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Genetic changes of *hOGG1* and the activity of oh8Gua glycosylase in colon cancer

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Abstract

oh8Gua glycosylase repairs DNA by removing oh8Gua, a highly mutagenic oxidative DNA adduct. Recently, the gene for human oh8Gua glycosylase (*hOGG1*) was cloned and several mutational types have been reported. However, the implications of such mutations in human cancer have not been clearly demonstrated. To test the involvement of *hOGG1* mutation in colon carcinogenesis, we analysed the genetic changes of *hOGG1* and the activity of oh8Gua glycosylase in 15 paired normal and tumorous colon specimens. The activity of antioxidant enzymes (catalase and superoxide dismutase (SOD)) and extent of oxidative cellular damage (oh8Gua and malondialdehyde) were also assessed to compare the oxidative status of normal and tumour tissues. An Arg 154 to His mutation was detected in two tumour samples, but not in the corresponding normal tissues. A Ser 326 to Cys mutation (polymorphism) was found in both the normal and tumour tissues of 3 patients. However, neither the Arg 154 to His mutation nor the polymorphism at codon 326 significantly decreased the oh8Gua glycosylase activity. The mean activity of oh8Gua glycosylase was significantly higher in the tumours than in normal tissues ($P=0.022$). Antioxidant enzyme activities were decreased (catalase; $P=0.004$ and SOD; $P=0.002$), and the extent of oxidative damage correspondingly increased in the tumour tissues (oh8Gua; $P=0.007$ and malondialdehyde; $P=0.046$). Although the sample size was limited, these results suggest that the somatic mutation or the polymorphism of *hOGG1* is less likely to be involved in colon carcinogenesis. Nevertheless, the greater oxidative DNA damage in the tumour tissues, as a possible result of impaired antioxidant activity, implies an important role for oxygen free-radicals in colon carcinogenesis.

Keywords: Oxidative DNA damage; Colon cancer; oh8Gua glycosylase; *hOGG1*

1. Introduction

Oxygen free-radicals react with guanosine residues in cellular DNA to form 8-hydroxyguanine (8-oxoguanine; oh8Gua). In DNA, oh8Gua has been shown to be involved in mutagenesis/carcinogenesis, since it induces the GC > TA transversion which causes the activation of oncogenes [1,2]. Previously, we isolated an oh8Gua-repairing enzyme, oh8Gua endonuclease, from *Escherichia coli* and later confirmed its activity in mammalian tissue [3,4]. We also found that the enzyme has two activities, a glycosylase and a endonuclease activity; the former activity removes oh8Gua residues as a free base and the latter cleaves phosphodiester bonds 3′ and 5′ to the resulting apurinic (AP) site [3–5]. Furthermore, oh8Gua DNA glycosylase in *E. coli* was demonstrated to be identical to formamidopyrimidine (Fapy) DNA glycosylase, as the enzyme removes, not only oh8Gua, but also Fapy in DNA [6]. These findings indicate that oh8Gua DNA glycosylase is a mutM protein, since the *mutM* gene, a mutator gene specifically leading to GC > TA transversion in *E. coli*, is identical to the *fpg* gene encoding Fapy DNA glycosylase.

Recently, the *OGG1* gene of *Saccharomyces cerevisiae* (*yOGG1*) was cloned as the functional yeast homologue of the bacterial *mutM* gene [7]. Subsequently, a human homologue of *yOGG1*, *hOGG1* was also isolated [8–12]. Two main splice-variant forms of the hOGG1 protein have been reported including α-hOGG1 and β-hOGG1.

