Role of MUTYH and MSH2 in the Control of Oxidative DNA Damage, Genetic Instability, and Tumorigenesis

Maria Teresa Russo,1 Gabriele De Luca,1 Ida Casorelli,1 Paolo Degan,2 Sara Molatore,3 Flavia Barone,1 Filomena Mazzei,1 Tania Pannellini,1 Piero Musiani,1 and Margherita Bignami1

1Department of Environment and Primary Prevention, Istituto Superiore di Sanità, Rome, Italy; 2Department of Translational Oncology, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy; 3Department of Genetics and Microbiology, University of Pavia, Pavia, Italy; and 4Centro Studi per l’Invecchiamento, Università degli Studi “G. d’Annunzio,” Chieti-Pescara, Italy

Abstract
Mismatch repair is the major pathway controlling genetic stability by removing mispairs caused by faulty replication and/or mismatches containing oxidized bases. Thus, inactivation of the Msh2 mismatch repair gene is associated with a mutator phenotype and increased cancer susceptibility. The base excision repair gene Mutyh is also involved in the maintenance of genomic integrity by repairing premutagenic lesions induced by oxidative DNA damage. Because evidence in bacteria suggested that Msh2 and Mutyh repair factors might have some overlapping functions, we investigated the biological consequences of their single and double inactivation in vitro and in vivo. Msh2+/− mouse embryonic fibroblasts (MEF) showed a strong mutator phenotype at the hprt gene, whereas Mutyh inactivation was associated with a milder phenotype (2.9 × 10−6 and 3.3 × 10−7 mutation/cell/generation, respectively). The value of 2.7 × 10−6 mutation/cell/generation in Msh2−/−Mutyh−/− MEFs did not differ significantly from Msh2−/− cells. When steady-state levels of DNA 8-oxo-7,8-dihydroguanine (8-oxoG) were measured in MEFs of different genotypes, single gene inactivation resulted in increases similar to those observed in doubly defective cells. In contrast, a synergistic accumulation of 8-oxoG was observed in several organs of Msh2−/−Mutyh−/− animals, suggesting that in vivo Msh2 and Mutyh provide separate repair functions and contribute independently to the control of oxidative DNA damage. Finally, a strong delay in lymphomagenesis was observed in Msh2−/−Mutyh−/− when compared with Msh2−/− animals. The immunophenotype of these tumors indicate that both genotypes develop B-cell lymphoblastic lymphomas displaying microsatellite instability. This suggests that a large fraction of the cancer-prone phenotype of Msh2−/− mice depends on Mutyh activity. [Cancer Res 2009;69(10):4372–9]

Introduction
Mismatch repair (MMR) is the major repair pathway for removal of mispairs originating from errors of DNA polymerases. MMR is initiated by recognition of base-base mismatches and/or small insertion/deletion loops by heterodimeric complexes formed by Escherichia coli MutS homologues. The possible partners of Msh2 are Msb6 or Msh3 in the MutSα and MutSβ, respectively. Repair proceeds via interaction of these recognition complexes with heterodimers containing Mlh1 as a common subunit followed by Exo1-mediated excision of the tract with the mispair and resynthesis done most likely by polymerase β. Inactivation of MMR genes is invariably associated with a pronounced mutator phenotype (1). Germ-line mutations in the human MSH2 and MLH1 genes are associated with an autosomal recessive disease, the hereditary nonpolyposis colorectal cancer (2). Inactivation of the wild-type allele leads to cancer formation mostly but not exclusively of the gastrointestinal tract. Msh2−/− mice, in which both alleles have been inactivated, are cancer-prone, and die within the first year of their life (3–5). Although increased colorectal carcinogenesis is observed in Msh2−/− mice exposed to alkylating carcinogens (6), the vast majority of these animals succumb to lymphoma (3–5). This resembles the occurrence of cancer in the hematopoietic compartments of children carrying biallelic germ-line MSH2 mutations (7–10). In addition, although hematologic malignancies are rarely found in association with hereditary nonpolyposis colorectal cancer, B- or T-cell lymphomas have been reported in hereditary nonpolyposis colorectal cancer patients with heterozygous mutations in MSH2 or MLH1 genes (11, 12). MMR recognizes DNA mismatches containing oxidized bases. 8-Oxo-7,8-dihydroguanine (8-oxoG) is a major product of base oxidation, which frequently miscodes with adenine, and these unrepaired mismatches lead to GC→TA transversions. Mammalian MutSα can bind DNA duplexes containing 8-oxoG:A mismpairs (13, 14) and 2-hydroxyadenine–containing mismatches (15). It has been suggested that MMR prevents oxidative mutagenesis either by removing 8-oxoG directly or by removing adenine misincorporated opposite 8-oxoG or both. In anaerobically grown MMR-deficient bacteria, spontaneous mutagenesis is reduced when compared with aerobically grown same cells (16). In addition, MMR pathway removes 8-oxoGIMP incorporated from the oxidized deoxyx nucleotide triphosphate (dNTP) pool (17) and a considerable fraction of mutations occurring in Msh2−/− cells are due to the incorporation of oxidized bases (18, 19). These data are consistent with a role of MMR acting on oxidized DNA and preventing mutagenesis originating from an oxidized dNTP pool.

