Oxidative DNA Damage Induced by a Metabolite of 2-Naphthylamine, a Smoking-related Bladder Carcinogen

Shiho Ohnishi, Mariko Murata and Shosuke Kawanishi

Department of Hygiene, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507

2-Naphthylamine (2-NA), a bladder carcinogen, is contained in cigarette smoke. DNA adduct formation is thought to be a major cause of DNA damage by carcinogenic aromatic amines. We have investigated whether a metabolite of 2-NA, 2-nitroso-1-naphthol (NO-naphthol) causes oxidative DNA damage, using 32P-labeled DNA fragments. We compared the mechanism of DNA damage induced by NO-naphthol with that by N-hydroxy-4-aminobiphenyl (4-ABP(NHOH)), a metabolite of 4-aminobiphenyl, another smoking-related bladder carcinogen. NO-naphthol caused Cu(II)-mediated DNA damage at T>>>C>>>G residues, with non-enzymatic reduction by NADH. Catalase and bathocuproine, a Cu(I)-specific chelator, inhibited the DNA damage, suggesting the involvement of H2O2 and Cu(I). Some free •OH scavengers also attenuated NO-naphthol-induced DNA damage, while free •OH scavengers had no effect on the DNA damage induced by 4-ABP(NHOH). This difference suggests that the reactive species formed by NO-naphthol has more free •OH-character than that by 4-ABP(NHOH). A high-pressure liquid chromatograph equipped with an electrochemical detector showed that NO-naphthol induced 8-oxo-7,8-dihydro-2′′′′-deoxyguanosine formation in the presence of NADH and Cu(I). The oxidative DNA damage by these amino-aromatic compounds may participate in smoking-related bladder cancer, in addition to DNA adduct formation.

Key words: Naphthylamine — DNA damage — Copper — Hydrogen peroxide

Epidemiological studies have shown that occupational exposure to 2-naphthylamine (2-NA), is strongly associated with the occurrence of bladder cancer. Oral administration of 2-NA has been reported to produce bladder carcinomas in the dog and monkey, and at high dosage levels in the hamster. The International Agency for Research on Cancer (IARC) has classified 2-NA as a group 1 carcinogen that is carcinogenic to humans. The commercial production and usage of 2-NA have been prohibited, whereas smokers are exposed to 1–22 ng of 2-NA with every cigarette smoked. There is increasing evidence that the excess of bladder cancer in smokers is attributable to aromatic amines. Smoking is a risk factor for bladder cancer, in addition to lung cancer.

DNA adduct formation is thought to be a major cause of DNA damage by these carcinogenic aromatic amines. 2-NA is metabolically N-hydroxylated and glucuronidated in the liver and the N-glucuronide is transported to the urinary bladder. The hydrolysis of the glucuronide to N-hydroxy-naphthylamine and subsequent DNA adduct formation are thought to be important for causing bladder cancer. 2-Nitroso-1-naphthol (NO-naphthol) may be produced as a metabolite of 2-NA. NO-Naphthol is formed by conversion of the N-hydroxy-2-naphthylamine (N-OH-NA) into nitroso compounds, followed by hydroxylation. Nakayama et al. reported that metabolites of 2-NA, such as N-OH-NA and 2-amino-1-naphthol, generated reactive oxygen species and that N-OH-NA induced DNA lesions.

In this study, we investigated site-specific DNA damage induced by a metabolite of 2-NA, NO-naphthol, using 32P-labeled human DNA fragments from the p53 tumor suppressor gene and the c-Ha-ras-1 protooncogene. We analyzed 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation, a marker of oxidative DNA damage, in calf thymus DNA treated with NO-naphthol. It has been reported that 8-oxodG formation can lead to DNA misreplication, resulting in mutation and cancer.

Previously, we reported Cu(II)-mediated oxidative DNA damage induced by N-hydroxy-4-aminobiphenyl (4-ABP(NHOH)), which is another bladder carcinogen contained in cigarette smoke. We compared the mechanism of DNA damage induced by NO-naphthol with that by 4-ABP(NHOH).