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The α-hOGG1 is the most abundant form and is targeted to the nucleus (β-hOGG1 protein is targeted to the mitochondrion). The human *α-hOGG1*-transcribed region spans less than 7 kb of genomic sequences and has seven exons. In contrast, β-*hOGG1* shares the six first exons with the α-form, but as a consequence of alternative splicing, the seventh exon is replaced by a new one located downstream of the last exon of the α-form [13]. We found a functional loss of OGG1 occurred in senescence-accelerated mice, developed by Takeda and colleagues, due to a single base mutation at codon 304 of the *OGG1* gene from CGG (Arg) to TGG (Trp) [14]. The mutational inactivation of this enzyme appears to be related to the accelerated accumulation of somatic mutations and the early aging phenotype observed in these mice [14]. Recently, attention has been focused on *OGG1* as a potential tumour suppressor, since inactivation of the *OGG1* gene in human cells could lead to a mutator phenotype that is vulnerable to DNA mutations. Indeed, somatic mutations in *hOGG1* have been found in human lung and kidney cancers [15], and a polymorphic mutation at codon 326 of *hOGG1* (Cys326), which is known to have a lower activity than Ser326, was found slightly more frequently in patients with lung cancer [16,17]. However, these observations seem to be insufficient to support the anticarcinogenic role of the hOGG1 enzyme, since decreased activity due to these genetic changes was only determined in a few cases, and the activity difference between the two polymorphic enzymes was not large. Thus, what is needed most to validate this hypothesis is confirmation that the activity is decreased in cancer tissues rather than details of genotypic changes. In the present study, this attempt was made in colon cancer, since cancer transformation due to a defective DNA repair process has already been confirmed in hereditary non-polyposis colorectal cancer, which was shown to have a defective mismatch repair process [18]. To test the involvement of *hOGG1* mutation in colon carcinogenesis, the activity of the hOGG1 protein (oh8Gua glycosylase), genotypes of this gene and also oxidative status of normal and tumour tissues of colon cancer patients were compared.

2. Patients and methods

2.1. Tissue harvesting

Tissue samples were obtained from operations on 15 colorectal cancer patients. Immediately after surgical removal of the specimens, approximately 0.5 gram of tissue was removed from the viable growing edge of the tumour and from normal colonic mucosa. The samples were quickly frozen and stored in liquid nitrogen until the time of assay.

2.2. oh8Gua Glycosylase assay

Tissue (100 mg) was homogenised in 2 volumes of homogenisation buffer (50 mM Tris–HCl, 50 mM KCl, 1 mM EDTA, 5% glycerol and 0.05% 2-mercaptoethanol, pH 7.5). The homogenised suspension was mixed with streptomycin (final concentration 1.5% w/v), to remove the nucleic acids and centrifuged. The supernatant was dialysed extensively against homogenisation buffer and used as tissue extracts for an oh8Gua glycosylase assay. All the procedures were performed below 4°C and the amount of protein was measured using the bicinchoninic acid method with bovine serum albumin as a standard [38].

The oh8Gua glycosylase assay was performed by the quantitative detection of the oh8Gua released from substrate DNA, according to a previously published method [19]. Briefly, substrate DNA containing oh8Gua (5′-GGTGGCCTGACG*CATTCCCCAA-3′; G*, oh8Gua) and its complementary DNA were prepared as previously described [20]. The [^{14}C(U)]-oh8Gua, an internal standard for the quantification of oh8Gua, was prepared by the method described by Degan and colleagues [21]. The duplex substrate DNA (10 pmol) was incubated at room temperature for 1 h with 5 mg of tissue extract, prepared in a 1 ml reaction mixture containing 50 mM Tris–HCl, 50 mM KCl, 1 mM EDTA, pH 7.5. The reaction was terminated by heating at 90°C for 3 min and centrifuged at 12 000*g* for 10 min. Supernatant (900 μl) was mixed with 100 μl of [^{14}C(U)]-oh8Gua solution (approximately 400 cpm) as an internal standard. The mixture was loaded onto an immunoaffinity column prepared with a monoclonal antibody raised against oh8Gua (provided by B. Ames, UC, Berkeley) as previously described [22]. Purified oh8Gua was dissolved in 50 μl of water and injected into a high performance liquid chromatography (HPLC) machine equipped with a Beckman Ultrasphere ODS column (5 μm, 4.6 mm×25 cm) and an electrochemical detector (ECD; Coulchem Model-5100 A, ESA). The activity of oh8Gua glycosylase was calculated as previously described [19], and was expressed as the amount of oh8Gua (pmol) released from the substrate DNA under the reaction conditions described above.

