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Citation	DNA repair, 9(5), 542-550 https://doi.org/10.1016/j.dnarep.2010.02.004
Issue Date	2010-05-04
Doc URL	http://hdl.handle.net/2115/43021
Type	article (author version)
File Information	DNAREp.pdf

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Effects of base excision repair proteins on mutagenesis by 8-oxo-7,8-dihydroguanine (8-hydroxyguanine) paired with cytosine and adenine

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Abstract

8-Oxo-7,8-dihydroguanine (8-oxo-Gua, also known as 8-hydroxyguanine) is a major base lesion that is generated by reactive oxygen species in both the DNA and nucleotide pool. The role of DNA glycosylases, which initiate base excision repair, in the mutagenic processes of 8-oxo-Gua in DNA and 8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP, also known as 8-hydroxy-2'-deoxyguanosine 5'-triphosphate) were investigated using *supF* shuttle plasmids propagated in human cells. The DNA glycosylases, OGG1, MUTYH, NTH1, and NEIL1, in 293T cells were individually knocked-down by siRNAs and plasmid DNAs containing an 8-oxo-Gua:C/8-oxo-Gua:A pair, and 8-oxo-dGTP plus unmodified plasmid DNA were then introduced into the knocked-down cells. The knock-down of OGG1, MUTYH, NTH1, and NEIL1 resulted in a significant increase in G:C → T:A transversions caused by the 8-oxo-Gua:C pair in the shuttle plasmid. The knock-down of MUTYH resulted in a reduction in A:T → C:G transversions induced by 8-oxo-dGTP and the 8-oxo-Gua:A pair, but the knockdown of OGG1, NTH1, and NEIL1 had no effect on mutagenesis. These results indicate that all of the above DNA glycosylases suppress mutations caused by 8-oxo-Gua:C in DNA. In contrast, it appears that MUTYH enhances A:T → C:G mutations caused by 8-oxo-dGTP.

Keywords: 8-oxo-7,8-dihydroguanine; 8-hydroxyguanine; base excision repair; DNA glycosylase

1. Introduction

Reactive oxygen species (ROS) are generated through endogenous cellular oxygen metabolism and by exogenous environmental factors [1,2]. ROS react with DNA, RNA, and their precursors. The oxidized nucleic acids cause mutations, resulting in carcinogenesis, neurodegeneration, and aging [3].

8-Oxo-7,8-dihydroguanine (8-oxo-Gua, also known as 8-hydroxyguanine) is one of the most important oxidized bases produced by ROS [4,5]. The oxidation of G bases result in the formation of 8-oxo-Gua in DNA and 8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate (8-oxo-dGTP, also known as 8-hydroxy-2'-deoxyguanosine 5'-triphosphate) in the nucleotide pool. In addition to dCTP, dATP is incorporated opposite 8-oxo-Gua in template DNA by the action of DNA polymerases, resulting in an 8-oxo-Gua:A mispair [6]. Thus, the formation of 8-oxo-Gua:C in DNA leads to G:C → T:A transversions in cells [7-15]. The incorporation of 8-oxo-dGTP opposite A is catalyzed by a variety of DNA polymerases, and the 8-oxo-Gua:A pairs thus formed induce A:T → C:G transversions [8,16-18].

In vitro studies indicate that DNA duplexes containing base pairs involving 8-oxo-Gua serve as substrates for DNA glycosylases, which play an important role in the base excision repair (BER) pathway. The DNA glycosylases OGG1 and MUTYH excise 8-oxo-Gua bases paired with C, and A bases paired with 8-oxo-Gua, respectively [19-29]. Thus, both of these enzymes would be expected to suppress G:C → T:A transversions caused by the oxidation of G bases in DNA. It has been reported that the overexpression of OGG1 and MUTYH suppress mutations by 8-oxo-Gua in cultured cells [30,31]. The NTH1 and NEIL1 proteins are homologues of *Escherichia coli* Nth and Nei, DNA glycosylases that are specific for oxidized pyrimidine bases [32-39]. The observation that NTH1 can be efficiently trapped to an oligonucleotide duplex containing 8-oxo-Gua:C by treatment with NaBH₄, suggests that NTH1 binds to 8-oxo-Gua:C DNA [40]. However, no evidence has been reported to show that NTH1 catalyzes the cleavage of 8-oxo-Gua:C oligonucleotides *in vitro* [39,40]. In addition, the activity of NEIL1 for the 8-oxo-Gua:C pair, as well as 8-oxo-Gua in single-stranded (ss) DNA, is very low [39,41-44]. However, these results may not reflect the *in vivo* situation, where

its activity could be regulated by the presence of other proteins. The issue of whether these DNA glycosylases also have activities for suppressing mutations induced by 8-oxo-Gua *in vivo* is currently unclear. Moreover, it is unclear whether the human BER system acts as a defense against mutations induced by 8-oxo-dGTP, although the Nth protein plays a preventive role in bacterial cells [45].

