**Table 3. Influences of the *CMA/B* and *ACE* genotype status on the development of two different histologic types of gastric cancer**

| Histologic type | Gene | | Adjusted OR (95% CI) | *P* |
|---|---|---|---|---|
| Intestinal type (*n* = 87) | *CMA/B* | G/G | 1.000 | |
| | | G/A | 1.899 (1.029-3.503) | 0.040 |
| | | A/A | 8.334 (1.832-37.906) | 0.006 |
| | | A allele | 2.181 (1.209-3.932) | 0.010 |
| | *ACE* | I/I | 1.000 | |
| | | I/D | 0.738 (0.467-1.341) | 0.319 |
| | | D/D | 0.434 (0.166-1.131) | 0.088 |
| | | D allele | 0.701 (0.396-1.240) | 0.222 |
| Diffuse type (*n* = 32) | *CMA/B* | G/G | 1.000 | |
| | | G/A | 1.983 (0.874-4.503) | 0.102 |
| | | A/A | 7.398 (1.203-45.491) | 0.031 |
| | | A allele | 2.250 (1.025-4.943) | 0.043 |
| | *ACE* | I/I | 1.000 | |
| | | I/D | 0.583 (0.254-1.339) | 0.203 |
| | | D/D | 0.544 (0.160-1.847) | 0.329 |
| | | D allele | 0.572 (0.263-1.246) | 0.160 |

NOTE: ORs were adjusted by age and sex.

## Discussion

The present study was designed to test the hypothesis that polymorphisms of enzymes involved in the local RA system, such as chymase and ACE, are associated with gastric carcinogenesis and peptic ulcer development. We showed that a significant association between the *CMA/B* A/G polymorphism and susceptibility to gastric cancer and gastric ulcer in Japanese patients with *H. pylori* infection. The A allele carriage of *CMA/B* (i.e., A/A and A/G) significantly increased the risk of gastric cancer development. Although the plasma ACE level of individuals with the *ACE* I/I genotype has been reported to be lower than those with the D/D genotype (15, 16), the *ACE* I/D polymorphism is not associated with susceptibility to, pathologic classification, and clinical stage of gastric cancer. However, we observed that the *ACE* I allele increased the gastric cancer risk in patients having the *CMA/B* A allele.

In carcinogenesis, angiotensin II/angiotensin II receptors signaling pathways are associated with cell proliferation, angiogenesis, and inflammation. First, AT1R induces cell proliferation in cancer cells through various intracellular protein cascades associated with growth factor stimulations, of which, the epidermal growth factor receptor–related kinase and protein kinase C are major mediators in cells (31, 32). Second, AT1R induces vascular endothelial growth factor, vascular endothelial growth factor-2 receptor, and angiopoietin II, resulting in the angiogenesis of cancer tissues (33, 34). Third, the activation of AT1Rs enhances the transcription of several proinflammatory cytokines (e.g., interleukin-1 and tumor necrosis factor-α) and chemokines via signaling pathways involving nuclear factor κB and activator protein-1 (10).

Recent studies have shown the local overexpression of chymase and ACE in various cancer cells and tissues (e.g., lung, pancreas, breast, prostate, skin, and cervix carcinoma), suggesting that local overexpression of several components of the RA system is associated with carcinogenesis, such as cell proliferation, angiogenesis, and inflammation (21, 35, 36). For example, the positivities and expressions of chymase are significantly higher in *H. pylori*-associated chronic gastritis and gastric cancer cells than the normal gastric mucosa without *H. pylori* infection (21, 36, 37). The overexpression of chymase observed in mucosa infected with *H. pylori* has been reported to be closely related to an infiltration of inflammation cells, such as neutrophils, macrophages, and T lymphocytes (37). Therefore, chymase is assumed to be associated with the pathogenesis of *H. pylori*-related disorders.

Angiotensin II is an important mediator of gastric vasoconstriction and contributes to physiologic maintenance (38). The systemic generation of angiotensin II from angiotensin I is mainly mediated by ACE. However, 60% to 80% of local, not systemic, generation of angiotensin II is mediated by chymase, and the remainder is mediated by the ACE pathway (24, 39). In a hamster-sponge model, both angiotensin I and angiotensin II injected directly into the sponge enhanced angiogenesis (40). The angiogenesis by angiotensin I was inhibited by chymase inhibitors, but that by angiotensin II was not (40). These findings suggest the importance of chymase-dependent angiotensin II formation in angiogenesis. With regard to the RA system, therefore, the *CMA/B* polymorphism, one of the determinant factors of local CMA levels, may become an important factor of interindividual differences in the susceptibility to cardiovascular diseases and neoplastic diseases, compared with the *ACE* polymorphism (41). In fact, Pfeufer et al. (41) reported that patients with *CMA/B* A/A and A/G genotypes had a 3.2-fold higher risk of hypertrophic cardiomyopathy. However, the role of the *CMA/B* polymorphism in tumor growth and angiogenesis in carcinogenesis has not been directly addressed in previous studies. Although the expressions of chymase in chronic gastritis and gastric cancer cells are significantly increased (21, 36, 37), the role of the differences in local chymase generation associated with *CMA/B* polymorphisms in the stomach has not been directly shown in previous studies. In the present study, we first showed that carriage of the A allele of *CMA/B* polymorphism and the *CMA/B* A/A and A/G genotypes significantly increased the risks of gastric cancer development compared with the carriage of the G allele and the G/G genotype. Therefore, *H. pylori* infection–induced local up-regulation of chymase might be related to gastric carcinogenesis.

