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MSH6 haploinsufficiency at relapse contributes to the development of thiopurine resistance in pediatric B-lymphoblastic leukemia

Nikki A. Evensen,¹ P. Pallavi Madhusoodhan,¹ Julia Meyer,² Jason Saliba,¹ Ashfiyah Chowdhury,¹ David J. Araten,³ Jacob Nersting,⁴ Teena Bhatla,¹ Tiffany L. Vincent,⁵ David Teachey,⁵ Stephen P. Hunger,⁵ Jun Yang,⁶ Kjeld Schmiegelow⁴ and William L. Carroll¹

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¹Departments of Pediatrics and Pathology, Perlmutter Cancer Center, NYU-Langone Medical Center, New York, NY, USA; ²Huntsman Cancer Institute, University of Utah Medical Center, Salt Lake City, USA; ³Department of Medicine, Perlmutter Cancer Center, NYU-Langone Medical Center, New York NY, USA; ⁴Department of Pediatrics and Adolescent Medicine, The University Hospital Rigshospitalet, Copenhagen, Denmark; ⁵Department of Pediatrics and the Center for Childhood Cancer Research, Children's Hospital of Philadelphia and The Perelman School of Medicine at The University of Pennsylvania, Philadelphia, PA, USA and ⁶St. Jude Children's Research Hospital, Memphis, TN, USA

ABSTRACT

Survival of children with relapsed acute lymphoblastic leukemia is poor, and understanding mechanisms underlying resistance is essential to developing new therapy. Relapse-specific heterozygous deletions in *MSH6*, a crucial part of DNA mismatch repair, are frequently detected. Our aim was to determine whether *MSH6* deletion results in a hypermutator phenotype associated with generation of secondary mutations involved in drug resistance, or if it leads to a failure to initiate apoptosis directly in response to chemotherapeutic agents. We knocked down *MSH6* in mismatch repair proficient cell lines (697 and UOCB1) and showed significant increases in IC50s to 6-thioguanine and 6-mercaptopurine (697: 26- and 9-fold; UOCB1: 5- and 8-fold) *in vitro*, as well as increased resistance to 6-mercaptopurine treatment *in vivo*. No shift in IC50 was observed in deficient cells (Reh and RS4;11). 697 *MSH6* knockdown resulted in increased DNA thioguanine nucleotide levels compared to non-targeted cells (3070 vs. 1722 fmol/μg DNA) with no difference observed in mismatch repair deficient cells. Loss of *MSH6* did not give rise to microsatellite instability in cell lines or clinical samples, nor did it significantly increase mutation rate, but rather resulted in a defect in cell cycle arrest upon thiopurine exposure. *MSH6* knockdown cells showed minimal activation of checkpoint regulator CHK1, γH2AX (DNA damage marker) and p53 levels upon treatment with thiopurines, consistent with intrinsic chemoresistance due to failure to recognize thioguanine nucleotide mismatching and initiate mismatch repair. Aberrant *MSH6* adds to the list of alterations/mutations associated with acquired resistance to purine analogs emphasizing the importance of thiopurine therapy.

Correspondence:

william.carroll@nyumc.org

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Introduction

Relapsed B-precursor acute lymphoblastic leukemia (B-ALL) is a leading cause of cancer mortality amongst children. Development of chemoresistance is a crucial factor contributing to relapse, therefore understanding the biological mechanisms underlying this resistance is imperative for discovering innovative treatment strategies.¹ Recent work has begun to highlight the direct role of relapse specific/enriched genetic alterations in the emergence of clones that have gained a selective advantage under the pressure of specific chemotherapeutics, such as *NT5C2*, *TBL1XR1*, *PRPS1*, and *CREBBP*.¹⁻⁵ Many of these mutations cause resistance specifically to thiopurines, which are the backbone of maintenance therapy

and have proven vital for achieving cures.⁶ Our analysis of copy number alterations (CNAs) in diagnosis/relapse pairs revealed a relapse specific hemizygous deletion on chromosome 2p16.3 involving MSH6 in 4-10% of patients.^{7,8}

MutS homolog 6 (MSH6) is a major component of the mismatch repair (MMR) system, which is a highly conserved biological process that recognizes and repairs errors in nascent DNA strands during replication to maintain genomic integrity. Initial recognition of replicative errors is carried out by protein heterodimers consisting of either MSH6 and MSH2 (hMutS α), or MSH3 and MSH2 (hMutS β). Upon recognition of a mismatch, hMutS α recruits MutL α (MLH1-PMS2) which engages downstream proteins and enzymes involved in DNA repair.^{9,10}

Constitutional defects in MMR, including monoallelic mutations in Lynch syndrome and biallelic loss in constitutional mismatch repair deficiency (CMMRD), are strongly linked to carcinogenesis, where loss of MMR functionality causes increased mutability and predisposition to malignancy.¹¹⁻¹⁵ Previous work has linked defects in MMR to drug resistance, including thiopurines, in various cancers.¹⁶⁻¹⁹ However, it is uncertain if resistance in MMR defective clones occurs through the acquisition of secondary mutations as a consequence of mutagenic therapy, or the outgrowth of clones that have intrinsic drug resistance. Our lab previously demonstrated that lower expression of MSH6 in patient samples was associated with increased *ex vivo* resistance to 6-mercaptopurine and prednisone,⁷ highlighting the clinical importance of understanding the role of this genetic alteration in B-ALL.

The mechanism of action of thiopurines is based upon the insertion of a false nucleotide, namely a thioguanine (TGN), into DNA that when thiomethylated pairs with a thymine instead of a cytosine.¹⁸ Cytotoxicity is thought to be dependent on the MMR machinery recognizing the mismatch and attempting to match the TGN on the parental strand with an appropriate base on the daughter strand.^{19,20} Whether the DNA damage induced by the repetitive, futile cycles of DNA excision and repair, or simply the recognition of mismatches by hMutS α is enough to initiate a signaling cascade culminating in cell cycle arrest and apoptosis is not entirely understood.

