumors (1, 3, 4).

Recent findings from The Cancer Genome Atlas (TCGA) implicate defects in the DNA mismatch repair (MMR) system, primarily involving mutations of MSH6, as additional mediators of temozolomide resistance (5). MMR proteins recognize O6-MeG adducts not removed by MGMT and initiate an apoptotic response that leads to cell death (6). Hence, sensitivity to temozolomide likely requires both
the absence of MGMT and the presence of functional MMR. Inactivation of MMR has been previously reported to be a consequence of temozolomide exposure. All four MSH6 mutations reported in the TCGA dataset were identified in consequence of temozolomide resistance. We investigated endogenous MSH6 mutations in GBM and anaplastic oligodendroglioma tumor tissue and corresponding brain tumor–initiating cell lines (BTIC). We documented 11 previously unreported MSH6 mutations, including three mutations in oligodendroglomas and two mutations in treatment-naïve gliomas. These previously undocumented findings expand the diversity of MSH6 mutations in glioma and the implications of temozolomide treatment for MSH6-mutated patients. Inhibition of MGMT with O^6^-benzylguanine and temozolomide treatment of BTIC xenografts confirmed endogenous MSH6 mutations influenced temozolomide, independent of MGMT promoter methylation status. Our results suggest that endogenous MSH6 mutations may be present before alkylator therapy, occur in at least two histologic subtypes of glioma, and influence temozolomide sensitivity both in vitro and in vivo.

The high frequency of G-C to A-T transitions at non-CpG sites (5, 8). However, the precise detail concerning the incidence of MSH6 mutations in acquired temozolomide resistance of GBM and other glial tumors remains unclear. Some studies have concluded that MSH6 mutations are not associated with MGMT promoter methylation status (9), and few studies have investigated MSH6 in glial tumors, other than GBM, that are also routinely treated with temozolomide (10).

Here, we report for the first time, the presence of endogenous MSH6 mutations in GBM and anaplastic oligodendroglioma and their impact on chemotherapeutic response in brain tumor–initiating cell (BTIC) lines derived from these tumors. We have identified 11 previously unreported nonsynonymous MSH6 mutations in nine different tumor samples and six paired BTIC lines. Moreover, in five tumor samples for which normal DNA from paired patient blood was available for comparison, we found that the MSH6 mutations were somatic, not germline, in nature. Furthermore, in previously treated recurrent cases, the presence of MSH6 mutations influenced response to temozolomide in both in vitro culture and in vivo xenografts of BTIC lines. We also report, for the first time, MSH6 mutations in three oligodendroglioma samples and two treatment-naïve glioma cases, thus demonstrating that MSH6 mutations may occur in at least two subtypes of glioma, including GBM, and be present before radiotherapy and alkylator therapy.

**Materials and Methods**

**Patient tumor samples and brain tumor–initiating cell culture**

Fresh tumor samples were obtained from 26 adult patients with GBM and anaplastic oligodendroglioma during their operative procedure, following informed consent and approval by the University of Calgary (Calgary, AB, Canada) Ethics Review Board. Tumor tissue was frozen at −80°C for molecular analysis or used to culture BTICs as previously described (11–14). In brief, BTIC lines were initiated in defined culture serum-free medium (SFM). All cultures grew as nonadherent spheres except for one line (BT117, which grew as a monolayer) and were expanded and cryopreserved in 10% DMSO (Sigma-Aldrich) in SFM for use in later experiments. Authentication and testing of all cell lines were performed per American Association for Cancer Research recommendations. Molecular characterization of all BTIC lines was performed as described below at initiation and before experimental use. Thirteen tumor samples, from which 11 paired BTIC lines were established, were obtained from patients at recurrence following chemoradiotherapy (Table 1). The remaining samples were from treatment-naïve patients. For tumor 61, tumor tissue was available both at initial presentation and recurrence following chemoradiotherapy.

