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Msh2 Deficiency Attenuates But Does Not Abolish Thiopurine Hematopoietic Toxicity in *Msh2*$^{-/-}$ Mice

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ABSTRACT

The amount of MSH2 protein, a major component of the mismatch repair system, was found to differ >10-fold in leukemia cells from children with newly diagnosed acute lymphoblastic leukemia, with a subgroup of patients (17%) having undetectable MSH2 protein. We therefore used a murine *Msh2* knockout model to elucidate the in vivo importance of MSH2 protein expression in determining thiopurine hematopoietic cytotoxicity. After mercaptopurine (MP) treatment (30 mg/kg/day for 14 days), there was a significantly greater decrease in circulating leukocytes in *Msh2*$^{+/+}$ and *Msh2*$^{+/-}$ mice when compared with *Msh2*$^{-/-}$ mice ($p < 0.002$). Likewise, the decrease in erythrocyte counts was more prominent in mice with at least one functional *Msh2* allele. MP doses of more than 50 mg/kg/day for 14 days resulted in treatment-related deaths, but *Msh2*$^{-/-}$ mice had a significant survival advantage ($p = 0.02$). Murine embryonic fibroblasts (MEFs) from *Msh2*$^{+/+}$ mice also exhibited increased sensitivity to MP when compared with MEFs from *Msh2*$^{-/-}$ mice (IC_{50}, 3.8 ± 0.1 μM versus 11.9 ± 1.3 μM, $p < 0.001$). After MP treatment, deoxythioguanosine incorporation into DNA was similar in mice and MEFs with each of the *Msh2* genotypes. Electromobility shift assay experiments identified an Msh2-containing GT- or G^{S}T-DNA-nuclear protein complex in *Msh2*$^{+/+}$ but not *Msh2*$^{-/-}$ MEFs. Together, these findings establish that hematopoietic toxicity in vivo after treatment with mercaptopurine is attenuated but not abolished by MSH2 deficiency.

The therapeutic outcome for children with acute lymphoblastic leukemia (ALL) has improved dramatically over the past two decades (Pui and Evans, 1998). However, the emergence of drug-resistant leukemia cells contributes to treatment failure in approximately 20 to 25% of patients with ALL (Chessells, 1998; Pui and Evans, 1998). Drug resistance can emerge from altered metabolism or cellular accumulation of antileukemic agents, from altered drug targets, or from changes in cellular responses downstream of drug-target interactions (Johnstone et al., 2002). For example, abnormalities of DNA repair proteins have been linked to both the pathogenesis of several human malignancies and the therapeutic effects of medications (Das-Gupta et al., 2000; Flores-Rozas and Kolodner, 2000; Olipitz et al., 2002). The postreplicative mismatch repair (MMR) system has been shown to modulate in vitro cytotoxicity of several anticancer chemotherapeutic agents, including busulfan, cisplatin, temozolomide, doxorubicin, etoposide, thiopurines, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Fink et al., 1998), each targeting DNA. These findings suggest that the MMR system plays an important role in recognizing DNA damage and triggering cell death under genotoxic stress. Characterized components of MMR include hMSH2 and hMSH6, which are associated with a protein complex interacting with mismatched DNA base pairs (Wang et al., 2000), and inactivation of MSH2 attenuates mismatch repair activity (Dewind et al., 1995).

Thiopurines (e.g., 6-mercaptopurine, MP, and 6-thioguanine, TG) are widely used medications for the treatment of pediatric ALL, representing an important component of essentially all modern treatment protocols (Elion, 1989; Pui and Evans, 1998). The incorporation of 6-thiodeoxyguanosine (dG^S) into DNA after MP or TG treatment results in DNA damage and is considered the major mechanism of thiopurine cytotoxicity (LePage, 1963; Maybaum and Mandel, 1983; Krynetskaia et al., 1999). It has been shown in vitro that G^S-inserts in DNA change DNA-protein interactions with restriction endonucleases, RNaseH and topoisomerase II (Iwaniec et al., 1991; Krynetskaia et al., 1999; Krynetskaia et al., 2000). The mechanisms of cellular response to DNA damaged by thiopurine incorporation are not well defined but

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ABBREVIATIONS: ALL, acute lymphoblastic leukemia; MMR, mismatch repair; MSH2, MutS homolog 2; MP, mercaptopurine; TG, 6-thioguanine; dG^S, deoxythioguanosine; MEF, murine embryonic fibroblast; HPRT, hypoxanthine guanine phosphoribosyl transferase; TPMT, thiopurine methyltransferase; WBC, white blood cell; RBC, red blood cell; EMSA, electromobility shift assay; RU, relative unit(s).

presumably involve cellular systems such as DNA repair, transcription control, cell cycle arrest, and/or apoptosis (Karran and Bignami, 1996; Das-Gupta et al., 2000). The putative mechanism by which the MMR system promotes thiopurine cytotoxicity involves the initiation of apoptosis after futile efforts to repair DNA containing thioguanine mismatch pairs (Karran and Bignami, 1996; Durant et al., 1999). In vitro experiments have demonstrated that the human MMR complex interacts with S^6-thioG · T mismatches (but not with S^6-thioG · C) in DNA (Branch et al., 1993; Krynetski et al., 2001) and with S^6-methylthioG · T mismatch pairs formed after nonenzymatic methylation of thioguanine in DNA (Swann et al., 1996).

The current studies were undertaken to evaluate the extent of heterogeneity in MSH2 protein expression in pediatric ALL cells and to assess the influence of MSH2 deficiency on thiopurine hematopoietic toxicity in an in vivo mouse model.

Materials and Methods

Patient Samples

Bone marrow samples were obtained from 63 patients with newly diagnosed ALL who were enrolled, after informed consent, on an Institutional Review Board-approved protocol at St. Jude Children's Research Hospital. Lymphoblasts from bone marrow aspirates were isolated using a Ficoll-Hypaque density gradient, and the final cell yield was determined by hemocytometer. The MSH2 protein level was estimated by Western blot analysis of total cellular lysates, as described below. MSH2 cDNA was prepared from ALL cells, cloned and sequenced as previously described (Krynetski et al., 1995).

In Vivo Model. Mice in which the *Msh2* gene had been disrupted by homologous recombination were generously provided by Dr. Tak Mak (Amgen Institute, Toronto, ON, Canada) (Reitmair et al., 1995). Heterozygous ($Msh2^{+/-}$) mice with a mixed C57BL/6J and 129/Ola genetic background were bred, resulting in a Mendelian ratio of viable wild-type ($Msh2^{+/+}$), heterozygous ($Msh2^{+/-}$), and knockout ($Msh2^{-/-}$) mice. These mice were genotyped by a previously described polymerase chain reaction technique (Reitmair et al., 1996), and the level of Msh2 protein expression was determined by Western blot analysis of bone marrow, liver, kidney, and spleen, using an MSH2 antibody (Ab-2) (Oncogene Research Products, San Diego, CA). Mice aged 6 to 14 weeks were used in all studies.

