

Oxidative DNA damage induced by hair dye components *ortho*-phenylenediamines and the enhancement by superoxide dismutase

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Abstract

There is an association between occupational exposure to hair dyes and incidence of cancers. Permanent oxidant hair dyes are consisted of many chemical components including *ortho*-phenylenediamines. To clarify the mechanism of carcinogenesis by hair dyes, we examined DNA damage induced by mutagenic *ortho*-phenylenediamine (*o*-PD) and its derivatives, 4-chloro-*ortho*-phenylenediamine (Cl-PD) and 4-nitro-*ortho*-phenylenediamine (NO₂-PD), using ³²P-labeled DNA fragments obtained from the human *p16* and the *p53* tumor suppressor gene. We also measured the content of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a marker of oxidative DNA damage, in calf thymus DNA with an electrochemical detector coupled to a high performance liquid chromatograph. Carcinogenic *o*-PD and Cl-PD caused Cu(II)-mediated DNA damage, including 8-oxodG formation, and antioxidant enzyme superoxide dismutase (SOD) enhanced DNA damage. *o*-PD and Cl-PD caused piperidine-labile and formamidopyrimidine-DNA glycosylase-sensitive lesions at cytosine and guanine residues respectively in the 5'-ACG-3' sequence, complementary to codon 273, a well-known hotspot of the human *p53* tumor suppressor gene. UV-vis spectroscopic studies showed that the spectral change of *o*-PD and Cl-PD required Cu(II), and addition of SOD enhanced it. This suggested that SOD enhanced the rate of Cu(II)-mediated autoxidation of *o*-PD and Cl-PD, leading to enhancement of DNA damage. On the other hand, mutagenic but non-carcinogenic NO₂-PD induced no DNA damage. These results suggest that carcinogenicity of *ortho*-phenylenediamines is associated with ability to cause oxidative DNA damage rather than bacterial mutagenicity.

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1. Introduction

Epidemiologic studies have revealed an association between occupational exposure to hair dyes and incidence of cancers [1]. Several cohort and case-control

studies have shown increased risk of bladder cancer among hairdressers and barbers who are occupationally exposed to hair dyes [1]. It has been reported that there is a statistically significant and a dose-dependent association between hair dyeing and risk of ovarian cancer [2]. Women with prolonged used of dark, particularly black, hair dyes may have increased risk of fatal non-Hodgkin's lymphoma and multiple myeloma [3,4].

The main components of permanent oxidant dyes are *para*-phenylenediamine, 2,5-diaminotoluene, 2,4-

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diaminoanisole, 2-amino-4-nitrophenol, and *ortho*-phenylenediamine (*o*-PD) [5]. *o*-PD and its derivatives including 4-chloro-*o*-phenylenediamine (Cl-PD) and 4-nitro-*o*-phenylenediamine (NO₂-PD) are used for the production of many dyes, and are also used directly as color-yielding compounds which include hair and fabric dyes. *o*-PD, Cl-PD and NO₂-PD have been tested for the evaluation of their carcinogenicity using experimental animals. It has been reported that *o*-PD and Cl-PD are carcinogenic, whereas NO₂-PD is not carcinogenic [6]. *o*-PD caused hepatocellular carcinomas in rats, and Cl-PD caused carcinomas of urinary bladder and liver in rats and mice [6]. The international Agency for Research on Cancer (IARC) has classified Cl-PD as possibly carcinogenic to humans (group 2B) [7], whereas NO₂-PD as not classifiable (group 3) [8]. IARC has not assessed *o*-PD to any group.

To clarify the mechanism of carcinogenesis by *o*-PD derivatives, we examined DNA damage induced by *o*-PD, Cl-PD and NO₂-PD in the presence of Cu(II), using ³²P-5'-end-labeled DNA fragments obtained from the human *p16* and the *p53* tumor suppressor gene. These two genes are known to be the targets for chemical carcinogens, and the damaged sites may lead to mutational hotspots [9]. In addition, we measured the content of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a marker of oxidative DNA damage, in calf thymus DNA with an electrochemical detector coupled to a high performance liquid chromatograph (HPLC-ECD). It has been reported that 8-oxodG formation can lead to DNA misreplication resulting in mutation and cancer [10,11]. To clarify the mechanism of oxidative DNA damage, spectral changes during the autoxidation of *o*-PD and Cl-PD were measured by UV-vis spectroscopy.