**MGMT promoter methylation assay**

DNA was extracted from tumor tissue and BTIC sphere/monolayer cultures using DNeasy (Qiagen) according to the manufacturer’s instructions. Of note, 500 ng of DNA was bisulfite converted using the Epitect Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. Two microliters of the Epitect product was used for methylation-specific PCR (MS-PCR) determination of MGMT promoter methylation as previously described (3). Thermocycling conditions for the MGMT MS-PCR were 95°C for 10 minutes initial denaturation, then 35 cycles of 95°C for 45-second denaturation, 45-second annealing, 72°C for 45-second extension, followed by 72°C for 10-minute final extension. The annealing temperatures were 61°C for MGMT-methylated and 58°C for MGMT-unmethylated MS-PCR. PCR products were visualized by agarose gel electrophoresis.
cDNA sequencing

RNA was extracted from tumor tissue and BTIC cultures with RNaseasy (Qiagen) according to the manufacturer’s instructions. Of note, 500 ng of RNA was reverse transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen) using poly-T primers according to the manufacturer’s instructions. Sequencing of \textit{EGFR}, \textit{PTEN}, and \textit{TP53} cDNA was performed as previously described (13). For sequencing of \textit{MSH6}, 2 μL of cDNA was then used in two 50-μL RT-PCR reactions (Platinum High Fidelity \textit{Taq}; Invitrogen) with primers designed in PrimerBlast (NCBI) to amplify overlapping 2100-bp fragments (fragment 1—forward: 5'-AGC TGC GTC ACG TAC CCC ACC AGG TGC TTA-3', reverse: 5'-CCA CCT AGA GCA GAG AGC GGC A-3'; fragment 2—forward: 5'-GGG TCA TGT TAC CCC AGG TGC TTA-3', reverse: 5'-CAC TTT TCT TCT CAG AAG TCA ACT CAA AGC-3'). Thermocycling conditions were 94°C for 5-minute initial denaturation, then 35 cycles of 94°C for 30-second denaturation, 62°C for 30-second annealing, 68°C for 3-minute extension, followed by 68°C for 7-minute final extension. The RT-PCR products were purified by agarose gel electrophoresis and isolated with the QIAquick Gel Extraction Kit (Qiagen). A series of three primers were used to sequence overlapping regions of each fragment (fragment 1: primer 1-1: 5'-TTG ATG ACA GCC CAA AGG AGG-3', primer 1-2: 5'-TTT CCT CAA TTC TTT AGG AAA ACG ACC-3', primer 1-3: 5'-TTT CCT CTC TAC CCC AGG TGC TTA-3'; fragment 2: primer 2-1: 5'-GATT TTT CCT TCT CCT CTG AGG G-3', primer 2-2: 5'-TAT GTG TCG CCC AGT AAT TCT GTT G-3', primer 2-3: 5'-TAC AGT CAA ATC AGG AAA ACG ACC-3'). For sequencing of \textit{MSH2}, 2-μL of cDNA was then used in two 50-μL RT-PCR reactions (Platinum High Fidelity \textit{Taq}; Invitrogen) with primers designed in PrimerBlast (NCBI) to amplify overlapping fragments (fragment 1—forward: 5'-GGA GAA CAG CAT TTG AGT ACA GGT CAA GGA-3', reverse: 5'-ATT GGT TTT CCT TTA GAG GAA AGG GCC-3'; fragment 2—forward: 5'-GGG AGG CAT TCT CAA TCT CTT GTT GAC CAA-3', reverse: 5'-TTG ACA GAG GAA AGG GCC-3').

### Table 1. Clinical and molecular characteristics of tumor samples

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Abbreviations: Adj, adjuvant mTOR inhibitor; GBM (WHO grade 4); Me, methylated promoter; OII, anaplastic oligodendroglioma (WHO grade 3); -r, recurrent; PCV, procabazine, CCNU, and vincristine; TMZ, temozolomide chemotherapy; U, unmethylated promoter; -2°, secondary.
CAG CT TGG GCA-3', primer 1-2: 5'-GCA CAT TGG ACA TTC CTT TTT CAG G-3', primer 1-3: 5'-TAC TTT GAT GAC GGA ACC CTT TGG-3'; fragment 2: primer 2-1: 5'-CGT GTC AAA TGG AGC ACC TGT TC-3', primer 2-2: 5'-CTT AAA ATA TTT ATT TTA GTA CAT C-3'. Automated DNA sequencing was performed at the University of Calgary Core DNA Services facility. All identified mutations were confirmed in at least two independently amplified RT-PCR products.

