Research Paper

Mismatch Repair Deficient Mice Show Susceptibility to Oxidative Stress-Induced Intestinal Carcinogenesis

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Abstract

We have previously established an experimental system for oxidative DNA damage-induced tumorigenesis in the small intestine of mice. To elucidate the roles of mismatch repair genes in the tumor suppression, we performed oxidative DNA damage-induced tumorigenesis experiments using *Msh2*-deficient mice. Oral administration of 0.2% Potassium Bromate, $KBrO_3$, effectively induced epithelial tumors in the small intestines of *Msh2*-deficient mice. We observed a 22.5-fold increase in tumor formation in the small intestines of *Msh2*-deficient mice compared with the wild type mice. These results indicate that mismatch repair is involved in the suppression of oxidative stress-induced intestinal tumorigenesis in mice. A mutation analysis of the *Ctnnb1* gene of the tumors revealed predominant occurrences of G:C to A:T transitions. The TUNEL analysis showed a decreased number of TUNEL-positive cells in the crypts of small intestines from the *Msh2*-deficient mice compared with the wild type mice after treatment of $KBrO_3$. These results suggest that the mismatch repair system may simultaneously function in both avoiding mutagenesis and inducing cell death to suppress the tumorigenesis induced by oxidative stress in the small intestine of mice.

Key words: HNPCC, oxidative DNA damage, Wnt signaling pathway, mutagenesis, cell death

Introduction

Reactive oxygen species (ROS) are generated by the normal cellular metabolism and also by exposure to environmental factors, such as radiation and chemicals. ROS constantly induce various lesions in the DNA of living organisms under physiological conditions, and the resulting DNA damage causes mutations and cell death, leading to aging-associated diseases, such as cancer and neurodegeneration (1). Among the various types of oxidative DNA damage, oxidized guanine, 8-oxo-7, 8-dihydroguanine (8-oxoG) is abundant and highly mutagenic because of its ambiguous base-pairing properties; it can be paired with adenine as well as with cytosine (2-6). Therefore, 8-oxoG in DNA causes G:C to T:A transversions after two rounds of DNA replication. In

mammalian cells, the base excision repair (BER) pathway initiated by OGG1 or MUTYH plays a role in the suppression of 8-oxoG-related mutagenesis. OGG1, an 8-oxoG DNA glycosylase, excises the 8-oxoG paired with cytosine from DNA (7, 8). MUTYH is an adenine DNA glycosylase that excises the adenine incorporated opposite 8-oxoG from DNA (9-12). The synergistic actions of OGG1 and MUTYH suppress the mutagenesis caused by 8-oxoG in DNA.

In addition to DNA repair, a nucleotide pool sanitizing enzyme, MTH1, suppresses the mutagenesis induced by oxidative stress (13, 14). This enzyme can hydrolyze oxidized purine nucleotides, such as 8-oxo-dGTP, 2-OH-dATP and 8-OH-dATP, to prevent the incorporation of mutagenic nucleotides into DNA

during replication (15, 16). In addition to 8-oxoG, a wide variety of oxidatively modified bases in DNA were removed by the BER pathway following initiation by various DNA glycosylases (17).

Besides BER, mismatch repair (MMR) is also involved in the repair of oxidative DNA damage. MMR is an evolutionarily conserved system that corrects replication errors such as mismatched bases and small insertions/deletions. The MSH2/MSH6 heterodimer (MutSα) and MSH2/MSH3 heterodimer (MutSβ) recognize mismatched bases and small insertions/deletions, respectively, and then recruit MutLα (MLH1 and PMS2 heterodimer) to initiate the MMR reaction (18). In addition to its role in correcting replication errors, MMR is known to involve in the induction of apoptosis in response to DNA lesions caused by alkylating agents (19-21). The human MMR genes are associated with hereditary non-polyposis colorectal cancer (HNPCC), which is a common cancer predisposition syndrome characterized by a dominant mode of transmission and high penetrance (22-25). deWeese *et al* reported that *Msh2*-deficient mouse embryonic stem (ES) cells showed the accumulation of oxidative DNA damage, such as 8-oxoG and thymine glycol, in their genomes, as well as tolerance to apoptosis caused by low dose gamma-ray irradiation (26). Based on the analysis of spontaneous mutational specificity of mice defective in the *Mth1* and/or the *Msh2* genes, we speculated that MMR might act to correct mispairs with the oxidized nucleotides (27). Furthermore, Colussi *et al*, and Russo *et al* reported that MMR could suppress the mutations caused by the incorporation of oxidized purine deoxynucleoside triphosphate (28, 29). These findings suggest the involvement of MMR in the suppression of oxidative stress-induced mutagenesis and tumorigenesis in mammals.