On the other hand, base excision repair pathway is the major pathway involved in the repair of oxidized bases. In particular, 8-oxoG:A mismatches are bound by Mutyh, a DNA glycosylase that removes adenine across the oxidized purine (20, 21). The resulting apurinic site is then incised by the apurinic/apyrimidinic endonuclease and resynthesis occurs via long-patch base excision repair. Insertion of cytosine across 8-oxoG creates a substrate for other DNA glycosylases, which are capable to remove 8-oxoG from 8-oxoG:C mismatches (22–24). Both bacterial and human enzymes are capable to remove 2-hydroxyadenine when this oxidized base is
paired with guanine, thus reducing another source of mutagenic lesions (25, 26). Inactivation of the E. coli MutY gene results in 10- to 20-fold increases in mutation rates mostly due to accumulation of GC>T-A transversions (27, 28).

In humans, biallelic germ-line mutations in MUTYH cause the autosomal recessive colorectal adenomatous polyposis (29, 30). This syndrome is characterized by the absence of inherited mutations in the APC gene and accumulation of somatic GC>T-A transversions in oncogenes probably due to defective repair of 8-oxoG:A mismatches (31). It is therefore expected that MUTYH inactivation in mammalian cells might also lead to a mutator phenotype. Thus, inactivation of Muty in murine embryo stem cells is associated with a modest increase in mutation rates (32). Muty-null mice show a limited susceptibility to spontaneous intestinal tumorigenesis mostly in the second year of life (33, 34).

Evidence in bacteria suggests that the MMR and MutY-dependent pathways might overlap in some respect. For example, overexpression of MutS decreased the frequency of GC>T-A transversions in a MutY mutant strain possibly because of repair of 8-oxoG:A mismatches (35). MutY, under certain conditions, might compete with MutS-dependent MMR in the processing of some A:C mismatches. Thus, in the presence of unbalanced dNTP pool because of mutations in the nucleotide diphosphate kinase gene (high levels of dCTP), MutY would wrongly remove the correct template adenine from A:C potentially fixing mutations (36). It has been suggested that the strand specificity of MutY might be lost in a MutS strain, thus causing increased misrepair at A:C or A:G:8-oxoG mismatches (37). Finally, the human MUTYH protein has been shown to physically interact with the MutS complex (38).

To investigate the biological consequences of these possibly overlapping functions, Msh2−/−/Mutyh−/− mice were constructed and mouse embryo fibroblasts (MEF) were derived from these and from singly defective mice. Accumulation of 8-oxoG and spontaneous mutation rates at the hprt gene were compared in the different genotypes. Whereas single or double inactivation of Msh2 and Muty leads to similar increases in steady-state levels of 8-oxoG in vitro, a synergistic accumulation of 8-oxoG was observed in vivo in Msh2−/−/Mutyh−/− mice. More importantly, the pronounced delay in lymphomagenesis observed in doubly defective animals suggests that part of the Msh2-dependent cancer proneness is strongly dependent on Muty activity.

Materials and Methods

Cell lines. MEFs were derived from Msh2−/−, Muty−/−, or Msh2−/−/Mutyh−/− embryos and their genotype were analyzed with appropriated primer pairs (39). PCR was carried out in buffer (50 μL) containing DNA (0.25-1 μg), 50 pmol of each primer, 2.5 mmol/L MgCl2, 0.2 mmol/L of each dNTP, and 2.5 units AmpliTaq DNA polymerase (Applied Biosystems). The PCR profile was 94°C (1 min), 58°C (2 min), and 72°C (2 min) for 35 cycles for Muty and 94°C (1 min), 55°C (2 min), and 72°C (2 min) for 40 cycles for Msh2. Spontaneously immortalized MEFs were grown in DMEM (Life Technologies) with 10% FCS, penicillin (100 units/mL), and streptomycin (100 μg/mL; Life Technologies) at 37°C in a 5% CO2 atmosphere.

Cell extracts, Western blotting, and binding assay. For Western blotting analysis of Muty, cell-free extracts were separated on 8.5% SDS-polyacrylamide gel, transferred to nitrocellulose membrane with Trans-Blot cell apparatus (Bio-Rad), and probed overnight with anti-Muty (Novus Biological) followed by the appropriate secondary antibody. Blots were developed using the enhanced chemiluminescence detection reagents (Pierce).