In Vitro Model. Primary cultures of $Msh2^{+/+}$ and $Msh2^{-/-}$ murine embryonic fibroblasts (MEFs) were produced from embryos of wild-type ($Msh2^{+/+}$) and knockout ($Msh2^{-/-}$) mice, collected between 12 and 14 days of gestation (Charles River Laboratories, Wilmington, MA). The washed cells were resuspended in growth media containing 1× Dulbecco's modified Eagle's medium (Cambrex Bio Science Walkersville, Inc., Walkersville, MD), 10 to 20% fetal bovine serum (Hyclone Laboratories, Logan, UT), 4 mM L-glutamate (Cambrex Bio Science Walkersville), and 1× nonessential amino acids (Irvine Scientific, Santa Ana, CA) and incubated at 37°C in 5% CO_2 in humidified air.

Western Blot Analysis of MSH2 Protein

Patient Samples. Human bone marrow cells (1×10^6) were lysed in 250 μl of triple-detergent lysis buffer (Sambrook et al., 1989), incubated on ice for 10 min, and then sheared by aspirating through a syringe with a 25-gauge needle. The lysate was centrifuged at +4°C for 10 min, and then concentrated in an Ultrafree concentration cartridge 10K (Millipore Corporation, Bedford, MA). All lysates were analyzed by 12% SDS-polyacrylamide gel electrophoresis with Laemmli buffer system (Bio-Rad, Hercules, CA). Separated proteins were electroblotted onto Hybond-P membranes in a Mini Trans-Blot electrotransfer cell (Bio-Rad). The membrane was then incubated with a monoclonal anti-hMSH2 antibody (Ab-2; Oncogene Science, Cambridge, MA) or with anti-GAPDH monoclonal antibody (Chemicon International, Temecula, CA), both at 1:500 dilution for 1 h at room temperature, and developed using secondary goat anti-mouse horseradish peroxidase-conjugated antibody at 1:5000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the ECL Plus protein detection system with a detection limit of about 20 ng of protein/band (Amersham Biosciences Inc., Piscataway, NJ). Lysates from 697 human ALL cells [German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany] were used for quality control in each membrane. Western blot of MSH2 protein in 697 human ALL cells demonstrated a linear increase of the signal corresponding to the amount of MSH2 (data not shown). Intensity of the band corresponding to MSH2 protein was normalized versus the GAPDH signal. Bands were visualized and quantified by PhosphorImager with the ImageQuaNT Software system (Amersham Biosciences Inc.), using blue fluorescence/chemifluorescence at 488 nm excitation.

TABLE 1
Demographic data for ALL patients at the time of initial diagnosis with different MSH2 status

	MSH2-Negative (n = 11)	MSH2-Positive (n = 52)	p Value[a]	
Age				
Median	6.7	4.9	0.35	
Range	3.7–10.4	0.3–15.2		
Sex				
Male	8	37	1.0	
Female	3	15		
Race				
W (NOS)	6	37	0.44	
W (Hispanic/Latino)	0	3		
B (NOS)	4	8		
A	0	1		
NOS	1	3		
ALL Subtype				
T-lineage	2 (18%)	11 (21%)	1.0	0.7
B-lineage	9 (82%)	41 (79%)		
Hyperdiploid	4 (36%)	11 (21%)	0.42	
Non-Hyperdiploid[b]	5 (54%)	30 (79%)		
TEL/AML	1 (9%)	13 (25%)	0.66	
BCR/ABL	0	1 (2%)		
E2A/PBX	0	2 (4%)		
MLLr	0	1 (2%)		
Other	4 (36%)	13 (25%)		

W, white; B, black or African American; A, Asian; NOS, not otherwise specified.
[a] $Msh2^{+/+}$ versus$Msh2^{-/-}$: Mann-Whitney U Test for age; otherwise Fisher's exact test.
[b] Genetic abnormalities as defined in Pui and Evans (1998).

Murine Tissues. Mouse tissues (~200 mg of liver, spleen, or kidney) were homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at 4°C for 0.5 min at 5000 rpm in 5 volumes of ice-cold buffer, containing 50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol, 7 mM glutathione, 10% (v/v) glycerol. Complete protease inhibitor (1 tablet per 50 ml; Roche Diagnostics, Mannheim, Germany) and 0.2 mM phenylmethylsulfonyl fluoride were added. The lysate was centrifuged for 20 min at 10,000*g* (4°C); supernatant was transferred to a fresh tube and further centrifuged for 1 h at 100,000*g* at 4°C. Then, 20 μg of total protein was loaded onto the gel and analyzed by Western blot analysis using polyclonal anti-hMSH2 antibody (Santa Cruz Biotechnology, Inc.).

Cytotoxicity Studies

In Vivo Murine Model. *Msh2*$^{+/+}$, *Msh2*$^{+/-}$, and *Msh2*$^{-/-}$ mice were stratified according to age and gender, and randomized to receive i.p. injections of MP, 2.5 to 150 mg/kg/day (Sigma-Aldrich, St. Louis, MO) or 0.9% NaCl (American Pharmaceutical Partners Inc., Los Angeles, CA) up to 21 days. The solution of MP (2.64 mg/ml, pH 8.0) was prepared by dissolving MP in 1 N NaOH and then adjusting with 2 M Na_2HPO_4 to pH 7.8 to 8.0. This method of preparation was utilized for all subsequent experiments including the preparation of MP-free 0.9% NaCl. The i.p. route of administration was selected to minimize variability in MP systemic exposure. Complete blood count was obtained before and after MP treatment. Complete blood count was performed with the Hemavet 3700 (CDC Technologies Inc., Oxford, CT) using 100 μl of blood in EDTA obtained by orbital bleed. Mice were euthanized on day 15, approximately 12 to 16 h post-MP, according to a protocol approved by the Institutional Animal Care and Use Committee.

In Vitro Model. Cytotoxic effects of MP were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, after incubation of *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ primary MEFs with MP (0.001–100 μM) for 3 to 6 days (Pieters et al., 1990). The 96-well plates were read by a microplate spectrometer (Bio-Rad). The IC_{50} values were obtained by fitting a sigmoid E_{max} model to the cell viability (percentage) versus drug concentration (micromolar) data, determined in triplicate.

