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Abstract Oxygen radicals generated through normal cellular metabolism induce a variety of types of oxidative damage into DNA and its precursors. Among such types of oxidative damage, 7, 8-dihydro-8-oxoguanine (8-oxoG), an oxidized form of guanine, is known to be abundant and highly mutagenic. 8-OxoG can pair with adenine as well as cytosine, thus causing G: C to T: A transversions after DNA replication, if not repaired. Organisms are equipped with elaborate systems to avoid such mutations caused by 8-oxoG. In *Escherichia coli*, two DNA glycosylases have been identified to suppress these mutations. One is MutM, an 8-oxoguanine DNA glycosylase that removes 8-oxoG from 8-oxoG: C base pairs. The other is MutY, an adenine DNA glycosylase that excises adenine from 8-oxoG: A mismatches. Mammals also have such DNA glycosylases; OGG1 (Ogg1) is the functional counterpart of MutM, and MUTYH (Mutyh) is the MutY homologue. In order to investigate the roles of these two enzymes in the avoidance of 8-oxoG-related mutagenesis in mammals, we analyzed spontaneous mutagenesis in the small intestine of Ogg1-deficient (*Ogg1<sup>-/-</sup>* and Ogg1<sup>-/-</sup>), Mutyh<sup>-/-</sup> double deficient (*Ogg1<sup>-/-</sup>; Mutyh<sup>-/-</sup>) mice at the age of 4 ~ 5 weeks using the prokaryotic *rpsL* transgene as a reporter. The observed mutation frequency was 1.00 x 10<sup>-5</sup> in both wild type and *Ogg1<sup>-/-</sup>* mice, and 1.91 x 10<sup>-5</sup> in *Ogg1<sup>-/-</sup>; Mutyh<sup>-/-</sup> mice, indicating that the overall spontaneous mutation frequency was increased in *Ogg1<sup>-/-</sup>; Mutyh<sup>-/-</sup> mice, but not in *Ogg1<sup>-/-</sup>* mice. Analysis of the mutation spectrum revealed that the frequency of G: C to T: A transversions were significantly increased in both *Ogg1<sup>-/-</sup>* and *Ogg1<sup>-/-</sup>; Mutyh<sup>-/-</sup> mice; a 5-fold increase in *Ogg1<sup>-/-</sup>* mice, and a 41-fold increase in *Ogg1<sup>-/-</sup>; Mutyh<sup>-/-</sup> mice when compared with wild type mice. A previous study in our laboratory indicated that a defect in Mutyh caused a 4-fold increase in the frequency of G: C to T: A transversions in mice. Combined, these observations suggest that a cooperative function between Ogg1 and Mutyh exists to prevent 8-oxoG-related mutagenesis in mammals.

Introduction

In order to maintain species, as well as individuals, the genetic information contained in DNA must be accurately copied and transmitted to each daughter cell. However, DNA molecules are susceptible to alteration caused by a wide variety of environmental and endogenous compounds. Reactive oxygen species (ROS) may be generated by ionizing radiation in the environment, or endogenously by normal cellular metabolism or the metabolism of several chemical carcinogens<sup>3</sup>. ROS oxidize DNA and introduce various modifications in the bases of DNA<sup>7,39</sup>. Among the modified bases, 8-oxo-7, 8-dihydroguanine (8-oxoG) is most abundant, and seems to play a critical role in carcinogenesis and aging<sup>4</sup>. 8-OxoG can pair with both cytosine and adenine during DNA synthesis, and as a result, G: C to T: A transversions
are induced\(^5\). Oxidation of nucleotides also occurs in the cellular nucleotide pool\(^6\). 8-Oxo-dGTP, an oxidized form of dGTP, is a mutagenic substrate for DNA synthesis; it is equally incorporated opposite adenine and cytosine in DNA, resulting in both A: T to C: G and G: C to T: A transversions\(^6\). In addition, another mutagenic oxidative substrate, 2-OH-dATP can be incorporated opposite guanine during DNA replication, causing G: C to T: A transversions\(^9\).

Studies on mutator mutants of *Escherichia coli* revealed that three enzymes, encoded by the *MutT*, *MutM* and *MutY* genes, play important roles in avoiding 8-oxoG-related mutagenesis\(^10\). *MutT* hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP and pyrophosphate, thus preventing the incorporation of 8-oxo-dGTP into DNA during replication\(^6\). *MutM*, originally identified as a formamidopyrimidine DNA glycosylase, removes 8-oxoG paired with cytosine\(^11\)\(^{12}\). *MutY* is an adenine DNA glycosylase that excises adenine paired with guanine or 8-oxoG\(^13\)\(^{14}\). The functional cooperation of these three enzymes prevents mutations caused by 8-oxoG in bacteria\(^10\).