**DNA sequencing**

Genomic DNA was extracted from patient blood, tumor tissue, and BTIC cultures with DNeasy (Qiagen) according to the manufacturer’s instructions. Sequencing of *IDH1* was performed as previously described (14). For sequencing of *MSH6*, 30-ng of DNA was used in two 50-µL PCR reactions (Platinum High Fidelity *Taq*; Invitrogen) with primers designed in PrimerBlast (NCBI) to amplify regions of exons 4, 7, and 8 of *MSH6* containing mutations according to the cDNA sequence analysis (exon 4-1 forward: 5'-ATC CCA ACG CCA CGT TAG-3', reverse: 5'-TCA GTC TGT TCC ACT CGT GC-3'; exon 4-2 forward: 5'-CTG ACT ACA GTA AGA CTAC AGA GTA AG-3', reverse: 5'-CCA AAA CTG GGA GCC GGG TA-3'; exon 4-3 forward: 5'-CTTG TCTTTC TCA GGA AGG TC-3', reverse: 5'-AGC CAT TGG ACA TAA TTG TTT ATG TC-3'; exon 7 forward: 5'-GAC CTC ATG GTT GTG CCT GA-3', reverse: 5'-GGT ATT CCA GGA GCC TGT CT-3'; exon 7 forward: 5'-CCC GCC CAA TAA TTG CAT AGT C-3' reverse: 5'-TGCTGGA GTG CGT GCT CTA AA-3'; exon 8 forward: 5'-CCG ATG TTG CIT TCTGCT CCI-3', reverse: 5'-TGG CTG GGG TGT CTC TCA C-3'). The PCR products were purified as described above and sequenced bidirectionally using the forward and reverse primers for each respective fragment.

**Western blotting**

BTICs were treated with O6-benzylguanine (BG; Sigma-Aldrich) or DMSO (Sigma-Aldrich) for 72 hours and lysed in modified radioimmunoprecipitation assay buffer supplemented with Complete Mini Protease (Roche) and Halt Phosphatase (Thermo Scientific) inhibitor cocktails. Of note, 15 µg of protein was loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transblotted to nitrocellulose membranes. Blots were stained with mouse anti-MGMT (1:1,000; Santa Cruz Biotechnology) and goat anti-actin (1:2,500; Santa Cruz Biotechnology) antibodies diluted in 5% nonfat milk (Bio-Rad) in Tris-buffered saline with Tween 20. Horseradish peroxidase–conjugated donkey anti-mouse IgG and donkey anti-goat IgG (Millipore) were used at 1:5,000. Bands were visualized with the ECL Plus Western Blotting Detection System and Hyperfilm (Amersham).

**BTIC drug sensitivity assays**

Dissociated BTIC cultures were seeded at 2,500 to 5,000 cells per well in 96-well plates and treated with temozolomide (Merck), BG (Sigma-Aldrich), or vehicle (DMSO; Sigma-Aldrich). For MGMT depletion experiments, cells were allowed to rest for 24 hours after plating, pretreated with BG for 48 hours, and then treated with temozolomide at 72 hours. After 14 to 21 days, BTIC growth was assessed by performing the alamarBlue assay (Invitrogen) according to the manufacturer’s instructions. All culture experiments were performed in triplicate with a minimum of 3 wells per condition.