In this study, we examined roles of DNA glycosylases in mutagenesis by 8-oxo-Gua and 8-oxo-dGTP in human 293T cells. To achieve this, we introduced shuttle plasmids containing 8-oxo-Gua:C and 8-oxo-Gua:A pairs in the *supF* gene (Fig. 1) into 293T cells, in which the target DNA glycosylases, OGG1, MUTYH, NTH1, or NEIL1, were knocked-down by siRNAs. The knock-down of these DNA glycosylases resulted in an increase in 8-oxo-Gua:C-induced mutations. In contrast, mutations by 8-oxo-dGTP and the 8-oxo-Gua:A pair in DNA were reduced by the knock-down of MUTYH, but the knock-down of the other glycosylases had no effect on the mutations. These results suggest that OGG1, MUTYH, NTH1 and NEIL1 suppress mutations by 8-oxo-Gua in DNA, and MUTYH enhances mutations by 8-oxo-dGTP in the nucleotide pool.

2. Materials and methods

2.1. Materials

dGTP (FPLC-grade) was purchased from GE Healthcare Bio-Sciences (Piscataway, New Jersey, USA). 8-oxo-dGTP was obtained from TriLink BioTechnologies (San Diego, California, USA). Phosphorylated oligodeoxyribonucleotides containing 8-oxo-Gua and their control oligodeoxyribonucleotides were purchased from Nihon BioService (Asaka, Japan) and were purified by HPLC, as described previously [46]. Other oligodeoxyribonucleotides were obtained from Hokkaido System Science (Sapporo, Japan) and Sigma Genosys Japan (Ishikari, Japan) in purified forms. siRNAs (“stealth RNAi”, Invitrogen, Carlsbad, California, USA) were synthesized according to the BLOCK-iT RNAi Designer software, on the supplier’s website. The following siRNAs were used: *ogg1* sense, 5'-UCCAAGGUGUGCGACUGCUGCGACA; *ogg1* antisense 5'-UGUCGCAGCAGUCGCACACCUUGGA; *mutyh* sense,

5'-UCACAUCAAGCUGACAUUCAAGUA;	mutyh	antisense,
5'-UACUUGAUUAUGUCAGCUUGAUGUGA;	nth1	sense,
5'-GGAGCAAGGUGAAAUACAUCAAGCA;	nth1	antisense,
5'-UGCUUGAUGUAUUUCACCUUGCUC;	neil1	sense,
5'-GGCUCGCCCCAUGUUUCGUGGACAU;	neil1	antisense,

5'-AUGUCCACGAAACAUAAGGGCGAGCC. Stealth RNAi Negative Control Low and High duplexes (Invitrogen) were used as the negative controls, according to recommended GC contents. The *E. coli* strain KS40/pOF105 was provided by Dr. Tatsuo Nunoshiba, of International Christian University, and was used as an indicator strain of the *supF* mutants [47].

2.2. Construction of shuttle plasmids containing 8-oxo-Gua

The oligodeoxyribonucleotides indicated below were synthesized and chemically phosphorylated on the support.

oligo G*-122	5'-dCGACTTCGAAGG*TTCGAATCC;
oligo G-122	5'-dCGACTTCGAAGGTTCGAATCC;
oligo G*-96	5'-dGCAGACTCG*AAATCTGCCGTCAT;
oligo T-96	5'-dGCAGACTCTAAATCTGCCGTCAT;

where G* represents 8-oxo-Gua. The ss form of pZ189-StuI [48] was obtained as described [17]. The oligodeoxyribonucleotides were annealed with the ss form of pZ189-StuI, and the double-stranded (ds) DNAs containing 8-oxo-Gua were obtained as described previously [49].

2.3. Mutagenesis experiments for shuttle plasmids containing 8-oxo-Gua

293T cells (2.5×10^5 cells) were plated onto gelatin-coated 24-well dishes and were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37°C under a 5% CO₂ atmosphere for 24 h. The siRNA (7.2 pmol) was mixed with Lipofectamine (Invitrogen) and

introduced into the cultured 293T cells according to the supplier's recommendations. After 24 h, the plasmid containing 8-oxo-Gua (100 ng, 29 fmol) was introduced using Lipofectamine. After 48 h of culturing, the plasmid amplified in the cells was recovered by the method described by Stary and Sarasin [50]. The recovered DNA was treated with *Dpn* I (New England Biolabs, Ipswich, Massachusetts, USA), to digest unreplicated plasmids.

2.4. *Mutagenesis experiments for introduction of 8-oxo-dGTP*

At 24 h after the siRNA transfection described above, pZ189-StuI (200 ng, 58 fmol) was transfected using PolyFect (Qiagen, Hilden, Germany), according to the supplier's recommendations. After 24 h, dGTP or 8-oxo-dGTP (120 nmol) was introduced by osmotic pressure, using a buffer solution (50 mM KCl, 10 mM Hepes-KOH, pH 7.4) as described previously [48]. After 24 h of culture, the plasmid amplified in the cells was recovered and digested with *Dpn* I.