In mammalian cells, similar enzymatic activities have been found, suggesting that similar systems are employed to avoid 8-oxoG-related mutagenesis in mammals\(^15\)\(^{18}\). MTH1 (Mth1) and MUTYH (Mutyh) have been identified as a human (mouse) *MutT* homolog\(^19\)\(^{20}\) and *mutY* homolog\(^21\)\(^{22}\), respectively. MUTYH has been reported to excise 2-OH-A, in addition to adenine, paired with either guanine or 8-oxoG\(^23\). There is no *MutM* homolog in the human or the mouse genome. However, an ortholog for yeast 8-oxoG DNA glycosylase, Ogg1, a functional counterpart of *E. coli* MutM, has been identified in humans and mice\(^24\)\(^{30}\). Mice mutant for each of these genes have been generated using gene targeting techniques\(^22\)\(^{31}\)\(^{33}\)\(^{34}\). *Mth1*\(^{-/-}\) ES cells demonstrated a 2-fold increase in spontaneous mutation rates as compared with parental cell lines, and *Mth1*\(^{-/-}\) mice were found to be susceptible to spontaneous tumorigenesis\(^39\). Overall spontaneous mutation frequencies in *Mth1*\(^{-/-}\) mice were not significantly different from that of wild type mice\(^39\), however, in a mismatch repair system deficient background, a significant increase in the frequency of G: C to T: A transversions was observed\(^35\). *Ogg1*\(^{-/-}\) mice showed a greater extent of accumulation of 8-oxoG in genomic DNA and an elevated spontaneous mutation frequency\(^31\)\(^{32}\)\(^{33}\)\(^{34}\). Using the *gpt* transgene to analyze spontaneous mutations, it has been reported that the mutation frequency in the liver of *Ogg1*\(^{-/-}\) mice showed a 2.3-fold increase at the age of 16 ~ 20 weeks when compared with wild-type mice, and that G: C to T: A transversions were predominantly detected in *Ogg1*\(^{-/-}\) mice\(^32\). Sakumi *et al*\(^34\) revealed that *Ogg1*\(^{-/-}\) mice were highly susceptible to spontaneous lung adenoma and carcinoma. *Mutyh*\(^{-/-}\) ES cells showed a 2-fold increase in spontaneous mutation rates as compared with parental cell lines\(^22\). We have examined the tumorigenesis and mutagenesis in *Mutyh*\(^{-/-}\) mice. Sakamoto *et al* found that *Mutyh*\(^{-/-}\) mice showed a marked increase of susceptibility to intestinal adenoma and carcinoma as well as splenic angiosarcoma and hepatic hemangioma (manuscript in preparation). Yamauchi *et al* analyzed spontaneous mutations which occurred in the *rpsL* transgene of *Mutyh*\(^{-/-}\) mice. The frequency of G: C to T: A transversions was significantly increased in *Mutyh*\(^{-/-}\) mice at 24 weeks of age,
when compared with wild-type mice (manuscript in preparation). These data indicate that Mth1, Oggl and Mutyh play roles in the suppression of mutagenesis and tumorigenesis spontaneously caused by oxidative stress in mammals.

It has been shown that the cooperative action of MutM and MutY effectively suppresses 8-oxoG-related mutagenesis in E. coli\(^2\) \(^1\). In mutY mutM double mutants of E. coli, the rate of G: C to T: A transversions is 20 times higher than the sum of the mutation rates detected in each of the single mutant strains. Based on observed enzymatic similarities, it is logical to assume that a cooperative action between Oggl and Mutyh may be observed in mice. Furthermore, significant increases in the mutation frequency in Oggl\(^{−/−}\); Mutyh\(^{−/−}\) mice at the age of 4~5 weeks were analyzed using the bacterial \(rpsL\) transgene.