We sought to delineate whether reduced expression of MSH6 could give rise to chemoresistance in B-precursor ALL and elucidate the mechanism responsible for the resistance. Our data here support the view that reduced MSH6 directly results in an increased tolerance to incorporated TGN and subsequent mismatches through a failure to initiate MMR, thus allowing cells to proliferate and survive under thiopurine treatment both *in vitro* and *in vivo*. We demonstrate that ALL cell lines with a functional MMR trigger a CHK1-mediated cell cycle arrest in response to thiopurines that is followed by DNA damage and apoptosis. In contrast, upon reduction of MSH6, the MMR signaling cascade is not fully activated and cells do not undergo apoptosis.

Methods

Cells and reagents

The B-lineage leukemia cell lines RS4;11 (ATCC, Manassas, VA, USA), Reh (ATCC), 697 (DSMZ, Braunschweig, Germany), and UOCB1 (a kind gift from Dr. Terzah Horton at Texas Children's Cancer Center/Baylor College of Medicine) were grown in

RPMI1640 medium. HEK293T (ATCC) cells were grown in DMEM medium. All media were supplemented with 10% FBS, 1% penicillin/streptomycin under 5% CO₂ at 37°C.

Drug preparation, viral preparation, immunoblotting, apoptosis assays, and cell cycle

Standard protocols were followed and have been previously described.^{3,21} More detailed information is provided in the *Online Supplementary Appendix*.

Patients' samples

Cryopreserved pediatric B-ALL specimens were obtained from the Children's Oncology Group (COG) ALL cell bank. All patients were treated on COG protocols for newly diagnosed ALL. All subjects provided consent for banking and future research use of these specimens in accordance with the regulations of the institutional review boards of all participating institutions.

Microsatellite instability analysis

Microsatellite instability (MSI) analysis was performed using MSI Analysis System, v.1.2 (Promega, Madison, WI, USA) following the manufacturer's protocol. Detailed information is provided in the *Online Supplementary Appendix*.

Measurement of mutation rate

Spontaneous mutation rate was measured using a flow cytometry assay previously described by Araten *et al.*²² that detects the presence of numerous glycosylphosphatidylinositol-linked (GPI) membrane proteins (see *Online Supplementary Appendix*). Briefly, GPI(+) isolated clones from the NT and MSH6-KD cell lines were expanded either untreated or treated with 6-TG (0.040 μ g/mL and 0.100 μ g/mL, respectively, based on IC50 values determined for clones). Cells were then stained for GPI-dependent markers including FLAER-Alexa 488 (Pinewood), CD48, CD52, and CD59 (Serotec),²³ and analyzed by flow cytometry. The mutant frequency (f) was calculated as the number of GPI(-) events divided by the total number of live events, and mutation rate (μ) was calculated as f divided by cell divisions.²²

Thioguanine quantification assay

Cells were treated with 6-thioguanine (6TG) and collected every day for four days. DNA was extracted using Puregene Core Kit A (QIAGEN). DNA TGN levels were measured using liquid chromatography-tandem mass spectrometry as described previously.²⁴

In vivo mouse model of chemoresistance

All experiments were conducted on protocols approved by the Institutional Animal Care and Use Committee and Institutional Review Board of the Children's Hospital of Philadelphia. Briefly, 1 million UOCB1 NT GFP-CBG or MSH6 shRNA1 GFP-CBR cells were injected into NSG mice *via* tail vein on day 0 (total 20 mice; 10 per cell line). On day 6 leukemic burden was confirmed *via* bioluminescence imaging (BLI) (IVIS Spectrum imaging system, Perkin Elmer) and animals were randomized to treatment groups [PBS vehicle or Purixan (50 mg/kg) diluted in PBS]. Mice were treated on day 7 by gavage (0.2 mL/mouse). For BLI, 3 mg of luciferin was injected intraperitoneally and mice were imaged ten minutes post injection. Quantification of total flux was determined by analyzing the BLI images using Living Image Software (Perkin Elmer) (see *Online Supplementary Appendix*).

Statistical analysis

Statistical significance was calculated using unpaired *t*-test for IC50s, paired *t*-test for mutation rates, one-way ANOVA for cell