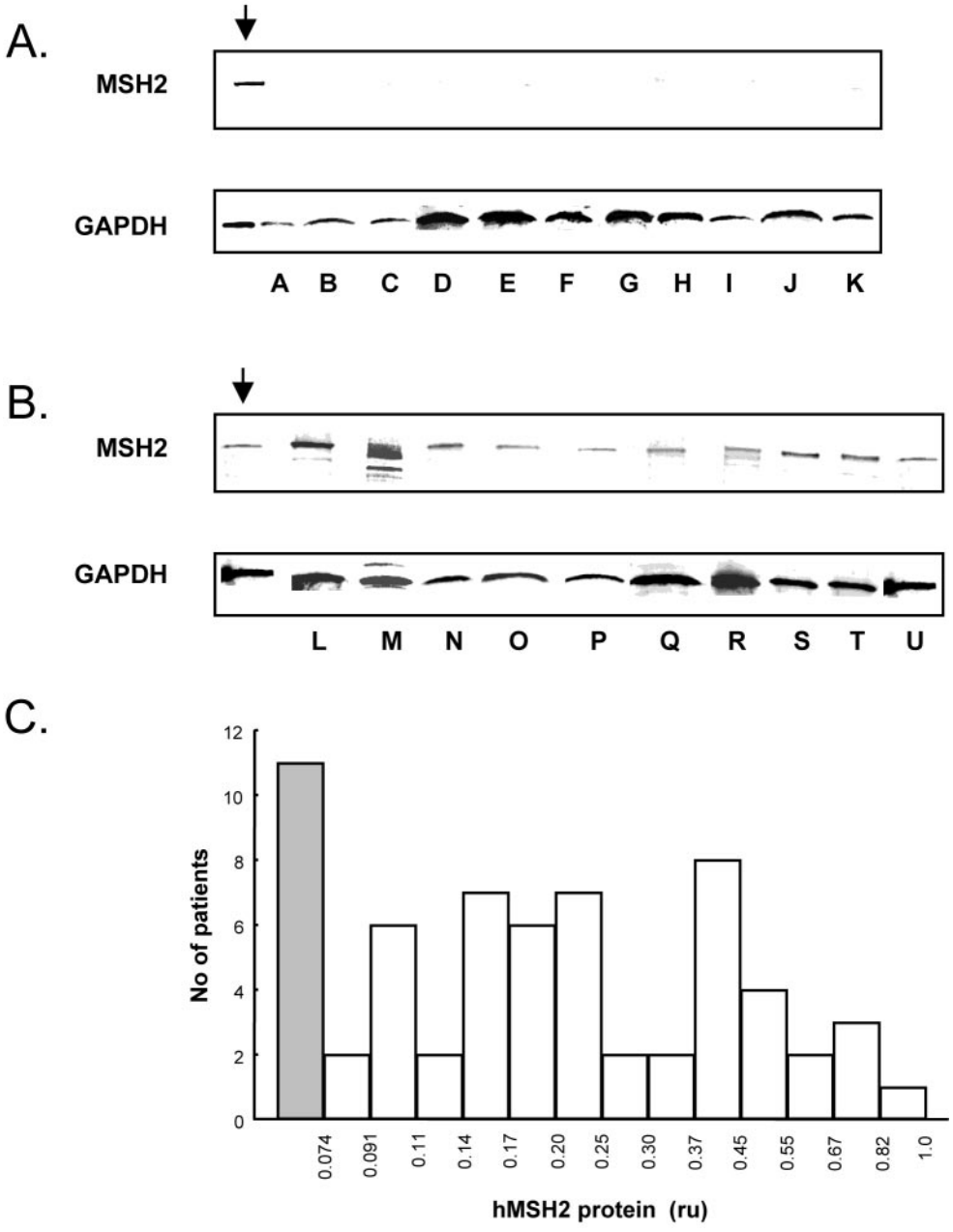

Fig. 1. Western blot analysis of MSH2 protein in human acute lymphoblastic leukemia cells. In each experiment, total protein extracted from 1×10^6 cells was loaded per lane and developed with anti-MSH2 antibody or anti-GAPDH antibody. A, no MSH2 protein was found in 11 ALL patient samples (A–K). B, representative view of 10 from 52 samples with positive MSH2 expression in ALL patient samples (L–U). Arrows indicate MSH2 and GAPDH proteins in human 697 ALL cell line (positive quality control). C, histogram of relative amount of MSH2 protein normalized versus GAPDH for 63 ALL patient samples. The gray bar represents the number of patients with an undetectable level of MSH2. Expression of GAPDH was shown for all 63 patients.

Hypoxanthine Guanine Phosphoribosyl Transferase (HPRT) and Thiopurine Methyltransferase (TPMT) Activity

HPRT activity in WBC lysates was determined by formation of [^{14}C]inosine monophosphate from [^{14}C]hypoxanthine, as previously reported (Krynetskaia et al., 1999). TPMT activities in WBC and RBC lysates were determined by the nonchelated radiochemical assay (Weinshilboum et al., 1978).

DNA Modification

Mice. *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ mice were injected i.p. with a single dose of [^{14}C]MP solution (11.4 mg/kg, 80 μCi; Moravek Biochemicals, Brea, CA) that was prepared as described above. Whole blood was collected and bone marrow was harvested from the femurs and sternum after 24, 72, or 168 h. DNA was extracted with the QIAGEN Blood and Cell Culture DNA Midi Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. DNA (10 μg) was used for analysis. ^{14}C incorporation into DNA was determined in duplicate using a Beckman LS 6500 Scintillation Counter (Beckman Coulter Inc., Fullerton, CA).

MEFs. Genomic DNA was isolated from 2 $\times$ 10^7 primary MEFs (*Msh2*$^{+/+}$ and *Msh2*$^{-/-}$) after 24- to 120-h incubation of cells with 10 μM MP, using the QIAGEN Blood and Cell Culture DNA Midi Kit, per the manufacturer's instructions. DNA (10 μg) was used for analysis. The level of 2′-deoxy-6-thioguanosine (dG^S) incorporation into DNA after MP treatment was determined in triplicate by high-performance liquid chromatography analysis, as previously described in detail (Krynetskaia et al., 1999).

DNA-Protein Interaction by Electromobility Shift Assay (EMSA)

Nuclear extracts were prepared from the primary MEF cells (2.5 $\times$ 10^8) as previously described (Dignam et al., 1983). Protein concentrations of nuclear extracts were determined by the Bradford dye-binding procedure, using the Bio-Rad Protein assay (Bio-Rad). Aliquots (25 μl) of nuclear protein extracts were stored at −70°C. Nuclear extracts from HeLa cells were used as positive controls (Promega, Madison, WI). The oligodeoxyribonucleotides d(ACTCTTGCCTTTAAG**G**AAAGTATCTAAATGCTTC), the complementary strands d(GAAGCATTTAGATACTTT**C**CTTAAAGGCAAGAGT) to form GC-duplex, and d(GAAGCATTTAGATACTTT**T**CTTAAAGGCAAGAGT) to form GT-duplex were synthesized using standard protocols with an automatic synthesizer (380B, Applied Biosystems, Foster City, CA) in the Hartwell Center of St. Jude Children's Research Hospital. Modified strand d(ACTCTTGCCTTTAAG**G^S**AAAGTATCTAAATGCTTC) to form G^{S}T-duplex, containing one thioguanosine insert (dG^S), was synthesized by standard phosphoramidite chemical methods with S6-DNP-dG-CE phosphoramidite (Krynetskaia et al., 1999). The 5′-ends of the single-stranded oligodeoxyribonucleotides were labeled using [^{32}P]ATP and the RTS T4 Kinase Labeling System (Invitrogen, Carlsbad, CA). Oligodeoxyribonucleotide duplexes containing GC-, GT- and G^{S}T-pairs were prepared by annealing complementary single strands and template strand. DNA duplexes were then purified by nondenaturing gel electrophoresis on a 12% polyacrylamide gel at 4°C, as previously described (Krynetski et al., 2001) or by fast protein liquid chromatography using a Superdex 75 column (Amersham Biosciences Inc.). DNA-protein binding assays (EMSA) were performed as described