Materials and Methods

1. Mice

\(Oggl^{+/−}\) mice and \(Mutyh^{+/−}\) mice were previously established\(^2\) \(^3\) \(^4\). The heterozygotes were backcrossed with C57BL / 6J for over 10 generations to establish congenic lines with C57BL / 6J genetic backgrounds. The \(rpsL\) transgenic C57BL / 6J mouse line was used to analyze mutagenesis in mice\(^5\). \(Oggl^{+/−}\) mice or \(Mutyh^{+/−}\) mice were mated with a \(rpsL\) transgenic mice (\(rpsL\) hemizygotes) to produce \(Oggl^{+/−}\) or \(Mutyh^{+/−}\) mice with a hemizygous \(rpsL\) gene. To obtain \(Oggl^{+/−}\); \(Mutyh^{+/−}\) mice with a hemizygous \(rpsL\) transgene, we prepared mating pairs of combinations of \(Oggl^{+/−}\) mice with a hemizygous \(rpsL\) transgene and \(Mutyh^{+/−}\) mice or combinations of \(Oggl^{+/−}\) mice and \(Mutyh^{+/−}\) mice with hemizygous \(rpsL\) transgene. In order to obtain the mice for analysis, \(Oggl^{+/−}\); \(Mutyh^{+/−}\) mice with a hemizygous \(rpsL\) transgene were crossed with \(Oggl^{+/−}\); \(Mutyh^{+/−}\) mice. For the analysis of spontaneous mutations, two mice with each genotype (wild-type, \(Oggl^{−/−}\), and \(Oggl^{+/−}\); \(Mutyh^{−/−}\)) were sacrificed at the age of 4~5 weeks. Mice were maintained under specific pathogen-free conditions at the Station for Collaborative Research, Kyushu University. All experiments were carried out in accordance with the Guidelines for Animal Experiments of the Faculty of Medical Sciences, Kyushu University.

2. Genotyping

To determine the genotype of Oggl-deficient mice, polymerase chain reaction (PCR) was performed with the following primers: OG3 : 5’-GAAGGACTGTCCAGAAGCTA-3’ (20-mer), OG5 : 5’-GTTAAGCTTCAACGTGCCTC-3’ (21-mer), OGN : 5’-AAAGTCTCTCATTTAGTATCC-3’ (22-mer). PCR was performed at 94 °C for 1 min and 35 cycles of [94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 5 min]. For PCR determination of the Mutyh genotype: MY3 : 5’-GCAGTAGACACAGCTGCA T-3’ (19-mer), MY5 : 5’-GTTAAGCTTCAACGTGCCTC-3’ (21-mer), OGN : 5’-AAAGTCTCTCATTTAGTATCC-3’ (22-mer). PCR was performed at 94 °C for 1 min and 35 cycles of [94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 40 sec] and 72 °C for 5 min. For PCR determination of the \(Mutyh\) genotype: MY3 : 5’-GCAGTAGACACAGCTGCA T-3’ (19-mer), MY5 : 5’-CCTGTCGCAAAGCTGCTGA-3’ (18-mer), MYN : 5’-CTACGTCAGATTAAAGG-3’ (20-mer) were used. PCR was performed at 94 °C for 1 min and 38 cycles of [94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec] and 72 °C for 5 min. PCR products were analyzed by electrophoresis on a 2 % agarose
gelled in TAE buffer (40 mM Tris, 40 mM glacial acetic acid, 1 mM EDTA) at 100 V for 35 min. The agarose gel was stained with ethidium bromide and the DNA fragments were visualized under ultraviolet light (Fig. 1).

3. rpsL mutation assay
Genomic DNA was extracted from small intestine samples according to the QIAGEN 'Genomic DNA Handbook For Tissue'. The small intestine was homogenized in QIAGEN-G2 buffer containing RNaseA. The homogenate was incubated with QIAGEN protease at 50 °C for 2 hrs to prepare a lysate. The lysate was loaded onto the QIAGEN genome tip. After isopropanol precipitation, the DNA pellet was dissolved in TE buffer.

Genomic DNA (10 µg) was digested with 35.2 units of Ban II (Takara, Kyoto) at 37 °C for 2.5 hrs in a 200 µl reaction volume. To circularize the plasmid DNA containing the rpsL gene (pSSW), 5 µg of the DNA digested with Ban II was treated with 350 units of T4 DNA ligase (Takara, Kyoto) at 16 °C for 2 hrs in a 600 µl reaction mixture. After each reaction step, DNA was extracted once with phenol / chloroform, twice with chloroform, and then precipitated with isopropanol.