Recent reports showed that the *ACE* polymorphism also has a strong association with risk of development of several cancers, such as breast cancer and prostate cancer (19, 20, 22, 42). In gastric carcinogenesis, Ebert et al. (22) reported that the risks for early gastric cancer development was significantly lower in patients with *ACE* I/I and I/D genotypes than those with D/D genotype (ORs, 0.20 and 0.55, respectively). Goto et al. (23) reported that the *ACE* I/D polymorphism was associated with the incidence of gastric cancer and *H. pylori*-positive patients with atrophic gastritis. However, Rocken et al. (21) reported that although the *ACE* polymorphisms for patients with gastric cancer correlated with the number of lymph node metastasis and clinical stage, the distribution of the *ACE* genotype status did not differ significantly from the non–gastric cancer group. On the other hand, many authors have shown that the *ACE* I/D polymorphism was not likely to be a strong predictor of cancer risk (43), and that ACE inhibitors had no preventive effects on tumor growth and angiogenesis in cancer cells (44). In this study, we found no significant association between the *ACE* I/D polymorphism and susceptibility to gastric cancer in Japan. Although the number of chymase-positive mast cells was significantly

**Table 4. Influences of the combination of allele carriage of *CMA/B* and *ACE* on the development of gastric cancer**

| Allele combination | | Control/Cancer, *n* | Adjusted OR (95% CI) | *P* |
|---|---|---|---|---|
| *CMA/B* | *ACE* | | | |
| G | I | 78/26 | 1.000 | |
| G | D | 103/32 | 1.073 (0.539-2.134) | 0.841 |
| A | I | 23/27 | 4.749 (2.050-11.001) | <0.001 |
| A | D | 88/34 | 1.120 (0.539-2.250) | 0.750 |

NOTE: High-producer allele combination of *CMA/B* and *ACE* polymorphisms were shown as follows: GI, GG/II; GD, GG/ID or DD; AI, GD or AA/II; and AD, GA, AA/ID, or DD. ORs were adjusted by age and sex.

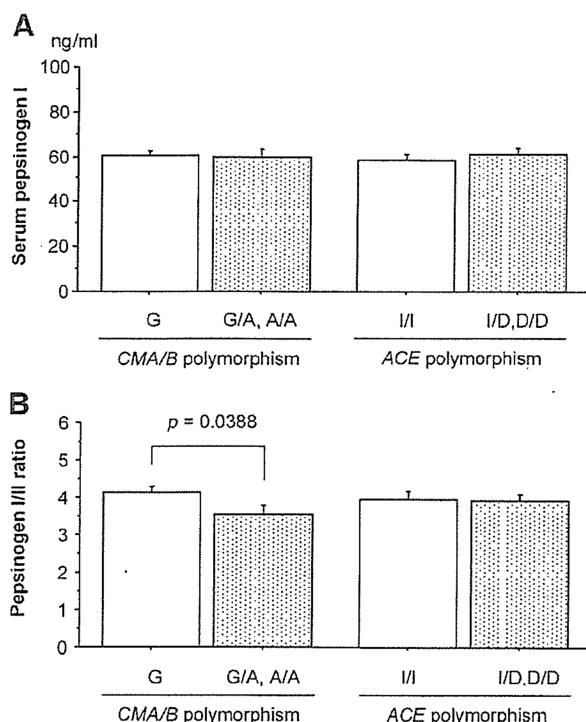


**Figure 1.** Mean serum PG I levels (**A**) and PG I/PG II ratios (**B**) in *CMA/B* and *ACE* polymorphisms of >50-year-old patients with *H. pylori*-infection. The mean serum PG I levels did not depend on different *CMA/B* and *ACE* genotypes (**A**). The mean PG I/PG II ratios of subjects with *CMA/B* A/G and A/A genotypes was significantly lower compared with those with the *CMA/B* G/G genotype, whereas no significant difference was observed when subjects were classified based on the ACE polymorphism (**B**).

higher in chronic gastritis with *H. pylori* infection than in normal stomach without *H. pylori* infection, the expression of ACE was not up-regulated in *H. pylori*-associated chronic gastritis (37). Moreover, we observed that the *ACE* I allele had the tendency to increase the risk of gastric cancer in subjects with the *CMA/B* A allele. Because the local generation of angiotensin II is mainly mediated by chymase (24, 39), we thought that the role of the *ACE* polymorphism in gastric carcinogenesis seems to be *CMA/B* polymorphism-dependent. Further studies are, however, required to determine the exact role of the *ACE* polymorphism in the pathogenesis of gastric cancer.