MATERIALS AND METHODS

Materials

T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). [γ-32P]ATP (222 TBq/mmol) was obtained from New England Nuclear. Restriction enzymes (ApaI, Aval, EcoRI, HindIII and PstI) and alkaline phosphatase from calf intestine were purchased from Roche Molecular Biochemicals (Mannheim, Germany).
DNA Damage by a 2-Naphthylamine Metabolite

Germany). NO-Naphthol was purchased from Aldrich Chem. Co. (Milwaukee, WI). 4-ABP(NHOH) was a kind gift from Dr. Mariko Tada (Aichi Shukutoku University). Copper(II) chloride dihydrate was purchased from Nacalai Tesque, Inc. (Kyoto). Diethylenetriamine-\(N,N,N',N'',N''',N''''\)-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were purchased from Dojin Chemicals Co. (Kumamoto). Piperidine was purchased from Wako Chemical Industries, Ltd. (Osaka). Methional (3-(methylthio)propionaldehyde) was purchased from Tokyo Kasei Co. (Tokyo). Calf thymus DNA, \(\beta\)-nicotinamide adenine dinucleotide disodium salt (reduced form) (NADH), superoxide dismutase (SOD) (3000 units/mg from bovine erythrocytes) and catalase (45 000 units/mg from bovine liver) were purchased from Sigma Chemical Co. (St. Louis, MO). Nuclease \(P_1\) (400 units/mg) was purchased from Yamasa Shoyu Co. (Chiba).

Preparation of \(^{32}\text{P}\)-5'-end-labeled DNA fragments obtained from the p53 gene and the c-Ha-ras-1 gene

DNA fragments were obtained from the human p53 tumor suppressor gene and the c-Ha-ras-1 protooncogene.\(^{20,21}\) A singly \(^{32}\text{P}\)-5'-end-labeled double-stranded 443-bp p53 fragment (ApaI 14 179– EcoRI 14 621) and 211-bp p53 fragment (\(\text{HindIII}^*\) 13 972–ApaI 14 182) were prepared from the pUC18 plasmid according to a method described previously.\(^{22}\) A 337-bp c-Ha-ras-1 fragment (\(\text{PstI} 2345–\text{AvalI} 2681\)) was prepared from plasmid pbcNI, which carries a 6.6-kb \(\text{BamHI}\) chromosomal DNA restriction fragment.\(^{23}\) The asterisk indicates \(^{32}\text{P}\)-labeling. The 443-bp DNA fragment was singly labeled on the 5'-end of the sense strand. The 337-bp fragment was singly labeled on the 5'-end of the antisense strand of the c-Ha-ras-1 gene.

Detection of DNA damage

Detection of DNA damage and analysis of its site-specificity were performed according to a previously described method.\(^{20,21}\) A standard reaction mixture (in a microtube; 1.5 ml) contained \(\text{CuCl}_2\), NADH, NO-naphthol or 4-ABP(NHOH), the \(^{32}\text{P}\)-labeled double-stranded DNA fragments and calf thymus DNA in 200 \(\mu\)l of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 \(\mu\)M DTPA. After incubation at 37°C, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min and treated as described previously.\(^{20,21}\) The treated DNA fragments were electrophoresed on an 8% precast polyacrylamide/8 M urea gel and an autoradiogram was obtained by exposing X-ray film to the gel. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure using a DNA-sequencing system (LKB 2010 Macrophor).\(^{20}\) A laser densitometer (LKB 2222 Ultrascan XL) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

Analysis of 8-oxodG formation by NO-naphthol

Calf thymus DNA was incubated with NO-naphthol, NADH and \(\text{CuCl}_2\). After ethanol precipitation, the treated DNA was digested to its component nucleosides with nuclease \(P_1\) and calf intestine phosphatase and analyzed by a high-pressure liquid chromatograph equipped with an electrochemical detector (HPLC-ECD), as described by Kasai et al.\(^{27,28}\)