2. Materials and methods

2.1. Materials

Restriction enzymes (*EcoRI*, *MroI* and *ApaI*) and calf intestine phosphatase were purchased from Boehringer Mannheim (Mannheim, Germany). Restriction enzymes (*HindIII* and *AvaI*) and T₄ polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). [γ -³²P]-ATP (222 TBq/mmol) was from New England Nuclear (Boston, MA). Superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes) and catalase (45,000 units/mg from bovine liver) were from Sigma Chemical Co (St. Louis, MO). *o*-PD and NO₂-PD were from Tokyo Kasei Co. (Tokyo, Japan). Cl-PD was from Sigma-Aldrich Chemical Co (Milwaukee, WI). Nuclease P₁ was from Yamasa Shoyu Co. (Chiba, Japan). Formamidopyrimidine-DNA glycosylase (Fpg, 20,000 units/mg from *Escherichia*

coli) was from Trevigen Inc. (Gaithersburg, MD). Bathocuproinedisulfonic acid was from Dojin Chemicals Co. (Kumamoto, Japan).

2.2. Preparation of ³²P-5'-end-labeled DNA fragments

DNA fragments obtained from the human *p53* tumor suppressor gene [12] containing exons were prepared, as described previously [13]. The 5'-end-labeled 650 bp fragment (*HindIII**13972–*EcoRI**14621) was obtained by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ -³²P]ATP and T₄ polynucleotide kinase (*, ³²P-labeled). The 650 bp fragment was further digested with *ApaI* to obtain a singly labeled 443 bp fragment (*ApaI* 14179–*EcoRI**14621) and a 211 bp fragment (*HindIII**13972–*ApaI* 14182). DNA fragment was also obtained from the human *p16* tumor suppressor gene [14]. The 5' end-labeled 490 bp fragment (*EcoRI**5841–*EcoRI**6330) containing exon 1 of the human *p16* tumor suppressor gene obtained from pGEM-T Easy Vector (Promega Corporation) was prepared as mentioned above. The 490 bp fragment was further digested with *MroI* to obtain a singly labeled 328 bp fragment (*EcoRI**5841–*MroI* 6168) and a 158 bp fragment (*MroI* 6173–*EcoRI**6330) as described previously [15].

2.3. Detection of DNA damage by *o*-PDs

The standard reaction mixture in a 1.5 mL microtube contained *o*-PD, Cl-PD or NO₂-PD, 20 μ M CuCl₂, ³²P-labeled DNA fragment and calf thymus DNA (20 μ M/base) in 200 μ L of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μ M DTPA. After incubation at 37 °C for 2 h, the DNA fragments were treated in 10% (v/v) piperidine at 90 °C for 20 min, or treated with 6 units of Fpg protein in 20 μ L of reaction buffer (10 mM HEPES-KOH (pH 7.4), 100 mM KCl, 10 mM EDTA and 0.1 mg/mL BSA) at 37 °C for 2 h. The treated DNA was electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing X-ray film to the gel [16].

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 [17] using a DNA-sequencing system (LKB 2010 MacroPhor). The relative amounts of oligonucleotides from the treated DNA fragments were measured with a laser densitometer (LKB 2222 UltraScan XL).

2.4. Analysis of 8-oxodG formation in calf thymus DNA by *o*-PDs

Calf thymus DNA fragments (100 μ M/base) were incubated with *o*-PD, Cl-PD or NO₂-PD, and 20 μ M CuCl₂ at 37 °C for 2 h. After ethanol precipitation, DNA was digested to the nucleosides with nuclease P₁ and calf intestine phosphatase, and analyzed by an HPLC-ECD, as described previously [18].