2.3. Analysis of oh8Gua formation

The analysis was performed as previously described [39]. DNA was extracted from tissues using a WB DNA Extractor Kit (Wako Chemical, Richmond, VA, USA). The extracted DNA was digested with nuclease P1 and acid phosphatase in a 10 mM sodium acetate solution (37°C for 30 min). After incubation, the mixture was centrifuged at 15 000*g* for 5 min. Supernatant was transferred to a filter tube (Millipore; Samprep C; 0.2 μm) and centrifuged at 5000*g* for 5 min, then injected

onto a HPLC column equipped with an ECD. A 20-μl solution containing deoxyguanosine (0.5 mg/ml) and 8-hydroxydeoxyguanosine (5 ng/ml) solutions were injected as standards. The amount of oh8Gua was calculated as the number of oh8Gua per 1×10^5 guanines.

2.4. Measurement of catalase, superoxide dismutase (SOD) and malondialdehyde

Tissue was homogenised in 4 volumes of homogenisation buffer (10 mM KH_2PO_4, 30 mM KCl, 1 mM EDTA, pH 7.4) and centrifuged at 10 000*g* for 30 min. The protein content of the homogenate was estimated as described above. Catalase activity was assayed using the method of Claiborne, which measures the rate of decomposition of hydrogen peroxide spectrophotometrically at 240 nm [23]. SOD activity was measured as described by Misra and colleagues [24], who measured the inhibition rate of the auto-oxidation of epinephrine. The extent of lipid peroxidation was determined by measuring malondialdehyde formed by the reaction between lipid peroxide and thiobarbituric acid, as previously described [25].

2.5. Mutational assay of hOGG1 gene

The DNA for the mutational analysis was extracted by the same method described in the oh8Gua analysis. Only the growing edge of tumor was sharply removed and used as the material for DNA extraction. Exons 1 to 7 of the *hOGG1* gene were amplified by the polymerase chain reaction (PCR) for single strand conformation polymorphism (SSCP) analysis, using six sets of primers as previously described [16]. Briefly, PCR was carried out using 50 ng of DNA as a template, in a 10 μl reaction mixture containing 0.25 μl of [α-32P] dCTP (3000 Ci/mmol, 10 Ci/ml). After 35 cycles of reaction at an annealing temperature of 58°C, the PCR products were denatured and electrophoresed on a 5% w/v polyacryamide gel. Amplified exons corresponding to the shifted band were re-amplified by PCR. The PCR products were purified and directly sequenced using the Taq dideoxy terminator cyclic sequencing kit with ABI 377 DNA sequencers (Perkin–Elmer, Foster City, CA, USA).

Six different sizes of DNA fragments (233, 355, 276, 295, 475 and 198 bp) were produced by the primers used in this study. Among these, the longest fragment (475bp) which contains the exons 5, 6 and the interlacing intron was regarded to be too long for the optimal sensitivity of the SSCP analysis. Thus, the longest fragment was analysed by direct sequencing as well as SSCP.

2.6. Statistical analysis

Results are presented as means±standard deviations (S.D.). Differences between tumour and normal tissue were tested using the paired Student *t*-test.

3. Results

A total of 15 colon cancer patients with ages ranging from 28 to 72 years (mean 55 years) were included in the study. The patients and their tumour-related characteristics are briefly summarised in Table 1. The mean

Table 1
Characteristics of patients and tumours

Serial number of patients	Sex/age	Tumour site	Stage[a]	Differentiation[b]	Family history[c]	MSI status[d]	*hOGG1* change
1	Female/72	Sigmoid	C2	MD	No	MSS	No
2	Male/50	Ascending	B2	Mucinous	No	MSS	Ser326Cys
3	Female/60	Ascending	D	MD	No	MSS	No
4	Male/55	Rectum	B1	WD	No	MSS	No
5	Female/54	Rectum	B1	MD	No	MSS	Ser326Cys
6	Female/46	Ascending	C2	MD	No	MSS	No
7	Female/28	Sigmoid	B2	MD	No	MSS	Ser326Cys
8	Male/70	Ascending	B1	MD	No	MSS	No
9	Female/51	Rectum	C1	WD	No	MSS	No
10	Female/71	Sigmoid	B2	MD	No	MSS	No
11	Male/58	Rectum	D	MD	No	MSS	Arg154His
12	Female/57	Ascending	B2	WD	No	MSS	No
13	Male/49	Rectum	A	MD	No	MSS	No
14	Male/44	Rectum	C2	MD	No	MSS	No
15	Male/61	Ascending	B2	PD	No	MSS	Arg154His

MSI, microsatellite instability; MSS, microsatellite stability.
[a] Aster–Coller staging system.
[b] WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.
[c] At least 2 colorectal cancer patients within first degree relatives, one should be diagnosed under the age of 50 years [26].
[d] Tested using *BAT26* marker.