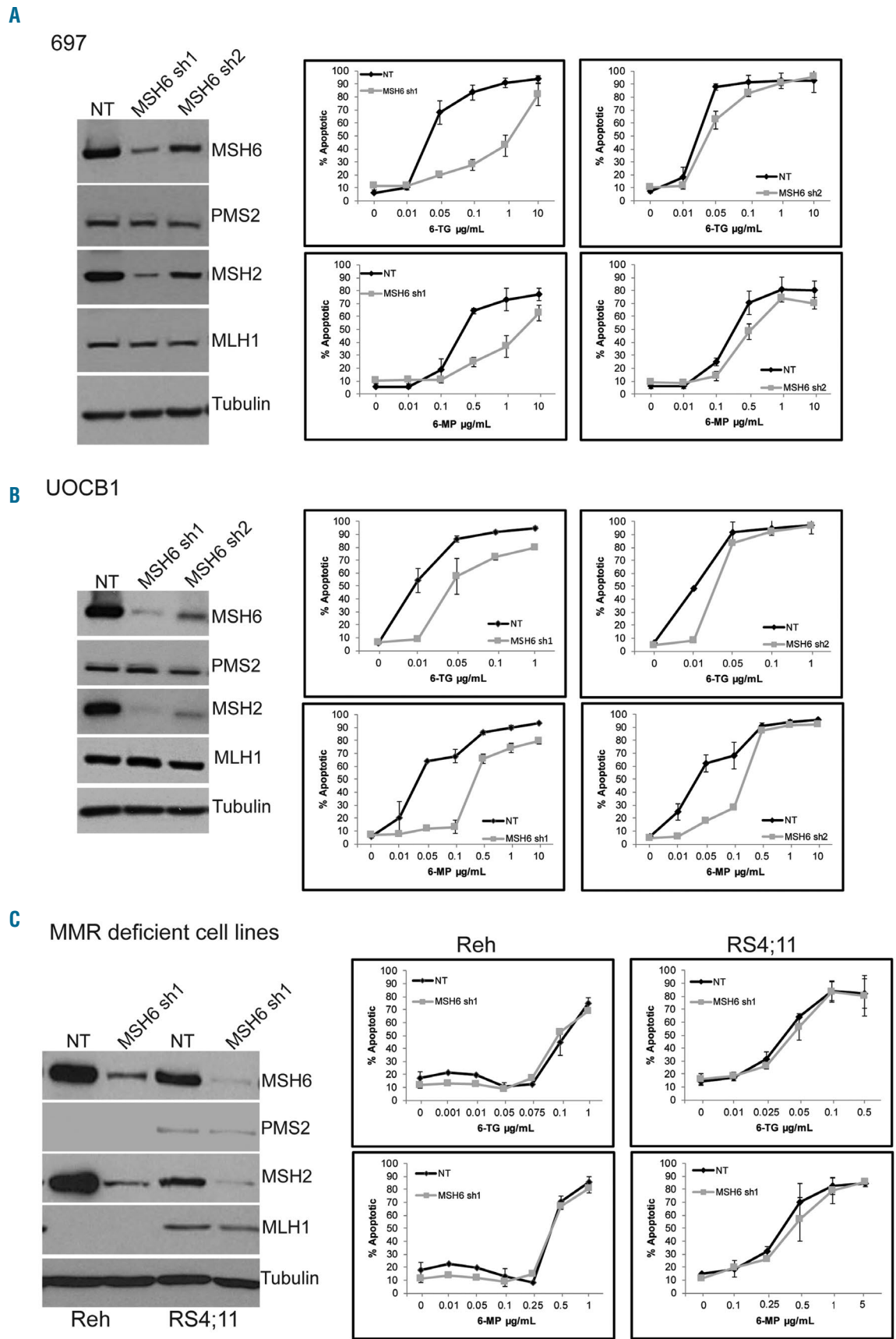


Figure 1. Knockdown of MSH6 in mismatch repair (MMR) proficient cells lead to decreased sensitivity to thiopurines. (A-C, left) Western blot analysis of whole cell lysates from 697 (A), UOCB1 (B), and Reh and RS4;11 (C). (A-C, right) Apoptotic cells measured by Annexin V and 7AAD staining followed by flow cytometry after 5 days of treatment. Graphs represent 3 experiments each performed with duplicates. Bars indicate mean+Standard Deviation.

cycle analysis, and two-way ANOVA for mutation rates with and without treatment as well as *in vivo* studies.

Results

Previously we noted relapse-specific heterozygous deletions in *MSH6* in 4 out of 76 patients that were near identical and deleted *MSH6* only for 3 patients while one harbored a larger deletion involving more genes within the region (Online Supplementary Figure S1). To begin to elucidate the impact of *MSH6* deletion on the development of relapsed disease, we knocked down expression of *MSH6* using shRNA in 697 cells, a B-ALL cell line that expresses all four MMR proteins (Figure 1A) and is MMR proficient,²⁵ and tested for changes in chemosensitivity. We observed approximately 80-90% (shRNA1) and 50-60% (shRNA2) knockdown of *MSH6* expression, as well as decreased expression of *MSH2*, compared to non-targeting (NT) control cells (Figure 1A), which is consistent with literature on the loss of protein stability of *MSH2* and *MSH6* when not dimerized.^{17,26} Knockdown of *MSH6* with both shRNA1 and shRNA2 leads to a significant decrease in apoptotic cells when treated with thiopurines for five days (Figure 1A). A 26-fold increase in IC₅₀ with 6-TG (NT: 0.027 vs. shRNA1: 0.716 $\mu\text{g}/\text{mL}$; $P=0.007$) and 8.5-fold increase for 6-MP (NT: 0.340 vs. shRNA1: 2.89 $\mu\text{g}/\text{mL}$; $P=0.006$) was observed for shRNA1 (Online Supplementary Figure S2). A 1.7-fold (NT: 0.015 vs. shRNA2: 0.025 $\mu\text{g}/\text{mL}$; $P=0.015$) and a 2.6-fold (NT: 0.143 vs. shRNA2: 0.373 $\mu\text{g}/\text{mL}$; $P=0.032$) increase in IC₅₀ for 6-TG and 6-MP, respectively, were observed for shRNA2 cells compared to NT cells (Online Supplementary Figure

S2). However, no significant differences were observed when cells were treated with prednisolone (Pred), doxorubicin (Doxo), cytarabine (Ara-c), or methotrexate (MTX) (Online Supplementary Figure S3A). Interestingly, we found that knockdown of *MSH6* also resulted in decreased sensitivity to temozolomide (TMZ), an alkylating agent used to treat glioblastomas, as reported previously (Online Supplementary Figure S3B).^{27,28}

To further support the role of *MSH6* in chemoresistance, we knocked down expression in UOCB1 cells, another B-ALL cell line that expresses all four MMR proteins (Figure 1B). Similar to the effect observed in 697 cells, depletion of *MSH6* with either shRNA significantly reduced the induction of apoptosis upon treatment with thiopurines (Figure 1B) [fold increase in IC₅₀ as compared to NT with 6TG: 4.8 for shRNA1 ($P=0.007$) and 3 for shRNA2 ($P<0.001$); 6MP: 8.3 for shRNA1 ($P<0.001$) and 9.2 shRNA2 ($P<0.001$)] (Online Supplementary Figure S4). Additionally, a similar impact on TMZ resistance was observed with UOCB1 *MSH6* shRNA1 expressing cells compared to NT control cells, although shRNA2 did not show the same effect, possibly due to less depletion by shRNA2 (Online Supplementary Figure S3B).

To determine the specificity of the phenotype observed for *MSH6* depletion versus defects in other MMR proteins, we assessed the effect of *MSH6* knockdown in MMR deficient B-ALL cell lines Reh and RS4;11.^{25,29} Both Reh and RS4;11 have minimal to no expression of *MLH1* and *PMS2* (Figure 1C). Knockdown of *MSH6* expression had no effect on the sensitivity of either Reh or RS4;11 to 6-TG or 6-MP (Figure 1C and Online Supplementary Figure S5).