The 36 bp DNA substrate was a 3′-end-labeled 6-carboxyfluorescein duplex with an 8-oxoG:A mismatch (5′-GCAAAGACCTTATAGCCCCCTTGAGCACACAGGGG-3′ and 5′-CCTTCTGTGCTCAAGGGGGG-3′; Thermo). Whole-cell extracts were prepared as described (40). Extracts (0-50 μg) were incubated with 0.14 mmol duplexes at 37°C for 1 h in 12 μL reaction buffer [20 mmol/L Tris-HCl (pH 8), 1 mmol/L DTT, 1 mmol/L EDTA, 100 mg/mL bovine serum albumin]. Samples were analyzed by PAGE on discontinuous 6% to 20% non-denaturing gel at 80 V for 18 h and 4°C.

Mutation rates. Cells were plated at low density (100 per dish) to effectively guarantee the absence of preexisting mutants. Cultures (50-60 independent cultures) were grown in complete medium to a density of ~1 × 106 cells per dish before plating each culture into five dishes containing 6-thioguanine (5 μg/mL; Sigma). The mean number of mutations per culture (M) was calculated as M = −ln(P0), where P0 is the proportion of cultures with no mutants. The mutation rate was calculated as μ = M/CN, where C is the number of cells at selection time. The variance of m was estimated as variance m = (ln2)(e^(-t) - 1)N^-1C^-2, where N is the number of tested cultures.

8-OxoG determinations. 8-oxodG was measured by high-performance liquid chromatography/electrochemical detection as described previously (41). Following DNA extraction, RNase treatment, and enzymatic hydrolysis, DNA hydrolysate was analyzed by high-performance liquid chromatography/electrochemical detection (Coulochem I) using a C18 250 × 46 mm 5 μm Uptisphere column (Interchim) equipped with a C18 guard column. The eluent was 50 mmol/L ammonium acetate (pH 5.5) containing 0.9% methanol at a flow rate of 0.7 mL/min. Deoxyguanosine was measured in the same run of corresponding 8-oxoG with a UV detector (model SPD-2A; Shimadzu) at 256 nm.

Microsatellite instability. Genomic DNA isolated from normal tissue and tumor of Msh2−/− and Muty−/−/Msh2−/− mice was analyzed at mononucleotide L24372 and dinucleotide DM1662 and D6Mit59 microsatellites. Primers labeled with the fluorescent compound 6-carboxyfluorescein were described previously (42, 43). PCR was carried out in a reaction buffer containing DNA (100 ng), primers (2 pmol/μL), dNTP (200 mmol/mL), and 0.5 units/μL Taq polymerase (Applied Biosystems). After denaturation (95°C for 2 min), DNA was amplified by 25 cycles (30 s at 95°C, 30 s at 55°C, and 30 s at 72°C). Amplification products (10 μL) were digested with 0.4 units/μL T4 DNA polymerase (Roche; 30 min, 37°C) denatured in deionized formamide (2 min, 95°C) and analyzed with an ABI Prism 310 automatic sequencer by the Genecode software (Applied Biosystems).

Results

Spontaneous mutation rates in MEFs derived from Muty−/−, Msh2−/−, and Msh2−/−/Muty−/− MEFs. Spontaneously immortalized MEFs derived from Muty−/− MEFs were characterized for...
MutYh expression and activity. No expression of the MutYh protein was detectable by Western blotting (Fig. 1A) and cell-free extracts from MutYh MEFs were defective in binding to a duplex oligonucleotide containing a single 8-oxoG:A mismatch (Fig. 1B).

When spontaneous mutation rates/cell/generation were measured in MutYh−/− MEFs, the observed value of 33 × 10−8 (Table 1) was at least 10-fold higher than rates observed in human tumor HeLa or A2780 cell lines (1.7 × 10−8–2.5 × 10−8/cell/generation; ref. 41) or to estimates of mutation rates in mouse (44). Because no wild-type MEFs in which hprt mutations could be measured in our laboratory, spontaneous mutation rates were compared in MutYh−/− cells and the same cell line complemented with a human MUTYH cDNA (+hMUTYH). These cells were obtained by transfection of MutYh−/− cells with the pYMv200-flaghisN expression vector containing a wild-type human MUTYH cDNA (45). Western blotting analysis indicated that +MUTYH cells express the MUTYH protein (Fig. 1A) and cell-free extracts from +MUTYH cells were able to efficiently bind an 8-oxoG:A−containing duplex, indicating that the expressed MUTYH was functional in these cells (Fig. 1B).