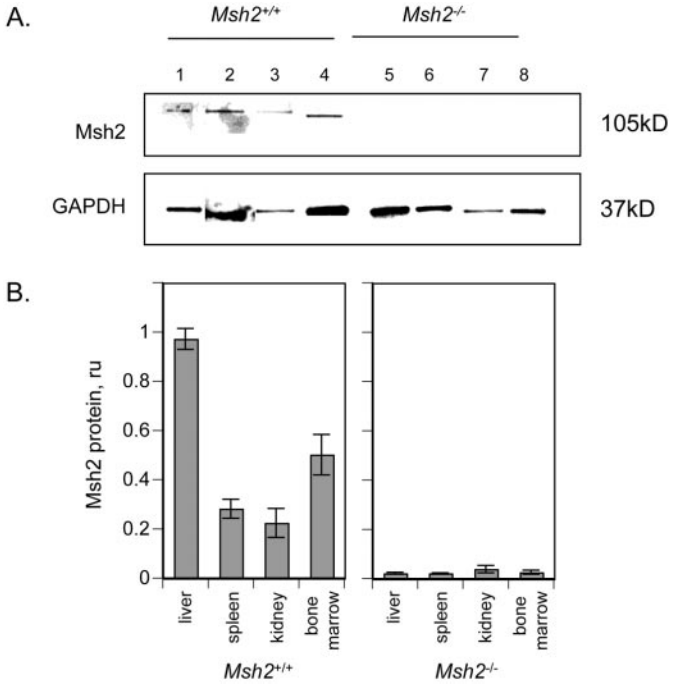

Fig. 2. Msh2 expression in mice. A, Western blot analysis of Msh2 protein in murine tissues obtained from *Msh2*$^{+/+}$ (lanes 1–4) and *Msh2*$^{-/-}$ mice (lanes 5–8). Lanes 1 and 5, liver; lanes 2 and 6, spleen; lanes 3 and 7, kidney; lanes 4 and 8, bone marrow. Total protein (20 μg) was loaded per lane and the membranes were developed with anti-MSH2 antibody or anti-GAPDH antibody (control). The expression of GAPDH was shown in all samples including samples from *Msh2*$^{-/-}$ mice. Histogram of the relative amount of Msh2 protein in mouse tissues, normalized versus GAPDH.

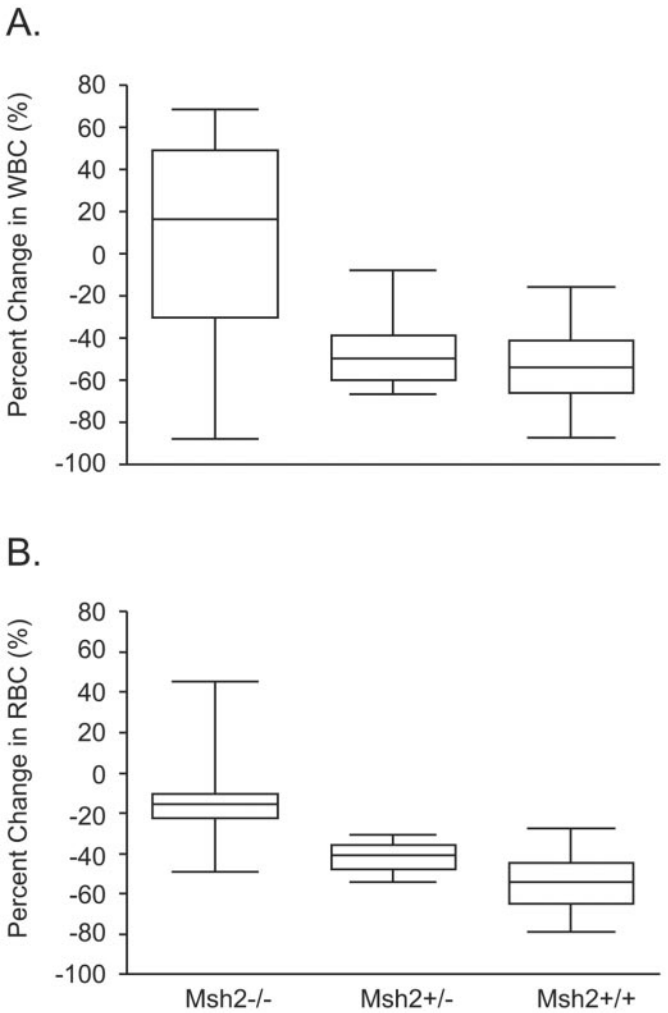

Fig. 3. Hematological toxicity studies in *Msh2*$^{+/+}$, *Msh2*$^{+/-}$, and Msh2$^{-/-}$ mice. Changes of WBC count (A) and changes in RBC count (B) in mice after i.p. administration of MP (30 mg/kg/day, 14 days). Each point represents the result of three parallel experiments (mean ± S.E.).

A.

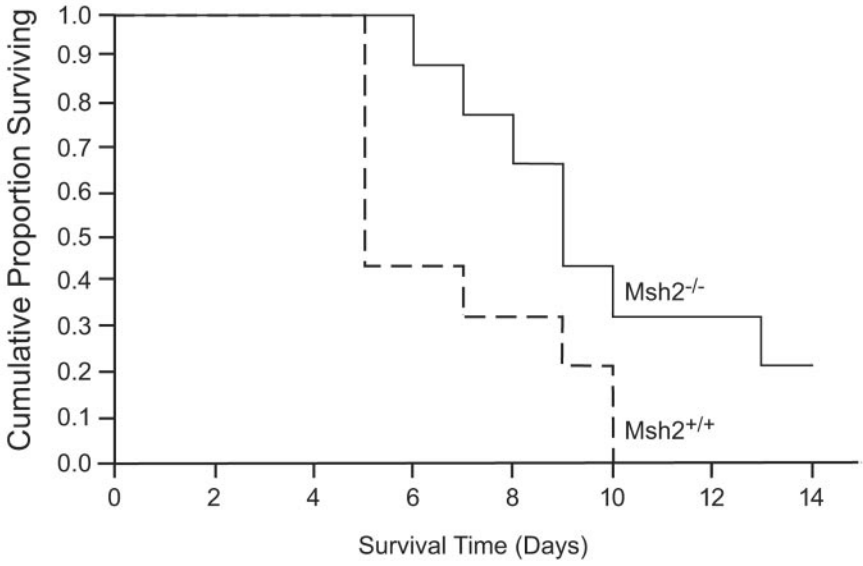

Fig. 4. Mortality of *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ mice after daily treatment with high-dose MP (50, 100, or 150 mg/kg) by i.p. administration. A, Kaplan-Meier analysis of proportion surviving in *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ mice. B, cumulative dose of MP and cumulative survival days for each group of mice with different doses of MP.

B.

		50/mg/kg/day	100/mg/kg/day	150/mg/kg/day
Cumulative MP Dose (mg)	Msh2$^{-/-}$	2050	2500	3600
	Msh2$^{+/+}$	1350	1900	2250
Cumulative Survival Days	Msh2$^{-/-}$	41	25	24
	Msh2$^{+/+}$	27	19	15

(Griffin et al., 1994) using 10 to 100 nM ^{32}P-labeled DNA duplex, 5× "cold" GC-duplex, and 10 to 50 μg of total protein.