To begin to elucidate the mechanism of resistance, we

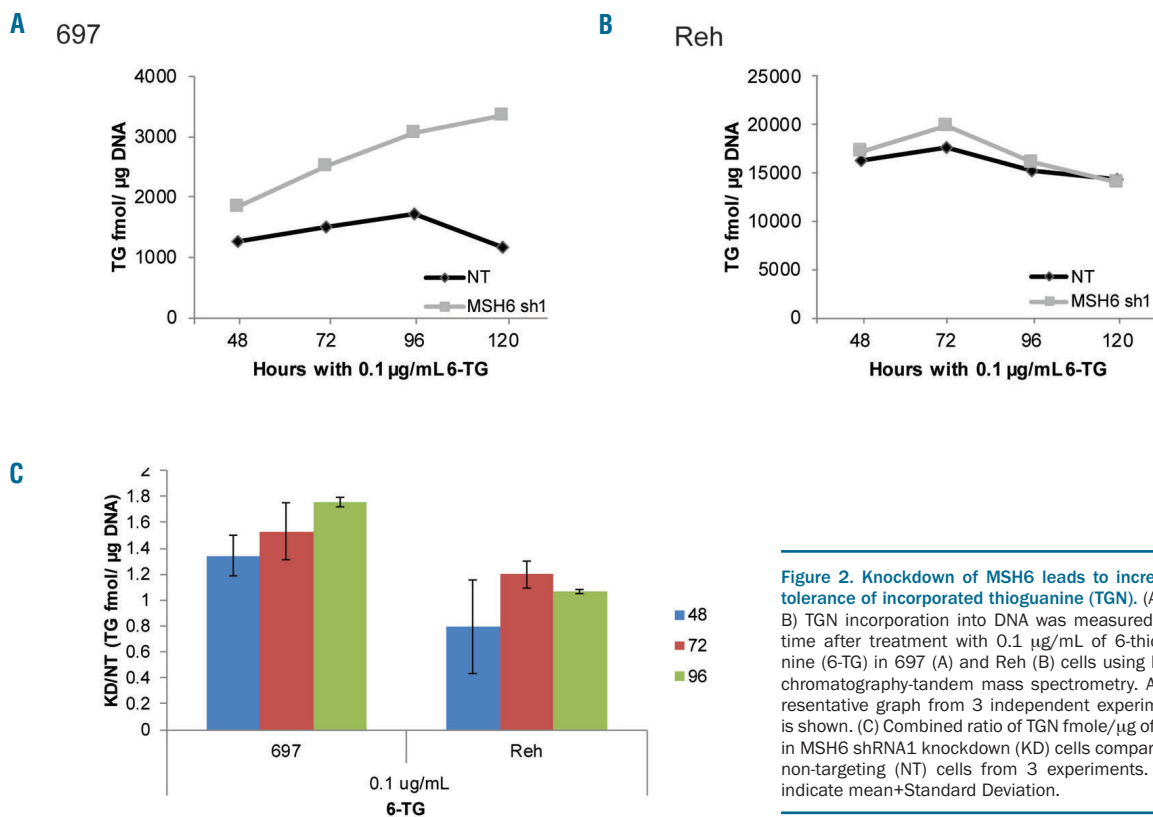


Figure 2. Knockdown of *MSH6* leads to increased tolerance of incorporated thioguanine (TGN). (A and B) TGN incorporation into DNA was measured over time after treatment with 0.1 $\mu\text{g}/\text{mL}$ of 6-thioguanine (6-TG) in 697 (A) and Reh (B) cells using liquid chromatography-tandem mass spectrometry. A representative graph from 3 independent experiments is shown. (C) Combined ratio of TGN fmole/ μg of DNA in *MSH6* shRNA1 knockdown (KD) cells compared to non-targeting (NT) cells from 3 experiments. Bars indicate mean+Standard Deviation.

measured the level of TGN incorporation into DNA upon treatment with 6-TG. 697 MSH6 shRNA1 cells accumulated more TGN/ μg DNA over time than NT cells (NT 1722 and KD 3070 fmol/ μg DNA) (Figure 2A and C). In contrast, no difference in TGN levels was observed in Reh cells (Figure 2B and C). Additionally, Reh cells had approximately 10-fold higher TGN levels compared to 697 cells (Figure 2A and B), highlighting the difference between MMR deficient and proficient cells in their ability to

respond to and survive thiopurine exposure. Thus, MMR proficient cells with high TGN succumb to the damage and therefore display less TGN/ μg DNA over time, meanwhile deficient cells tolerate higher levels of TGN.

We next tested whether or not a change occurs in cell cycle progression upon treatment. 697 NT cells slowed their growth and had a significantly higher proportion of cells in S phase and less cells in G1 beginning at 96 hours (h) (6-TG, $P=0.014$; 6-MP, $P=0.051$) and progressing