RESULTS

Damage to \(^{32}\text{P}\)-labeled DNA

Fig. 1 shows an autoradiogram of a DNA fragment treated with NO-naphthol in the presence of NADH and/or Cu(II). Oligonucleotides that formed after DNA cleavage were detected on the autoradiogram. In the presence of both NADH and Cu(II), NO-naphthol caused DNA damage, with 2–5 \(\mu\)M NO-naphthol being most damaging. Piperidine treatment increased the number of oligonucleotides produced, suggesting that NO-naphthol induced not only strand breakage, but also base modification and/or liberation. NO-naphthol did not cause DNA damage in the absence of NADH or Cu(II). NO-naphthol plus NADH caused no DNA damage with Fe(II) or Fe(III) (data not shown).

Analysis of 8-oxodG formation by NO-naphthol

Calf thymus DNA was incubated with NO-naphthol, NADH and \(\text{CuCl}_2\). After ethanol precipitation, the treated DNA was digested to its component nucleosides with nuclease \(P_1\) and calf intestine phosphatase and analyzed by a high-pressure liquid chromatograph equipped with an electrochemical detector (HPLC-ECD), as described by Kasai et al.\(^{27,28}\)

RESULTS

Damage to \(^{32}\text{P}\)-labeled DNA

Fig. 1 shows an autoradiogram of a DNA fragment treated with NO-naphthol in the presence of NADH and/or Cu(II). Oligonucleotides that formed after DNA cleavage were detected on the autoradiogram. In the presence of both NADH and Cu(II), NO-naphthol caused DNA damage, with 2–5 \(\mu\)M NO-naphthol being most damaging. Piperidine treatment increased the number of oligonucleotides produced, suggesting that NO-naphthol induced not only strand breakage, but also base modification and/or liberation. NO-naphthol did not cause DNA damage in the absence of NADH or Cu(II). NO-naphthol plus NADH caused no DNA damage with Fe(II) or Fe(III) (data not shown).

Analysis of 8-oxodG formation by NO-naphthol

Calf thymus DNA was incubated with NO-naphthol, NADH and \(\text{CuCl}_2\). After ethanol precipitation, the treated DNA was digested to its component nucleosides with nuclease \(P_1\) and calf intestine phosphatase and analyzed by a high-pressure liquid chromatograph equipped with an electrochemical detector (HPLC-ECD), as described by Kasai et al.\(^{27,28}\)

RESULTS

Damage to \(^{32}\text{P}\)-labeled DNA

Fig. 1 shows an autoradiogram of a DNA fragment treated with NO-naphthol in the presence of NADH and/or Cu(II). Oligonucleotides that formed after DNA cleavage were detected on the autoradiogram. In the presence of both NADH and Cu(II), NO-naphthol caused DNA damage, with 2–5 \(\mu\)M NO-naphthol being most damaging. Piperidine treatment increased the number of oligonucleotides produced, suggesting that NO-naphthol induced not only strand breakage, but also base modification and/or liberation. NO-naphthol did not cause DNA damage in the absence of NADH or Cu(II). NO-naphthol plus NADH caused no DNA damage with Fe(II) or Fe(III) (data not shown).

Analysis of 8-oxodG formation by NO-naphthol

Calf thymus DNA was incubated with NO-naphthol, NADH and \(\text{CuCl}_2\). After ethanol precipitation, the treated DNA was digested to its component nucleosides with nuclease \(P_1\) and calf intestine phosphatase and analyzed by a high-pressure liquid chromatograph equipped with an electrochemical detector (HPLC-ECD), as described by Kasai et al.\(^{27,28}\)

RESULTS

Damage to \(^{32}\text{P}\)-labeled DNA

Fig. 1 shows an autoradiogram of a DNA fragment treated with NO-naphthol in the presence of NADH and/or Cu(II). Oligonucleotides that formed after DNA cleavage were detected on the autoradiogram. In the presence of both NADH and Cu(II), NO-naphthol caused DNA damage, with 2–5 \(\mu\)M NO-naphthol being most damaging. Piperidine treatment increased the number of oligonucleotides produced, suggesting that NO-naphthol induced not only strand breakage, but also base modification and/or liberation. NO-naphthol did not cause DNA damage in the absence of NADH or Cu(II). NO-naphthol plus NADH caused no DNA damage with Fe(II) or Fe(III) (data not shown).