Modeling, Statistics, and Parameter Estimation. Differences among genotypes regarding percentage change in hematopoietic cell numbers were determined by Kruskal-Wallis analysis of variance. Differences in cumulative doses in *Msh2*$^{-/-}$ mice were determined by the Mann-Whitney *U* test. The survival curves were determined by Kaplan-Meier estimation. Differences in survival between *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ mice were determined by the Cox proportional hazard regression model. Incorporation of dG^{S} into DNA was modeled using the sigmoid E_{max} model. Model parameter estimates including the IC_{50} were determined by the maximum likelihood

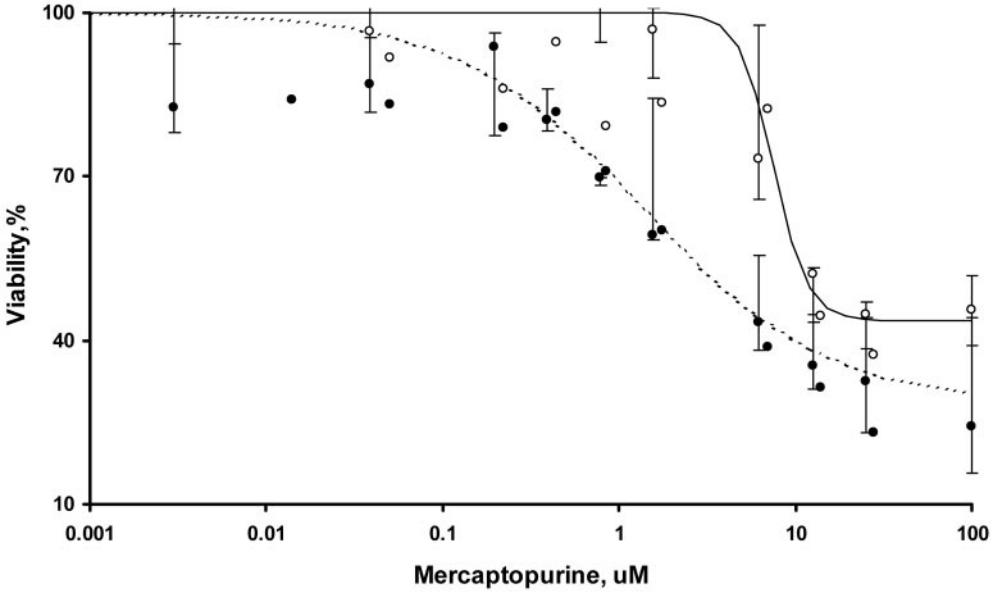

Fig. 5. Viability of *Msh2*$^{-/-}$ and *Msh2*$^{+/+}$ MEFs after 6 days of mercaptopurine treatment, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay. Closed circles, *Msh2*$^{+/+}$ cells; open circles, *Msh2*$^{-/-}$ cells. Each point represents the result of three parallel experiments (mean ± S.E.).

method, using the Adapt II software. (D'Argenio and Schumitzky, 1997) The *t* test was used to determine significant differences in the IC_{50} between $Msh2^{+/+}$ and $Msh2^{-/-}$ mice.

Results

MSH2 Expression. Sixty-three patients were studied, the demographics of whom are summarized in Table 1. MSH2 protein was undetectable in ALL blasts (Fig. 1A) from 11 of 63 patients (17.5%), whereas a GAPDH signal was detectable in all samples (Fig. 1, A and B). Comparison of the normalized MSH2 signal across the group of MSH2-positive samples revealed a 10-fold difference in the level of MSH2 protein (median 0.21 RU, range 0.074–0.82 RU), as shown in Fig. 1C. Cloning and sequencing of MSH2 cDNA isolated from leukemia cells of three patients with undetectable MSH2 protein and three patients with high (0.51, 0.58, and 0.82 RU) MSH2 protein expression failed to identify MSH2 coding region sequence variants that differed in ALL blasts with low or high MSH2 protein levels.

The *Msh2* phenotype in mice was confirmed by Western analysis of several murine tissues, including bone marrow, liver, spleen, and kidney (Fig. 2). Msh2 was expressed in all analyzed tissues of $Msh2^{+/+}$ mice (see relative Msh2 protein level normalized per GAPDH, Fig. 2B), whereas no Msh2 protein was detected in any tissues from $Msh2^{-/-}$ mice (Fig. 2).

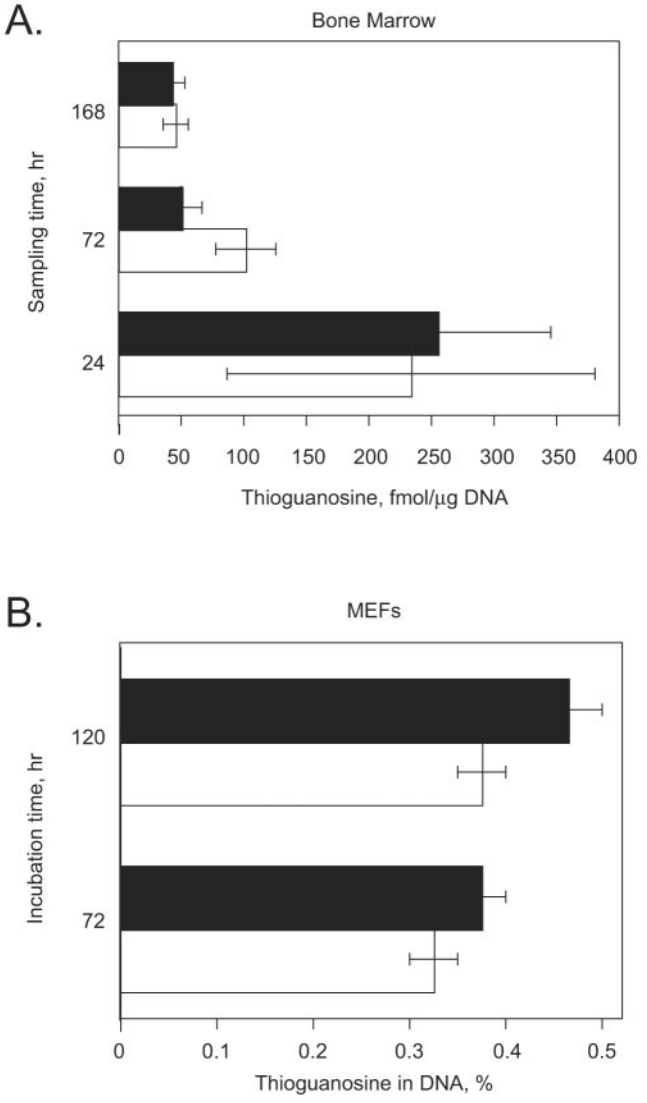

Fig. 6. Thioguanosine incorporation into genomic DNA. A, DNA extracted from bone marrow of mice ($Msh2^{+/+}$, closed bars; or $Msh2^{-/-}$, open bars) after treatment with a single dose of 11.4 mg/kg [^{14}C]MP for 24, 72, and 168 h. B, DNA extracted from MEFs ($Msh2^{+/+}$, closed bars; or $Msh2^{-/-}$, open bars) after incubation with 10 μM MP for 72 h and 120 h. Each point represents the result of three parallel experiments (mean ± S.E.).