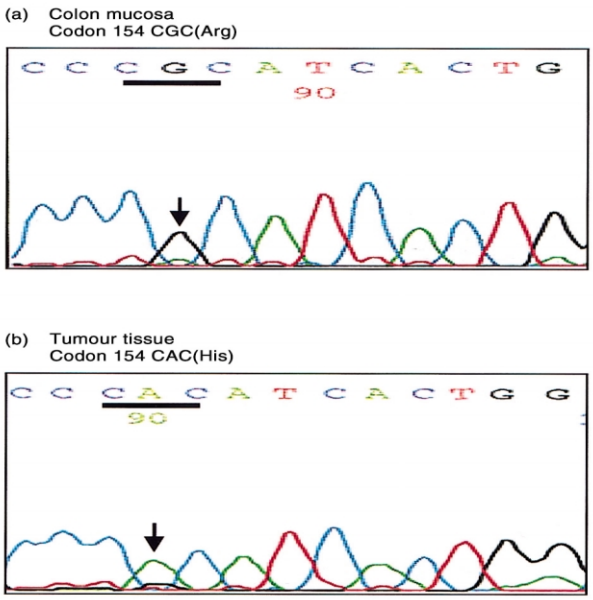

Fig. 1. A base substitution at codon 154 from (a) CGC (Arg) to (b) CAC (His). The mutation was detected in two tumour tissues, whereas DNA from corresponding normal mucosas showed the wild-type base.

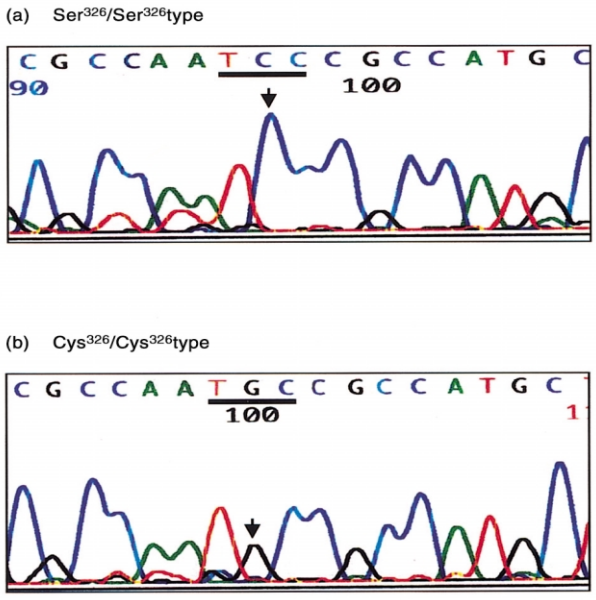

Fig. 2. (a) 12 patients showed Ser326/Ser326 genotype in both normal and tumour tissues, whereas 3 patients were regarded to have the Cys326/Cys326 genotype (b) in both normal and tumour tissues since the line representing cytosine was not seen.

activity of oh8Gua glycosylase for tumour samples was 4.3±1.3 (mean±S.D.) pmol oh8Gua released/h, whereas the mean value for mucosal samples was 2.7±1.2. By paired Student *t*-test analysis, the activity of oh8Gua glycosylase was significantly higher in the tumour samples than in the normal mucosas ($P=0.022$). The activity of oh8Gua glycosylase was not influenced by the age of the patients, site, stage or differentiation of the tumours.

A base transition at codon 154 of the *hOGG1* gene (CGC→CAC) that leads to an amino acid replacement (Arg→His) was detected in two tumour samples (Fig. 1). The mutated allele was not found in the corresponding normal tissues. A previously reported polymorphism at codon 326, TCC(Ser)→TGC(Cys), was detected in 3 patients (Fig. 2). The activity of oh8Gua glycosylase was not compromised by the Arg154 to His mutation. On the contrary, it was enhanced in the tumour tissues harbouring the mutated allele compared with the corresponding normal tissues, consistent with a trend towards higher activity in tumour tissue (Fig. 3). The oh8Gua glycosylase activities in both the normal and tumour samples with the Cys 326 polymorphism were not significantly different from those with Ser 326.