Serum PG levels are well known as a surrogate biomarker of gastric atrophy and inflammation induced by *H. pylori* infection (29, 30). The serum PG I level and the low PG I/PG II ratio are decreased with the progression of atrophic gastritis (29, 30), which is considered to be one of major risk factors of gastric cancer. In the present study, we reconfirmed that serum PG I levels and PG I/PG II ratios in the gastric cancer group were significantly decreased compared with those in the control group, and that gastric mucosal atrophy determined by PG methods differed among different *CMA/B* and *ACE* genotypes. The mean serum PG I/PG II ratio in the *CMA/B* A allele carriage group was significantly lower than those from the non-carriage group, which is consistent with our result that the *CMA/B* A allele carriage was at a higher risk for the development of intestinal types of gastric cancer. Therefore, we anticipate that the increased local angiotensin II generation mediated by chymase in subjects with the *CMA/B* A allele carriage enhances gastric inflammation, which leads to or accelerates the severe gastric atrophy and intestinal metaplasia, and finally, to the development of the intestinal type of gastric cancer.

In conclusion, we showed that *CMA/B* A/G polymorphisms in the RA system were associated with an increased risk of the development of gastric cancer and gastric ulcer. We also found the possible association of the *ACE* I/D polymorphisms with *CMA/B* A/G polymorphisms in this process. Therefore, genotyping tests of RA system–related genes (i.e., *CMA/B* A/G polymorphisms and *ACE* I/D polymorphisms) seem to be useful in screening individuals at a higher risk for gastric cancer development. Based on this genotyping test, individuals at a higher risk for gastric cancer seem to be good candidates for chemoprevention with chymase inhibitor and AT1R antagonist or eradication of *H. pylori*. However, the clinical usefulness of this genotyping test must be evaluated in future studies under the appropriate study design.

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# Efficient and erroneous incorporation of oxidized DNA precursors by human DNA polymerase η

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Running title: Erroneous incorporation of oxidized dNTPs by human Polη

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**Abbreviations:** ROS, Reactive oxygen species; 8-OH-dGTP, 7,8-dihydro-8-oxo-dGTP; A, adenine; 2-OH-dATP, 1,2-dihydro-2-oxo-dATP; G, guanine; Pols, DNA polymerases; C, cytosine; TLS, translesion DNA synthesis; T, thymine; Polη, DNA polymerase η; Polι, DNA polymerase ι; HPLC, high performance liquid chromatography

**Textural Footnotes**


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ABSTRACT

Altered oxidative metabolism is a property of many tumor cells. Oxidation of DNA precursors, i.e., dNTP pool, as well as DNA is a major source of mutagenesis and carcinogenesis. Here, we report remarkable nature of human DNA polymerase η that incorporates oxidized dNTPs into nascent DNA strand in an efficient and erroneous manner. The polymerase almost exclusively incorporated 8-hydroxy-dGTP (8-OH-dGTP) opposite template adenine (A) at 60% efficiency of normal dTTP incorporation, and incorporated 2-hydroxy-dATP (2-OH-dATP) opposite template thymine (T), guanine (G) or cytosine (C) at substantial rates. The synthetic primers having 8-hydroxy-G paired with template A or 2-hydroxy-A paired with template T, G or C at the termini were efficiently extended. In contrast, human DNA polymerase ι incorporated 8-OH-dGTP opposite template A with much lower efficiency and did not incorporate 2-OH-dATP opposite any of template bases. It did not extend the primers having the oxidized bases at the termini either. We propose that human DNA polymerase η may participate in oxidative mutagenesis through the efficient and erroneous incorporation of oxidized dNTPs during DNA synthesis.

Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen, are produced through normal cellular metabolism, and formation of such radicals is further enhanced by irradiation or chemical exposure (*1;2*). ROS generates a variety of altered purines and pyrimidines in DNA (*3;4*), and oxidation of DNA plays important roles in mutagenesis, carcinogenesis and aging (*5;6*). Several lines of evidence indicate, however, that oxidation of DNA precursors in the nucleotide pool, i.e., dNTPs, is another important cause of genome instability (*7-9*). Indeed, the frequency of A:T-to-C:G transversion mutations increases more than a 1000-fold over the wild-type level in *Escherichia coli mutT* mutants, which are deficient in the ability to hydrolyze oxidized dGTP, i.e., 7,8-dihydro-8-oxo-dGTP (8-hydroxy-dGTP, 8-OH-dGTP, Fig. 1A) (*10;11*). 8-OH-dGTP leads to A:T-to-C:G mutations when it is incorporated opposite adenine(A) in the template DNA because the incorporated 8-OH-G in DNA can pair with incoming dCMP in the next round of DNA replication (*8;12*). The high spontaneous A:T-to-C:G mutations in the *mutT* strain are almost completely suppressed when the *mutT* cells are cultured in anaerobic conditions, indicating the essential role of oxygen in the mutagenesis (*13*). Another oxidized nucleotide, i.e., 1,2-dihydro-2-oxo-dATP (2-hydroxy-dATP, 2-OH-dATP, Fig. 1 B), can induce G:C-to-T:A transversions when it is incorporated opposite guanine (G) in the template (*14;15*). The sanitizing enzyme, i.e., Orf135, in *E. coli* degrades 2-OH-dATP, and G:C-to-T:A mutations occur in the *orf135*-deficient strain more frequently than in the wild-type strain (*16;17*).