DNA pellets were dissolved in 0.1 x TE buffer and introduced into E. coli DH10B (F- mcrA (mrr-hsdRMS-mcrBC) φ80 lacZ ΔM15 ΔlacX74 deoR recA1 endA1 ara D139 Δ(arə, lev) 7697 galU galK Δ− rpsA1 nupG) by electroporation with a MicroPulser™ (BIO-RAD) according to the manu-

Fig. 1 Genotyping of mice analyzed in this study
(A) Schematic representation of the targeting of the Ogg1 and Mutyh genes. The arrows indicate PCR primers for genotyping of mice. (B) PCR analysis for the Ogg1 and Mutyh genes. For Ogg1 (left), the sizes of PCR products are 0.8 kb for the wild type allele and 0.7 kb for the mutated allele. For Mutyh (right), the size of PCR products are 0.45 kb for the wild type allele and 0.3 kb for the mutated allele.
facturer's instructions. A 40-µl aliquot of DH10B competent cells was mixed with 2 µl of the DNA (1 µg), and loaded into an electroporation chamber on ice. Electroporation was performed at 1.8 kV/cm. Immediately after electroporation, 960 µl of SOC medium was added to the chamber, and the cell suspension was incubated with vigorous shaking at 28°C for 1 hr. A portion of the culture was then plated onto LB plates containing kanamycin (Km, 50 µg/ml), and the remainder was plated onto LB plates containing Km (50 µg/ml) and streptomycin (Sm, 200 µg/ml). The plates were incubated at 28°C for 48 hrs. Mutation frequencies were determined by calculating the ratio of Km- resistants (Km r) transformants to Km-resistant (Kmr) transformants.

4. Mutation-spectrum analysis

After single-colony-isolation of Km r, Sm r transformants, DNA fragments containing the rpsL gene were amplified by PCR from the colony. Each colony was suspended into 200 µl of TE buffer and boiled for 5 min. A 1-µl aliquot of the supernatant from the boiled colony solution was used as a template for PCR with a pair of rpsL primers. The primer sequences used are as follows: pSSW-F2: 5'-GACGAATTCCGGTTTGGACTGGTC-3' (24-mer) and pSSW-R1: 5'-GAGTGAGCTGATACCGCTCG-3' (20-mer). The PCR conditions were 40 cycles of (95°C for 1 min, 55°C for 30 sec, and 72°C for 1 min). A portion of the PCR product was subjected to 2% agarose gel electrophoresis to detect apparent insertions or deletions in the rpsL sequence. The remainder of each PCR reaction was purified and applied to direct sequencing with ABI Big Dye Terminator DNA sequencing kit. The nucleotide sequences of PCR products were determined using an ABI3100 or ABI3700 sequencer. Mutants with large deletions encompassing the region corresponding to the rpsL primer F2 or R1 were subjected to PCR with another set of primers localized in the Km gene and the replication origin of pSSW. The primer sequences used are as follows; Del-F2: 5'-CACCTGATTGGCCACATTA-3' (20-mer) and DEL-R1: 5'-CAGGGTGCAGAACAGGAGGC-3' (20-mer).

Results

1. Production of Ogg1+/-; Mutyh-/- mice

In order to obtain the mice used for analyzing spontaneous mutagenesis, Ogg1+/-; Mutyh+/- mice with a hemizygous rpsL transgene were crossed with Ogg1+/-; Mutyh+/- mice. The genotype of each mouse was determined by PCR as described in Material and Methods (Fig. 1). Figure 2 depicts the distribution of the progeny with each genotype. Intercrossing Ogg1+/-; Mutyh+/- mice yielded 12 Ogg1-/-; Mutyh-/- mice out of 204 progeny from 36 litters. The ratio of Ogg1-/-; Mutyh-/- mice in the progeny is perfectly matched to a ratio expected for normal Mendelian distribution. Thus, the deficiency of both Ogg1 and Mutyh caused no apparent effect on the in utero development of these mice.

2. Mutation frequency

In order to examine the contribution of Ogg1 and Mutyh in a cooperative action against spontaneous mutagenesis, we analyzed spontaneous mutations in wild type, Ogg1+/-, and Ogg1-/-; Mutyh+/- mice using the rpsL transgene as a reporter for mutations. We determined mutation frequencies in the small intestine of these mice at the age of 4 ~ 5 weeks. The experimental
approach for mutation analysis using the *rpsL* transgenic mouse system is illustrated in Figure 3. The *rpsL* plasmid recovered from the small intestine was introduced into *E. coli*, and the mutation frequency of each mouse was calculated as the ratio of the number of Km$^r$ and Sm$^r$ transformants to the total number of Km$^r$ transformants. Table 1 shows the results obtained from 2 animals of each genotype. The mean mutation frequency in wild-type and *Ogg1*–/– mice was $9.99 \times 10^{-6}$ and $10.04 \times 10^{-6}$, respectively. No significant effect of *Ogg1* deficiency on the overall spontaneous mutation frequency was observed. On the other hand, the mean mutation frequency in *Ogg1*–/–; *Mutyh*–/– mice was $19.12 \times 10^{-6}$, indicating that the combined defect of *Ogg1* and *Mutyh* gives rise to a mutator effect in mice.