The extent of oxidative tissue damage was evaluated by measuring the concentrations of oh8Gua and a thiobarbituric reactive compound (malondialdhyde). Both were present at higher levels in the tumour tissues than in normal mucosas. To assess the oxygen free-radical removing ability, the activity of catalase and SOD were measured. The mean activities of both enzymes were found to be significantly decreased in the tumour tissues with respect to their surrounding cancer-free tissues, suggesting that oxygen free-radicals are less

Table 2
Summary of results

	Normal mucosa (mean±S.D.)	Tumour tissue (mean±S.D.)	*P* value
Catalase (U mg^{-1} protein)	24.8±6.2	19.3±7.4	0.004
SOD (U mg^{-1} protein)	7.5±2.6	4.9±2.2	0.002
oh8Gua (number oh8Gua/10^5 Gua)	1.6±0.6	2.4±1.1	0.007
Malondialdehyde (nmol mg^{-1} protein)	69.8±12.8	81.1±17.8	0.046
oh8Gua Glycosylase (pmol of oh8Gua) released from DNA/1 h	2.7±1.2	4.3±1.3	0.022

SOD, superoxide dismutase; SD, standard deviation.

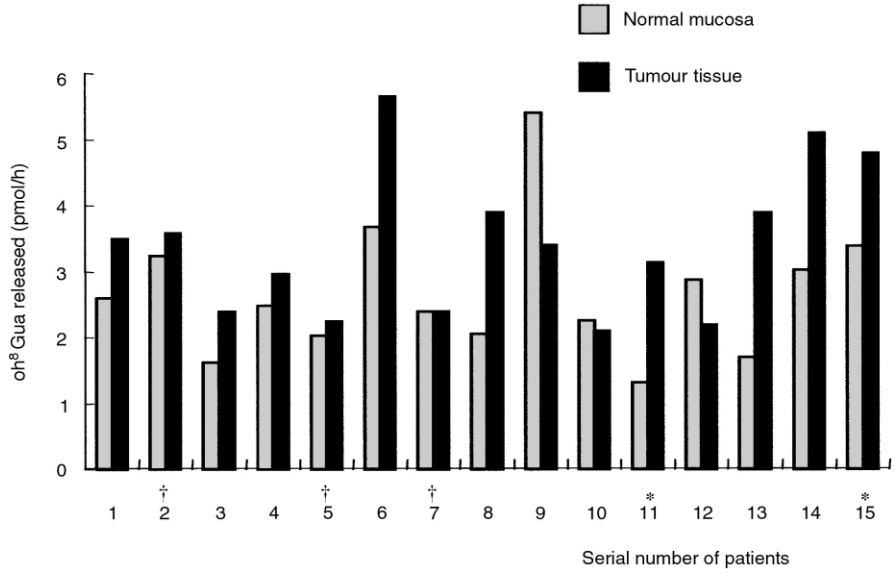

Fig. 3. Activities of oh8Gua glycosylase in paired normal and tumour tissues. * Tumour tissues with Arg 154 to His mutation; † patients with Cys326 polymorphism. No significant differences between the patients with cys and ser polymorphisms but there are some high values for patients 6 and 9.

efficiently removed from the tumour tissue. These results are summarised in Table 2.

4. Discussion

A base substitution at codon 154 (Arg154 to His) of the *hOGG1* gene shown in this study was previously reported in a gastric cancer cell line (MKN1) [27]. However, it was not clearly determined whether the genetic change in MKN1 was a somatic mutation or a rare polymorphism, and further, whether it affected the activity of the hOGG1 protein, because the corresponding normal tissue of cell line was unavailable. The result of the present study suggests that Arg154 to His is a somatic mutation rather than a polymorphism since the mutated allele is found only in the tumour tissues. The oh8Gua glycosylase activity was not compromised by the mutation. This result is in agreement with the finding that the Arg154 to His mutation does not diminish the ability of hOGG1 protein to remove oh8Gua paired to cytosine in *E. coli* [28], and implies that the Arg154 to His mutation is less likely to be involved in colon carcinogenesis. However, the possible involvement of this mutation in colon carcinogenesis cannot be clearly ruled out since the mutation is known to cause a substantial increase in its activity to remove oh8Gua paired with adenine, which can cause premutagenic GA→TA conversions [28].