Oxidized dNTPs also cause genome instability in mammalian cells. Spontaneous tumorigenesis in the mice deficient in *Mth1*, a mammalian counterpart of *mutT*, is much enhanced in lung, liver and stomach, and the MTH1 protein hydrolyzes both 8-OH-dGTP and 2-OH-dATP (*18;19*). Recent studies with mismatch repair defective cells suggest that the majority of mutations in human cells that are deficient in mismatch repair functions do not arise from spontaneous replication errors, but from the incorporation of oxidized dNTPs (*20;21*). Thus, it is of great interest in the mechanisms as to how these oxidized dNTPs induce genome instability and oxidative carcinogenesis.

When the oxidized dNTPs escape from the sanitation by the enzymes, they will be incorporated into nascent DNA by DNA polymerases (Pols). However, oxidized dNTPs are in general poor substrates for

Pols (*22*). For example, the efficiency of incorporation of 8-OH-dGTP by Pol δ is more than $10^4$-fold lower than that of incorporation of normal dGTP, and the enzyme prefers to incorporate 8-OH-dGTP opposite template cytosine (C) (*23*). 8-OH-dGTP is poorly incorporated into DNA by T7 Pol $exo^-$, HIV reverse transcriptase, *E. coli* Pol II and Klenow $exo^-$ as well (*24*). An exception may be human Pol β, which incorporates 8-OH-dGTP into DNA with an efficiency of 10-20% of normal dGTP incorporation and favors to incorporate it opposite template A (*25*). 2-OH-dATP is also a poor substrate for mammalian Pols. The efficiencies of incorporation of 2-OH-dATP opposite template T and C by Pol α are more than 100-fold and 1000-fold, respectively, lower than those of incorporation of normal dATP and dGTP (*26*).

The Y-family Pols are recently recognized Pols that comprise proteins from different species, including members of Archaea, Bacteria and Eukarya (*27*). The most distinct feature of this family of enzymes is their ability to bypass various lesions, such as ultraviolet light photoproducts, in template DNA (*28-30*). Some bypass reactions, i.e., translesion DNA synthesis (TLS), catalyzed by these enzymes are error prone while others are error free (*31*). Thus, this family of Pols seems to be involved in mutagenesis and DNA-damage tolerance (*32*). Interestingly, some of Y-family Pols are shown to incorporate oxidized dNTPs into DNA in an erroneous manner. Archaeal Y-family Pols from *Sulfolobus* sp. and a bacterial Y-family Pol, i.e., DNA Pol IV (DinB) of *Escherichia coli*, almost exclusively incorporate 8-OH-dGTP opposite template A, and 2-OH-dATP opposite template G and thymine (T) (*33;34*). Further genetic analysis with *E. coli sod/fur* mutants suggests that both Y-family Pols of *E. coli*, i.e., Pol IV and Pol V (UmuD'C), are involved in oxidative mutagenesis caused by oxidized dNTPs (*34*). The enzymes may participate in sequential biochemical steps, such as incorporation of oxidized dNTPs into DNA and extension of primers having oxidized bases at the termini. Pol IV is also shown to be involved in induction of G:C-to-T:A mutations when 2-OH-dATP is directly introduced into *E. coli* cells by $CaCl_2$ treatment (*35*). Collectively, these results led us to postulate that certain human Y-family Pols might be involved in oxidative mutagenesis through incorrect and efficient incorporation of

oxidized dNTPs into nascent DNA. In fact, human Polη incorporates oxidized dNTPs into DNA in an erroneous manner like the archaeal and bacterial Y-family Pols (*33*).

In this study, we quantitatively compared two human Y-family Pols, i.e., Polη and Polι, in the specificity and efficiency of incorporation of 8-OH-dGTP and 2-OH-dATP into nascent DNA strand. Surprisingly, Polη incorporated 8-OH-dGTP opposite template A at almost the same efficiency as incorporation of normal dTTP, and incorporated 2-OH-dATP opposite template T, G and C at substantial rates. In contrast, Polι poorly incorporated 8-OH-dGTP opposite template A and did not incorporate 2-OH-dATP opposite any of the bases in template DNA. Since Polη is constitutively associated with replication factories (*36;37*) and involved in multiple DNA transactions (*38-40*), we propose that this enzyme may be involved in mutagenesis through the efficient and erroneous incorporation of oxidized dNTPs into DNA strand.

## EXPERIMENTAL PROCEDURES

*Materials.* Human Polη and Polι were purified as described (*41;42*). 8-OH-dGTP and 2-OH-dATP were prepared as described (*26;43*) and no discernible impurities were detected by high performance liquid chromatography (HPLC). dNTPs (ultrapure grade) were purchased from GE Healthcare Bio-Sciences KK (Tokyo).