KBrO$_3$ is an oxidizing agent that is known to induce 8-oxoG in the DNA of rats and mice, and is recognized as a renal carcinogen in rats (30-33). We previously established an experimental system for oxidative DNA damage-induced tumorigenesis in the intestinal tract of mice using this agent (34). The oral administration of KBrO$_3$ for 16 weeks effectively induced epithelial tumors in the small intestines of *Mutyh*-deficient mice, indicating the significance of Mutyh in the suppression of tumorigenesis induced by oxidative stress (34).

In this study, we performed KBrO$_3$-induced tumorigenesis experiments using *Msh2*-deficient mice to elucidate the roles of MMR in the suppression of oxidative stress-induced tumorigenesis. We found that MMR plays a significant role in the suppression of oxidative stress-induced intestinal tumorigenesis in mice.

Materials and Methods

Animals

The *Msh2*-deficient mice used in this study were generated as reported previously (27). The wild type, heterozygous and homozygous mutant mice used in this study were obtained by intercrossing the heterozygous mutant mice. All animals were maintained under specific pathogen free (SPF) conditions. All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of Kyushu University, and followed the Guideline for Proper Conduct of Animal Experiments, Science Council of Japan.

KBrO$_3$ treatment

KBrO$_3$ (Sigma-ALDRICH) was given to 6~8-week-old mice in their drinking water at a concentration of 0.2% for 16 weeks. The body weight and consumption of drinking water were measured every week. After 16 weeks, all the animals were sacrificed, and the intestines were fixed with 10% phosphate buffered-formalin, and then stored in 70% ethanol.

Histological analysis

The inspections for tumor formation in the intestinal mucosa were carefully performed under a dissecting microscope. The small intestinal tumors were carefully removed from intestines, embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin for the diagnosis of the tumors. The evaluation of the tumors was performed according to the Vienna classification (35).

Mutation analysis of the *Ctnnb1* gene

The small intestinal tumors were carefully removed from the mucosa under a dissecting microscope. Genomic DNA was extracted using a DNeasy Tissue Kit (QIAGEN) according to the manufacturer's protocol. Eighty-nine small intestinal tumors obtained from five *Msh2*-deficient mice were analyzed for mutations in the *Ctnnb1* (*β-catenin*) gene. Thirty to fifty nanograms of genomic DNA extracted from each small intestinal tumor was used as the template for PCR with rTaq DNA polymerase (TaKaRa). The entire coding sequence of the second exon of the *Ctnnb1* gene was amplified using primers 5'-TCCTTGGCTGCCTTTCTAACAGTA-3' (upper) and 5'-GCATGCCCTCATCTAGCGTCT-3' (lower). Amplified DNA containing exon 2 of the *Ctnnb1* gene was purified with a PCR purification kit (QIAGEN) according to the manufacturer's protocol. The purified DNA fragments were used as a template for direct sequencing with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and the se-

quences were determined with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

TUNEL analysis

The intestines were removed from the wild type and mutant mice treated with $KBrO_3$ for 16 weeks, and 3 μm sections were made after the samples were embedded in paraffin. We analyzed the cell death (apoptosis) in the crypts of the small intestine using a TUNEL kit (TaKaRa) as described in the manual supplied by the manufacturer. We counted the TUNEL-positive cells in more than 100 crypts from five mice of each genotype.

Results

Tumor formation induced by $KBrO_3$ treatment in *Msh2*-deficient mice

In order to examine whether oxidative stress increases the intestinal tumorigenesis in mismatch repair-deficient mice, congenic wild type, heterozygous and homozygous *Msh2*-deficient (5, 6 and 7 animals for each genotype, respectively) mice were administered 0.2% $KBrO_3$ in their drinking water for 16 weeks. At the same time, five mice of each genotype were kept under the same conditions except for the $KBrO_3$-treatment. As previously observed, the $KBrO_3$-treatment appeared to cause a slowdown in the increase of body weight at almost the same rate in all groups of animals during the period of $KBrO_3$-treatment. We dissected the mice after the 16-week treatment with $KBrO_3$, and inspected the intestines under a dissecting microscope. In the homozygous *Msh2*-deficient mice treated with $KBrO_3$, the formation of small intestinal tumors was dramatically increased (Figure 1, Table 1). The mean number of tumors induced in the small intestines of the seven *Msh2*-deficient mice was 27.0, whereas it was 1.2 and 1.5 in the five wild type and six heterozygous mice, respectively. Tumor formation was also observed in the untreated homozygous *Msh2*-deficient mice, albeit at a much lower frequency (mean: 1.2 tumor/mouse, n=5) compared with the treated homozygous mice. As previously observed in the *Mutyh*-deficient mice (34), the $KBrO_3$-induced tumors predominantly developed in the duodenum and in the upper region of the jejunum (Figure 1A). We found no other anomalies in the $KBrO_3$-treatment mice.