2.5. *supF* mutant frequency and sequencing analysis

The DNAs recovered from the 293T cells were introduced into *E. coli* KS40/pOF105 by electroporation, using a Gene Pulser II transfection apparatus with a Pulse Controller II (Bio-Rad, Hercules, California, USA). The *supF* mutant frequency was calculated, based on the numbers of white and faint blue colonies on Luria–Bertani agar plates containing nalidixic acid (50 µg/ml), streptomycin (100 µg/ml), ampicillin (150 µg/ml), chloramphenicol (30 µg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (80 µg/ml), and isopropyl-β-D-thiogalactopyranoside (23.8 µg/ml), and the number of colonies on agar plates containing ampicillin and chloramphenicol.

2.6. *Western blotting*

The cells were extracted with a radio immuno-precipitation assay buffer containing protease inhibitors. The whole cell extracts were fractionated on 12.5% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were blocked in 3-5% non-fat milk and probed with either a mouse anti-OGG1 antibody (American Research Products, Belmont, Massachusetts, USA), a mouse anti-MUTYH antibody (Abnova, Taipei, Taiwan), a mouse anti-NTH1 antibody (R&D Systems, Minneapolis, Minnesota, USA), or a rabbit anti-NEIL1 antibody (Abcam, Cambridge, Massachusetts, USA) overnight in Can Get Signal solution 1 (Toyobo, Osaka, Japan). After washing the membranes three times in phosphate-buffered saline containing 0.05% Tween 20, they were incubated with either anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences) for 1 h. The OGG1, MUTYH, NTH1, and NEIL1 DNA glycosylases were then visualized using the Enhanced Chemiluminescence (ECL) System (GE Healthcare Bio-Sciences).

2.7. Statistical analysis

Statistical significance was examined by the Student's *t*-test. Levels of $P < 0.05$ were considered to be significant.

3. Results

3.1. Knock-down of DNA glycosylases by siRNAs

OGG1, MUTYH, NTH1, and NEIL1 were selected as knock-down targets and were knocked-down in human 293T cells using siRNAs. Negative Control Low and High GC duplexes were used as control siRNAs for MUTYH and NTH1, and OGG1 and NEIL1, respectively. The knock-down of the target proteins was confirmed by Western blotting (Figs. 2 and 3). These target proteins were effectively knocked-down from 24 h (the time point of the shuttle plasmid

introduction) to 72 h (the time point of the plasmid rescue) after the introduction of the siRNAs.

3.2. Enhanced 8-oxo-Gua $\rightarrow$ T mutation by knock-down of DNA glycosylases

Plasmids containing single G:C and 8-oxo-Gua:C pairs at position 122 of the *supF* gene (Fig. 1) were first introduced into 293T cells that contained knocked-down DNA glycosylases. The replicated DNA was recovered from the treated 293T cells, and was then transfected again into the indicator *E. coli* strain, KS40/pOF105, in order to calculate the mutant frequencies for the *supF* gene.

Knock-down of the DNA glycosylases had no effect on the mutant frequencies of the control plasmid containing the G:C pair. In contrast, the mutant frequencies of the plasmid containing the 8-oxo-Gua:C pair in the OGG1- and MUTYH-knocked-down cells were 2.5- and 5.6-times, respectively, higher than those in the cells that had been treated with their control siRNAs (Table 1). Thus, OGG1 and MUTYH suppressed the mutagenesis by 8-oxo-Gua in DNA, as expected from previous reports [30,31]. Unexpectedly, the knock-down of NTH1 and NEIL1 also enhanced the mutant frequencies by 2.9- and 2.4-fold, compared to controls (Table 1). To exclude the possibility that the effects of NTH1- and NEIL1-knock-down were off-target ones, siRNAs against NTH1 and NEIL1 with different sequence contexts were used. Again, the mutant frequencies were similarly enhanced (data not shown), indicating that the reduction in NTH1 and NEIL1 actually induced the increased mutagenesis by 8-oxo-Gua:C.

The sequences of the *supF* gene in the mutant clones were analyzed (Supplementary Table 1), and the G:C $\rightarrow$ T:A transversion frequencies at position 122 of the gene were calculated (Table 1). Almost all of the mutations for the G:C plasmid were deletions, insertions and base substitutions at other positions. In contrast, more than 60% of the mutations for the 8-oxo-Gua:C plasmid were G:C $\rightarrow$ T:A transversions at the targeted position. The proportion of transversion in the cells with knocked-down DNA glycosylases was increased relative to the control cells. Therefore, the G:C $\rightarrow$ T:A transversion frequencies at position 122 in the knocked-down cells were higher than those of the corresponding control. These results suggest that the four DNA glycosylases suppress the mutations

induced by 8-oxo-Gua, which is produced by the oxidation of G in DNA.