3. Mutational spectrum

To determine the mutator effect on specific base substitutions, we performed sequence analysis of the mutant *rpsL* gene obtained from wild type, *Ogg1*–/–, and *Ogg1*–/–; *Mutyh*–/– mice, and determined the type and site of mutations. Table 2 shows

<table>
<thead>
<tr>
<th>ID</th>
<th>sex</th>
<th>genotype</th>
<th>age (days)</th>
<th>Km$^r$ colonies</th>
<th>Sm$^r$ and Km$^r$ colonies</th>
<th>mutation frequency ($\times 10^{-6}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1</td>
<td>♀</td>
<td><em>Ogg1</em>+/– <em>Mutyh</em>+/+</td>
<td>30</td>
<td>3393152</td>
<td>36</td>
<td>10.61</td>
</tr>
<tr>
<td>W2</td>
<td>♂</td>
<td><em>Ogg1</em>+/– <em>Mutyh</em>+/+</td>
<td>29</td>
<td>6812125</td>
<td>66</td>
<td>9.69</td>
</tr>
<tr>
<td>OG1</td>
<td>♀</td>
<td><em>Ogg1</em>–/– <em>Mutyh</em>+/+</td>
<td>41</td>
<td>5122740</td>
<td>50</td>
<td>9.76</td>
</tr>
<tr>
<td>OG2</td>
<td>♀</td>
<td><em>Ogg1</em>–/– <em>Mutyh</em>+/+</td>
<td>40</td>
<td>8221600</td>
<td>85</td>
<td>10.21</td>
</tr>
<tr>
<td>OGMY1</td>
<td>♀</td>
<td><em>Ogg1</em>–/– <em>Mutyh</em>–/–</td>
<td>31</td>
<td>5423068</td>
<td>135</td>
<td>24.89</td>
</tr>
<tr>
<td>OGMY2</td>
<td>♀</td>
<td><em>Ogg1</em>–/– <em>Mutyh</em>–/–</td>
<td>31</td>
<td>4777097</td>
<td>60</td>
<td>12.56</td>
</tr>
</tbody>
</table>
Fig. 3 Mutation analysis using the rpsL transgenic mouse system

A filled box and a filled box with a white circle show wild type and mutated rpsL gene, respectively. An open box shows the kanamycin resistance gene. Km': kanamycin resistance, Sm': streptomycin resistance.

type distributions of the mutations found in the three groups of mice, together with the scoring numbers and frequency.

In wild-type mice, only 7 base-substitutions were scored among 92 mutations. All of these base substitutions are G: C to A: T transitions (7.6%, MF=0.76 x 10^-6). No transversions were detected (the frequency of any transversion is less than 0.11 x 10^-6). Frame shift mutations were predominantly detected. Out of 92 mutations, 46 mutations (50.0 %, 5.0 x 10^-6) were 1-base-deletions and 38 mutations (41.3 %, 4.1 x 10^-6) were 2-base or more deletions.

In Ogg1-/- mice, 19 cases (15.1 %) of base-substitutions were detected in a total of 126 mutations. Among these base substitutions, 11 cases were G: C to A: T transitions (8.7 %, 0.88x10^-6), 7 cases were G: C to T: A transversions (5.6 %, 0.56x10^-6) and 1 case was a G: C to C: G transversion (0.8 %, 0.08x10^-6). Ogg1-/- mice showed more than a 5-fold increase in the frequency of G: C to T: A transversions when compared with wild-type mice. This difference is statistically significant (p<0.03, Fisher's exact test). All of the remaining mutations were frame shift mutations (104 cases, 84.9 %). Among frame-shift mutations, 48 cases (38.1 %, 3.83 x 10^-6) were 1-base-deletions, 56 cases (44.4 %, 4.46 x 10^-6) were 2-base or more deletions, and 3 cases (2.4 %, 0.24 x 10^-6) were 1-base-additions.