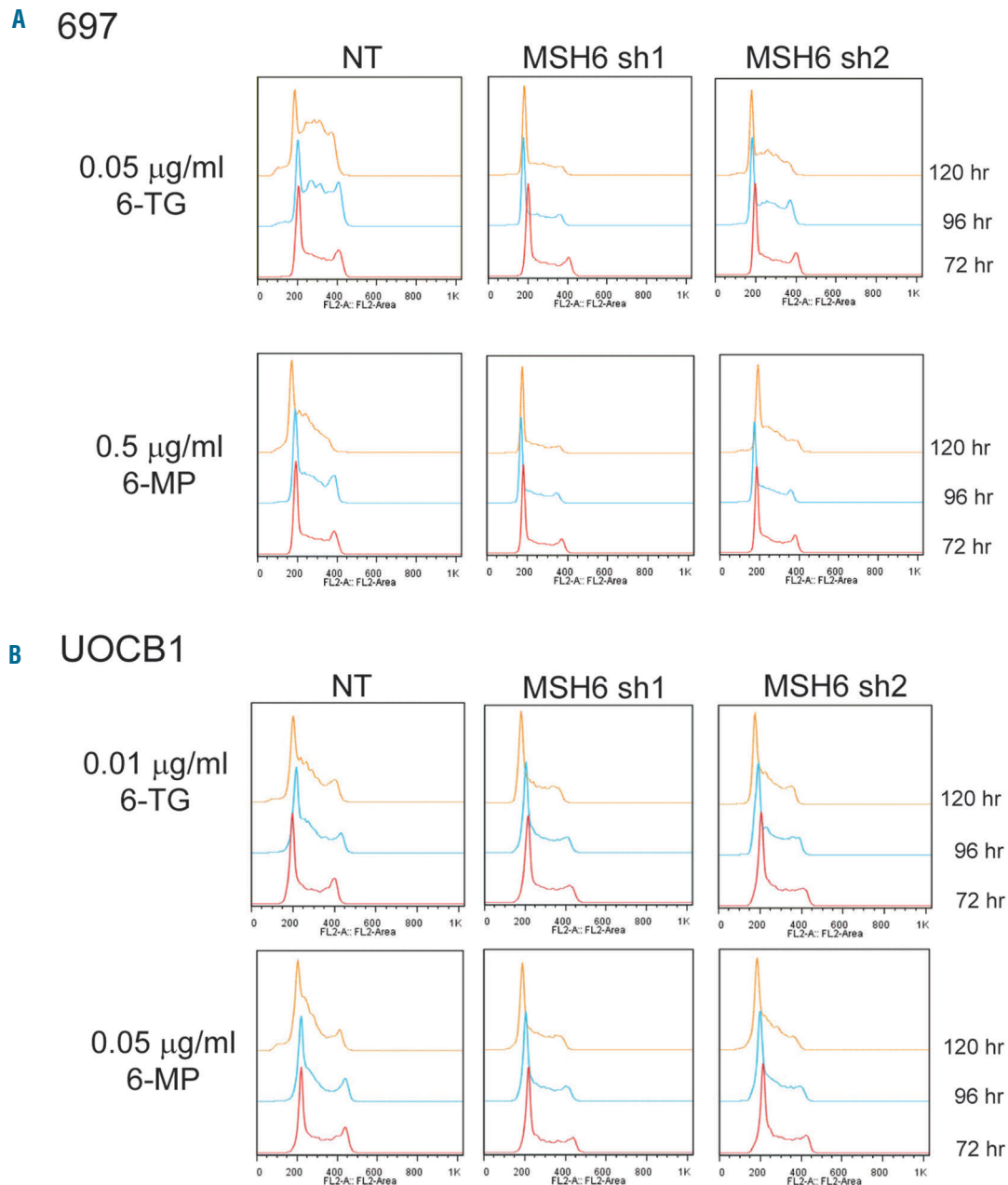


Figure 3. Thiopurine treatment resulted in an S phase arrest, which was abrogated upon knockdown of MSH6. 697 (A) and UOCB1 (B) NT and MSH6 shRNA1 and 2 expressing cells were treated with indicated drug for 5 days. Cells were fixed with 70% ethanol, treated with RNase, and then stained with propidium iodide. DNA content was analyzed by flow cytometry. Representative images from 3 individual experiments are shown. A one-way ANOVA was performed to determine statistical significance of the increase in % of cells in S phase at each time point.

through 120 h of thiopurine treatment (6-TG, $P=0.013$; 6-MP, $P=0.001$) compared to the MSH6 shRNA lines by one-way ANOVA (Figure 3A and *Online Supplementary Figure S6A*). MSH6 shRNA1 cells had only a modest decrease in growth with no clear S phase arrest (6-TG, $P=0.011$, and 6-MP, $P=0.001$, for percent of cells in S phase compared to NT at 120 h using Tukey's multiple comparison test), even at higher concentrations of 6-TG (Figure 3A and *Online Supplementary Figure S6A and B*). MSH6 shRNA2 cells had a more moderate accumulation of cells in S phase and drop of cells in G1 (Figure 3B and *Online Supplementary Figure S6A*), which is consistent with the modest levels of knockdown and apoptosis. Similar trends were observed with UOCB1 cells (6-TG, $P=0.31$; and 6-MP, $P=0.34$ at 120 h) (Figure 3B). This more moderate effect observed with the UOCB1 cells is consistent with the degree of impact MSH6 knockdown had on chemoresistance compared to the 697 cells. Neither NT nor MSH6 shRNA1 Reh cells showed alterations in cell cycle upon exposure (*Online Supplementary Figure S6B*).

To gain a more complete understanding of the mechanism leading to apoptosis following TGN incorporation, we analyzed downstream pathways in 697 NT and MSH6 shRNA1 cells after treatment with 6-TG. Based on the observed S phase arrest and previous research demonstrating activation of the ataxia telangiectasia and Rad3-related (ATR)-Chk1 pathway downstream of MMR,^{30,31} we first assessed the level of activation of Chk1 by probing for phosphorylation of serine 317 (pChk1). 697 NT cells had a low level of pChk1 at 48 h with a significant increase through 96 h of exposure. 697 MSH6 shRNA1 cells had minimal to low levels of pChk1 at 72 h with minimal increase over time (Figure 4A). The 697 cells expressing shRNA2 had a similar pattern but slightly lower levels of pChk1 compared to NT cells, which is consistent with the cell cycle data. We next assessed the level of phosphorylated H2AX (γ H2AX), a marker of DNA damage that is phosphorylated downstream of the ATR/ATM pathways following drug treatment,³² as well as levels of the apopto-

sis marker p53. There was a very modest level of γ H2AX starting at 72 h that increased to a higher level at 96 h after treatment in 697 NT cells compared to very modest levels in the MSH6 shRNA1-2 cells (Figure 4A), suggesting that the functional MMR system in the NT cells was attempting to repair the DNA, leading to nicks. Additionally, the levels of phosphorylated and total p53 were higher in NT cells at 72 and 96 h compared to MSH6 shRNA1-2 cells (Figure 4A) and the shRNA2 cells had higher levels than the shRNA1 cells. Similar results were found in UOCB1 cells (Figure 4B).