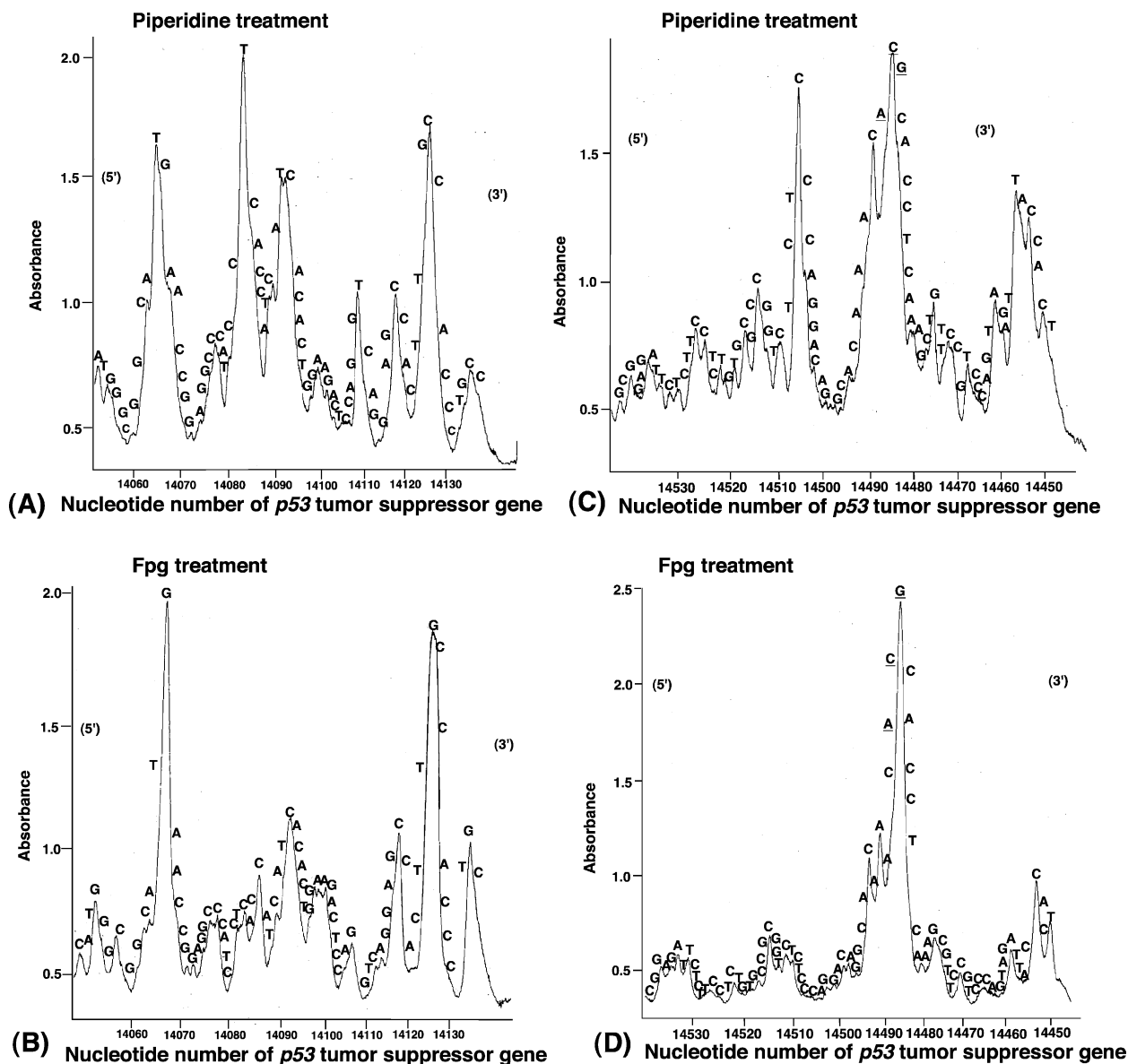


Fig. 2. Site specificity of DNA cleavage induced by *o*-PD in the presence of Cu(II). The reaction mixture contained the ^{32}P -5'-end labeled 211 bp (*Apa*I 13972–*Hind*III*14182) (A and B) and 443 bp (*Apa*I 14179–*Eco* RI*14621) (C and D) DNA fragment, calf thymus DNA (20 μM /base), 200 μM *o*-PD and 20 μM CuCl_2 in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated at 37 $^\circ\text{C}$ for 2 h, followed by hot piperidine (A and C) and Fpg treatment (B and D) as described in Section 2. The horizontal axis shows the nucleotide number of the human *p53* tumor suppressor gene, and underscoring shows complementary sequence to codon 273 (nucleotide numbers 14486–14488).