**Intracranial BTIC xenografts**

BTIC spheres from BT073 (MGMT methylated/MSH6 wild-type), BT134 (MGMT unmethylated/MSH6 wild-type), and BT143 (MGMT methylated/MSH6 mutant) were dissociated to single-cell suspensions and 1 x 10^5 cells were stereotactically implanted into the right striata of 6- to 8-week-old NOD/SCID mice as previously described (11–14). Seven days after BTIC implantation, mice were randomized to vehicle or temozolomide treatment cohorts (BT073: n = 7 for vehicle and n = 8 for temozolomide; BT134: n = 7 for vehicle and n = 7 for temozolomide; BT143: n = 11 for vehicle and n = 12 for temozolomide). Oral treatment with temozolomide (50 mg/kg; Merck) or vehicle (Ora-Plus; Galenova) was delivered daily 5 days a week for 3 weeks. Mice were sacrificed upon significant weight loss or presentation of neurologic symptoms necessitating euthanasia as per University of Calgary animal care guidelines. Necropsy and cranial section were performed to confirm presence of tumor in all animals.

**Statistical analyses**

Results are displayed as the mean ± standard deviation. Student *t* tests were performed for comparison of two conditions and one-way ANOVAs followed by Tukey multiple comparison tests were performed for comparison of multiple conditions. Fisher exact tests were used to test correlation between G-C to A-T transition frequency and *MSH6* status and temozolomide exposure. Statistically significant differences in median survival were determined by the log-rank test using GraphPad Prism.

**Results**

**MSH6 mutations are present in BTIC lines isolated from both MGMT-methylated and -unmethylated tumors before and following chemoradiotherapy**

To investigate *MSH6* mutations in malignant glioma, we sequenced *MSH6* cDNA in tumor samples and BTIC lines cultured from a series of 26 patients with GBM or anaplastic oligodendroglioma (Table 1). Point mutations (Fig. 1 and Table 2) resulting in nonsynonymous amino acid substitutions or protein truncation were present in nine of the samples. Most tumors contained only a single *MSH6* mutation except for samples 143 and 248, which had three and two mutations, respectively. *MSH6* mutations were heterozygous in all of the samples except for sample 88. A polymorphism involving a substitution of glutamate for glycine at codon 39, previously reported not to influence patient survival (15), was detected in BT042, 054, and 088 (data not shown). To exclude other uncommon polymorphisms, sequencing of paired patient blood samples from five tumors (119, 143, 157, 248, and 253) was
performed; seven of the eight MSH6 alterations were only found in the tumor (Supplementary Fig. S1). The sole exception was V878A in BT143, which was present in the blood sample, raising the possibility that it represents a germline polymorphism. With the exception of the T1219I mutation (5, 6) present in BT119, none of the other mutations have been previously reported in the glioma literature.

MSH6 mutations were detected in samples 054, 088, and 255 obtained from patients with anaplastic oligodendroglioma (Fig. 1A and B and Table 2), demonstrating that MSH6 mutations arise in other glioma subtypes besides GBM. Two samples of treatment-naïve tumors (054 and 061) harbored mutations in MSH6, and the mutation in sample 061 remained detectable at tumor recurrence following chemoradiotherapy (Fig. 1C and D), suggesting that MSH6 mutations exist in tumors before therapy. Similarly, MSH6 mutations were present in both MGMT-methylated and -unmethylated tumors, although there was a trend toward MSH6 mutations being more frequent in MGMT-methylated tumors. No mutations in MSH2 were present in our subset of MSH6-mutant samples. We further sequenced other commonly mutated genes in GBM (Table 1) and observed that all six MSH6-mutant GBMs also harbored a PTEN mutation and five of the six had a TP53 mutation. Conversely, only one of the six MSH6-mutant GBMs had either an EGRF or IDH1 mutation. The combinations of mutations present in MSH6-mutant GBMs suggest that they include at least three of the four recognized TCGA GBM subgroups, although expression analyses would be needed for definitive subtyping characterization. Nonetheless, it seems likely that the IDH1-mutant secondary GBM BT061 is of the proneural subgroup while the IDH1-wild-type GBMs BT012, 119, 143, 157, and 248 with different combinations of EGRF, PTEN, and TP53 mutations encompass molecular profiles of the classical and mesenchymal GBM subgroups. Taken together, these results demonstrate that MSH6 mutations occur in GBMs and oligodendrogliomas both before and following chemoradiotherapy and do not seem to be restricted to tumors that are MGMT-methylated or comprise a particular molecular subgroup of GBM.