The effects of the double knock-down of these DNA glycosylases were also examined (Table 2). The double knock-down of these DNA glycosylases had no effect on the background mutant frequencies (the G:C plasmid). The 8-oxo-Gua:C-induced mutant frequencies in cells that had been treated with the control plus the targeted siRNAs were similar to those for the case of a single knock-down. The double knock-down of OGG1 and MUTYH resulted in more than a simple additive effect on the mutant frequency induced by 8-oxo-Gua:C. The knock-down of OGG1 plus NEIL1, MUTYH plus NTH1, and MUTYH plus NEIL1 resulted in an additive increase in the induced mutant frequencies, compared with the single knock-down of these DNA glycosylases. However, the double knock-down of OGG1 plus NTH1 and NEIL1 plus NTH1 resulted in mutant frequencies similar to those for the single knock-down of OGG1 and NEIL1, respectively.

3.3. Decrease in 8-oxo-dGTP-induced mutations by knock-down of MUTYH

We next examined the effects of the knock-downs on mutations induced by 8-oxo-dGTP. Previously, we reported that A:T → C:G transversions were specifically induced by 8-oxo-dGTP in mammalian cells [48,51]. In this study, we examined the mutagenic potential of 8-oxo-dGTP in cells with knocked-down DNA glycosylases.

As shown in Table 3, the introduction of 8-oxo-dGTP into 293T cells enhanced the mutant frequency, compared to controls (no deoxyribonucleotides (none) and dGTP). The mutant frequencies upon treatment with siRNAs against OGG1, MUTYH, NTH1, and NEIL1 (and the control siRNAs) were similar in the control (none and dGTP) experiments. We then introduced 8-oxo-dGTP into the knocked-down cells. The 8-oxo-dGTP-induced mutant frequency was significantly decreased in cells in which MUTYH was reduced. In contrast, the knock-down of OGG1, NTH1 and NEIL1 had no effect on the mutant frequencies.

The mutation spectra in the knocked-down cells are shown in Table 4. In all cells that had been treated with siRNAs, 8-oxo-dGTP specifically induced A:T → C:G transversions. The frequency of

the A:T → C:G substitution was calculated by multiplying the mutant frequency by the proportion of mutants containing A:T → C:G substitution(s) (Table 3). The knock-down of MUTYH reduced the frequency of the A:T → C:G transversions. These results suggest that MUTYH, but not OGG1, NTH1, and NEIL1, is involved in the mutagenic event induced by 8-oxo-dGTP.

3.4. Effects of DNA glycosylase knock-down on mutations induced by 8-oxo-Gua:A pair

Finally, the effects of DNA glycosylase knock-down on mutations induced by the 8-oxo-Gua:A pair were evaluated, based on 8-oxo-dGTP experiments. We introduced plasmid DNAs with single T:A and 8-oxo-Gua:A pairs at position 96 of the *supF* gene (Fig. 1) into 293T cells with knocked-down DNA glycosylases. The frequencies of the *supF* mutants were then determined (Table 5).

Surprisingly, more than 80% of the progeny of the 8-oxo-Gua:A plasmid contained a mutant *supF* gene in cells that had been treated with the control siRNAs, and the A:T → C:G mutation at position 96 was found in all of the mutant plasmids examined (data not shown). Similar results were observed in the OGG1-, NTH1-, and NEIL1-knocked-down cells. Meanwhile, the targeted A:T → C:G mutation frequency in the MUTYH-knocked-down cells was two-fold less than that in the cells that had been treated with the control siRNA (Negative Control Low GC duplex). These results, together with those obtained in the 8-oxo-dGTP experiments, show that the 8-oxo-Gua:A pair is efficiently converted to the G:C pair by the excision of the A opposite 8-oxo-Gua by MUTYH. We therefore conclude that MUTYH suppresses mutations induced by 8-oxo-Gua:C in DNA, but enhances those generated by 8-oxo-dGTP.

4. Discussion

In this study, we investigated the issue of whether BER proteins, OGG1, MUTYH, NTH1, and NEIL1, affect the mutagenesis induced by 8-oxo-Gua in DNA and 8-oxo-dGTP, by their

knock-downs with siRNAs. We first examined the effects of the knock-down on mutagenesis by 8-oxo-Gua:C in DNA. The knock-down of all of the DNA glycosylases examined increased the G:C → T:A mutation frequencies by the 8-oxo-Gua:C pair in human cells (Table 1). These results show that OGG1, MUTYH, NTH1, and NEIL1 suppress the mutations induced by 8-oxo-Gua, which is produced by the direct oxidation of DNA. OGG1 and MUTYH are known to excise 8-oxo-Gua in 8-oxo-Gua:C pairs and A in 8-oxo-Gua:A pairs, respectively, *in vitro* [19-29]. Thus, our results are in agreement with the previous observations.