3.3. Formation of 8-oxodG in DNA by *o*-PDs in the presence of Cu(II) and SOD

We measured 8-oxodG contents in calf thymus DNA treated with *o*-PD, Cl-*o*-PD and NO₂-*o*-PD in the presence of Cu(II) and SOD using an HPLC-

ECD (Fig. 3). The amount of 8-oxodG increased with increasing concentrations of *o*-PD and Cl-*o*-PD. On the other hand, NO₂-*o*-PD generated no or little 8-oxodG in the presence of Cu(II). Addition of SOD dramatically enhanced the formation of 8-oxodG (Fig. 3B).

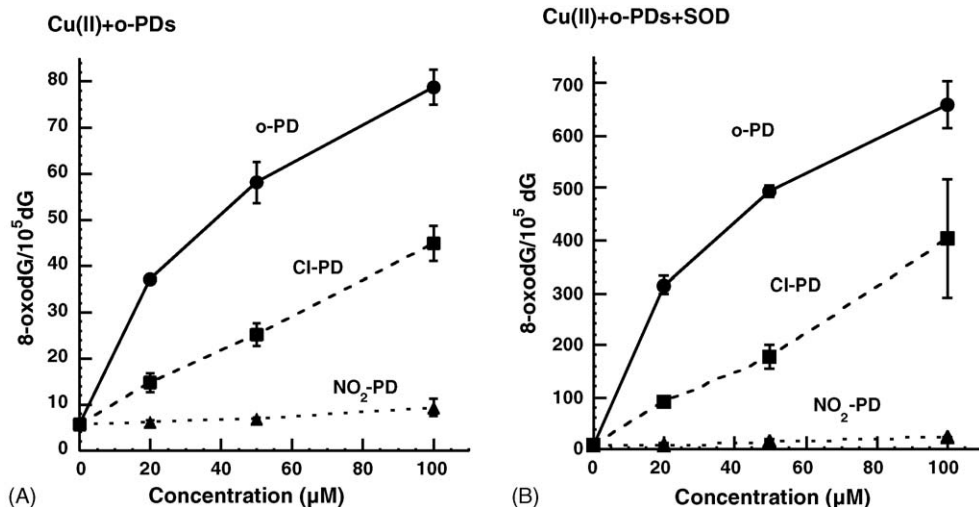


Fig. 3. Formation of 8-oxodG by *o*-PD and its derivatives in the presence of Cu(II) and SOD. The reaction mixture contained calf thymus DNA (100 μM /base) and 20 μM CuCl_2 , indicated concentrations of *o*-PD, Cl-PD and NO_2 -PD in 400 μL of 4 mM phosphate buffer (pH 7.8) containing 5 μM DTPA in the presence (B) and absence of 150 units/mL SOD (A). After incubation at 37 $^\circ\text{C}$ for 2 h, DNA fragment was enzymatically digested into nucleosides, and 8-oxodG formation was measured with an HPLC-ECD as described in Section 2. Results are expressed as means and S.D. of values obtained from three independent experiments.

3.4. UV-vis spectroscopic studies on autoxidation of *o*-PD

Fig. 4 shows change in UV-vis spectra of *o*-PD in the presence of Cu(II) and SOD. The spectra of *o*-PD did not

significantly change in the absence of Cu(II) (Fig. 4A). No spectral change suggests very slow autoxidation in the buffer solution (pH 7.8) in the absence of Cu(II). *o*-PD plus Cu(II) showed an increase in the absorbance maximum at about 420 nm, which can be attributed