Table 1. Tumor formation in the intestine of *Msh2*-deficient mice

Genotype	No treatment	$KBrO_3$-treatment	
	No. of tumors[a]	No. of tumors[a]	Ratio[b]
Wild type	0	1.20 ± 0.98	1.00
Heterozygote	0	1.50 ± 1.26	1.25
Homozygote	1.20 ± 0.75	27.00 ± 7.44	22.50

a: The no. of tumors is the mean number of tumors per mouse, with the standard deviation. b: ratio to tumors in wild type mice
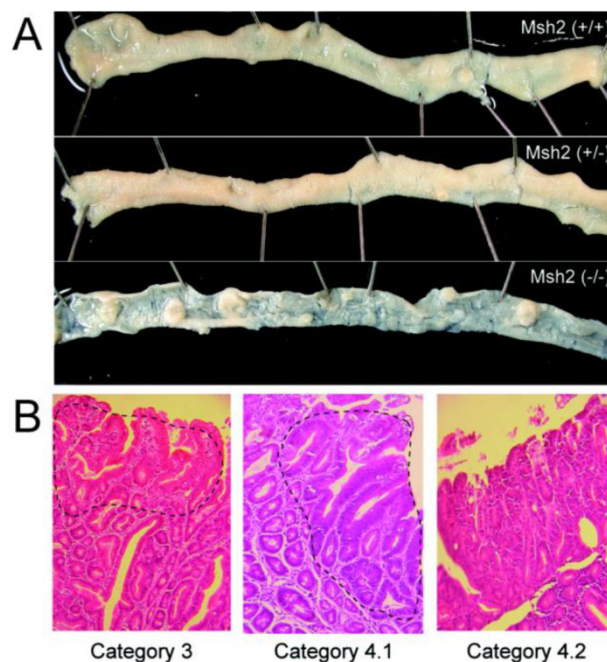
Figure 1. $KBrO_3$-induced tumors in the small intestine of *Msh2*-deficient mice. A. The proximal regions of the small intestines of $KBrO_3$-treated mice are shown; (+/+): wild type, (+/-): heterozygous *Msh2*-deficient, and (-/-): homozygous *Msh2*-deficient mice. Multiple polyp formations could be observed in the $KBrO_3$-treated homozygous *Msh2*-deficient mice. **B.** A section of a $KBrO_3$-induced tumor stained with hematoxylin and eosin (original magnification: objective 10X). The regions containing the neoplasia are encircled by a broken line.

Pathological analysis of tumors induced by $KBrO_3$ in the small intestines

We performed a pathological analysis of 25, three and two small intestinal tumors derived from five homozygous-deficient, three heterozygous *Msh2*-deficient mice and two wild type mice, respectively, according to the Vienna classification of gastrointestinal epithelial neoplasia (Table 2, Figure 1B). All tumors from homozygous *Msh2*-deficient mice were classified as category 4 (non-invasive high grade neoplasia), except for one case that was classified as category 3 (non-invasive low grade neoplasia). All tumors from wild type and heterozygous *Msh2*-deficient mice were also classified as category 4 (Table 2).

Table 2. Classification of $KBrO_3$-induced small intestinal tumors in mice

| Genotype	Category 3*	Category 4*			Total
		4.1	4.2	4.3	
Wild type	—	—	2	—	2
Heterozygote	—	—	3	—	3
Homozygote	1	3	21	—	25

*Tumors were categorized according to the Vienna classification of gastrointestinal epithelial neoplasia