In contrast, the suppression of the mutations by 8-oxo-Gua by NTH1 and NEIL1 has come under scrutiny lately, due to their lack of activity and weak activity, respectively, for DNA duplexes containing 8-oxo-Gua:C [39,41-44]. The findings reported herein provides the first evidence to demonstrate that NTH1 and NEIL1 suppress the mutagenesis induced by 8-oxo-Gua in living cells. It has been suggested that NTH1 binds to ds DNA containing an 8-oxo-Gua:C pair, but does not excise the 8-oxo-Gua base *in vitro* [39,40]. The activity of NTH1 for thymine glycol is enhanced in the presence of p53, XPG, YB-1, and APE1 [52-54]. Assuming that not only the activity for thymine glycol but also that for other modified bases including 8-oxo-Gua are enhanced, the possibility that NTH1 is activated by interactions with these proteins in 293T cells, resulting in the suppression of the mutations caused by 8-oxo-Gua:C, cannot be excluded, based on the findings reported here. In addition, no additive effects were detected in cells with NTH1 plus OGG1 and NTH1 plus NEIL1 double knock-downs. This result suggests that NTH1 may enhance the repair of lesions by OGG1 and NEIL1.

The non-additive effects of the double knock-down of NTH1 plus OGG1 and NTH1 plus NEIL1 might be due to a reduction in knock-down efficiencies, as compared to the cases of the single knock-downs. However, “double knock-down experiments” using control plus OGG1, control plus NEIL1, and control plus NTH1 siRNAs resulted in an enhancement in mutations caused by 8-oxo-Gua:C with frequencies similar to those of a single knock-down (Table 2). Furthermore, the knock-down of NTH1 plus MUTYH increased the mutant frequencies additively, compared with the single knock-down of these DNA glycosylases. Thus, the non-additive effects concerning NTH1

could be interpreted as the result of a currently unknown function(s) of the protein.

NEIL1 has the ability to excise not only 8-oxo-Gua in ds DNA [39,41-43] but also 8-oxo-Gua in ss DNA [44]. In addition, proliferating cell nuclear antigen (PCNA) interacts with NEIL1 and stimulates NEIL1 activity with respect to excising 5-hydroxyuracil from ss DNA sequences, including fork structures [55]. Thus, the 8-oxo-Gua-induced mutations may be suppressed by NEIL1, due to an elevated 8-oxo-Gua glycosylase activity of the protein during replication. Moreover, NEIL1 stimulated the turnover of OGG1 and increased the 8-oxo-Gua excision activity of OGG1 [56]. However, this putative mechanism may not adequately explain the observation that the double knock-down of NEIL1 and OGG1 increased the mutations caused by 8-oxo-Gua:C in an additive manner.

Chan et al. measured 8-oxo-Gua levels in the DNA in organs from young and old *Nth1^{-/-} Neil1^{-/-}* mice [57]. The 8-oxo-Gua levels in livers of double knock-out mice appeared to be higher than those in the control mice, although the differences were statistically insignificant (Figs. 5 and 6 in reference 57). In contrast, this trend was not observed in kidney and brain tissue. Thus, NTH1 and NEIL1 may function as repair enzymes for 8-oxo-Gua in the mouse liver and in cultured 293T cells (this study), depending on the level of expression of other protein(s) that stimulate NTH1 and NEIL1 activities.

We next examined the mutagenesis by 8-oxo-dGTP and 8-oxo-Gua:A in cells with knocked-down DNA glycosylases. The suppression of MUTYH expression decreased the mutation frequencies by 8-oxo-dGTP and 8-oxo-Gua:A, but no effects were observed for the other DNA glycosylases examined. These results suggest that MUTYH enhances the mutations induced by 8-oxo-dGTP by excising the A in 8-oxo-Gua:A pairs formed by the incorporation of 8-oxo-dGTP opposite A. Thus, MUTYH enhances the mutations by 8-oxo-dGTP although the protein suppresses the mutations by 8-oxo-Gua formed by the direct oxidation of DNA. The MutY protein plays the same role in *Escherichia coli* [58]. The biological significance of the enhancement in 8-oxo-dGTP-induced mutations is currently unclear.

The enhancement of the 8-oxo-dGTP-induced mutations by MUTYH and the lack of contribution of the other three DNA glycosylases to the suppression suggest the importance of sanitization

enzymes from the nucleotide pool, such as MTH1, as a defense against mutagenesis by the oxidized DNA precursor. Indeed, we observed that the knock-down of human nucleotide pool sanitization enzymes resulted in an increased mutagenicity of 8-oxo-dGTP [59]. In addition, cell death triggered by the accumulation of 8-oxo-Gua in MTH1-null cells was reported to be dependent on the MUTYH activity [60,61]. This suggests that MUTYH may be involved in another pathway, such as cell death triggered by the accumulation of 8-oxo-dGTP.