Expression of MUTYH in MutYh−/− MEFs resulted in a 10-fold reduction in spontaneous mutation rates (3.9 × 10−8 mutations/cell/generation; Table 1). These data indicate that lack of MutYh activity affects spontaneous mutation rates of mammalian cells and expression of the wild-type human protein is able to correct the mutator phenotype of MutYh−/− MEFs.

Mutation rates were also investigated in MEFs derived from Msh2−/− or Msh2−/−/MutYh−/− animals. Spontaneous mutation rates in Msh2−/−/MutYh−/− MEFs closely resembled those observed in Msh2-defective cells (273 × 10−8 and 299 × 10−8 mutation/cell/generation, respectively; Table 1; ref. 19).

We conclude that MutYh inactivation is associated with a milder mutator phenotype at the hprt gene in comparison with loss of Msh2. In addition, the strong mutator phenotype of Msh2−/− MEFs is not significantly affected by inactivation of the MutYh gene.

DNA 8-oxoG levels in MutYh−/−, Msh2−/−, and Msh2−/−/MutYh−/− MEFs. Both Msh2 and MutYh contribute to exclude 8-oxoG from DNA of mammalian cells, although the relative importance of these genes is not fully understood. Steady-state levels of this oxidized purine were analyzed in Msh2, MutYh, or doubly defective MEFs. A, protein levels of hMUTY in MutYh−/− cells expressing a wild-type human MUTYH cDNA (+MUTYH) or an empty vector (no ins) analyzed by Western blot. Actin is included as an internal control. B, binding to a 6-carboxyfluorescein–labeled duplex containing a single 8-oxoG:A mismatch by extracts prepared from MUTYH-expressing or MutYh−/− MEFs. Reaction products were analyzed by nondenaturing PAGE. Arrow, position of the hMUTYH complex. C, basal levels of 8-oxoG were measured by high-performance liquid chromatography/electrochemical detection in DNA extracted from wild-type (WT; white), Msh2−/− (black), MutYh−/− (gray), and MutYh−/−/Msh2−/− (striped) MEFs. Mean ± SD of 5 independent determinations. D, repair kinetics of 8-oxoG in MEFs of the various genotypes following 30 min exposure to 20 mmol/L KBrO3. Mean ± SE of 6 independent determinations.

Figure 1. Characterization, steady-state levels, and repair kinetics of 8-oxoG in MutYh−/−, Msh2−/−, and MutYh−/−/Msh2−/− MEFs. A, protein levels of hMUTY in MutYh−/− cells expressing a wild-type human MUTYH cDNA (+MUTYH) or an empty vector (no ins) analyzed by Western blot. Actin is included as an internal control. B, binding to a 6-carboxyfluorescein–labeled duplex containing a single 8-oxoG:A mismatch by extracts prepared from MUTYH-expressing or MutYh−/− MEFs. Reaction products were analyzed by nondenaturing PAGE. Arrow, position of the hMUTYH complex. C, basal levels of 8-oxoG were measured by high-performance liquid chromatography/electrochemical detection in DNA extracted from wild-type (WT; white), Msh2−/− (black), MutYh−/− (gray), and MutYh−/−/Msh2−/− (striped) MEFs. Mean ± SE of 5 independent determinations. D, repair kinetics of 8-oxoG in MEFs of the various genotypes following 30 min exposure to 20 mmol/L KBrO3. Mean ± SE of 6 independent determinations.
MEFs and compared with wild-type cells. Increased basal levels of DNA 8-oxoG were observed in all three defective genotypes, and no significant difference was identified in the doubly defective Msh2<sup>-/-</sup>/Mutyh<sup>-/-</sup> cells when compared with single mutants (Fig. 1C). These data are consistent with previous indications that both Msh2 and Mutyh are involved in the maintenance of low levels of oxidized bases (46).

To further investigate this point, kinetics of 8-oxoG removal from DNA were compared in wild-type or repair-defective MEFs following exposure to 20 mmol/L KBrO<sub>3</sub>. In wild-type cells, removal of 8-oxoG appeared to be fast and 50% of the oxidized purine disappeared from DNA in <1 h (Fig. 1D). Loss of either Msh2 or Mutyh leads to a much slower removal of 8-oxoG, with half-lives of 1.5 and 2 h, respectively. In addition, kinetics of removal in cells defective in both genes were comparable with those observed in singly defective Msh2<sup>-/-</sup> or Mutyh<sup>-/-</sup> MEFs.

These data indicate that both Mutyh and Msh2 contribute to the exclusion of 8-oxoG from DNA. Furthermore, the absence of additive effects when both genes are deleted suggests that, in the same pathway of 8-oxoG repair.