Analysis of 8-oxodG formation by NO-naphthol

Calf thymus DNA was incubated with NO-naphthol, NADH and \(\text{CuCl}_2\). After ethanol precipitation, the treated DNA was digested to its component nucleosides with nuclease \(P_1\) and calf intestine phosphatase and analyzed by a high-pressure liquid chromatograph equipped with an electrochemical detector (HPLC-ECD), as described by Kasai et al.\(^{27,28}\)
Effects of scavengers and bathocuproine on DNA damage induced by NO-naphthol or by 4-ABP(NHOH) in the presence of NADH and Cu(II)

Some typical free •OH scavengers, dimethylsulfoxide (DMSO) (data not shown) and ethanol efficiently inhibited the DNA damage induced by NO-naphthol, as shown in Fig. 2A, while none of the typical free •OH scavengers had any effect on the DNA damage induced by 4-ABP(NHOH), as shown in Fig. 2B. In Fig. 2A, another •OH scavenger, mannitol partially attenuated the NO-naphthol-induced DNA damage, whereas sodium formate did not. Bathocuproine, a Cu(I)-specific chelator, catalase and methional inhibited DNA damage induced by NO-naphthol and by 4-ABP(NHOH). SOD also attenuated NO-naphthol-induced DNA damage, but did not affect 4-ABP(NHOH)-induced DNA damage.

Comparison of site-specific DNA damage by NO-naphthol and Cu(II)

An autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensities of DNA cleavage products from the human p53 tumor suppressor gene (Fig. 3) and from the c-Ha-ras-1 protooncogene (Fig. 4). NO-naphthol was observed to induce piperidine-labile sites preferentially at T>C>G residues in the presence of NADH and Cu(II). 4-ABP(NHOH) was observed to induce piperidine-labile sites specifically at thymine residues in the presence of NADH and Cu(II) (Figs. 3B and 4B). NO-naphthol induced piperidine-labile sites at cytosine and guanine residues, in addition to thymine residues similar to those frequently damaged by 4-ABP(NHOH). We also detected guanine damage induced by NO-naphthol and by 4-ABP(NHOH) as 8-oxodG, which is a piperidine-inert base modification. 8-OxodG formation should occur adjacent to piperidine-labile T sites, since double base lesions at 5'-TG-3' or at 5'-GT-3' were previously reported in oxidative DNA base damage.29, 30)

In the cases of both NO-naphthol and 4-ABP(NHOH), the ACG sequence complementary to codon 273 of the p53 gene was significantly damaged. Codon 273 of the p53 gene is a well-known hotspot of lung cancer, and it is also reported to be a mutational hotspot of bladder cancer in relation to smoking.31)

Formation of 8-oxodG in calf thymus DNA

Using HPLC-ECD, we measured the 8-oxodG content of calf thymus DNA treated with NO-naphthol in the presence of...
NADH and Cu(II) (Fig. 5). NO-naphthol at 2–5 \( \mu \)M most effectively caused 8-oxodG formation on native DNA. The amount of 8-oxodG increased with the concentration of NADH. In the absence of NADH, NO-naphthol and Cu(II) together did not stimulate 8-oxodG formation above control levels (data not shown).