To investigate whether MSH6 mutations may be associated with a hypermutator phenotype in BTICs, we assessed the frequency of G/C to A/T transitions at non-CpG sites in our sequencing data for MSH6, EGFR, PTEN, TP53, and IDH1. Five of 12-point mutations in the MSH6 wild-type tumors were G/C to A/T transitions, whereas 33 of 38 point mutations in the MSH6-mutant tumors were G/C to A/T transitions (Fig. 2A). These results suggested that MSH6 mutation was associated with more

![Figure 1](https://example.com/figure1.png)

**Figure 1.** MSH6 mutations are present in both MGMT-methylated and -unmethylated tumors before and after chemoradiotherapy. A–D, cDNA sequencing revealed heterozygous MSH6 mutations in samples of oligodendroglial (A and B) and treatment-naïve tumors (A and C). The heterozygous R911STOP MSH6 mutation in tumor 61 was isointense with the wild-type allele at recurrence following chemoradiotherapy in this patient (D). TMZ, temozolomide; XRT, radiotherapy.

### Table 2. Distribution of MSH6 mutations by MGMT promoter methylation and treatment subgroups

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<tr>
<td>MGMT unmethylated</td>
<td>042 wt</td>
<td>068 wt</td>
<td>075 wt</td>
</tr>
<tr>
<td>pretreatment</td>
<td>090 wt</td>
<td>101 wt</td>
<td>134 wt</td>
</tr>
<tr>
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<td>012 A1021G</td>
<td>041 wt</td>
<td>117 wt</td>
</tr>
<tr>
<td>119 T1219I</td>
<td>156 wt</td>
<td>157 A555V</td>
<td>177 wt</td>
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frequent G-C to A-T transitions at non-CpG sites ($P = 0.004$, Fisher exact test). However, as our MSH6 wild-type subset was enriched in temozolomide-naïve tumors, whereas our MSH6-mutant tumor subset was heavily biased toward temozolomide-exposed tumors, we performed a subset analysis on the post-temozolomide tumors to control for the potential confounding effects of temozolomide exposure. Two of four-point mutations in the post-temozolomide MSH6 wild-type tumors were G-C to A-T transitions, whereas 28 of 32 point mutations in the post-temozolomide MSH6-mutant tumors were G-C to A-T transitions (Fig. 2B). Although there was a trend toward more frequent G-C to A-T transitions in the MSH6-mutant subset, it did not reach statistical significance in this small data subset ($P = 0.12$, Fisher exact test). Finally, we examined the frequency of G-C to A-T transitions in MSH6 versus EGFR, PTEN, TP53, and IDH1. In the temozolomide-naïve subset, one of two MSH6 mutations were G-C to A-T transitions and seven of 12 mutations in the other genes were G-C to A-T transitions. In the temozolomide-exposed subset, eight of 10 MSH6 mutations were G-C to A-T transitions and 22 of 26 mutations in the other genes were G-C to A-T transitions (Fig. 2C and D). Neither the G-C to A-T transition frequency in mutations of the MSH6 gene nor the other four genes was statistically correlated with temozolomide exposure ($P = 0.45$ and 0.089, respectively, Fisher exact tests). Taken together, these data suggest that MSH6 mutation predisposes tumors to an increased frequency of G-C to A-T transitions after temozolomide exposure.