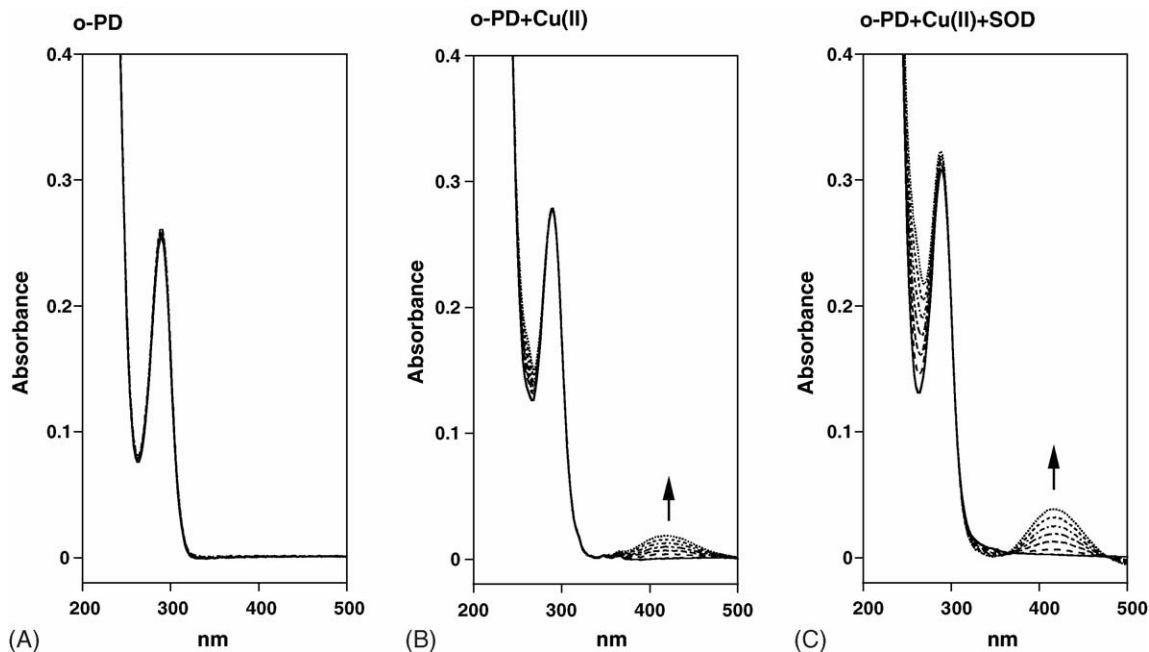


Fig. 4. Changes in UV-vis spectra of *o*-PD. (A) 100 μM *o*-PD alone, (B) 100 μM *o*-PD and 20 μM CuCl_2 and (C) 100 μM *o*-PD, 20 μM CuCl_2 and 150 units/mL SOD in 10 mM phosphate buffer (pH 7.8) containing 5 μM DTPA. The spectra were measured every 10 min for 60 min at 37 $^\circ\text{C}$.

to 2,3-diaminophenazine [20] (Fig. 4B, arrow). The increase of the absorbance at about 420 nm (Fig. 4C, arrow) was enhanced by the addition of SOD, suggesting that SOD enhanced the rate of Cu(II)-mediated autoxidation of *o*-PD. In the case of Cl-PD, similar effects were observed, although spectral changes were small compared with *o*-PD (data not shown). On the other hand, no or little spectral change was observed in the case of NO₂-PD even in the presence of Cu(II) and SOD (data not shown).

4. Discussion

Several studies [6–8,21] have demonstrated that bacterial mutagenicity of NO₂-PD, which is non-carcinogenic, is stronger than those of *o*-PD and Cl-PD, which are carcinogenic. The present study showed that carcinogenic *o*-PD and Cl-PD caused Cu(II)-mediated DNA damage, and non-carcinogenic NO₂-PD could not induce DNA damage under the condition used. Similarly, *o*-PD and Cl-PD significantly induced 8-oxodG formation in the presence of Cu(II), whereas NO₂-PD did not. Our previous study on other representative hair dyes, *m*-phenylenediamine derivatives [22] revealed that 4-methoxy-*m*-phenylenediamine (also known as 2,4-diaminoanisole) which is carcinogenic, caused Cu(II)-mediated DNA damage whereas mutagenic but non-carcinogenic *m*-phenylenediamine caused neither DNA damage nor 8-oxodG production. Our previous and the present studies indicate that oxidative DNA damage may be crossly related to carcinogenesis rather than bacterial mutagenicity.