In Ogg1-/- Mutyh-/- mice, out of 179 mutations, 54 cases (30.2 %) were base-substitutions and 125 cases (69.8 %) were frame-shift mutations. Among base-substitutions, 9 cases were G: C to A: T transitions (5.0 %, 0.96 x 10^-6), 42 cases were G:
Table 2  Class distribution of spontaneous mutations detected in wild type, Ogg1−/−, and Ogg1−/−; Mutyh−/− mice

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Ogg1−/−</th>
<th>Ogg1−/−; Mutyh−/−</th>
<th>ratio</th>
</tr>
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<tbody>
<tr>
<td>transition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G: C→A: T</td>
<td>7</td>
<td>0.76</td>
<td>11</td>
<td>0.88</td>
</tr>
<tr>
<td>A: T→G: C</td>
<td>0</td>
<td>&lt;0.11</td>
<td>0</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>transversion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G: C→T: A</td>
<td>0</td>
<td>&lt;0.11</td>
<td>7</td>
<td>0.56</td>
</tr>
<tr>
<td>G: C→G: T</td>
<td>0</td>
<td>&lt;0.11</td>
<td>1</td>
<td>0.08</td>
</tr>
<tr>
<td>A: T→T: A</td>
<td>0</td>
<td>&lt;0.11</td>
<td>0</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>A: T→C: G</td>
<td>0</td>
<td>&lt;0.11</td>
<td>0</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>1bp frame shift</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1bp deletions</td>
<td>46</td>
<td>5.00</td>
<td>48</td>
<td>3.83</td>
</tr>
<tr>
<td>1bp additions</td>
<td>0</td>
<td>&lt;0.11</td>
<td>3</td>
<td>0.24</td>
</tr>
<tr>
<td>≥2bp frame shift</td>
<td>38</td>
<td>4.13</td>
<td>56</td>
<td>4.46</td>
</tr>
<tr>
<td>others</td>
<td>1</td>
<td>0.11</td>
<td>0</td>
<td>&lt;0.08</td>
</tr>
<tr>
<td>total</td>
<td>92*</td>
<td>9.99</td>
<td>126*</td>
<td>10.04</td>
</tr>
</tbody>
</table>

W=Wild type  
OG = Ogg1−/−  
OGMY = Ogg1−/−; Mutyh−/−  
*includes double mutations from two mutants  
**includes double mutations from four mutants

C to T: A transversions (23.5 %, 4.49 x 10−6), 1 case was a G: C to C: G transversion (0.6 %, 0.11 x 10−6), and 2 cases were A: T to T: A transversions (1.1 %, 0.21 x 10−6). The frequency of G: C to T: A transversions in Ogg1−/−; Mutyh−/− mice was 41-fold and 8-fold increased when compared with wild-type mice and Ogg1−/− mice, respectively. Among frame-shift mutations, 78 cases (43.6 %, 8.33 x 10−6) were 1-base-deletions, and 47 cases (26.3 %, 5.02 x 10−6) were 2-base or more deletions.  

A characteristic difference was observed in the mutational spectrum of wild type, Ogg1−/− and Ogg1−/−; Mutyh−/− mice. The frequency of G: C to T: A transversions was not significantly different between the three animal groups. There are some differences in the frequency of 1-base-frame shift mutations among the three groups of mice, however, they were not found to be statistically significant.

4. Site-distribution of mutations  
The majority of the mutations scored in this study were frame shift mutations. Almost all of them were 1-base or more deletions, and were scattered throughout the coding region of the rpsL gene (data not shown). No apparent difference in the site distributions of the frame shift mutations was observed among the groups of mice.  

Figure 4 shows the site distribution of base substitutions scored from Ogg1−/− and Ogg1−/−; Mutyh−/− mice in the coding and
the flanking regions of the \textit{rpsL} gene. The positions of G: C to A: T transitions detected in wild type mice were at 233C or 272C (four and three cases, respectively). These two positions appear to be mutational hotspots for G: C to A: T transitions, because most of the G: C to A: T transitions found in \textit{Ogg1}^{-/-} and \textit{Ogg1}^{-/-}; \textit{Mutyh}^{-/-} mice were also localized to these sites. The mutation frequency of G: C to T: A transversions was increased in \textit{Ogg1}^{-/-} and \textit{Ogg1}^{-/-}; \textit{Mutyh}^{-/-} mice (Table 2). The majority of the G: C to T: A transversions were localized to a few specific sites, positioned at 3G, 73G, 81C, 178G, 184G and 285C. In \textit{Ogg1}^{-/-} mice, 3 out of 7 G: C to T: A transversions were localized to 184G. The G: C to T: A transversions at \textit{rpsL} were commonly observed in both \textit{Ogg1}^{-/-} and \textit{Ogg1}^{-/-}; \textit{Mutyh}^{-/-} mice, while mutational hotspots positioned at 3G, 73G, 81C and 178G appeared to be specific for \textit{Ogg1}^{-/-}; \textit{Mutyh}^{-/-} mice.