**MSH6 mutations and MGMT promoter methylation status both influence sensitivity to temozolomide in vitro and in vivo**

To determine whether the mutations in MSH6 identified in this study were functionally significant, we assessed sensitivity to temozolomide in tumors for which we had corresponding BTIC lines. All MGMT-methylated/MSH6 wild-type BTIC lines demonstrated a significant response to temozolomide at the clinically relevant dose of 10 μg/mL temozolomide (16) by the alamarBlue assay ($P < 0.05$, Student $t$ test; Fig. 3A). Conversely, MGMT-methylated/MSH6-mutant BTICs were much less responsive to temozolomide; only BT054 demonstrated a dramatic reduction in alamarBlue conversion, whereas BT088 and BT143 were modestly or minimally responsive. MGMT-unmethylated BTICs were unresponsive to temozolomide regardless of MSH6 status.

To investigate the influence of MSH6 on temozolomide sensitivity in MGMT-unmethylated BTICs, we used BGC to deplete the MGMT enzyme (17). A concentration of 50-μmol/L BGC did not significantly reduce BTIC viability (Fig. 3B), but dramatically depleted MGMT protein after 72 hours (Fig. 3C). Pretreatment of MGMT-unmethylated/MSH6 wild-type BTICs with 50-μmol/L BGC for 24 hours significantly increased sensitivity to temozolomide ($P < 0.01$, Tukey multiple comparison test; Fig. 3D)

Figure 2. MSH6 mutations are associated with increased frequency of G-C to A-T transitions. A–D, the frequency of G-C to A-T transitions was associated with MSH6 mutation in the entire dataset (A), but did not reach statistical significance in the subset of temozolomide (TMZ)-exposed tumors (B). The frequency of G-C to A-T transitions in the MSH6 gene (C) or in the EGFR, PTEN, TP53, and IDH1 genes (D) was not associated with temozolomide exposure. $P$ values were determined by the Fisher exact test.
consistent with depletion of MGMT protein. BG pretreatment had no discernible effect on temozolomide response in MGMT-methylated BTICs, regardless of MSH6 mutation status (Fig. 3D). In all three of the MGMT-unmethylated/MSH6 wild-type BTICs, BG pretreatment did not confer temozolomide sensitivity (Fig. 3D), suggesting that the MSH6 mutations in BT012, BT119, and BT157 were functionally significant. Most endogenous MSH6 mutations in GBM and oligodendroglioma therefore seem to be associated with decreased temozolomide response independently of MGMT; and in this study, dramatic sensitivity to temozolomide required the combination of low levels of MGMT activity and functional MSH6.

To further evaluate the influence of MSH6 and MGMT on temozolomide response in vivo, we treated animals bearing intracranial xenografts of BT073, 134, and 143 with an oral dose of 50-mg/kg temozolomide five times a week for 3 weeks. This treatment regimen was able to dramatically increase the survival time of animals xenografted with the MGMT-methylated/MSH6 wild-type cell line BT073 (P = 0.0002; Fig. 4A). Median survival was at least doubled with more than half of the treated animals demonstrating no systemic or neurologic signs of illness when the predetermined endpoint of 100 days was reached. As expected, temozolomide did not significantly increase median survival in xenografts of the MGMT-unmethylated/MSH6 wild-type cell line BT134 (45 vs. 49 days; P = 0.0772; Fig. 4B). Similarly, temozolomide only modestly increased median survival in xenografts of the MGMT-methylated/MSH6-mutant cell line BT143 (83 vs. 99 days; P = 0.0025; Fig. 4C). These data thus support the hypothesis that MSH6 status influences xenograft survival with the presence of mutant MSH6 dramatically attenuating benefit of temozolomide in MGMT-methylated tumors.

To investigate the possibility that temozolomide treatment may induce de novo MMR mutations, we sequenced...
MSH6 and MSH2 in our xenograft tumors. De novo MSH6 mutations were not identified in any of the xenograft tumors. Interestingly, several different de novo MSH2 mutations were present in the BT073 xenografts treated with temozolomide, but absent in the xenografts treated with vehicle (data not shown). No MSH2 mutations were identified in either the BT134 or 143 temozolomide-treated xenografts. These data demonstrate that MSH2 mutations can arise during the course of temozolomide treatment, but did not seem to abrogate temozolomide response in the MGMT-methylated/MSH6 wild-type BT073 xenografts within the time frame of the experiment.