**DISCUSSION**

The present study showed that NO-naphthol, a metabolite of 2-NA caused oxidative DNA damage including 8-oxodG in the presence of NADH and Cu(II). NO-naphthol plus Cu(II) did not cause DNA damage in the absence of NADH, indicating that the reduction of NO-naphthol by NADH was essential. To clarify the kinds of reactive species at relatively low concentrations of NO-naphthol, we examined the effects of scavengers on the DNA damage induced by NO-naphthol. Both catalase and bathocuproine inhibited the DNA damage, suggesting the involvement of \( \text{H}_2\text{O}_2 \) and Cu(I). The formation of 8-oxodG by 2–5 \( \mu \)M NO-naphthol was more efficient in double-stranded DNA than in single-stranded DNA (data not shown). This sug-

---

**Fig. 3.** Comparison of site-specific DNA damage by NO-naphthol and 4-ABP(NHOH). The reaction mixture contained \(^{32}\text{P}\)-5'-end-labeled 443-bp fragment, calf thymus DNA [(A) 50 \( \mu \)M, (B) 5 \( \mu \)M], 2 \( \mu \)M NO-naphthol (A) or 1 \( \mu \)M 4-ABP(NHOH) (B), 200 \( \mu \)M NADH and 20 \( \mu \)M CuCl\(_2\) in 200 \( \mu \)l of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 \( \mu \)M DTPA. The 443-bp fragment was singly labeled on the 5'-end of the antisense strand of the human \( \beta^\text{33} \) tumor suppressor gene. The reaction mixture was incubated at 37°C for 1 h (A) or 30 min (B). After piperidine treatment, the DNA fragments were analyzed as described in the legend to Fig. 1. The horizontal axis shows the nucleotide number. (A) NO-naphthol. (B) 4-ABP(NHOH).

**Fig. 4.** Comparison of site-specific DNA damage by NO-naphthol and 4-ABP(NHOH). The reaction mixture contained \(^{32}\text{P}\)-5'-end-labeled 337-bp fragment, calf thymus DNA [(A) 50 \( \mu \)M, (B) 5 \( \mu \)M], 2 \( \mu \)M NO-naphthol (A) or 1 \( \mu \)M 4-ABP(NHOH) (B), 200 \( \mu \)M NADH and 20 \( \mu \)M CuCl\(_2\) in 200 \( \mu \)l of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 \( \mu \)M DTPA. The 337-bp fragment was singly labeled on the 5'-end of the antisense strand of the human c-Ha-ras-1 protooncogene. The reaction mixture was incubated at 37°C for 1 h (A) or 30 min (B). After piperidine treatment, the DNA fragments were analyzed as described in the legend to Fig. 1. The horizontal axis shows the nucleotide number. (A) NO-naphthol. (B) 4-ABP(NHOH).
suggests that double-stranded structure is important for DNA damage. At relatively high concentrations of NO-naphthol, the amount of DNA damage decreased, suggesting that two molecules of NO-naphthol may bischelate with one molecule of copper to generate an inactive form. This is supported by the report that NO-naphthol chelates metal ions, such as iron, copper and zinc.\(^{32}\)

Based on these results and references, we propose the following possible mechanism (Fig. 6). 2-NA is metabolized to NO-naphthol through several metabolic steps.\(^{10, 12, 13}\) NO-naphthol is non-enzymatically reduced to hydronitroxide radical by NADH. This is supported by an electron-spin resonance (ESR) study that has confirmed the existence of the radical.\(^{33, 34}\) We previously reported that aromatic nitroso compounds can be easily reduced to hydronitroxide radicals by NADH, forming a redox cycle.\(^{19, 35, 36}\) Auto-oxidation of hydronitroxide radical to the nitroso compound (NO-naphthol) occurs coupled with generation of \(O_2^-\), and \(O_2^-\) mediated reduction of Cu(II) to Cu(I). Then auto-oxidation of Cu(I) to Cu(II) occurs coupled with generation of \(O_2^-\), which is dismutated to \(H_2O_2\). When iron was used instead of copper, NO-naphthol with NADH did not cause DNA damage in this system, though it remains possible that iron ions mediate DNA damage.