Lee and Pfeifer introduced plasmids that had been treated with methylene blue plus light (MBL), which predominantly produces 8-oxo-Gua in DNA, into human cells [62]. They found that the knock-down of OGG1 led to a two-fold increase in mutations in the replicated plasmid, in agreement with our present results. However, the spectra of mutations induced by MBL between the knocked-down cells and the control cells were similar. In contrast, the enhanced mutant frequency by OGG1 knock-down was determined to be due to an increased G:C $\rightarrow$ T:A mutation at the modified position in this study (Table 1). The unexpected mutation spectra observed by Lee and Pfeifer might arise from DNA lesions other than the 8-oxo-Gua formed by the MBL treatment.

The transfection of the plasmid containing the 8-oxo-Gua:A pair resulted in a high mutation frequency (~85%) in 293T cells (Table 5). Since the A:T $\rightarrow$ C:G mutation was induced at the modified site and the knock-down of MUTYH reduced the mutation frequency, the A base paired with 8-oxo-Gua would be removed by the glycosylase, and DNA polymerase(s) would insert dCTP opposite 8-oxo-Gua in the gap-filling process. DNA polymerase (pol) λ was recently shown to be ~150-fold more efficient than DNA pol β for the insertion of dCTP opposite 8-oxo-Gua in gapped DNA, in the presence of replication protein A (RP-A) [63]. Moreover, PCNA inhibited the insertion of dCTP by DNA pol β , although little, if any, effect was observed for DNA pol λ . Thus, the activities of MUTYH and gap-filling DNA polymerase(s), such as pol λ , were determinants of the induced A:T $\rightarrow$ C:G mutation. In contrast to our result, only ~35% mutation frequencies were reported using simian COS-7 and human MRC5V1 cells [64]. This discrepancy could be due to differences in MUTYH and/or DNA pol λ activities in the cells.

In this study, we established that OGG1, MUTYH, NTH1 and NEIL1 suppressed mutations by

8-oxo-Gua:C, and MUTYH enhanced the mutations by 8-oxo-dGTP in human cells. The four DNA glycosylases each function in the suppression of mutations by 8-oxo-Gua in DNA. The overlapped recognition of 8-oxo-Gua could explain the fact that single knock-out mice deficient in OGG1, MUTYH, NTH1, and NEIL1 show few tumor phenotypes [57,65-71]. In contrast, there is evidence to support the view that polymorphisms in these DNA glycosylases are associated with human carcinogenesis (reviewed in 72). Therefore, further studies of these DNA glycosylases should be undertaken, if we are to better understand human carcinogenesis.

Disclosure

The authors declare that neither full nor part of their work is under consideration for publication in any other journals.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We are grateful to Dr. T. Nunoshiba for *E. coli* strain KS40/pOF105. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Takeda Science Foundation, and the Research Foundation for Pharmaceutical Sciences.

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Figure legends

Figure 1. Structure of the pZ189-StuI plasmid containing 8-oxo-Gua in the *supF* gene. 8-oxo-Gua was introduced to either position 96 (8-oxo-Gua:A) or 122 (8-oxo-Gua:C). X's indicate the positions where 8-oxo-Gua is incorporated.

Figure 2. Knock-down of the target proteins by siRNAs. The OGG1, NTH1, MUTYH, and NEIL1 proteins were detected by Western blot analyses.

Figure 3. Double knock-down of the (A) OGG1, (B) MUTYH, (C) NTH1, and (D) NEIL1 proteins by siRNAs. 293T cells were treated with two kinds of siRNAs indicated and the target proteins were detected by Western blot analyses. Low and High represent Stealth RNAi Negative Control Low and High duplexes used as the negative controls.

96 122
CTCXAAAT CTGCCGTCAT CGACTTCGAA GXTTC
GAGATTTA GACGGCAGTA GCTGAAGCTT CCAAG

Fig. 1

Fig. 2

Fig. 3

Table 1

Table 1. Frequencies of *supF* mutants induced by 8-oxo-Gua:C in 293T cells with knocked-down DNA glycosylases^a

Plasmid	siRNA	Mutant frequency ($\times 10^{-3}$)	122 G:C --> T:A transversion frequency ($\times 10^{-3}$) ^b
122 G:C	None	1.7 (0.3)	0
	Control-Hi	2.0 (0.4)	0
	OGG1	1.5 (0.3)	0
	NEIL1	1.7 (0.4)	0
	Control-Lo	1.7 (0.7)	0
	MUTYH	2.0 (0.4)	0
	NTH1	2.0 (0.5)	0.042
122 G*:C ^e	None	10 (3.0)	5.9
	Control-Hi	9.9 (3.0)	5.3
	OGG1	25 (7.0) ^c	21
	NEIL1	24 (5.9) ^d	23
	Control-Lo	5.2 (1.8)	3.2
	MUTYH	29 (6.5) ^d	27
	NTH1	15 (4.0) ^c	11

^aExperiments were repeated four times. The data are expressed as means and numbers in parentheses represent standard deviations.