Mutation analysis of the *Ctnnb1* gene

The β-catenin protein encoded by *Ctnnb1* gene is a transcriptional activator functioning in the Wnt-signaling pathway (36). The phosphorylation of β-catenin by GSK3β in a complex with Axin and Apc is required for the ubiquitin-mediated degradation of β-catenin. Therefore, the presence of mutations affecting the phosphorylation of the protein lead to its stabilization and the accumulation of β-catenin in nuclei, inducing the expression of target genes such as c-myc and cyclin D1 without Wnt signaling. The mutations at four putative GSK3β-phosphorylation sites (S33, S37, T41, S45) and amino acids adjacent these sites have been detected in a wide variety of human cancers including HNPCC, as well as in chemically-induced tumors in model animals. Therefore, we analyzed the mutations in exon 2 of the *Ctnnb1* gene encoding the GSK3β-phosphorylation sites of β-catenin. Among 89 tumors from five homozygous *Msh2*-deficient mice, 27 tumors (30.3%) showed a mutation in this region (Figure 2, Table 3). All the mutations were base substitutions and occurred at or in the vicinity of the codons for S33, S37 and T41. No mutations were observed at the codon for S45. Among them, G:C to A:T transitions predominantly occurred; 20 mutations (74.1%) were identified as G:C to A:T transitions, and the others were three A:T to G:C transitions (11.1%), two G:C to T:A transversions and two G:C to C:G transversions (7.4%, respectively). There is no clear hotspot for G:C to A:T transitions in this region, with there being seven at D32, five at S37, four at G34 and four at T41. However, besides one G:C to T:A transversion at S33, three other types of mutations were observed only at the codon for S37. The base substitutions observed at the codon for S37 were as follows; five G:C to A:T transitions, three A:T to G:C transitions, two G:C to C:G transversions and one G:C to T:A transversion.
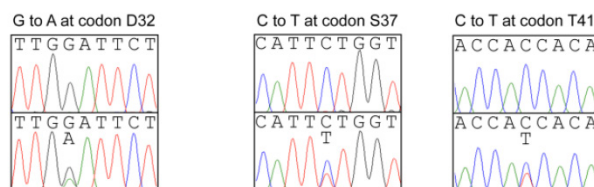

Figure 2. Somatic mutations found in the *Ctnnb1* gene of tumors. The amino acid sequence and the corresponding nucleotide sequence of GSK3β phosphorylation sites are shown at the top. The amino acids at phosphorylation sites are depicted in bold. The somatic mutations found in the KBrO$_3$-induced intestinal tumors are shown below the nucleotide sequence of the *Ctnnb1* gene; upper and lower panels show the nucleotide sequencing results from normal tissues and tumors, respectively.

Table 3. Mutations found in the *Ctnnb1* gene

Mouse ID	Sample ID	Nucleotide position	Wild type	Mutant	Mutation	Amino acid change
22	1	94	GAT	AAT	G:C→A:T	D32N
38	1	109	TCT	CCT	A:T→G:C	S37P
	4	109	TCT	CCT	A:T→G:C	S37P
	5	122	ACC	ATC	G:C→A:T	T41I
	7	101	GGA	GAA	G:C→A:T	G34E
	8	110	TCT	TAT	G:C→T:A	S37Y
	12	94	GAT	AAT	G:C→A:T	D32N
	13	100	GGA	AGA	G:C→A:T	G34R
	18	110	TCT	TTT	G:C→A:T	S37F
	20	110	TCT	TTT	G:C→A:T	S37F
	21	122	ACC	ATC	G:C→A:T	T41I
	22	94	GAT	AAT	G:C→A:T	D32N
48	4	110	TCT	TGT	G:C→C:G	S37C
	6	110	TCT	TTT	G:C→A:T	S37F
	10	94	GAT	AAT	G:C→A:T	D32N
	15	100	GGA	AGA	G:C→A:T	G34R
	22	109	TCT	CCT	A:T→G:C	S37P
45	1	122	ACC	ATC	G:C→A:T	T41I
	3	110	TCT	TGT	G:C→C:G	S37C
	9	110	TCT	TTT	G:C→A:T	S37F
	10	94	GAT	AAT	G:C→A:T	D32N
50	5	122	ACC	ATC	G:C→A:T	T41I
	8	100	GGA	AGA	G:C→A:T	G34R
	12	94	GAT	AAT	G:C→A:T	D32N
	14	94	GAT	AAT	G:C→A:T	D32N
	16	110	TCT	TTT	G:C→A:T	S37F
	24	98	TCT	TAT	G:C→T:A	S33Y