**In vivo accumulation of 8-oxoG.** Steady-state levels of 8-oxoG were also analyzed in vivo. It was previously shown that increased 8-oxoG levels are observed in Mutyh<sup>-/-</sup> mice only in liver DNA and in ageing mice (39). We confirmed these observations and steady-state levels of 8-oxoG in several tissues of young Mutyh<sup>-/-</sup> animals (3-6 months) did not differ significantly from wild-type mice (Fig. 2A-E). In contrast, Msh2<sup>-/-</sup> animals show small increases in oxidative DNA damage in several organs even at a young age (46) and 8-oxoG levels in lung, liver, kidney, and spleen of Msh2<sup>-/-</sup> animals were moderately increased (1.3- to 1.5-fold) when compared with wild-type mice (Fig. 2A-E).

Inactivation of both genes in Msh2<sup>-/-</sup>/Mutyh<sup>-/-</sup> mice further increased 8-oxoG levels in lung, liver, kidney, and spleen (2.4- to 2.5-, 2.2-, and 3-fold higher than in wild-type mice; Fig. 2A-D). In addition, even organs in which the single inactivation of Msh2 did not (bone marrow) or mildly (small intestine and thymus) affected basal levels of 8-oxoG showed increased levels of the oxidized purine in Msh2<sup>-/-</sup>/Mutyh<sup>-/-</sup> animals (range, 1.6- to 1.9-fold; Fig. 2E-G).

We can conclude that loss of Mutyh in a Msh2-defective background produces a significant increase in the amount of oxidative DNA damage in several organs. Thus, at variance with in vitro data using MEFs derived from these mice, in vivo observations indicate that Msh2 and Mutyh do contribute independently one from another to the control of DNA oxidation.

**Tumorigenesis in Msh2<sup>-/-</sup> and Msh2<sup>-/-</sup>/Mutyh<sup>-/-</sup> mice.** Similarly to previous reports (3, 47), tumors that arose in our colony of Msh2<sup>-/-</sup> knockout mice are lymphoblastic lymphomas. In 2 of 5 Msh2<sup>-/-</sup> mice, a retroperitoneal sarcoma and a skin spino-cellular carcinoma were found in association with lymphoblastic lymphomas. The times at which tumor-bearing animals died or were killed are summarized in Fig. 3, with a median time to death of 140 days (95% confidence interval, 111-169 days). This value is very similar to our previous report in this colony of Msh2<sup>-/-</sup> mice (6).

Msh2<sup>-/-</sup>/Mutyh<sup>-/-</sup> mice were healthy at birth and fertile. However, all of them developed metastasizing lymphoma with a peak at 9 months. Surprisingly, the median time to death of Msh2<sup>-/-</sup>/Mutyh<sup>-/-</sup> mice is significantly different from that observed in Msh2<sup>-/-</sup> mice (262 days; 95% confidence interval, 229-295 days; P<0.0001, log-rank, Wilcoxon, and Mann-Whitney U tests; Fig. 3). As expected, in this time interval (up to 9 months), Mutyh<sup>-/-</sup> mice were not prone to cancer development (33, 34).

Three doubly defective Msh2<sup>-/-</sup>/Mutyh<sup>-/-</sup> mice developed tumors macroscopically and histologically very similar to those originated in Msh2<sup>-/-</sup> mice. Lymphomas arising in both genotypes were composed of large lymphoid cells (lymphoblasts) arranged in a diffuse growing pattern. Numerous mitoses and aberrant mitotic forms as well as apoptotic cells were present at a high rate (Fig. 4A and 4B). Many of these tumors originated in lymphoid organs (lymph nodes, spleen, and thymus) that were infiltrated or replaced by tumor cells; residual shrink lymphoid B follicles and scattered T lymphocytes aggregates were left among malignant cells. Widespread tumor infiltration was sometimes found into nonlymphoid organs (liver and gut; Table 2). To determine whether the lymphoblastic lymphomas found in Msh2<sup>-/-</sup> and Msh2<sup>-/-</sup>/Mutyh<sup>-/-</sup> mice were a single histopathologic entity or displayed heterogeneity with respect to lineage, tumor samples obtained from the two groups of animals were analyzed by immunohistochemistry. Lymphomas