^bThe mutant frequency is multiplied by the ratio of the targeted G:C --> T:A transversion shown in Supplemental Table 1.

^c $P < 0.05$ (significant difference versus control).

^d $P < 0.01$ (significant difference versus control).

^eG* represents 8-oxo-Gua.

Table 2

Table 2. Frequencies of *supF* mutants induced by 8-oxo-Gua:C in 293T cells with double knocked-down DNA glycosylases^a

Plasmid	siRNA	Mutant frequency (x 10 ⁻³)	122 G:C --> T:A transversion frequency (x 10 ⁻³) ^b
122 G:C	Control Low + High	2.0 (0.7)	0
	OGG1 + Low	2.0 (0.3)	0.17
	MUTYH + High	2.0 (0.1)	0
	NTH1 + High	2.3 (0.3)	0
	NEIL1 + Low	1.9 (0.2)	0
	OGG1 + MUTYH	1.9 (0.3)	0
	OGG1 + NTH1	2.0 (0.2)	0.06
	OGG1 + NEIL1	1.7 (0.1)	0
	MUTYH + NTH1	2.4 (0.1)	0
	MUTYH + NEIL1	2.2 (0.2)	0.13
	NTH1 + NEIL1	2.0 (0.6)	0
122 G*:C ^e	Control Low + High	7.2 (1.1)	4.6
	OGG1 + Low	20 (4.5)	17
	MUTYH + High	33 (3.4)	32
	NTH1 + High	14 (1.0)	12
	NEIL1 + Low	20 (3.8)	17
	OGG1+MUTYH	79 (5.9) ^d	79
	OGG1+NTH1	19 (2.1) ^d	15
	OGG1+NEIL1	40 (9.4) ^c	39
	MUTYH+NTH1	48 (1.6) ^d	44
	MUTYH+NEIL1	55 (4.0) ^d	55
	NTH1+NEIL1	25 (6.8) ^c	22

^aExperiments were repeated four times. The data are expressed as means and numbers in parentheses represent standard deviations.

^bThe mutant frequency is multiplied by the ratio of the targeted G:C --> T:A transversion shown in Supplemental Table 1.

^c*P*<0.05 (significant difference versus control).

^d*P*<0.01 (significant difference versus control).

^eG* represents 8-oxo-Gua.

Table 3. Frequencies of *supF* mutants induced by 8-oxo-dGTP in 293T cells with knocked-down DNA glycosylases^a

siRNA	Mutant frequency ($\times 10^{-5}$)			A:T --> C:G transversion frequency ($\times 10^{-5}$) ^b		
	None	dGTP	8-oxo-dGTP	None	dGTP	8-oxo-dGTP
None	4.2 (0.7)	5.2 (1.2)	23 (5.3)	0.089	0	17
Control-Hi	4.9 (2.4)	6.1 (1.6)	22 (6.7)	0.32	0.26	14
OGG1	4.8 (0.8)	4.3 (0.7)	25 (4.6)	0.10	0.090	18
NEIL1	6.8 (1.8)	6.5 (1.8)	24 (6.4)	0.16	0.44	17
Control-Lo	5.1 (2.2)	5.1 (1.5)	23 (7.9)	0.23	0.47	19
MUTYH	4.8 (1.1)	4.3 (1.0)	9.3 (2.3) ^c	0.31	0.36	5.8
NTH1	7.3 (2.2)	7.6 (4.7)	26 (4.7)	0	0.80	18

^aExperiments were repeated four times. The data are expressed as means and numbers in parentheses represent standard deviations.

^bThe mutant frequency is multiplied by the ratio of A:T --> C:G transversion shown in Table 4.

^c $P < 0.05$ (significant difference versus control).

Table 4. Spectra of mutations induced by 8-oxo-dGTP in 293T cells with knocked-down DNA glycosylases

	none (no nucleotide)						
	none	Control-Hi	OGG1	NEIL1	Control-Lo	MUTYH	NTH1
Single base substitution							
Transition							
A:T to G:C	5 (11)	6 (13)	12 (26)	4 (10)	3 (7)	6 (13)	3 (7)
G:C to A:T	4 (9)	4 (9)	10 (21)	5 (12)	3 (7)	3 (7)	6 (14)
Transversion							
A:T to T:A	3 (6)	2 (4)	2 (4)	1 (2)	1 (2)	1 (2)	0 (0)
A:T to C:G	1 (2)	3 (6)	1 (2)	1 (2)	2 (5)	3 (7)	0 (0)
G:C to T:A	10 (21)	5 (11)	6 (13)	10 (24)	8 (18)	4 (9)	4 (10)
G:C to C:G	4 (9)	2 (4)	4 (9)	2 (5)	6 (14)	4 (9)	3 (7)
Deletions/Insertions							
1-2 bases	2 (4)	7 (15)	3 (7)	2 (5)	1 (2)	3 (7)	4 (10)
>3 bases	14 (30)	14 (30)	7 (15)	14 (33)	17 (39)	20 (44)	20 (48)
Complex	1 (2)	1 (2)	0 (0)	3 (7)	1 (2)	0 (0)	0 (0)
Others	3 (6)	2 (4)	1 (2)	0 (0)	2 (5)	1 (2)	2 (5)
Total	47 (100)	46 (100)	46 (100)	42 (100)	44 (100)	45 (100)	42 (100)