A

Wild type   Msh2

B
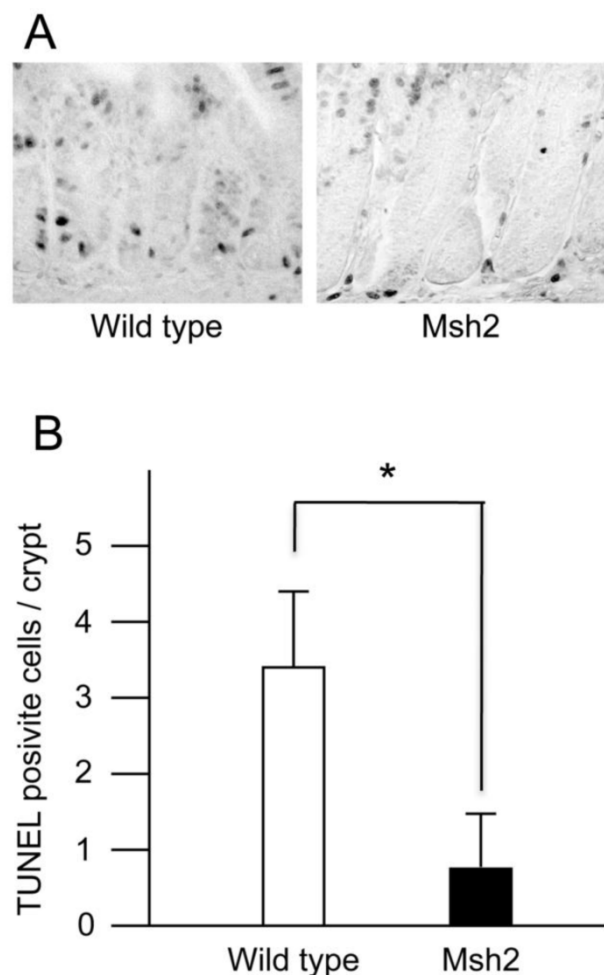
Figure 3. TUNEL-positive crypt cells in the small intestines from KBrO₃-treated mice. ***A.*** The sections stained with TUNEL. The crypts of small intestines from wild type (left) and *Msh2*-deficient (right) mice treated with KBrO₃. ***B.*** The number of TUNEL-positive cells in the crypts. The mean numbers of TUNEL-positive cells with standard deviations are indicated by white (wild type mice) and black (homozygous *Msh2*-deficient mice) bars. * $p < 0.002$ (Student's *t*-test).

Analysis of cell death

MMR is involved in the signaling for cell death induced by genotoxic chemicals, such as alkylating agents (19-21). A previous study showed that ES cells carrying disrupted *Msh2* alleles displayed an increased survival following exposure to low-level ionizing radiation compared with wild type ES cells (26). The increased survival could be attributed to a failure of the cells to efficiently execute apoptosis in response to oxidative DNA damage induced by radiation exposure. These findings suggested that MMR is involved in the induction of apoptosis caused by oxidative DNA damage. It has been shown that intestinal cancer originates in the stem cells resided in the bottom of intestinal crypts (37). Thus, in the present study, we analyzed the cell death in the crypts of small intestines from wild type and *Msh2*-deficient

mice treated with KBrO₃ using a TUNEL method. A few TUNEL-positive cells were detected in the crypts of wild type mice, but not in those of *Msh2*-deficient mice (Figure 3A). We counted the TUNEL-positive cells in more than 100 crypts from each genotype of mice. The average numbers of crypts per mouse were as follows: wild type, 21.2 (min 14, max 31) and *Msh2*-deficient, 21.6 (min 17, max 30). We found that 3.36 ± 0.96 (mean $\pm$ SD) TUNEL-positive cells per crypt were present in wild type mice, while 0.80 ± 0.46 (mean $\pm$ SD) TUNEL-positive cell per crypt were present in *Msh2*-deficient mice (Figure 3B). This difference was statistically significant ($p < 0.002$; *t*-test).

Discussion

In the present study, we performed KBrO₃-induced tumorigenesis experiments using *Msh2*-deficient mice to examine the involvement of mismatch repair (MMR) in the suppression of oxidative stress-induced tumorigenesis. The oral administration of KBrO₃ at a dose of 0.2% in drinking water dramatically increased the formation of intestinal tumors in *Msh2*-deficient mice compared to untreated *Msh2*-deficient mice and treated wild type mice. Thus, we concluded that MMR plays a significant role preventing the intestinal tumorigenesis induced by oxidative stress in mice.