To clarify the kinds of reactive species participating in site-specific DNA damage induced by *o*-PD and Cl-PD in the presence of Cu(II), the effects of scavengers and bathocuproine on DNA damage were examined. The DNA damage was inhibited by both catalase and bathocuproine, indicating the participation of H₂O₂ and Cu(I). Typical •OH scavengers showed no inhibitory effects on DNA damage by *o*-PD, suggesting that •OH does not play an important role in the DNA damage. Little involvement of •OH is supported by the site-specific DNA damage observed with *o*-PD and Cl-PD, because •OH causes DNA cleavage at any nucleotides with little site specificity [23,24]. Relevantly, Youngman and Elstner [25] proposed a concept of crypto-hydroxyl radical on the basis of reactions that were much less sensitive to the inhibition by traditional •OH scavengers e.g. ethanol and mannitol. Interestingly, SOD enhanced *o*-PD-induced DNA damage in the presence of Cu(II). UV–vis spectroscopic studies showed that the spectral changes of *o*-PD and Cl-PD required Cu(II), and addition

of SOD enhanced it. This suggests that SOD enhances the rate of Cu(II)-mediated autoxidation of *o*-PD and Cl-PD, leading to enhancement of DNA damage.

On the basis of our results, a possible mechanism of Cu(II)-dependent DNA damage induced by *o*-PD and Cl-PD can be explained as follows: *o*-PD and Cl-PD undergoes Cu(II)-mediated autoxidation to generate phenylenediamine-derived radicals and Cu(I). O₂•⁻ is generated probably by the reaction of O₂ with the radicals and/or Cu(I), and then is dismutated to H₂O₂. H₂O₂ interacts with Cu(I) to form the metal–oxygen complex, such as Cu(I)-OOH, capable of causing DNA damage. The metal–oxygen complex behaves like Fenton reaction rather than ionizing radiation. It is noteworthy to find that SOD, which should protect organelle from oxidative stress, enhances both DNA damage and autoxidation of *o*-PD in the presence of Cu(II). SOD accelerates the process of H₂O₂ generation by removing O₂•⁻. As the result, H₂O₂ by O₂•⁻ dismutation may participate in the formation of copper–oxygen complex responsible for DNA damage. It has been reported that copper occurs in the mammalian cell nucleus [26], and copper mediates production of reactive oxygen species and DNA damage [27]. Therefore, it is concluded that SOD and Cu(II)-mediated oxidative DNA damage may have an important role in carcinogenesis of *o*-PD and Cl-PD.

Site-specific and characteristic mutations were found in human cancers as molecular mutational fingerprints associated with chemical carcinogens [28]. We showed that *o*-PD and Cl-PD induced Cu(II)-mediated DNA damage at guanine and an adjacent pyrimidine residues such as TG and GC sites. There are some reports that reactive oxygen species induce double base lesions involving guanine and an adjacent pyrimidine base [29]. Therefore, our results may be interpreted as double base lesion, although our system cannot directly detect double base lesion at once. It is noteworthy that CG in the ACG sequence complementary to codon 273, a well-known hotspot [9,19], in the *p53* gene was damaged intensively. Feng et al. [30] reported that preferential DNA damage and poor repair determine mutational hotspot in human cancer. Recent studies showed that 8-oxo-guanine retarded the activity of DNA ligase during the repair of a single-strand break, when present within a clustered DNA damage site [31], and mutation frequencies were found to be significantly higher for clustered damage sites than for single lesions [32]. These literatures and our results indicate the possibility of carcinogenicity by *o*-PD due to clustered DNA damage containing 8-oxodG.

Hair dyes contain many chemical components. Severin et al. [33] showed 2,4-diaminotoluene induced

DNA damage. Our present and previous studies demonstrated that *o*-phenylenediamine derivatives, 2,4-diaminoanisole [22] and nitro-2-aminophenols [34] induced oxidative DNA damage. Further carcinogenic studies on the components of hair dyes are needed to prevent cancer among people exposed to hair dyes, occupationally and personally.

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