Fig. 5. Cu(II)-mediated formation of 8-oxodG in calf thymus DNA by NO-naphthol in the presence of NADH. Calf thymus DNA (50 \(\mu\)M/base) was incubated with the indicated concentrations of NO-naphthol and 20 \(\mu\)M CuCl\(_2\) in the presence of various concentrations of NADH for 1 h at 37\(^\circ\)C. After ethanol precipitation, DNA was enzymatically digested into nucleosides, and 8-oxodG formation was measured with an HPLC-ECD as described in “Materials and Methods.” ○ NADH 200 \(\mu\)M, ▲ NADH 100 \(\mu\)M.

Fig. 6. A proposed mechanism for oxidative DNA damage induced by a 2-NA metabolite in the presence of Cu(II) and NADH.
damage by NO-naphthol in vivo. In the case of iron, even if Fe(III) is reduced to Fe(II) by the formed O₂⁻, the reaction of Fe(II) with O₂ is much slower than that of Cu(I). Therefore, it can be one of the reasons why iron does not work in this system.

Typical •OH scavengers partially inhibited DNA damage induced by NO-naphthol, while they showed no inhibitory effect on DNA damage induced by 4-ABP(NHOH). Generally, it is known that copper exists in the nucleus and is closely associated with chromosomes and DNA bases in vivo. H₂O₂ interacts with Cu(I) to form DNA-copper-hydroperoxo complexes, causing DNA damage. The non-inhibitory effect of typical free •OH scavengers observed in the case of 4-ABP(NHOH), can be explained by assuming that DNA damage is induced by •OH generated in very close proximity to the DNA by the bound metal ion. On the other hand, NO-naphthol which intercalated in DNA, may react with the bound metal ion via its two functional groups and keep the metal ion relatively far from DNA. The DNA base damage by 4-ABP(NHOH) was more specific than that by NO-naphthol. It has been reported that free •OH caused DNA damage at any nucleotide, showing little site specificity. Therefore, it can reasonably be considered that the reactive species formed by NO-naphthol has a free •OH-like character to a greater extent than in the case of 4-ABP(NHOH).

Carcinogenicity of amino-aromatic compounds has been explained in terms of DNA-adduct formation. The DNA adducts derived from NA, formed by covalent binding through the activated nitrogen, have been identified. On the other hand, Nagao et al. pointed out that there was no direct correlation between DNA-adduct levels and cancer incidences induced by heterocyclic amines. In addition, free radicals and subsequently formed reactive oxygen species may participate in aromatic amine carcinogenesis. It has been reported that the levels of 8-oxodG and its repair activity are increased with cigarette smoking, and that 8-oxodG levels were increased in tissues of lung cancer patients. Smoking is a risk factor for bladder cancer as well as lung cancer. The present study has demonstrated that a metabolite of 2-NA induces oxidative DNA damage, as does the 4-ABP metabolite. NO-naphthol is likely to be involved in bladder carcinogenesis through metabolite formation around the bladder, although the genotoxicity of NO-naphthol is probably not specific to urothelial cells. The oxidative DNA damage by these amino-aromatic compounds may participate in smoking-related bladder cancer, in addition to DNA adduct formation.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Dr. Mariko Tada (Aichi Shukutoku University) for supplying 4-ABP(NHOH).
A. Formation of uterine and hepatic DNA adducts from the carcinogen 2-naphthylamine. *Carcinogenesis*, 2, 467–470 (1981).

12) Boyd, J. A. and Eiling, T. E. Prostaglandin H synthase-catalyzed metabolism and DNA binding of 2-naphthylamine. *Cancer Res.*, 47, 4007–4014 (1987).

13) Manson, D. Oxidation of N-naphthylhydroxylamines to nitrosothiols by air. *J. C. S. Perkin I*, 2, 192–194 (1974).