Several lines of evidence suggest that oxidative stress could be generated in the intestines of animals under physiological conditions. For example, it was reported that the incidence of G:C to T:A transversions increases significantly in the intestines of older mice compared with younger mice (38). Because G:C to T:A transversions are mainly caused by 8-oxoG, a major oxidative DNA damage, these observations indicate that the mutations caused by oxidative DNA damage would tend to accumulate in the intestines during the course of aging. Consistent with this notion, defects in MUTYH, the human DNA glycosylase suppressing 8-oxoG-induced mutagenesis, lead to a susceptibility to colorectal cancers with excess G:C to T:A transversions in humans (39). Furthermore, *Mutyh*-deficient mice also show susceptibility to spontaneous and KBrO₃-induced intestinal adenoma/carcinoma (34). Therefore, based on these previous observations and our present results, it is likely that on a MMR-defective genetic background, oxidative stress generated in the intestine may enhance tumor development, thus leading to HNPCC in humans.

The mutation analyses of the tumor-related gene, *Ctnnb1*, revealed that more than 30% of the tumors that developed in KBrO₃-treated *Msh2*-deficient mice had somatic mutations in the coding region for GSK3β phosphorylation sites. All the mutations detected

were base substitutions; 20 G:C to A:T transitions (74.1%), three A:T to G:C transitions (11.1%), two G:C to T:A transversions and two G:C to C:G transversions (7.4%). These observations are consistent with the results obtained from mutation analyses of the *CTNNB1* gene in cancers from HNPCC patients; 12 reported base substitutions were as follows: TCT to TTT, CCT, or TGT at codon S45; ACC to GCC at codon T41; TCT to TGT at codon S37; GGA to GAA at codon G34; GAC to GGC or TAC at codon D32 (40). The G:C to A:T transition at codon G34 and G:C to C:G transversion at codon S37 were commonly observed in both human and mouse tumors. The difference observed between human and mouse mutation spectra may due to the different nucleotide sequence context in this locus of these two species. The similarity of the mutation types and spectra suggests that the KBrO$_3$-treatment of mice may mimic the oxidative stress in human to induce DNA damage in the intestine. We detected only two G:C to T:A transversions, thus suggesting that the DNA repair enzymes, including Ogg1 and Mutyh, may suppress 8-oxoG-related mutagenesis to some extent in KBrO$_3$-treated *Msh2*-deficient mice. Besides the one G:C to T:A transversion at the codon for S33, three types of mutations other than the G:C to A:T transition were observed at only the codon for S37; three A:T to G:C transitions, two G:C to C:G transversions, and one G:C to T:A transversion. Because these types of mutations were suppressed by the overexpression of MTH1, which hydrolyzes oxidized purine nucleotide triphosphates, in *MSH2*-deficient human cells (28, 29), the nucleotide sequence context around the codon for S37 might be competent to enhance the incorporation of oxidatively-damaged purine nucleotide triphosphates by DNA polymerase.

MMR factors are well known to be involved in the induction of apoptosis caused by O[6]-methylguanine (O[6]-mG), a type of DNA damage produced by alkylating agents (19-21). O[6]-mG can pair with thymine during DNA replication, forming O[6]-mG:T mispairs. MutSα recognizes this mispair and forms a complex with MutLα and PCNA to induce apoptosis (20). The *Msh2*-deficiency also caused a failure to induce apoptosis effectively in response to oxidative DNA damage (26). Thus, in the present study, we analyzed the cell death of the crypts of small intestines from wild type and *Msh2*-deficient mice treated with KBrO$_3$ using a TUNEL assay. A larger number of TUNEL-positive cells were observed in the crypts from wild type mice compared with *Msh2*-deficient mice, suggesting that the crypt cells with MMR-deficiencies showed an increased chance of surviving protracted exposure to KBrO$_3$. The better survival of MMR-deficient cells with pre-mutagenic

lesions in the genome induced by oxidative stresses may contribute to the increased cancer risk characteristic of the hereditary non-polyposis colorectal cancer syndrome.

In conclusion, we herein demonstrated that oxidative stress enhanced the tumor formation in the small intestines of *Msh2*-deficient mice, thereby providing experimental evidence of the association between oxidative stress and hereditary non-polyposis colorectal cancer caused by MMR-deficiency in humans. We propose that MMR suppresses spontaneous tumorigenesis in the intestines of mammals by simultaneously preventing the occurrence of mutations and the removal of precancerous cells containing pre-mutagenic oxidative lesions in the genome.

Acknowledgements

Competing Interests

The authors have declared that no competing interest exists.

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