**Discussion**

In this study, we have shown that a simultaneous defect of \textit{Ogg1} and \textit{Mutyh} dramatically induces G: C to T: A transversions in mice. It has been shown that both \textit{Ogg1} and \textit{Mutyh} contribute to the suppression of spontaneous G: C to T: A transversions in mice (Ref: 31, 32 and Yamauchi \textit{et al} (manuscript in preparation)). Based on the enzymatic characteristics of these proteins, it is likely that \textit{Ogg1} and \textit{Mutyh} would function cooperatively to suppress 8-oxoG-related mutagenesis in mice. In addition, \textit{Mutyh} may be involved in the suppression of mutagenesis caused by 2-HO-A, an oxidative form of adenine. Figure 5 shows a model for oxidative damage induced G: C to T: A transversions and its avoidance systems in mammals. This hypothetical pathway is based on the fact that \textit{Ogg1} removes 8-oxoG from 8-oxoG: C base pairs, while \textit{Mutyh} excises adenine and 2- OH-A paired with guanine and 8-oxoG in \textit{vitro} \cite{18,23,42,46,36}. The oxidation of DNA (a) and the incorporation of 8-oxo-dGTP

![Fig. 4 Comparison of site-distribution of base substitutions detected in \textit{Ogg1}^{-/-}; \textit{Mutyh}^{-/-} mice (upper) and \textit{Ogg1}^{-/-} mice (lower)](image-url)
opposite cytosine (b) forms an 8-oxoG: C base pair that would induce a G: C to T: A transversion if not repaired. Ogg1 could remove 8-oxoG from 8-oxoG: C base pairs, and thus, primarily suppress G: C to T: A transversion in pathway (a) and (b). If the replication fork passes through the lesion before Ogg1 removes the 8-oxoG, dATP would be inserted opposite 8-oxoG. In such a case, Mutyh excises the adenine from the resulting 8-oxoG: A base pair and facilitates to reform the 8-oxoG: C base pair, a good substrate for Ogg1. These dual defense mechanisms were originally proposed by Michaels et al.9) based on the findings that the combination of mutM and mutY mutations causes a synergistic mutator effect specific for G: C to T: A transversions in E. coli. In addition to 8-oxoG, the incorporation of 2-OH-dATP opposite guanine also would induce G: C to T: A transversions (Fig. 5c). In this case, Mutyh could remove 2-OH-A from the 2-OH-A: G base pair to avoid G: C to T: A transversion.

In Ogg1−/− mice, 8-oxoG is not removed from 8-oxoG: C base pairs, resulting in the accumulation of 8-oxoG in DNA313233). We, and others3132, have observed an increased frequency of G: C to T: A transversions in Ogg1−/− mice. The persistent 8-oxoG: C pairs continuously produce 8-oxoG: A pairs in every S phase of the cell cycle. Mutyh excises the adenine from the 8-oxoG: A base pair and reverses-back to an 8-oxoG: C base pair. However, the reversed-back 8-oxoG: C base pair is never repaired, and turns to 8-oxoG: A base pairs again in the next round of DNA replication in Ogg1−/− cells. Therefore, the accumulation of persistent 8-oxoG: C may lead to a failure of repair by Mutyh to some extent, resulting in an increased frequency of G: C to T: A transversion in Ogg1−/− mice. Alternatively, because 8-oxoG is more receptive to oxidative attacks than guanine,
the persistent 8-oxoG may turn to several another types of premutagenic lesions. These lesions, such as oxaluric acid, potentially induce G: C to T: A transversions and are shown to be removed by *E. coli* MutM, the functional counterpart of Oggl (see ref. 37 as a review). Thus, the increased frequency of G: C to T: A transversions might be attributed to a failure of the repair of such oxidative products of 8-oxoG in *Ogg1*-/- mice.