| Table 1. Spontaneous mutation rates at the hprt gene in Msh2<sup>-/-</sup>, Mutyh<sup>-/-</sup>, and doubly defective Msh2<sup>-/-</sup>/Mutyh<sup>-/-</sup> MEFs |
|----------------|----------------|--|----------------|----------------|----------------|
| Cells                  | No. cultures | Final cells x 10<sup>6</sup> | Fraction of culture P<sub>0</sub> | Mutation rate μ ± SD (10<sup>-8</sup>) | Mean of μ   |
| Mutyh<sup>-/-</sup> | Experiment 1  | 60 | 2.9 | 51/60 | 39 ± 12 | 33 |
|                       | Experiment 2  | 46 | 10.2 | 31/46 | 27 ± 7  | 3   |
| Mutyh<sup>-/-</sup> + MUTHY cDNA | Experiment 1  | 60 | 15.9 | 55/60 | 3.9 ± 1.7 | 3.9 |
| Msh2                  | Experiment 1  | 50 | 7.2 | 3/50 | 269 ± 40 | 299 |
|                       | Experiment 2  | 50 | 5.3 | 15/50 | 157 ± 30 | 30  |
|                       | Experiment 3  | 44 | 4.3 | 2/44 | 429 ± 30 | 30  |
|                       | Experiment 4  | 60 | 13  | 31/60 | 340 ± 63 | 63  |
| Msh2<sup>-/-</sup>/Mutyh<sup>-/-</sup> | Experiment 1  | 50 | 2.4 | 20/50 | 260 ± 50 | 273 |
|                       | Experiment 2  | 60 | 1.8 | 37/50 | 188 ± 40 | 40  |
|                       | Experiment 3  | 60 | 0.7 | 41/60 | 370 ± 80 | 80  |

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showed a high proliferation index as assessed by proliferating cell nuclear antigen staining (data not shown), indicating that these tumors were rapidly growing. Lymphoblasts were negative for T and monocyte/macrophage markers (CD3, CD4, CD8, and CD11b), whereas they were positive for PAX-5, a nuclear marker for B lineage-committed cells (48). In association with PAX-5, the pan B-cell marker B220 was strongly expressed in three cases (two from Msh2−/−/− group and one from Msh2−/−/Mutyh−/− group; Fig. 4C and D; Table 2). The coexpression of PAX-5 and B220 clearly indicates that these tumors belong to the B-cell compartment. The positivity for PAX-5 in absence of or associated with a weak B220 expression as observed in five cases (three from Msh2−/−/− group and two from Msh2−/−/Mutyh−/− group; Fig. 4E and F; Table 2) indicates that the neoplastic lymphoblasts are very early B-cell in origin, preceding pre-pro-B stage.

We can conclude that, even if the lymphomagenesis is delayed in the double-knockout animals, morphologic analyses indicate that both Msh2−/−/− and Msh2−/−/Mutyh−/− mice develop B-cell lymphoblastic lymphomas.

To investigate whether simultaneous inactivation of Msh2 and Mutyh affected maturation of lymphoid organs and peripheral blood cells, healthy Msh2−/−/Mutyh−/− mice were analyzed at age 2 months. Histologic and immunohistochemical analysis of lymphoid organs using anti-CD3, CD4, CD8, B220, CD11b, and CD68 antibodies showed a normal organ architecture with the expected cellular composition of T cells, B cells, and monocytes/macrophages. In addition, the peripheral blood white cell count and the percentages of lymphocytes and granulocytes were similar to those previously reported in C57BL/6J mice (49).

**Microsatellite instability.** The microsatellite instability status of tumors from Msh2−/−/− and Msh2−/−/Mutyh−/− mice was determined by analyzing two mononucleotide (L24372 and Aa006036) and two dinucleotide (D1Mit62 and D6Mit59) repeat markers. The size of the predominant alleles of mononucleotide or dinucleotide microsatellites was compared in tumor samples and the matching normal tissue (tail) (examples of microsatellite instability are shown in Supplementary Fig. S1). All tumors exhibited some degree of microsatellite instability, with the majority showing mutations in ≥50% of the repeat markers. In a single case, mouse 4, instability was limited to the Aa006036 locus. In accordance with other reports using the same microsatellite probes in MMR-defective mice (42, 43), both gains and losses of bases occurred at these loci.

Overall, we conclude that lymphomas arising in Msh2−/−/− and Msh2−/−/Mutyh−/− mice all display microsatellite instability and no
significant differences in the frequency of mutations between mononucleotide and dinucleotide markers was identified in the two genotypes.

Discussion

The Msh2 and Mutyh proteins are involved in the initial steps of their respective repair pathways, MMR and base excision repair, and both provide major contributions to the maintenance of genomic stability. To identify distinctiveness as well as possible overlapping repair functions of these genes in mammalian cells, we compared the biological consequences of their single or simultaneous inactivation. We report here that loss of Msh2 leads to a much more pronounced mutator phenotype at the hprt gene than Mutyh inactivation. This is not surprising in view of the broad substrate specificity of Msh2-containing complexes (1). The Mutyh glycosylase displays a more restricted recognition capacity, 8-oxoG:A and 2-hydroxyadenine:G mispairs, and, with a lower efficiency, G:A and AC mismatches (50). The 10-fold increase in spontaneous mutation rates we report here in Mutyh−/− MEFs is, however, not negligible and suggests that mispairs derived from oxidized purines contribute significantly to mutagenesis of mammalian cells. Secondly, the simultaneous inactivation of mammalian Mutyh and Msh2 did not significantly modify the strong mutator phenotype of Msh2-defective cells. This is at variance from results obtained in E. coli where inactivation of MutY in a MutS background reduced the mutational load of a MutS strain (37). It is possible that these observations in bacteria refer to processing of a subset of base-base mismatches formed under particular conditions of bacterial growth (36) that are not present in mammalian cells.