	dGTP						
	none	Control-Hi	OGG1	NEIL1	Control-Lo	MUTYH	NTH1
Single base substitution							
Transition							
A:T to G:C	10 (22)	9 (19)	11 (23)	6 (14)	8 (19)	11 (23)	4 (11)
G:C to A:T	6 (13)	5 (11)	4 (9)	7 (16)	2 (5)	3 (6)	5 (13)
Transversion							
A:T to T:A	3 (7)	2 (4)	2 (4)	1 (2)	3 (7)	0 (0)	0 (0)
A:T to C:G	0 (0)	2 (4)	1 (2)	3 (7)	4 (9)	4 (9)	4 (11)
G:C to T:A	10 (22)	7 (15)	6 (12)	10 (23)	3 (7)	7 (15)	3 (8)
G:C to C:G	1 (2)	2 (4)	3 (6)	3 (7)	5 (12)	5 (11)	4 (11)
Deletions/Insertions							
1-2 bases	4 (9)	2 (4)	3 (6)	4 (9)	2 (5)	3 (6)	2 (5)
>3 bases	10 (22)	18 (38)	16 (34)	8 (18)	13 (30)	14 (30)	13 (34)
Complex	1 (2)	0 (0)	0 (0)	2 (5)	1 (2)	0 (0)	2 (5)
Others	0 (0)	0 (0)	1 (2)	0 (0)	2 (5)	0 (0)	1 (3)
Total	45 (100)	47 (100)	47 (100)	44 (100)	43 (100)	47 (100)	38 (100)

Table 4. Spectra of mutations induced by 8-oxo-dGTP in 293T cells with knocked-down DNA glycosylases (continued)

	8-oxo-dGTP						
	none	Control-Hi	OGG1	NEIL1	Control-Lo	MUTYH	NTH1
Single base substitution							
Transition							
A:T to G:C	1 (2)	1 (2)	3 (6)	1 (2)	0 (0)	2 (4)	1 (2)
G:C to A:T	1 (2)	2 (4)	1 (2)	1 (2)	0 (0)	0 (0)	2 (4)
Transversion							
A:T to T:A	0 (0)	0 (0)	0 (0)	2 (4)	0 (0)	0 (0)	0 (0)
A:T to C:G	35 (73)	32 (65)	34 (72)	33 (70)	39 (85)	29 (62)	32 (70)
G:C to T:A	3 (6)	3 (6)	1 (2)	1 (2)	2 (4)	6 (13)	1 (2)
G:C to C:G	0 (0)	1 (2)	1 (2)	2 (4)	0 (0)	3 (6)	0 (0)
Deletions/Insertions							
1-2 bases	0 (0)	1 (2)	0 (0)	1 (2)	2 (4)	1 (2)	1 (2)
>3 bases	6 (13)	6 (12)	3 (6)	4 (9)	2 (4)	4 (9)	8 (17)
Complex	0 (0)	1 (2)	1 (2)	1 (2)	0 (0)	1 (2)	1 (2)
Others	2 (4)	1 (2)	4 (8)	1 (2)	1 (2)	1 (2)	0 (0)
Total	48 (100)	49 (100)	48 (100)	47 (100)	46 (100)	47 (100)	46 (100)

All data are represented as cases found (%).

Table 5. Frequencies of *supF* mutants induced by 8-oxo-Gua:A in 293T cells with knocked-down DNA glycosylases^a

Plasmid	siRNA	Mutant frequency
96 T:A	None	1.6 (0.4) x 10 ⁻³
	Control-Hi	2.1 (0.5) x 10 ⁻³
	OGG1	1.7 (0.3) x 10 ⁻³
	NEIL1	1.9 (0.1) x 10 ⁻³
	Control-Lo	1.6 (0.2) x 10 ⁻³
	MUTYH	1.8 (0.2) x 10 ⁻³
	NTH1	2.4 (0.9) x 10 ⁻³
96 G*:A ^c	None	0.87 (0.05)
	Control-Hi	0.85 (0.02)
	OGG1	0.87 (0.02)
	NEIL1	0.81 (0.05)
	Control-Lo	0.86 (0.03)
	MUTYH	0.40 (0.03) ^b
	NTH1	0.87 (0.05)

^aExperiments were repeated three times. The data are expressed as means and numbers in parentheses represent standard deviations.

^b*P*<0.01 (significant difference versus control).

^cG* represents 8-oxo-Gua.