14) Nakayama, T., Kimura, T., Kodama, M. and Nagata, C. Generation of hydrogen peroxide and superoxide anion from active metabolites of naphthylamines and aminoazo dyes: its possible role in carcinogenesis. *Carcinogenesis*, 4, 765–769 (1983).

15) Kaneko, M., Nakayama, T., Kimura, T., Kodama, M. and Nagata, C. Detection of DNA lesions in cultured human fibroblasts induced by active oxygen species generated from a hydroxylated metabolite of 2-naphthylamine. *Gann*, 75, 349–354 (1984).

16) Shibutani, S., Takeshita, M. and Grollman, A. P. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxoG. *Nature*, 349, 431–434 (1991).

17) Kamiya, H., Murata-Kamiya, N., Fujimuro, M., Kido, K., Inoue, H., Nishimura, S., Masutani, C., Hanaoka, F. and Ohtsuka, E. Comparison of incorporation and extension of nucleotides *in vitro* opposite 8-hydroxyguanine (7,8-dihydro-8-oxoguanine) in hot spots of the c-Ha-ras gene. *Jpn. J. Cancer Res.*, 86, 270–276 (1995).

18) Kamiya, H., Murata-Kamiya, N., Koizume, S., Inoue, H., Nishimura, S. and Ohtsuka, E. 8-Hydroxyguanine (7,8-dihydro-8-oxoguanine) in hot spots of the c-Ha-ras gene: effects of sequence contexts on mutation spectra. *Carcinogenesis*, 16, 883–889 (1995).

19) Murata, M., Tamura, A., Tada, M. and Kawaniishi, S. Mechanism of oxidative DNA damage induced by carcinogenic 4-aminobiphenyl. *Free Radic. Biol. Med.*, 30, 765–773 (2001).

20) Chumakov, P. EMBL Data Library, accession number X54156 (1990).

21) Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H. and Goeddel, D. V. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature*, 302, 33–37 (1983).

22) Kawaniishi, S. and Yamamoto, K. Mechanism of site-specific DNA damage induced by methylhydrazines in the presence of copper (II) or manganese (III). *Biochemistry*, 30, 3069–3075 (1991).

23) Otkawa, S. and Kawaniishi, S. Detection of DNA damage and analysis of its site-specificity. In “Experimental Protocols for Reactive Oxygen and Nitrogen Species,” pp. 229–235 (2000). Oxford University Press, New York.

24) Kawaniishi, S., Inoue, S. and Sano, S. Mechanism of DNA cleavage induced by sodium chromate (VI) in the presence of hydrogen peroxide. *J. Biol. Chem.*, 261, 5952–5958 (1986).

25) Ohnishi, S., Murata, M., Degawa, M. and Kawaniishi, S. Oxidative DNA damage induced by an N-hydroxy metabolite of carcinogenic 4-dimethylaminobenzene. *Jpn. J. Cancer Res.*, 92, 23–29 (2001).

26) Maxam, A. M. and Gilbert, W. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.*, 65, 499–560 (1980).

27) Kasai, H., Crain, P. F., Kuchino, Y., Nishimura, S., Ootsuyama, A. and Tanooka, H. Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. *Carcinogenesis*, 7, 1849–1851 (1986).

28) Tchou, J., Kasai, H., Shibutani, S., Chung, M.-H., Laval, J., Grollman, A. P. and Nishimura, S. 8-Oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. *Proc. Natl. Acad. Sci. USA*, 88, 4690–4694 (1991).

29) Bourdat, A.-G., Douki, T., Frelon, S., Gasparutto, D. and Cadet, J. Tandem base lesions are generated by hydroxyl radical within isolated DNA in aerated aqueous solution. *J. Am. Chem. Soc.*, 122, 4549–4556 (2000).

30) Ohnishi, S. and Kawaniishi, S. Double base lesions of DNA by a metabolite of carcinogenic benz[a]pyrene. *Biochem. Biophys. Res. Commun.*, 290, 778–782 (2002).