*DNA substrates.* Oligonucleotides containing 8-OH-G and 2-OH-A were prepared by the phosphoramidite method on controlled pore glass supports (1 μmol) by using a Beckman OLIGO1000 DNA synthesizer. After automated synthesis, the oligomers were detached from the support, deprotected, and purified by HPLC. The oligomers were identified by complete digestion of the oligomers with alkaline phosphatase and P1 nuclease to 2'-deoxymononucleosides. Other oligonucleotides were purchased from BEX Corp. (Tokyo). A Cy3-labeled 18mer primer (5'-CGCGCGAAGACCGGTTAC-3') or Cy3-labeled 19mer primers (5'-CGCGCGAAGACCGGTTAC$\underline{N}$-3' where N represents either 8-OH-G or 2-OH-A) were annealed to 36-mer templates (5'-GAAGGGATCCTTAAGAC$\underline{X}$GTAACCGGTCTTCGCGCG-3' where X represents A, C, G or T) at a

molar ratio of 1:1 to generate substrates. The 5'-Cy3-primer/templates generated by annealing the 18mer primer to the 36mer template were named substrate 1, and those generated by annealing the 19mer primer having 8-OH-G or 2-OH-A to the 36mer templates were named substrates 2 or 3, respectively. In control reactions, Cy3-labeled 19mer primers (5'-CGCGCGAAGACCGGTTAC<u>N</u>-3' where N represents either G or A) were annealed to the 36-mer templates. The 5'-Cy3-primer/templates generated by annealing the 19mer primer having G or A to the 36mer templates were named substrate 4 or 5, respectively.

*Steady-state kinetic analyses.* The standard reaction mixtures (10 μl) contained 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM dithiothreitol, 250 μg/ml bovine serum albumin, 60 mM KCl, 2.5% glycerol, 1 nM Polη or Polι, 100 nM primer/template DNA (substrate 1, 2 or 3) and a single oxidized dNTP (8-OH-dGTP or 2-OH-dATP) or normal dNTP (dATP, dTTP, dGTP or dCTP). The activity of Polι was also measured in the same reaction mixtures but without KCl (*44*). In the case, the concentration of $MgCl_2$ was reduced to 1 mM instead of 5 mM. Six different concentrations of oxidized dNTP or normal dNTP were used to determine the kinetic parameters, i.e., $k_{cat}$ and $K_m$ (see below). The mixtures were incubated at 37°C, and the reactions were terminated by adding 10 μl of stop solution (98% formamide, 10 mM EDTA, 10mg/ml Blue Dextran). After heat denaturation for 10 min at 100ºC, the samples were separated by electrophoresis with 15% denaturing polyacrylamide gel containing 8M urea and visualized by the Molecular Imager FX Pro System (Bio-Rad Laboratories, CA).

When the kinetic parameters for incorporation of oxidized or normal dNTPs into DNA were determined, the reaction mixtures contained substrate 1. The reaction time and the concentrations of oxidized dNTP and normal dNTP varied depending on the rates of reactions and the $K_m$ values. For example, the incubation time was 1 min and the concentrations of 8-OH-dGTP varied from 0.05 to 10 μM for the analysis of incorporation of 8-OH-dGTP opposite template A by Polη. Identical reaction conditions were used for the analyses of incorporation of a single normal dNTP opposite a template canonical base by Polη. On the other hand, the incubation time was 10 min and the range of concentrations of 8-OH-dGTP was 5 to 100 μM to determine the parameters for incorporation of 8-OH-

dGTP opposite template A by Polι. For the kinetic analyses of extension of primers having oxidized bases at the 3'-termini by Polη, the incubation time was 5 min and the concentrations of dGTP varied from 0.05 to 10 μM with the template/primer DNAs of substrate 2 or 3.

*Data analyses.* Gel band intensities were measured using the Molecular Imager FX Pro System and Quantity One software (Bio-Rad Laboratories, CA) and nucleotide incorporation parameters were determined (*45*). Less than 20% of the primers were extended in these steady-state kinetic analyses, ensuring single-hit kinetics (*46*). For each DNA substrate, the rate of incorporation was plotted as a function of dNTP concentration, and the $V_{max}$ and $K_m$ values were determined by nonlinear regression fitting using the SigmaPlot software (Systat Software Inc., CA). $k_{cat}$ was calculated by dividing $V_{max}$ by the enzyme concentration. The fidelity of incorporation, i.e., $F_{inc}$, was calculated using the equation $F_{inc} = (k_{cat}/K_m)_{incorrect} / (k_{cat}/K_m)_{correct}$. All values are means ± standard errors from three experiments.