In Vivo Thiopurine Cytotoxicity. MP hematopoietic toxicity was assessed in the $Msh2^{+/+}$, $Msh2^{+/-}$, and $Msh2^{-/-}$ mice with two treatment protocols: 2.5 to 20 mg/kg/day (i.p.) for 21 days and 30 to 150 mg/kg/day (i.p.) for 14 days (Hara et al., 1989). Administration of 2.5 to 20 mg/kg/day of MP (i.p.) for 21 days caused negligible changes in leukocyte and erythrocyte counts (data not shown). Therefore, 30 mg/kg/day of MP (i.p.) for 14 days was further utilized for assessing hematological toxicity, because it did not result in treatment-related deaths in either group and consistently decreased WBC count in $Msh2^{+/+}$ and $Msh2^{+/-}$ mice by more than 50%. Cytotoxicity data were obtained from three independent studies using 14 mice in each group ($Msh2^{+/+}$, $Msh2^{+/-}$, and $Msh2^{-/-}$ mice) after treatment with MP (30 mg/kg/day) and 12 to 13 mice in each control group (treated with 0.9% NaCl). $Msh2^{+/+}$ and $Msh2^{+/-}$ mice exhibited a significant drop in total leukocytes [median (quartiles): −53.6% (−64.5%, −41.0%) and −49.6% (−60.0%, −39.7%)] following 14 days of MP (30 mg/kg/day i.p.), compared with the $Msh2^{-/-}$ mice [median (quartiles): +16.3% (−25.9%, 48.6%)] (Fig. 3A, $p < 0.002$). It is noteworthy that similar changes were found in neutrophils [median: −72.5% and −68.7% in $Msh2^{+/+}$ and $Msh2^{+/-}$ compared with +18.8% in $Msh2^{-/-}$ mice, $p = 0.0025$] and in lymphocytes [median: −50.8% and −50.53 in $Msh2^{+/+}$ and $Msh2^{+/-}$ compared with −1.4% in $Msh2^{-/-}$ mice, $p = 0.016$], but no significant differences were found in monocytes [median: −8.7% and +45.2% in $Msh2^{+/+}$ and $Msh2^{+/-}$ compared with +37.9% in $Msh2^{-/-}$ mice, $p = 0.15$]. Likewise, $Msh2^{+/+}$ and $Msh2^{+/-}$ mice demonstrated a significantly greater decrease in erythrocyte count [median (quartiles): −54.0% (−62.2%, −44.7%) and −41.1% (−48.1%, −36.8%)]

TABLE 2
TPMT and HPRT activity (mean ± S.E.M.) in RBC and WBC from untreated $Msh2^{+/+}$, $Msh2^{+/-}$, and $Msh2^{-/-}$ mice

Genotype	RBC	WBC		Murine Embryonic Fibroblasts	
	TPMT	TPMT	HPRT	TPMT	HPRT
	U/ml packed RBC	*nmol/h/10^9 cells*	*nmol/h/10^6 cells*	*nmol/h/10^9 cells*	*nmol/h/10^6 cells*
$Msh2^{-/-}$	7.6 ± 3.1	10.4 ± 3.5	8.2 ± 0.9	6.1 ± 3.9	17.6 ± 0.8
$Msh2^{+/-}$	6.8 ± 1.9	8.3 ± 3.2	5.2 ± 1.1	N/A	N/A
$Msh2^{+/+}$	7.4 ± 1.9	16.8 ± 3.0	8.2 ± 1.9	26.2 ± 5.3	16.2 ± 1.4

following MP treatment compared with −15.6% (−22.3%, −10.6%) [median (quartiles)] in *Msh2*$^{-/-}$ mice (Fig. 3B, $p < 0.0001$).

As depicted in Fig. 4, MP doses of more than 50 mg/kg/day (50, 100, and 150 mg/kg/day with three mice in each group and three in each control group treated with 0.9% NaCl) for 14 days resulted in treatment-related deaths. However, *Msh2*$^{-/-}$ mice had a survival advantage compared with *Msh2*$^{+/+}$ mice (Fig. 4A, $p = 0.02$). In addition, *Msh2*$^{-/-}$ mice tolerated significantly higher cumulative doses of MP after treatment with 50 mg/kg/day and 150 mg/kg/day (Fig. 4B, $p < 0.05$).

In Vitro Thiopurine Cytotoxicity. After 4 days of MP treatment (0.001–100 μM), only *Msh2*$^{+/+}$ MEFs revealed cytotoxicity ($IC_{50} = 31.4 \pm 15.1$ μM). After 5 to 6 days of MP treatment, *Msh2*$^{-/-}$ fibroblasts were 3- to 4-fold less sensitive, compared with MEFs from *Msh2*$^{+/+}$ mice ($IC_{50\text{-day5}} = 18.4 \pm 6.8$ versus 6.8 ± 1.9 and $IC_{50\text{-day6}} = 11.9 \pm 1.3$ versus 3.8 ± 0.1, $p = 0.0001$). Cytotoxicity for MEFs with different Msh2 genotypes after 6 days of MP treatment are shown in Fig. 5.

ThioG Incorporation in DNA and Nuclear Protein-DNA Interactions. Figure 6 shows the level of 6-thiodeoxyriboguanosine in genomic DNA after [^{14}C]MP administration in a single dose (11.4 mg/kg) to mice with each *Msh2* genotype, and in MEFs after 10 μM MP treatment. No statistically significant differences in G^S-insert accumulations into genomic DNA were found between *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ mice after treatment for 24, 72, or 168 h ($p = 0.83$, 0.08, and 0.28, respectively). Also, there were no significant differences in the activity of thiopurine-activating (i.e., HPRT, $p = 0.6$) or -inactivating (i.e., TPMT) enzymes in leukocytes ($p = 0.17$) and erythrocytes ($p = 0.8$) from *Msh2*$^{+/+}$ and Msh2$^{-/-}$ mice (Table 2). HPRT activities are similar for MEFs from *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ mice ($p = 0.6$). Note that TPMT activity was higher in MEFs from *Msh2*$^{+/+}$ mice versus MEFs from *Msh2*$^{-/-}$ mice (26.2 ± 5.3 versus 6.1 ± 3.9 nmol/h/10^9 cells; Table 2; $p = 0.043$).

Western blot analysis of Msh2 protein in nuclear extracts from Msh2-proficient and -deficient MEFs is shown in Fig. 8A. Using [^{32}P]GT-duplex (positive control) and [^{32}P]GC-duplex (negative control), we demonstrated that only nuclear proteins from *Msh2*$^{+/+}$ MEFs interact with [^{32}P]GT-duplex (Fig. 7), corroborating previously published data (Dewind et al., 1995). Likewise, a DNA-protein complex containing Msh2 protein was formed only with *Msh2*$^{+/+}$ nuclear extracts and [^{32}P]G^ST-duplex (Fig. 8B, lane 2, band a). This complex was attenuated by 2.5 mM ATP (Fig. 8B, compare lanes 1 and 2), had mobility similar to that of the GT-DNA-protein complex from human nuclear extract (Fig. 8B, band a, lane 6), and contained Msh2 protein as documented by Western analysis of the EMSA gel using anti-MSH2 antibody (Fig. 8C, lanes 1 and 2). Titration of the G^ST-DNA duplex with increasing amounts of total nuclear protein from *Msh2*$^{+/+}$ MEFs resulted in an increase of Msh2-containing DNA-protein complex formation (Fig. 8E). In contrast, no such G^ST-DNA-protein complex was formed in the *Msh2*$^{-/-}$ MEFs (Fig. 8B, lanes 3 and 4). However, the increased formation of another DNA-protein complex was observed with *Msh2*$^{-/-}$ nuclear extracts (Fig. 8B, band b, lanes 1–2 versus 3–4; and Fig. 8D, open bars). The intensity of bands (percentage of total radioactivity) corresponding to complexes a and b is shown in Fig. 8D.