We next examined the impact of *MSH6* knockdown on mutation rate by performing two assays that measure genomic instability and mutation burden. Microsatellite instability (MSI) is a marker for genomic instability and has been observed in cases where expression of MLH1 or MSH2 is lost.^{25,33} We investigated MSI on 2 patient sample pairs that we previously found to have deletions of *MSH6* at relapse, as well as on 697 NT and MSH6 shRNA1 cells treated with 6-TG for 120 h. No MSI was observed in the patient samples comparing diagnosis to relapse or in the 697 cells comparing either untreated to 6-TG treated or NT to MSH6 shRNA1 cells (Figure 5A). These data are consistent with previous literature that found alterations in MSH6 expression alone do not lead to high MSI.³⁴ To investigate the effect of *MSH6* disruption on the rate of spontaneous mutations in *PIG-A*, which is required for expression of GPI, we used a flow cytometry-based assay that measures surface expression of several GPI-dependent markers (CD48, CD52, and CD59).^{22,35} Although there was a trend to suggest that 697 MSH6 shRNA1 cells had a slightly higher mutation rate, statistical significance was not achieved (Figure 5B). Furthermore, treatment of the clones from each cell line with 6-TG did not lead to an increased mutation rate (Figure 5B).

To investigate the clinical relevance of reduced MSH6 expression and drug resistance, we utilized an *in vivo* mouse model. We injected mice with either UOCB1 NT or UOCB1 MSH6 shRNA1 cell lines (knockdown con-

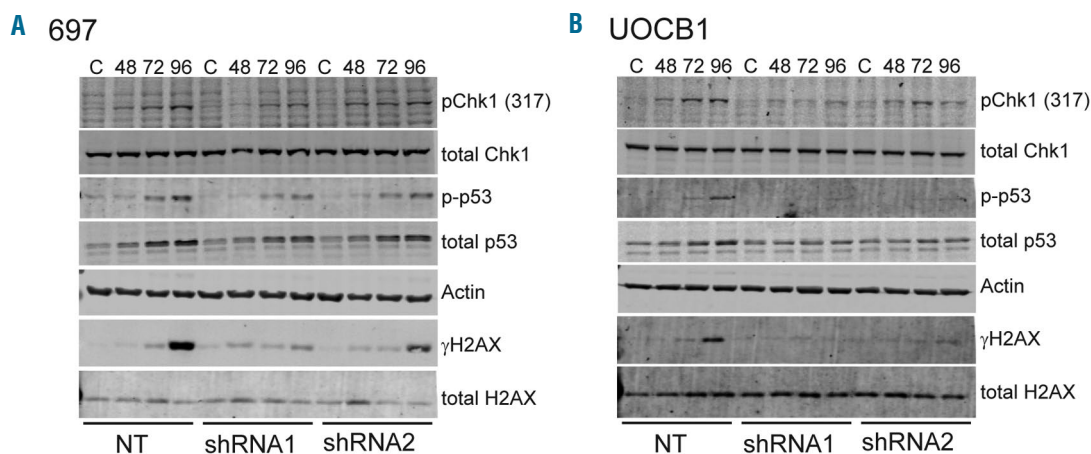


Figure 4. Thiopurine treatment leads to activation of cell cycle regulator Chk1 and DNA repair that ultimately resulted in DNA damage and cell death. Western blot analysis of whole cell lysates from 697 (A) and UOCB1 (B) non-targeting (NT), MSH6 shRNA1, and shRNA2 cells after treatment with 6-thioguanine (0.1 μ g/mL, and 0.025 μ g/mL, respectively). (C) Untreated cells; numbers are hours after treatment. Blots were probed for Chk1 activation, γ H2AX for DNA damage, and apoptosis marker p53. Total Chk1, actin, and total H2AX were used as loading controls. Images are representative of 3 individual experiments.

firmed day of injection; Figure 6C) and, following confirmation of leukemic burden on day 6, the mice were treated with PBS (control) or purixan (an oral suspension form of 6-MP). Following the 10-day course of purixan treatment, the leukemic burden was significantly diminished in the mice harboring NT cells compared to that observed in the NT PBS treated mice ($P=0.0001$), suggesting that these cells were unable to survive and expand under the selective pressure of the purixan (Figure 6A and B). In contrast, the MSH6 shRNA1 mice treated with purixan were not significantly different from the NT PBS group ($P=0.828$). Although purixan also had a statistically significant impact on MSH6 shRNA1 cells compared to MSH6 shRNA1 PBS control ($P=0.0005$), these cells were able to continue proliferating under the selective pressure, unlike the NT cells (Figure 6A and B). Finally, a comparison between PBS MSH6 shRNA1 and PBS control NT cells at day 17 showed that MSH6 depleted cells also had a growth advantage *in vivo* ($P<0.0001$).

Discussion

In recent years, there has been an abundance of evidence demonstrating the outgrowth of clones at relapse in ALL that are associated with unique or enriched relapse

specific mutations that confer drug resistance. Some of the most common relapse specific mutations found thus far occur in *NT5C2* and *PRPS1* and lead to the outgrowth of thiopurine resistant clones.²⁴ Our data presented here demonstrate that reduction of MSH6 in ALL also leads to decreased sensitivity to purine analogs due to a failure to initiate the apoptotic cascade directly in response to nucleotide mismatches. Even with only 50-60% reduced expression, which potentially mimics levels in patients with heterozygous loss, we demonstrate a significant decrease in sensitivity to thiopurines. Our data are consistent with the recent work of Diouf *et al.* who showed that lower levels of MSH2 in cell lines were associated with resistance to 6-TG and 6-MP. They found 11% of ALL samples showed decreased protein levels of MSH2 through copy number loss of genes controlling MSH2 degradation.¹⁷ Thus defects in MMR, including heterozygous deletion of *MSH6*, can be added to the list of genetic alterations that result in the development of resistance to purine analogs, the foundation of maintenance therapy. The variety of mutations that lead to selective outgrowth of such clones in a substantial number of patients underscores the selective pressure of thiopurines on tumor cells.