31) Warren, W., Biggs, P. J., El-Baz, M., Ghoneim, M. A., Stratton, M. R. and Venitt, S. Mutations in the p53 gene in schistosomal bladder cancer: a study of 92 tumours from Egyptian patients and a comparison between mutational spectra from schistosomal and non-schistosomal urotheelial tumours. *Carcinogenesis*, 16, 1181–1189 (1995).

32) Kokocinski, C. W., Brundage, D. J. and Nicol, J. D. A study of the use of 2-nitroso-1-naphthol as a trace metal detection reagent. *J. Forensic Sci.*, 25, 810–814 (1980).

33) Takahashi, N., Fischer, V., Schriever, J. and Mason, R. P. An ESR study of nonenzymatic reactions of nitroso compounds with biological reducing agents. *Free Radic. Res. Commun.*, 4, 351–358 (1988).

34) Fischer, V. and Mason, R. P. Formation of iminoyl and nitrooxide free radicals from nitrosothiols: an electron spin resonance study. *Chem. Biol. Interact.*, 57, 129–142 (1986).

35) Ohnishi, S., Murata, M., Fukuhara, K., Miyata, N. and Kawaniishi, S. Oxidative DNA damage by a metabolite of carcinogenic 1-nitropyrene. *Biochem. Biophys. Res. Commun.*, 280, 48–52 (2001).

36) Okhuma, Y. and Kawaniishi, S. Oxidative DNA damage by a metabolite of carcinogenic and reproductive toxic nitrobenzene in the presence of NADH and Cu(II). *Biochem. Biophys. Res. Commun.*, 257, 555–560 (1999).

37) Otkawa, S. and Kawaniishi, S. Distinct mechanisms of site-specific DNA damage induced by endogenous reductants in the presence of iron(III) and copper(II). *Biochim. Biophys. Acta*, 1399, 19–30 (1998).

38) Burkitt, M. J. Copper-DNA adducts. *Methods Enzymol.*, 234, 66–79 (1994).

39) Celander, D. W. and Cech, T. R. Iron(II)-ethylenediaminetetraacetic acid catalyzed cleavage of RNA and DNA oligonucleotides: similar reactivity toward single- and double-
stranded forms. Biochemistry, 29, 1355–1361 (1990).
40) Nagao, M., Ochiai, M., Okochi, E., Ushijima, T. and Sugimura, T. LacI transgenic animal study: relationships among DNA-adduct levels, mutant frequencies and cancer incidences. Mutat. Res., 477, 119–124 (2001).
41) Maeda, H., Sato, K. and Akaike, T. Superoxide radical generation from heterocyclic amines. Proc. 23rd Int. Symp. Princess Takamatsu Cancer Res. Fund, 103–112 (1995).
42) Asami, S., Hirano, T., Yamaguchi, R., Tomioka, Y., Itoh, H. and Kasai, H. Increase of a type of oxidative DNA damage, 8-hydroxyguanine, and its repair activity in human leukocytes by cigarette smoking. Cancer Res., 56, 2546–2549 (1996).
43) Asami, S., Manabe, H., Miyake, J., Tsurudome, Y., Hirano, T., Yamaguchi, R., Itoh, H. and Kasai, H. Cigarette smoking induces an increase in oxidative DNA damage, 8-hydroxydeoxyguanosine, in a central site of the human lung. Carcinogenesis, 18, 1763–1766 (1997).
44) Lodovici, M., Casalini, C., Cariaggi, R., Michelucci, L. and Dolara, P. Levels of 8-hydroxydeoxyguanosine as a marker of DNA damage in human leukocytes. Free Radic. Biol. Med., 28, 13–17 (2000).
45) Inoue, M., Osaki, T., Noguchi, M., Hirohashi, S., Yasumoto, K. and Kasai, H. Lung cancer patients have increased 8-hydroxydeoxyguanosine levels in peripheral lung tissue DNA. Jpn. J. Cancer Res., 89, 691–695 (1998).