Discussion

Temozolomide has improved the prognosis for patients with GBM (1). Although the addition of temozolomide to radiotherapy (XRT) has lengthened median survival only modestly, those with MGMT-methylated tumors seem to have a greater degree of benefit from chemoradiotherapy. This enhanced benefit is reflected in the significantly greater proportion of 3- and 5-year survivors after temozolomide/XRT, than XRT alone (18). Unfortunately, regardless of MGMT methylation status, most patients eventually recur and these recurrences are often resistant to temozolomide. Recent evidence indicates that there are several potential mechanisms that contribute to temozolomide resistance in adult and pediatric GBM, including activation of the base excision DNA repair pathway (19, 20) and phosphoinositide-3-kinase-mediated upregulation of the HOXA9/HOXA10 genes (21). Findings from the TCGA and other groups implicate defects in the MMR pathway, frequently through mutation of MSH6, as a mediator of temozolomide resistance in alkylator-exposed, MGMT-methylated GBMs (5–7). Here, we reaffirm the role of MSH6 mutations as a mechanism of temozolomide resistance and also make the previously unreported observation of mutations in other subgroups of patients, including those that have oligodendrogliomas or GBMs that are treatment-naïve or MGMT-unmethylated. Although our dataset is relatively small, these novel findings potentially implicate preexisting non-germline MSH6 mutations in early temozolomide resistance in some patients with oligodendrogloma and GBM.

The MSH6 mutations in cases 054, 088, and 255 are the first such alterations to be reported in oligodendrogliomas. The consequences of these alterations for response to temozolomide were evaluated in BTICs derived from 054 and 088. In BT088, the T521I substitution seemed to result in obvious resistance to temozolomide compared with BTIC lines with methylation of the MGMT promoter and wild-type MSH6. In contrast, the L601V substitution in BT054 was not associated with resistance to temozolomide. It is possible that this mutation is simply another functionally neutral polymorphism similar to G39E (15); however, normal DNA from the patient was not available for comparison to address this possibility. In addition, the observed failure to confer resistance to temozolomide may have been a result of the nature of a leucine to valine substitution. Both amino acids are small, nonpolar, and neutral and hence may not have dramatically affected MSH6 folding or function. Nevertheless, the results with BT088 demonstrate that MSH6 mutations can functionally influence response to temozolomide in oligodendrogloma. These findings suggest that MSH6 mutations may be a potential mechanism for resistance.
for temozolomide resistance in oligodendrogliomas, as well as GBMs.

MMR defects have a well-established role in familial GBMs in patients with Turcot Syndrome type II (22) or constitutional MMR-deficiency syndrome (23). However, in sporadic adult GBM, MMR mutations do not seem to be an early, initiating event in tumor development. Previous reports of MSH6 mutations in nonfamilial adult gliomas have been almost exclusively confined to post-treatment tumors (5, 7, 9, 10), with the exception of one mutation in the MMR gene MSH2 in a treatment-naïve GBM sample in the TCGA dataset (5). Analysis of several TCGA samples for which tissue was available both at initial presentation and at recurrence following chemotherapy seemed to demonstrate that MSH6 mutations were detectable only after exposure to radio- and alkylator therapy (7). However, it is unclear whether MSH6 mutations may develop spontaneously and are then selected for by alkylating agents or if they are in fact induced as a side effect of alkylator therapy. Our results with tumor 061 suggest that at least in some tumors it is the former possibility. In tumor 061, we demonstrate a weak, but unequivocal, signal for the R911STOP mutation that was present before temozolomide exposure. As such, early in tumor evolution before exposure to alkylator chemotherapy, MSH6 mutations may already be present, even if it is potentially only in a small fraction of tumor cells and below the level of detection by current methods. Our results thus provide some of the first evidence that MSH6 mutations occur in treatment-naïve tumors, and may help to explain the paradoxical failure of temozolomide and rapid recurrence in some MGMT-unmethylated gliomas.