Discussion

Defects in apoptotic machinery or DNA repair can promote drug resistance by mechanisms downstream of drug-target interactions, permitting genotoxic agents to induce nonlethal genetic alterations, setting the stage for "damage without death" (Johnstone et al., 2002). This is consistent with the increased rate of secondary malignancies after treatment of ALL patients with topoisomerase II inhibitors following MP therapy (Blanco et al., 2001), which may be influenced by DNA repair competence.

In the current study, we initially established that MSH2 protein levels exhibit substantial interindividual differences in primary leukemia cells from children with newly diagnosed ALL, with the absence of detectable MSH2 protein in 17% of patients and more than a 10-fold range in MSH2 in ALL blasts with detectable MSH2 protein (Fig. 1). These findings are consistent with earlier studies in adults and children with ALL and acute myelogenous leukemia (Matheson and Hall, 1999; Zhu et al., 1999) and suggest that therapeutic effects could differ if MSH2 protein is an important determinant of cytotoxicity with genotoxic chemotherapy. There were no statistically significant differences in patient demographics, ALL lineage, or molecular subtypes, or MSH2

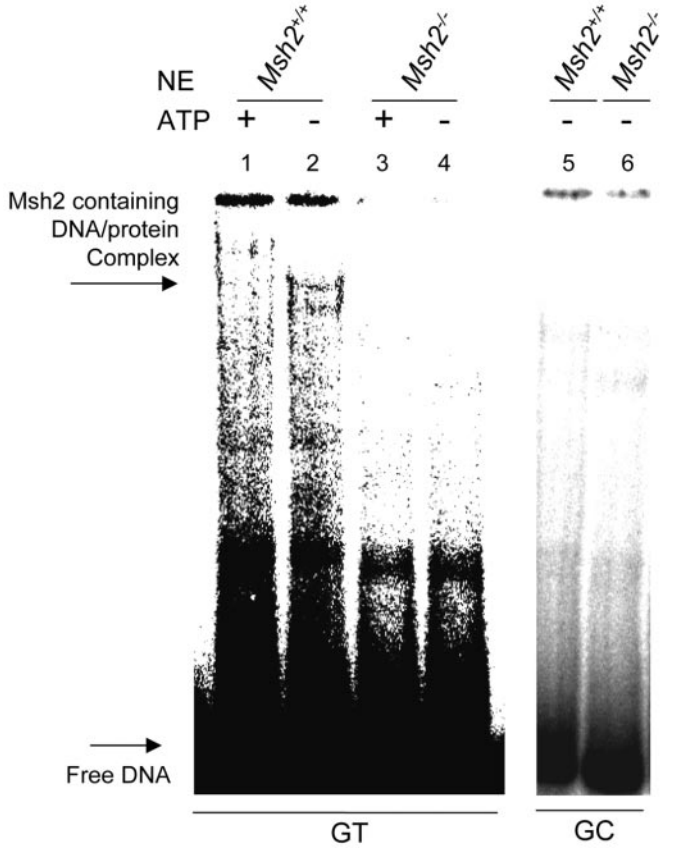

Fig. 7. DNA protein interactions by EMSA in the presence of nuclear extracts from *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ MEFs and ^{32}P-labeled GT-DNA (positive control, lanes 1–4) or GC-DNA (negative control, lanes 5 and 6). ATP treatment abrogates the GT-DNA-protein complex (compare lanes 1 and 2). Arrows indicate mobility of Msh2-containing DNA-protein complex and free DNA.