## RESULTS

*Efficient and erroneous incorporation of 8-OH-dGTP and 2-OH-dATP into DNA by Polη.* Human Polη almost exclusively incorporates 8-OH-dGTP opposite template A and incorporates 2-OH-dATP opposite template T, G and C (*33*). To compare two human Y-family Pols, we at first examined the specificity of human Polι incorporating 8-OH-dGTP and 2-OH-dATP into DNA. To this end, we used substrate 1 having four different bases in the N position in the template and incubated it with Polι in the presence of either 8-OH-dGTP or 2-OH-dATP. For the incorporation of 8-OH-dGTP, the primer annealed to the template strand having A in the N position was elongated, and no other primers annealed to templates having C, G or T in the N position were extended (Fig. 2A). These results indicate that Polι almost exclusively incorporates 8-OH-dGTP opposite template A as in the case of Polη. For the incorporation of 2-OH-dATP, no primers were elongated regardless of the bases in the template strand (Fig. 2B). The activity of Polι is enhanced by omission of KCl from the reaction mixtures (*44*). We conducted, therefore, the experiments in the absence of KCl. Although the activity was enhanced

several times (Table 1), the specificity was unchanged. 8-OH-dGTP was almost exclusively incorporated opposite template A and no 2-OH-dATP was incorporated into DNA regardless of the template bases (data not shown). In control reactions, Polι incorporated normal dGTP opposite template C and T and incorporated normal dATP opposite template T and C (Fig. 2C and D). Thus, we conclude that, although the incorporation of 8-OH-dGTP opposite template A is a common feature to Y-family Pols, the specificity incorporating 2-OH-dATP into DNA is markedly distinct even within two human homologues.

To compare the efficiency of two Pols for the incorporation of oxidized dNTPs quantitatively, we determined the steady-state kinetic parameters, i.e., $K_m$ and $k_{cat}$ (Table 1). The most remarkable feature of the kinetic data is the high efficiency of Polη incorporating 8-OH-dGTP opposite template A. The $K_m$ and $k_{cat}$ values (4.2 μM and 10.1 $min^{-1}$, respectively) were similar to those of incorporation of normal dTTP opposite template A (3.8 μM and 15.4 $min^{-1}$, respectively). Thus, the $F_{inc}$, i.e., $(k_{cat}/K_m)_{8\text{-OH-dGTP}}/(k_{cat}/K_m)_{dTTP}$, was about 0.6 (= 2.4/4.1), which suggests that Polη incorporates 8-OH-dGTP opposite template A with the efficiency of about 60% of that of incorporation of normal dTTP. In addition, the incorporation of 8-OH-dGTP by Polη was strongly biased opposite template A compared to template C. The $k_{cat}/K_m$ value of incorporation of 8-OH-dGTP opposite template A was more than 180 times greater than that of incorporation of 8-OH-dGTP opposite template C (2.4 versus 0.013). In contrast, Polι incorporates 8-OH-dGTP opposite template A with much lower efficiency. The $k_{cat}/K_m$ value of incorporation of 8-OH-dGTP opposite template A was more than 200 and 30 times, respectively, lesser than that of incorporation of 8-OH-dGTP opposite template A by Polη (0.011 versus 2.4) and normal dTTP by Polι (0.011 versus 0.35). The $k_{cat}/K_m$ value of incorporation of normal dTTP and 8-OH-dGTP opposite template A by Polι was enhanced several times by omission of KCl from the reaction mixtures (0.35 versus 2.6 and 0.011 versus 0.045). Nevertheless, the $k_{cat}/K_m$ value of incorporation of 8-OH-dGTP opposite template A was more than 50 times lesser than that of incorporation of 8-OH-dGTP opposite template A by Polη (0.045 versus 2.4) and normal dTTP by Polι (0.045 versus 2.6).

We then determined the kinetic parameters of incorporation of 2-OH-dATP opposite template C, G or T by Polη (Table 1). We did not determine the parameters for Polι because the enzyme did not incorporate 2-OH-dATP into DNA (Fig. 2 B). The $K_m$ values for the incorporation of 2-OH-dATP by Polη were indistinguishable from those for the incorporation of normal dNTPs opposite template C, G or T. However, the $k_{cat}$ values for the incorporation of 2-OH-dATP were 20 to 30 times lesser than those of incorporation of normal dNTPs. Nevertheless, the $F_{inc}$ values, i.e., $(k_{cat}/K_m)_{2\text{-OH-dATP}}/(k_{cat}/K_m)_{dNTP}$, opposite template C, G and T were 0.038, 0.017 and 0.062, respectively, suggesting that Polη incorporates 2-OH-dATP opposite multiple template bases (except for A) with efficiency of 2 to 6 % of that of incorporation of normal dNTPs. The order of $k_{cat}/K_m$ values of incorporation of 2-OH-dATP opposite template bases was T (0.18) > G (0.10) > C (0.06).

*Efficient extension of primers having 8-OH-G or 2-OH-A at the 3'-termini by Polη.* To investigate whether Polη and Polι can extend the primers upon incorporation of oxidized dNTPs during DNA synthesis, we examined whether these Pols extend primers having either 8-OH-G or 2-OH-A at the 3'-termini. We annealed the primers to the template strands where the terminal oxidized base was paired with template A, C, G or T and incubated the primer/templates, i.e., substrate 2 or 3, with Polη or Polι in the presence of four normal dNTPs. Polη extended the primer having 8-OH-G when the terminal oxidized base was paired with template A or C (Fig. 3A). Virtually no extension was observed, however, when the terminal 8-OH-G was paired with template G or T. Polη could also extend primers having 2-OH-A when the terminal oxidized base was paired with template C, G or T (Fig. 3C). In contrast, Polι was unable to extend any primers containing either 8-OH-G or 2-OH-A at the termini regardless of the pairing template bases (Fig. 3B and D). In control reactions, Polη extended the primers having G at the termini when the terminal base was paired with template C or T (Fig. 3E) and extended the primers having A at the termini when the terminal base was paired with template T (Fig. 3G). Weak extension was observed when the terminal A was paired with template C. Polι was a poor extender even without oxidized bases at the termini of primers. It weakly extended primers when the terminal G or A was

paired with canonical template bases (Fig. 3F and H). These results suggest that Polη extends the primers upon incorporation of 8-OH-dGTP opposite template A or C, and extends the primers upon incorporation of 2-OH-dATP opposite template C, G or T. Two Y-family Pols are distinct for their ability to extend the primers having oxidized bases at the termini.