The outgrowth of *MSH6* deleted/mutated clones not found at diagnosis has been observed at relapse in malignant gliomas following treatment with temozolo-

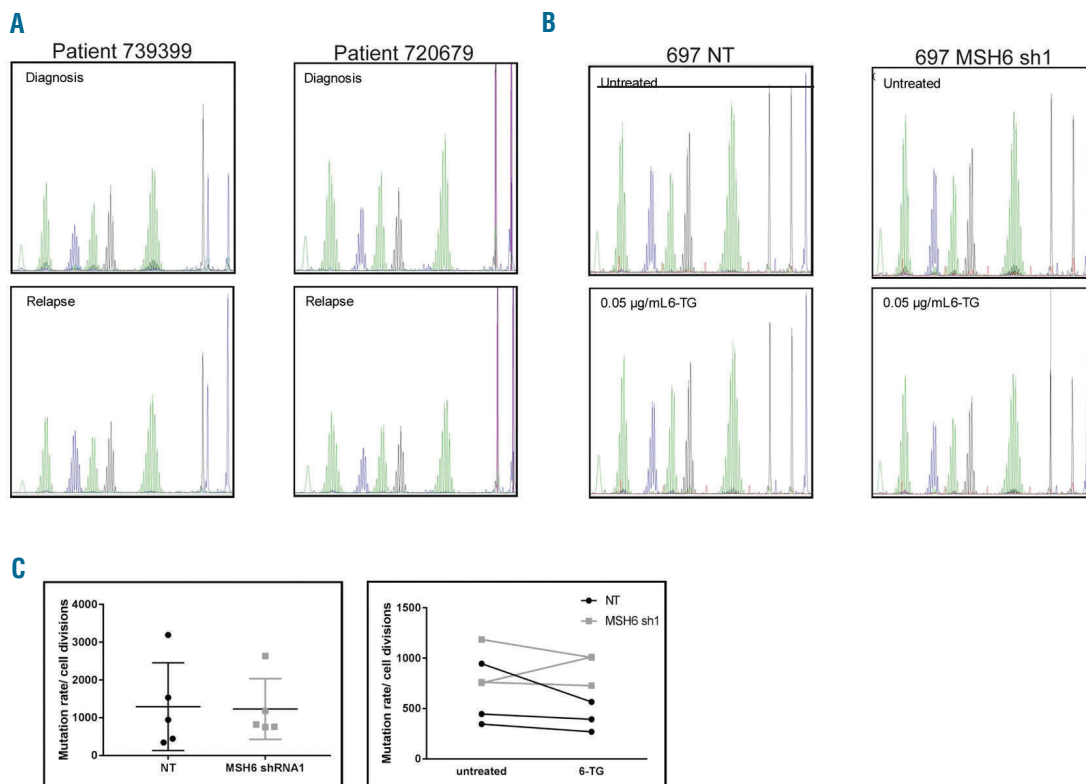


Figure 5. Knockdown of MSH6 did not lead to a mutator phenotype or increased mutation rate. (A) Microsatellite instability (MSI) was measured in diagnosis/relapse pairs that had relapse specific, heterozygous *MSH6* deletions and in 697 non-targeting (NT) and MSH6 shRNA1 cells left untreated or treated with 0.05 µg/mL of 6-thioguanine (6-TG) for 5 days. (B) Mutation rate in the *PIC-A* gene was measured in 697 NT and MSH6 shRNA1 clones that were expanded for 2-3 weeks with or without 6-TG. The cells were analyzed for loss of GPI-dependent cell surface markers, including FLAER, CD48, CD52, and CD59 using flow cytometry. (Left) Individual mutation rates/cell divisions for each clone; the line represents mean+Standard Deviation. (Right) Mutation rates/cell divisions for three clones with and without 6-TG treatment.

mide,^{28,36,37} which produces DNA O6-methylguanine, a lesion structurally similar to 6-TG.³⁸ Our data demonstrating decreased sensitivity of *MSH6* knockdown cells to temozolomide support the hypothesis that MMR deficient clones gain an advantage under this selective pressure leading to resistant recurrences.^{27,39} The difference in TMZ sensitivity between the two UOCB1 shRNA knockdown cell lines could be due to the interplay between *MSH6* and *SETD2* protein levels since UOCB1 cells have a copy number loss of *SETD2* (NA Evensen *et al.*, 2018, unpublished data) and there is a greater reduction of *MSH6* with shRNA1 compared to shRNA2. *SETD2*, the gene that codes for the methyltransferase responsible for the trimethylation of H3K36 that serves as the docking site for *MSH6*,⁴⁰ is among the epigenetic regulators commonly found mutated in relapse patients.⁴¹ Ongoing studies in our lab are focused on identifying the relationship of epigenetic readers, writers, and erasers, such as *SETD2*, *MSH6*, and *WHSC1* in chemoresistance.

Mechanistically, our *in vitro* and *in vivo* data support the hypothesis that the delayed cytotoxic response to thiopurines is due to the MMR system recognizing a mismatch and initiating futile, damaging DNA repair that ultimately

leads to apoptosis.^{20,42,43} This pathway is not fully activated in cells with reduced *MSH6* because the mismatch goes undetected, allowing these cells to tolerate excess TGN mismatches and, ultimately, to continue to survive and proliferate while under treatment. Our data provide evidence that, upon recognition of mismatches, NT cells slow their progression through S phase by activating Chk1 as they begin to repair their DNA. Due to the mismatch being on the daughter strand, the excision/repair process is unsuccessful, and over time nicks build up in the DNA, demonstrated by increased levels of γ H2AX. Eventually, the damage becomes overwhelming and cells initiate apoptosis, as shown by increased p53. *MSH6* shRNA1 cells exhibited minimal to no change in cell cycle, activation of Chk1, or increased γ H2AX and p53. The moderate changes observed with the *MSH6* shRNA2 cells highlight the idea that even a more modest reduction in *MSH6* expression could lead to subtle changes that have a significant impact on chemoresistance. The MMR deficient Reh cells also had no alteration in their cell cycle, suggesting that recognition of mismatches by MutS α is not sufficient for full activation of this cascade, but rather damage induced by the repair, which is orchestrated by