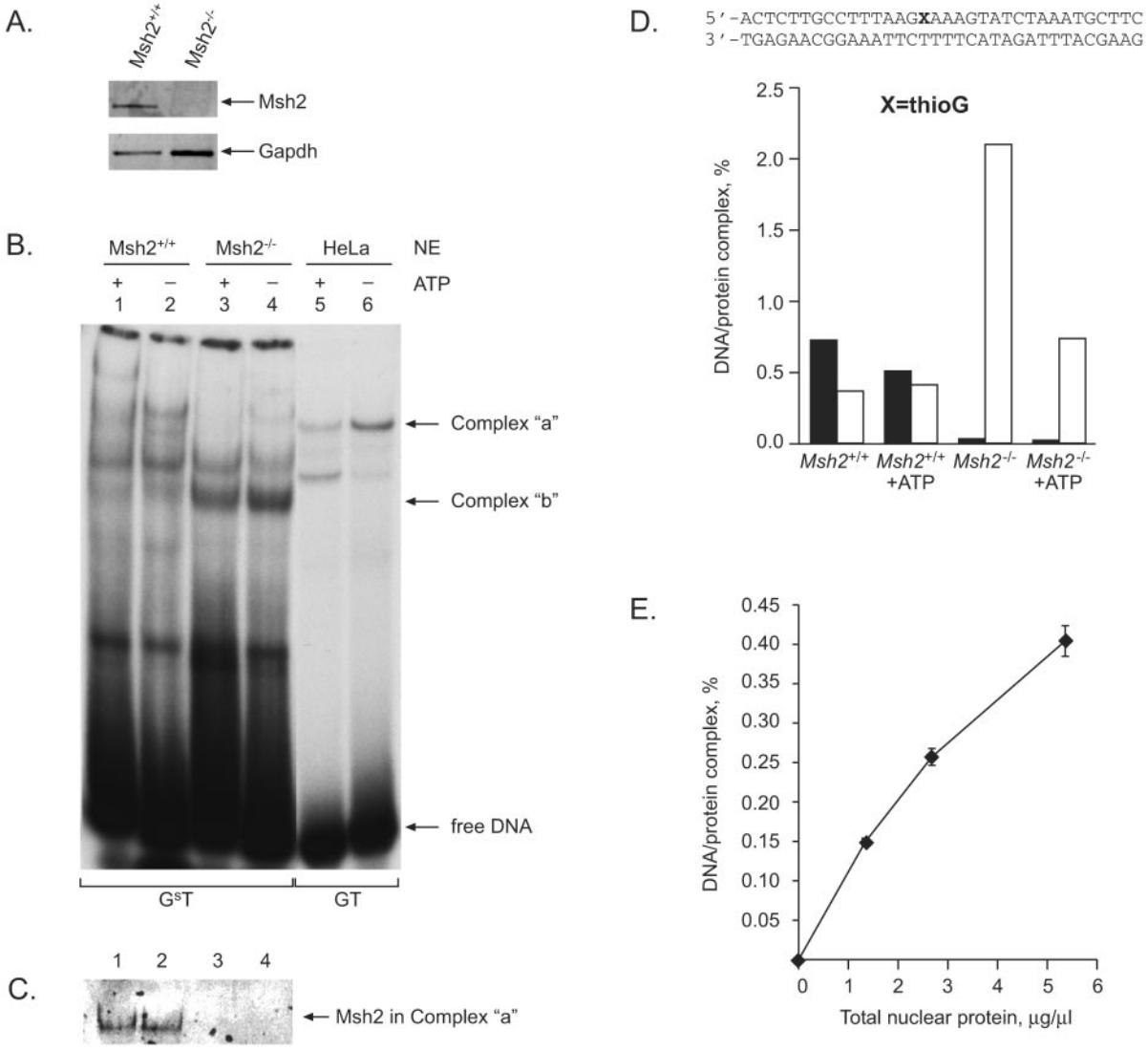

Fig. 8. DNA-protein complex formation in the presence of G^ST-DNA. A, Western blot analysis of nuclear protein extracts from *Msh2*$^{+/+}$ or *Msh2*$^{-/-}$ MEFs using anti-MSH2 antibody and anti-GAPDH antibody. B, EMSA in the presence of 10 nM 5′-^{32}P-labeled G^ST-DNA duplexes with protein extracts from *Msh2*$^{+/+}$ or *Msh2*$^{-/-}$ MEFs (lanes 1–4) and 5′-^{32}P-labeled GT-duplex with protein extract from human HeLa cells (lanes 5 and 6). C, Western blot analysis of EMSA gel transferred onto a polyvinylidene difluoride membrane and developed with anti-MSH2 antibody. Msh2-protein in complex "a" was found only in Msh2$^{+/+}$ cells (lanes 1 and 2). D, quantification of DNA-protein complexes "a" and "b" (as indicated by arrows in B; percentage of total radioactivity). Closed bars depict Msh2-containing complexes; open bars depict complexes that do not contain Msh2 protein. E, G^ST-DNA-protein complex formation (percentage of total radioactivity) containing Msh2 protein in the presence of 10 nM 5′-^{32}P-labeled G^ST-duplex and increasing amounts of nuclear proteins from *Msh2*$^{+/+}$ MEFs.

cDNA sequences between patients who had detectable versus undetectable MSH2 protein in ALL blasts (Table 1), although the relatively small number of patients limits the power of these comparisons.

In vitro experiments have indicated that cells with inactive components of the mismatch repair system (i.e., MSH2, MSH6, or MLH1), have greater resistance to thiopurines than do their MMR-proficient counterparts (Berry et al., 2000). To determine whether MSH2 was essential for hematopoietic toxicity in vivo, we performed cytotoxicity studies using an *Msh2* knockout mouse model and mercaptopurine treatment. Thiopurines have been used for the treatment of human leukemia for more than 50 years (Elion, 1989), yet the molecular events underlying their therapeutic effects remain obscure. Both MP and TG are inactive prodrugs that require intracellular anabolism to nucleoside triphosphates, with subsequent incorporation of fraudulent nucleoside (dG^S) into DNA, to induce cytotoxicity (Pennington and Bronk, 1995; McLeod et al., 2000; Chen et al., 2001). dG^S-inserts in DNA-template result in an increased frequency of G^ST-mismatch pair formation compared with nonmodified DNA-template, although in vitro replication showed that formation of G^SC-pairs was 300-fold preferential compared with G^ST-pairs (Ling et al., 1992; Rappaport, 1993; Krynetski et al., 2001). We recently showed that the presence of G^SC-pairs in DNA results in local alterations of DNA structure (Somerville et al., 2003) distinct from DNA structural changes caused by GT-mismatches (Roongta et al., 1990). Nonenzymatic alkylation of the thio group in G^S-DNA was hypothesized to convert thioguanosine to a highly toxic *S*-methylthioguanosine moiety (Swann et al., 1996; Waters and Swann, 1997), increasing the formation of G^{Sme}T mismatch pairs

during DNA replication across *S*-methyl deoxythioguanosine template (Spratt and Levy, 1997). In both scenarios, the MSH2-MSH6 complex plays an important role in detecting the mismatched base pair formed at the site of thioguanosine incorporation.

To study the role of MSH2 protein in determining thiopurine hematopoietic toxicity, we compared treatment-induced changes in leukocytes (i.e., neutrophils, lymphocytes, and monocytes) and erythrocytes in Msh2-deficient and -proficient mice. We found significantly greater reduction of total WBCs (Fig. 3A) including neutrophils and lymphocytes, and RBCs in MMR-proficient mice compared with $Msh2^{-/-}$ mice following MP treatment (30 mg/kg/day; Fig. 3B), but no changes were found with 2.5 to 20 mg/kg/day of MP for 21 days. These results indicate that MMR proficiency is important for cytotoxicity in hematopoietic cells at 30 mg/kg/day of MP. Likewise, in vitro experiments revealed a 3-fold difference in MP cytotoxicity in $Msh2^{+/+}$ versus $Msh2^{-/-}$ murine embryo fibroblast cells (Fig. 5, $p = 0.0001$). No differences in nutrition or general well being were observed between the untreated or low-dose (2.5 to 30 mg/kg/day) MP-treated MMR-deficient and MMR-proficient mice. Furthermore, treatment with higher MP doses (50–150 mg/kg/day i.p.) resulted in mortality of mice with each Msh2 genotype (Fig. 4). However, MMR-deficient mice survived longer while receiving higher MP dosages (Fig. 4A, $p = 0.02$), and they tolerated higher cumulative doses of MP compared with MMR-proficient mice (Fig. 4B, $p < 0.05$).

Because the MMR system interacts with mismatches generated due to thioguanosine incorporation into DNA, we compared the level of G^S-inserts in DNA of mice with both *Msh2* genotypes. Fig.6 demonstrates accumulation of similar levels of G^S-inserts in genomic DNA after MP treatment in mice and in MEFs with different genotypes, yet $Msh2^{+/+}$ mice and $Msh2^{+/+}$ MEFs had greater cytotoxicity compared with $Msh2^{-/-}$ mice and $Msh2^{-/-}$ MEFs after MP treatment (Figs. 3 and 5). It has been hypothesized that futile DNA repair of G^ST or G^{Sme}T mismatches eventually triggers apoptosis via mechanisms that remain unknown (Fink et al., 1998; Berry et al., 2000). Our in vivo findings are consistent with the hypothesis that the MMR system is unable to remove G^S-inserts from DNA in mice treated with MP.

To confirm that G^ST-DNA is recognized by MMR, we performed DNA-protein interaction studies using EMSA. These experiments demonstrated that nuclear proteins extracted from $Msh2^{+/+}$ MEFs, but not $Msh2^{-/-}$, recognized GT- as well as G^ST-mismatch pairs of DNA, forming DNA-protein complexes containing Msh2 (Figs. 7 and 8) No similar DNA-protein complexes were found in experiments with nuclear extracts from $Msh2^{-/-}$ MEF cells. However, another DNA-protein complex formed between G^ST-containing DNA duplex and nuclear proteins from $Msh2^{-/-}$ MEFs (Fig. 8B, lanes 3 and 4), indicating the existence of alternative proteins recognizing thioguanosine-modified DNA in $Msh2^{-/-}$ mice. An alternative G^S-DNA-protein complex, distinct from the known DNA-mismatch repair protein complex, has recently been found in human MSH2- and MSH6-negative ALL cells (Krynetski et al., 2001; Krynetski et al., 2003).

In summary, the current studies have documented that a subgroup of patients (17%) have undetectable MSH2 protein in their ALL cells and revealed marked heterogeneity of MSH2 protein in leukemia cells from the remainder of children with newly diagnosed acute lymphoblastic leukemia. In vivo experiments with $Msh2^{+/+}$ and $Msh2^{-/-}$ mice revealed a significant effect of MSH2 protein on the cytotoxicity of genotoxic thiopurine agents. Our in vivo findings indicate that MMR cannot repair newly synthesized DNA, consistent with the hypothesis that futile mismatch repair triggers apoptosis (Berry et al., 2000). The difference in thiopurine cytotoxicity and delayed mortality in Msh2-deficient mice indicates the in vivo importance of Msh2 in thiopurine hematopoietic toxicity and provides the first in vivo evidence that MMR deficiency attenuates, but does not abolish, the cytotoxicity of thiopurines.

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