Analysis of steady-state 8-oxoG levels in mammalian cells defective in either Msh2 or Mutyh support the notion that both repair proteins contribute to the maintenance of low levels of oxidative DNA damage. Thus, loss of either Mutyh or Msh2 in MEFs cultivated in vitro results in comparable increases in the steady-state levels of this oxidized purine. In addition, endogenous levels of 8-oxoG were not significantly augmented by simultaneous inactivation of both genes. These results are consistent with the two proteins cooperating in the same pathway of 8-oxoG removal.

This conclusion is supported by the similarities in the kinetics for 8-oxoG repair in singly and doubly defective MEFs exposed to an oxidant.

Important differences in the relative effect of these proteins on repair efficiency were, however, identified when in vitro and in vivo experimental conditions were compared. Msh2 seems to play a more prominent role than Mutyh in controlling 8-oxoG levels in vivo. Thus, single inactivation of Msh2 in young animals leads to a moderate accumulation of 8-oxoG in several organs, whereas this is not the case in Mutyh-defective animals. In addition, simultaneous inactivation of Msh2 and Mutyh resulted in much larger increases of oxidative DNA damage than loss of single genes. This synergistic increase in 8-oxoG levels stresses an independent role for these repair proteins in controlling oxidative DNA damage in vivo.

One major difference between in vitro and in vivo experimental conditions is the larger fraction of proliferating cells in the former one. It is possible therefore that in vitro cell growth emphasizes the cooperation of Msh2 and Mutyh in the removal of 8-oxoG from specific sources, which are maximized during replication. An obvious possibility is a partnership of MutSα and Mutyh in the recognition of 8-oxoG incorporated from an oxidized dNTP pool and located in the newly replicated strand. In this regard, MUTYH has been reported to interact with the MSH6 subunit of the MutSα (38). Which are the sources of oxidative damage and the mechanism underlying the autonomous role played by Msh2 and

Figure 3. Kaplan-Meier survival curve analysis in Msh2−/− and Mutyh−/−Msh2−/− mice. Survival of Mutyh−/−Msh2−/− mice (n = 29) was compared with Msh2−/− mice (n = 20) and found to be significantly different by log-rank test analysis (P < 0.0001).

Figure 4. Histology (A and B) and immunohistochemistry (C-F) of lymphoblastic lymphomas occurring in Msh2−/− and Mutyh−/−Msh2−/− mice. H&E staining of two cases (A and B) shows large lymphoid cells with irregular nuclei (lymphoblasts) arranged in diffuse growing pattern. Immunohistochemistry reveals that the lymphoblasts of the case illustrated in C and D are PAX-5 and B220 positive, respectively. The lymphoblasts of the case illustrated in E and F are PAX-5 positive and B220 negative, respectively (<1,000).

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Msh2 reports and the recent observation indicating that a lack of MMR resulting in tumorigenesis. This is in agreement with previous incorrectly regulated T- and B-cell receptor gene rearrangement (pre-B stage) when the first immunoglobulin gene rearrangement occurs early in B-cell differentiation (preceding pre-pro-B or rat pre-pro-B stage) (characterized by PAX-5 positivity in the absence of or with low levels of B220), the transformation events probably occurred at early stages in B-cell differentiation (preceding pre-pro-B or at pre-pro-B stage) when the first immunoglobulin gene rearrangement begins. These data suggest that Msh2 deficiency leads to an incorrectly regulated T- and B-cell receptor gene rearrangement resulting in tumorigenesis. This is in agreement with previous reports and the recent observation indicating that a lack of MMR on an athymic background predisposes to B-cell lymphoblastic lymphomas (47).

Although Msh2−/−/Mutyh−/− mice display a lifespan similar to Ogg1−/−/Mutyh−/− mice, tumor spectra in the last ones are different and lung, ovarian, and gastrointestinal tract tumors were observed (33). We found no evidence of these tumors in Msh2−/−/Mutyh−/− mice where only lymphomas were recorded. Triple inactivation of Myh/Ogg1/Msh2 mice drastically accelerated tumorigenesis and these mice did not differ from Msh2−/− mice in lifespan and tumor type (lymphomas; ref. 33). Whether the protective role of Mutyh against Msh2-dependent lymphomagenesis requires Ogg1 activity remains to be clarified.