Allelic change at codon 326 (Ser326 to Cys) has been reported as a genetic polymorphism [16]. This polymorphism has been reported to diminish slightly the activity of *hOGG1* gene based on the complementation assay in *E. coli*, and the activity assay of the recombinant hOGG1 protein *in vitro* [16,29]. However, the relevance of this polymorphism for the susceptibility to cancer is controversial. No significant association was found between allele types and gastric cancer susceptibility, but the lung cancer risk was higher in carriers of the Cys326 allele [27,30]. In the present study, the Cys326-type allele did not significantly affect the activity of oh8Gua glycosylase in both normal and tumour tissues. Thus, this polymorphism is also less likely to be involved in colon carcinogenesis.

The carcinogenic effect of the mutated *hOGG1* gene or the loss of heterozygosity (LOH) at chromosome 3p, where the *hOGG1* gene was mapped, has not been resolved, and the results are inconsistent, controversial, and depend upon the organs examined and the genetic changes found. A relevant correlation was observed in certain organs including lung, but not found in other organs [30,31]. The present study supports an innocent role for these two relatively well-defined genetic changes of *hOGG1* gene, at least in colon carcinogenesis. However, further studies are necessary to define the role of *hOGG1* mutation in colon carcinogenesis since the sample size used and the genetic changes found in our work were limited.

It is well known that a defect in DNA mismatch repair genes such as *hMSH2* or *hMLH1* results in colon cancers which characteristically display the microsatellite instability (MSI) phenotype [32]. Thus, we investigated the MSI status as well, since the DNA glycosylase (a base-excision DNA repair enzyme) and mismatch repair enzyme are similar in that both enzymes repair defects in DNA, although the targeted DNA

defect and mode of actions are completely different. However, none of the samples showed the MSI phenotype and, thus, there seems to be no interrelation between these two DNA repair systems.

Previously, we found an elevated oh8Gua glycosylase activity in response to a chemically-induced oxidative stress in mammalian tissues [19]. However, it is still unclear whether such inducibility is preserved after a cell is transformed to malignancy, and, thus, properly copes with the increased oxidative stress in cancer cell. The higher activity of oh8Gua glycosylase in tumour tissue shown in this study suggests that a compensation mechanism via oh8Gua glycosylase to inhibit mutagenesis is preserved even after the malignant transformation of cell. This further implies that inactivation of oh8Gua glycosylase may not be the primary cause of the increased accumulation of oh8Gua found in tumour tissues. However, such compensation seems to be insufficient since the oh8Gua level was still elevated in tumour tissue, despite the higher oh8Gua glycosylase activity.

In this work, the activities of antioxidant enzymes were significantly compromised in the tumour tissues and the levels of oh8Gua and malondialdehyde were higher in the tumour tissues. The defective antioxidant activity is possibly the primary cause of the oh8Gua accumulation in the tumours, since the activity of oh8Gua glycosylase, an eliminator of oh8Gua, was generally slightly elevated in the tumour tissue. It is still not conclusive from the data currently available whether increased DNA damage is merely the result of the disease (cancer) or whether it plays a role in colon carcinogenesis. However, several lines of evidence support the latter view. Firstly, treatment of laboratory animals with carcinogenic agents causes the formation of oh8Gua and the subsequent mutation of carcinogenesis-related genes in target organs before tumour formation occurs [33–35]. Secondly, the possible causal relationship between oxygen free-radicals and carcinogenesis has been reported mainly in organs under a high oxidative stress, including the lungs of chronic smokers and stomachs with *H. pylori* infection. The colon is also considered to be an organ under high oxidative stress since the fecal flora continuously produce large amounts of superoxide which, in the presence of chelated iron, can generate highly reactive hydroxyl radicals [36]. Thirdly, epidemiological data shows an inverse relationship between colon cancer incidence and the intake of antioxidant substances such as alpha-tocopherol [37].

Overall, these results indicate that the genetic changes of the *hOGG1* gene found in this study are not likely to significantly diminish the ability to remove oh8Gua from DNA, and thus, their role in colon carcinogenesis is likely to be limited. However, increased oxidative DNA damage caused by defective antioxidant activity may be involved in colon carcinogenesis.

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