Finally, in addition to the results reported by the TCGA, our data illustrate that MSH6 mutations can occur in both MGMT-methylated and -unmethylated tumors. The potential role of MMR inactivation in MGMT-methylated tumors is a seemingly intuitive mechanism for tumors lacking MGMT expression to ignore unrepaired O6-MeG lesions that otherwise would induce apoptosis. In tumors displaying the hypermutator phenotype (5) in which frequent G-C to A-T transitions arise at non-CpG sites, MSH6 deficiency would seem integral to both the accumulation of these mutations and ongoing survival of the tumor cells. Conversely, the presence of MSH6 mutations in MGMT-unmethylated tumors seems controversial and functionally redundant given that these tumors should be able to readily repair O6-MeG lesions before an apoptotic response is initiated. However, two recent studies with paired GBM specimens from patients at initial presentation and at recurrence have demonstrated reduced expression of MSH6, MSH2, and PMS2 (24) or MLH1 (25) in the recurrent tumors compared with their corresponding primaries. Although neither study further subdivided the results for MMR protein expression based on MGMT methylation status, given that nearly 75% of GBMs are unmethylated (5), it would seem likely that alterations in MSH6 expression could occur with reasonable frequency in MGMT-unmethylated tumors. The results presented here thus build on these findings by documenting MSH6 mutations in three GBMs with MGMT promoters that clearly are unmethylated and express detectable MGMT protein on Western blotting (data not shown). Depletion of MGMT protein in the three BTIC lines derived from these tumors failed to confer temozolomide sensitivity. As such, MSH6 mutations likely are a mechanism for enhanced temozolomide resistance in some MGMT unmethylated tumors. This may be of particular clinical significance for dose-dense temozolomide or other treatment strategies aimed at increasing response to alkylator chemotherapy in MGMT-unmethylated tumors.

Temozolomide is a cornerstone of the current standard of care for malignant glioma. However, the near universal development of resistance to alkylator therapies remains a major obstacle to durable tumor control. Our findings suggest that MMR deficiency may be more prevalent than previously appreciated, including in treatment-naïve and MGMT-unmethylated GBMs and also in oligodendrogliomas. These findings may thus help to explain seemingly paradoxical resistance to temozolomide in some tumor subgroups and reinforce the importance of developing additional nonalkylator therapeutics to deal with MSH6-deficient glioma.

Disclosure of Potential Conflicts of Interest

D.L. Senger and S.M. Robbins have ownership interest (including patents) in Arch Biopartners Inc. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: O.D.M. Stechishin

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O.D.M. Stechishin, D.L. Senger, S.M. Robbins

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.A. Nguyen, O.D.M. Stechishin, H.A. Luchman, D.L. Senger, J.C. Cairncross

Writing, review, and/or revision of the manuscript: S.A. Nguyen, O.D.M. Stechishin, H.A. Luchman, J.G. Cairncross, S. Weiss

Study supervision: H.A. Luchman, J.G. Cairncross, S. Weiss

Other (conducted animal experiments): X.Q. Liu

Acknowledgments

The authors thank Rozina Hassam, Dorothea Livingstone, and Orsolya Cseh for technical assistance. The patient samples were obtained through the Calgary Brain Tumour and Tissue Bank, generously supported by funds from the Clark H. Smith Family.

Grant Support

This work was supported by grants to S. Weiss from the Stem Cell Network and Alberta Cancer Foundation. S.A. Nguyen was the recipient of an Alberta Cancer Foundation scholarship.

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Received July 5, 2013; revised July 7, 2014; accepted July 14, 2014; published OnlineFirst July 30, 2014.
References

Clinical Cancer Research

Novel *MSH6* Mutations in Treatment-Naïve Glioblastoma and Anaplastic Oligodendroglioma Contribute to Temozolomide Resistance Independently of *MGMT* Promoter Methylation


*Clin Cancer Res* Published OnlineFirst July 30, 2014.

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