Analyses of spontaneous mutation rates at the hprt gene in Msh2−/−/Mutyh−/− do not support the hypothesis that the delayed lymphomagenesis in these mice is due to major changes in the rates of accumulation of base substitutions and/or frameshifts associated with loss of Msh2. Although no information is available on the role of Mutyh in the immunoglobulin gene rearrangement (occurring firstly during the pre-pro-B-cell stage), it is tempting to speculate that the cancer-prone phenotype of Msh2−/− mice might depend on a Mutyh activity affecting this specialized recombinogenic process.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### References

2. Lagerstedt Robinson K, Liu T, Vandrovcova J, et al. Mutyh in vivo remains to be established. It is important to stress that Mutyh contributes only indirectly to removal of oxidized bases and this probably relies on other repair factors (Ogg1, replication-associated NEIL glycosylases, and NER proteins; ref. 22).

Simultaneous inactivation of Mutyh and Msh2 is associated with an apparent paradox: increased levels of oxidative DNA damage but retarded lymphomagenesis. Several observations support a causative role of oxidative stress in cancer formation. For example, tumor predisposition of Mutyh−/−/Ogg1−/− mice is paralleled by accumulation of 8-oxoG in the target organs for enhanced carcinogenesis (33, 39). In addition, MUTYH-null mice show an increased susceptibility to oxidative stress-induced intestinal tumorigenesis (34). The data we present here show, however, that this relationship is not straightforward. Lymphomas originating in MMR-defective mice are generally explained by accumulation of genetic events underlying lymphocyte malignant transformation and aberrant expression of some proto-oncogenes (RB1N-2, TAL1, and HOX-11) has been identified both in lymphoblastic lymphomas of Msh2−/− mice and in human T-cell neoplasms (7). Msh2−/− and Msh2−/−/Mutyh−/− mice kept in our animal facility developed lymphoblastic lymphomas with histologic features similar to those previously reported (predominantly of T-cell origin; refs. 3, 7). However, frequently, their lineage was not thoroughly investigated. The immunophenotype of lymphomas arising in Msh2−/− and Msh2−/−/Mutyh−/− mice is identical in both genotypes and the lymphomagenesis involved B-committed cells. In at least five cases (characterized by PAX-5 positivity in the absence of or with low levels of B220), the transformation events probably occurred at early stages in B-cell differentiation (preceding pre-pro-B or at pre-pro-B stage) when the first immunoglobulin gene rearrangement begins. These data suggest that Msh2 deficiency leads to an incorrectly regulated T- and B-cell receptor gene rearrangement resulting in tumorigenesis. This is in agreement with previous reports and the recent observation indicating that a lack of MMR

#### Table 2. Immunohistochemistry and histopathologic classification of lymphoid tumors in Msh2−/− and Msh2−/−/Mutyh−/− mice

<table>
<thead>
<tr>
<th>Mouse ID Cage (mouse no.)</th>
<th>Organ</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>B220</th>
<th>PAX-5</th>
<th>Tumor type</th>
<th>Tumor latency (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Msh2−/−</td>
<td>182 (0) Lymph nodes, spleen, thymus</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>Lymphoblastic B-cell lymphoma</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>86 (0) Lymph nodes, liver, thymus, gut</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>Lymphoblastic B-cell lymphoma</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>24 (3) Lymph nodes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>Lymphoblastic B-cell lymphoma</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>20 (4) Lymph nodes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Lymphoblastic B-cell lymphoma</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>75 (1) Lymph nodes, thymus</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/-</td>
<td>Lymphoblastic B-cell lymphoma</td>
<td>173</td>
</tr>
<tr>
<td>Msh2−/−/Mutyh−/−</td>
<td>48 (0) Lymph nodes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>Lymphoblastic B-cell lymphoma</td>
<td>236</td>
</tr>
<tr>
<td></td>
<td>4 (0) Lymph nodes, salivary glands, thymus</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>Lymphoblastic B-cell lymphoma</td>
<td>258</td>
</tr>
<tr>
<td></td>
<td>47 (1) Lymph nodes</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Lymphoblastic B-cell lymphoma</td>
<td>328</td>
</tr>
</tbody>
</table>

NOTE: −, negative staining in the tumor; +, positive staining in the tumor; +/-, <20% of cell staining positive in the tumor.


Role of MUTYH and MSH2 in the Control of Oxidative DNA Damage, Genetic Instability, and Tumorigenesis

Maria Teresa Russo, Gabriele De Luca, Ida Casorelli, et al.


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