We then determined the steady-state kinetic parameters for the extension, i.e., incorporation of next dGTP opposite template C, by Polη from the primers having either 8-OH-G or 2-OH-A at the 3'-termini (Table 2). We did not determine the parameters for Polι because it did not extend the primers having 8-OH-G or 2-OH-A (Fig. 3 B and D).The terminal 8-OH-G in the primer was paired with template A or C while the terminal 2-OH-A was paired with template C, G or T. The primer/templates, i.e., substrates 2 or 3, were incubated with Polη in the presence of dGTP without other dNTPs. For the extension from the primer having 8-OH-G at the termini, Polη extended the primer more efficiently when the terminal oxidized base was paired with template A than template C. The $K_m$ value for the extension from the primer having 8-OH-G paired with template A was 2.5 times lesser than that for the extension from the primer having the oxidized base paired with template C (0.98 versus 2.5 μM) although the $k_{cat}$ values were very similar between the two substrates (2.4 versus 2.6 $min^{-1}$). Thus, the ratio, i.e., $(k_{cat}/K_m)_{8\text{-OH-G:A}}/(k_{cat}/K_m)_{8\text{-OH-G:C}}$, was calculated 2.4 (= 2.4/1.0), suggesting that Polη more than two times more efficiently extends the primer having 8-OH-G paired with template A compared to that having 8-OH-G paired with template C. For the extension from the primer having 2-OH-A at the 3'-temini, Polη extended the primer most efficiently when the oxidized base was paired with template T followed by template C and template G. The $K_m$ value for the extension from the primer having 2-OH-A paired with template T (2.8 μM) was 2-to-3 times lesser than those for the extension from the primers having 2-OH-A paired with template G or template C (6.9 or 7.3 μM) and the $k_{cat}$ values were not very different among the three substrates (8.9, 7.3 or 9.9 $min^{-1}$). Thus, the ratio of $k_{cat}/K_m$ values, i.e., $(k_{cat}/K_m)_{2\text{-OH-A:T}}$: $(k_{cat}/K_m)_{2\text{-OH-A:G}}$: $(k_{cat}/K_m)_{2\text{-OH-A:C}}$ was 1: 0.34: 0.44, which suggests that Polη extends the primers

having terminal 2-OH-A paired with template G or C at rates of 30 to 40% of that of extension from the primer having 2-OH-A paired with template T.

DISCUSSION

Oxidation is a major cause of spontaneous DNA damage that may contribute to mutagenesis, carcinogenesis and aging (*5;6*). Oxidative damage in DNA is caused not only by direct oxidation of bases in DNA but also by incorporation of oxidized dNTPs in the nucleotide pool into DNA during chromosome replication (*7;47*). In this study, we have revealed the remarkable nature of Polη that incorporates 8-OH-dGTP opposite template A almost as efficient as the incorporation of normal dTTP (Table 1). In addition, Polη extends the primer having 8-OH-G paired with template A more than two times more efficiently than the same primer having 8-OH-G paired with template C (Table 2). Calculation of the total efficiency by multiplying the individual $k_{cat}/K_m$ values suggests that Polη prefers template A more than 400 times (446 = 5.8/0.013) to template C for the incorporation and extension (Fig. 4). The efficient and erroneous incorporation of 8-OH-dGTP and the following extension may lead to A:T-to-C:G transversions because the incorporated 8-OH-G opposite template A may pair with dCMP in the next round of DNA replication (*8;43;48*). Besides 8-OH-dGTP, Polη incorporates 2-OH-dATP opposite template T, G and C with efficiency of 2 to 6 % of that of incorporation of normal dNTPs (TABLE 1). The $F_{inc}$ values of incorporation of 2-OH-dATP opposite template T and C are about 20 and 50 times higher than the corresponding values by calf thymus Polα (*26*). Moreover, Polη can extend the primers having terminal 2-OH-A paired with template T, G and C (Fig. 3, Table 2). The relative preference of Polη to template T: G: C for incorporation and extension is calculated as 1: 0.19: 0.14 (Fig. 4). Incorporation of 2-OH-dATP opposite template G or C and the following extension may lead to G:C-to-T:A and C:G-to-T:A mutations since the incorporated 2-OH-A can pair with dTMP during the next round of DNA replication (*14;49*). Thus, we suggest that Polη has a potential to enhance mutagenesis through the efficient and erroneous incorporation of 8-OH-dGTP and 2-OH-dATP and the

following extension during DNA replication (see below). It is possible, however, that other Pols may be involved in extension steps once oxidized dNTPs are inserted into nascent DNA by Pol η.