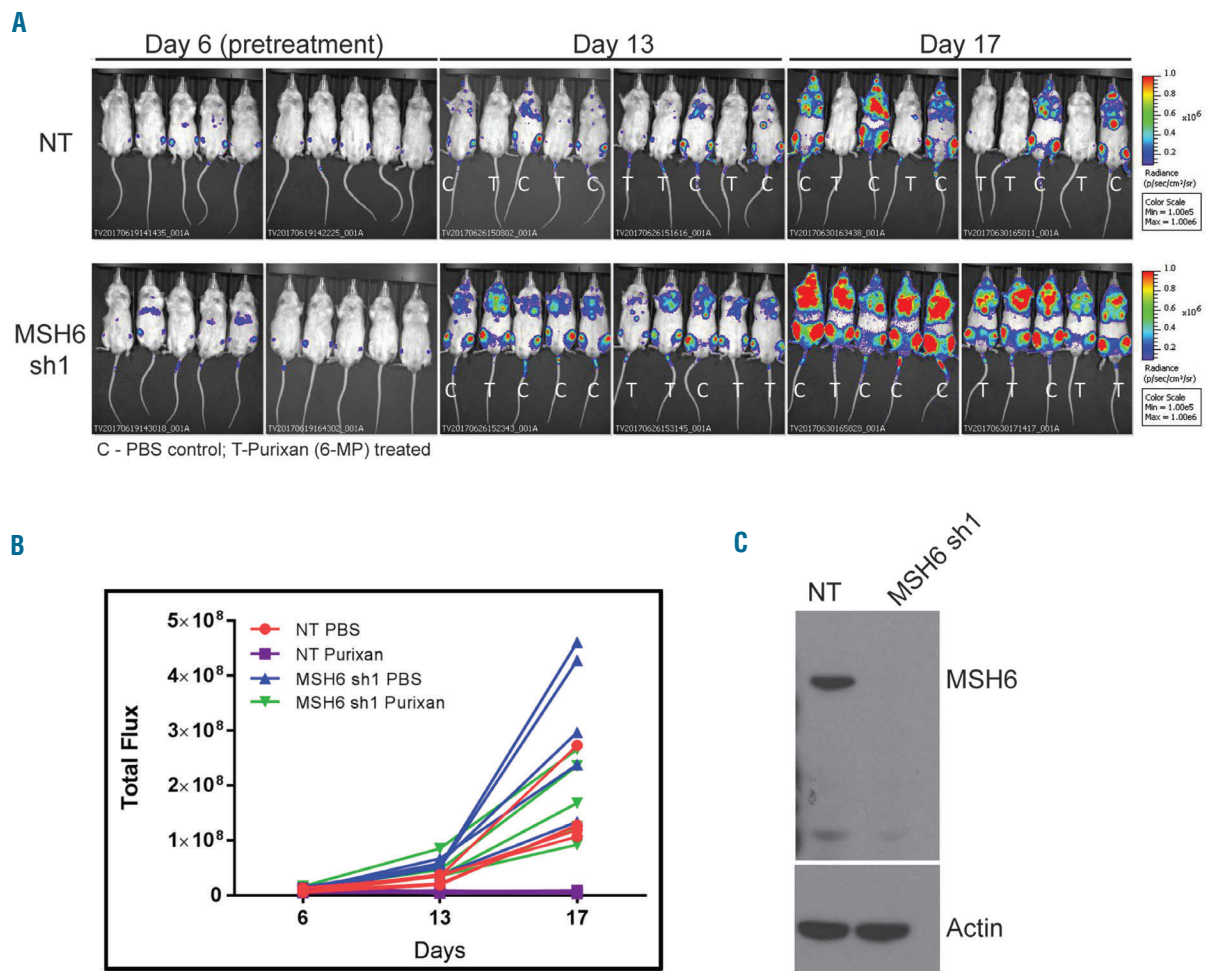


Figure 6. Knockdown of *MSH6* leads to decreased sensitivity to purixan *in vivo*. (A) Bioluminescence imaging (BLI) of mice injected with UOCB1 non-targeting (NT) or *MSH6* shRNA1 cells. Six days after injection mice were imaged and then randomized to treatment. Treatment was started on day 7 and images were taken again on days 13 and 17. C: PBS control treatment; T: purixan treatment. (B) Quantification of total flux was determined by analyzing the BLI images using Living Image software. (C) Western blot to confirm knockdown of *MSH6* in cells used to inject mice. Actin was used as loading control.

MutL α , is what initiates apoptosis. Our data demonstrating the involvement of the ATR-Chk1-H2AX signaling cascade is supported by the work of Eich *et al.* which demonstrated activation of this pathway upon treatment with temozolomide.³² Interestingly, understanding how MMR deficient cells respond to thiopurines in terms of TGN incorporation could prove essential given the emerging idea of measuring these parameters in patients on maintenance therapy.⁴⁴

The data presented here do not support the hypothesis of increased mutation burden, genomic instability, or MSI when MSH6 is reduced. Our inability to demonstrate MSI-high, which is considered a standard method for clinical testing of MMR deficiencies in tumors,⁴⁵ in MSH6 depleted cell lines and clinical samples is consistent with the lack of MSI in glioma samples with MSH6 deletions or mutations.^{46,47} Haploinsufficiency of MSH6 or compensation by MSH2/MSH3 may account for this observation.^{25,48} In addition, ALL clonal evolution from diagnosis to relapse is not associated with increased mutation burden supporting our mutation rate analysis, although Ma *et al.* reported a subset of hypermutated relapse cases.⁴⁹ Of these, one had a biallelic mutation of PMS2, another had multiple damaging MSH6 mutations as well as an MLH1 splice site mutation, while the others harbored no MMR mutations.⁴⁹ Furthermore, one case demonstrated that a heterozygous deletion of MSH6 at diagnosis was not sufficient to cause a hypermutator phenotype, but the acquisition of a second hit in the WT allele at relapse was.⁴⁹ Likewise, the majority of hypermutated gliomas at relapse show defects in multiple MMR genes or loss of heterozy-

gosity.⁵⁰ Thus, our work supports a model whereby haploinsufficiency of MSH6 results in TGN tolerance and resistance directly rather than by generation of secondary mutations. However, it does not rule out the possibility that haploinsufficiency, along with other defects in the MMR pathway, may result in a mutator phenotype.

Overall, it has become increasingly evident that the genetic and epigenetic landscape of cancer cells is vital to the overall effectiveness of treatment. These studies illustrate yet another example of a mutation/deletion found at relapse that directly influences the response to a therapeutic agent that is currently heavily relied on. Continuous efforts to elucidate the potential functions and mechanisms of genes found mutated at relapse will help lead us to novel treatment strategies.

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