Yamauchi in our laboratory found that *Mutyh*<sup>-/-</sup> mice displayed a significant increase in the frequency of G: C to T: A transversions. Since besides Mutyh, no other enzymes or mechanisms have been identified to remove 2-0H-A from 2-0H-A: G base pairs so far, 2-0H-A would not be removed in *Mutyh*<sup>-/-</sup> cells, resulting in an increase in G: C to T: A transversions (Fig. 5c). It is also possible that if *Ogg1* fails to remove a minor fraction of 8-oxoG from 8-oxoG: C base pairs, the frequency of G: C to T: A transversions would be increased in *Mutyh*<sup>-/-</sup> mice.

The present study indicates that the combined defect of *Ogg1* and *Mutyh* result in a synergistic mutator effect specific for G: C to T: A transversions in mice. The frequency of G: C to T: A transversions in *Ogg1*<sup>-/-</sup>; *Mutyh*<sup>-/-</sup> mice is much higher than the sum of those observed in *Ogg1*<sup>-/-</sup> mice and *Mutyh*<sup>-/-</sup> mice alone. This synergistic mutator effect suggests that *Ogg1* and *Mutyh* function in two independent processes to prevent G: C to T: A transversions caused by 8-oxoG, which is perfectly matched to the model depicted in Figure 5(a) (b), and similar in function to the mutation-avoidance mechanism of *E. coli*<sup>39</sup>. Thus, we conclude that mammalian *Ogg1* and *Mutyh* cooperatively function to avoid G: C to T: A transversions caused by 8-oxoG in DNA.

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**References**

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マウス個体における G:C → T:A トランスバージョン型
突然変異の抑制に対する Ogg1 と Mutyh の機能的調和

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生体内の代謝活動に伴い発生する活性酸素は DNA 等に酸化的損傷を与える。酸化型 DNA 損傷の中でも、グアニンの酸化により生成する 8-オキソグアニンはその強い変異原性からも注目されている。錬型 DNA 中の 8-オキソグアニン (8-oxoG) に対して、DNA 複製時に通常の対合相手であるシトシンとほぼ同じ効率でアデニン塩基も取り込まれるために、8-oxoG:A の誤塩基対合が生じる。8-oxoG:A はこのままの状態では次の複製段階を経て G:C → T:A トランスバージョン型突然変異を引き起こすが、大腸菌においては MutM (8-オキソグアニン DNA グリコシラーゼ) 及び MutY (アデニン DNA グリコシラーゼ) の働きにより、DNA 中の 8-オキソグアニンに誘発される突然変異が抑制されることが示されている。本研究では、哺乳動物における 8-オキソグアニン等の酸化的 DNA 損傷に対する修復系の役割を解明することを目的に、mutM 遺伝子の機能的ホモログである Ogg1 遺伝子の単独欠損マウス、及び Ogg1 遺伝子と mutY 遺伝子ホモログである Mutyh 遺伝子との二重欠損マウスを用いて、fpsL トランスジェンを指標に自然突然変異の解析を行なった。4～5 週令のマウス小腸での自然突然変異頻度は、野生型と Ogg1 欠損マウスでは 1.0×10^{-5}, Ogg1/Mutyh 二重欠損マウスでは 1.9×10^{-5} であり、野生型や Ogg1 欠損マウスに比べると、二重欠損マウスでは突然変異頻度が約 2 倍上昇していた。最近の研究で、ヒトの MUTYH 遺伝子産物は 8-オキソグアニンと対合したアデニンだけでなく、アデニンの酸化型である 2-ハイドロキシアデニンがアデニン塩基と対合した場合に、DNA から 2-ハイドロキシアデニンを除去する活性が認められている。マウスにおける自然突然変異のスペクトラムの解析の結果、野生型マウスでは検出されない（突然変異頻度は 0.11×10^{-6} 以下）8-オキソグアニンや 2-ハイドロキシアデニン等の酸化的 DNA 損傷に起因すると考えられる G:C → T:A トランスバージョンが、Ogg1 欠損マウスにおいて 0.56×10^{-6} （野生型マウスの 5.1 倍以上）の頻度で、Ogg1/Mutyh 二重欠損マウスでは 4.49×10^{-6} の頻度（野生型マウスの 41 倍以上で Ogg1 欠損マウスの 8 倍）で検出された。以上の結果から、今回解析したマウスの小腸では 8-オキソグアニン等の酸化的 DNA 損傷に起因する突然変異の抑制が、Ogg1 と Mutyh の協調により効率良く行われていることが示唆された。