Although Polη was initially identified as an error-free bypass Pol across a *cis-syn* thymine-thymine dimer in DNA (*28;29*), it appears to play important roles in several DNA transactions besides TLS (*50*). Polη interacts with RAD51 and has an activity to extend D loop, which is an intermediate of homologous recombination (*38;39*). It also participates in mutagenesis at A:T base-pairs in immunoglobulin genes during somatic hypermutation (*40*). In addition, Polη is identified in replication factories in cells not deliberately exposed to DNA damaging agents (*36;37*), suggesting that it might constitutively gain access to the genomic DNA and contribute to mutagenesis and/or damage avoidance even without external DNA damage. Interestingly, *E. coli* Y-family Pols, i.e., Pol IV (DinB) and variants of Pol V (UmuD'C), are shown to be involved in the chromosome replication when the cells are treated with hydroxyurea, which does not induce DNA damage but depletes the nucleotide pool (*51*). In this case, the Y-family Pols appear to rescue the stalled replication by efficient incorporation of dNTPs in the depleted nucleotide pool at an expense of increased mutations. By analogy, we envisage that Polη might be localized in replication factories even without external DNA damage and contribute to rescue the stalled replication by incorporation of oxidized dNTPs when the nucleotide pool is heavily oxidized and/or the ratio of oxidized versus normal dNTPs is substantially enhanced. The replicative Pols might have difficulty to extend the primers without Polη in the situation since the oxidized dNTPs are generally difficult substrates for replicative Pols. However, the incorporation by Polη is erroneous and thus may induce mutations as described above. In fact, the mutagenicity of 8-OH-dGTP directly incorporated into human cells is reduced significantly when the expression of Pol η is suppressed by RNAi technique (Kamiya et al., unpublished results). It is demonstrated that both 8-OH-dGTP and 2-OH-dATP are significant contributors to mutations in mismatch-repair-defective cells and incorporation of oxidized DNA precursors is a significant influence on microsatellite instability in repair-defective human tumor cells (*21*). We suggest, therefore, that Polη may be involved in mutagenesis by the

incorporation of oxidized dNTPs in various DNA transactions when the nucleotide pool is oxidized and imbalanced.

Unlike Polη, Polι has a limited ability to incorporate oxidized dNTPs and extend the primers having the oxidized bases at the termini. It could not incorporate 2-OH-dATP into DNA and extend the primers having 8-OH-G or 2-OH-A regardless of the template bases under the experimental conditions (Fig. 2 B, Fig. 3 B and D). The only activity we could detect in this study was the incorporation of 8-OH-dGTP opposite template A (Fig. 2 A, Table 1). Polι is known to form Hoogsteen base pairing in the active site where the template A is rotated to the *syn* conformation while the incoming dTTP is in the *anti* conformation (*52*). Thus, the template A may be driven to the *syn* conformation when 8-OH-dGTP is incorporated. The Hoogsteen base pairing, i.e., template A (*syn*): 8-OH-dGTP (*syn* or *anti*), may be the basis for the limited catalysis of incorporation of 8-OH-dGTP opposite template A. Nevertheless, Polι could not extend the primers having 8-OH-G at the termini. This may resemble the situation where Polι fails to further elongate the primers upon incorporation of C or T opposite 1, $N^2$-propano-2'-deoxyguanosine, which forms a *syn* conformation in the template DNA strand (*53*). Although it is possible that other Pols such as Polκ may help the extension step upon incorporation of 8-OH-G opposite template A by Polι, it seems unlikely that Polι is heavily involved in oxidative mutagenesis through the incorporation of oxidized dNTPs into nascent DNA.

In this study, we have revealed the remarkable nature of Polη that incorporates 8-OH-dGTP and 2-OH-dATP into DNA in an efficient and erroneous manner. We assume that the incoming 8-OH-dGTP adopts the *syn* conformation in the active site of Polη, which allows to form Hoogsteen base pairing with the *anti* form of template A. The active site large enough to accommodate bulky DNA adducts may permit the *syn* conformation of incoming 8-OH-dGTP, which is favored when it pairs with A in a DNA duplex (*54*). The Hoogsteen base pairing, i.e., 8-OH-dGTP (*syn*) and template A (*anti*), which might be opposite to the conformation of pairing bases in the active site of Polι, could be the basis for the efficient and erroneous incorporation of 8-OH-dGTP by Polη. Interestingly, yeast and human Polη can

bypass 8-OH-G in DNA in an efficient but error-free manner by insertion of dCMP opposite the oxidized base in template strand (*55*). In these cases, the large active site of Polη is thought to allow the *anti* conformation of 8-OH-G in the template strand, which introduces barriers for other Pols (*56*). Since oxidative damage in the nucleotide pool as well as DNA is a major source of spontaneous mutagenesis and carcinogenesis, the present study warrants the validity of further research on the roles of Polη in the oxidative mutagenesis in human cells.

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