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Evidence for upregulated repair of oxidatively induced DNA damage in human colorectal cancer

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ABSTRACT

Carcinogenesis may involve overproduction of oxygen-derived species including free radicals, which are capable of damaging DNA and other biomolecules *in vivo*. Increased DNA damage contributes to genetic instability and promote the development of malignancy. We hypothesized that the repair of oxidatively induced DNA base damage may be modulated in colorectal malignant tumors, resulting in lower levels of DNA base lesions than in surrounding pathologically normal tissues. To test this hypothesis, we investigated oxidatively induced DNA damage in cancerous tissues and their surrounding normal tissues of patients with colorectal cancer. The levels of oxidatively induced DNA lesions such as 4,6-diamino-5-formamidopyrimidine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 8-hydroxyguanine and (5′*S*)-8,5′-cyclo-2′-deoxyadenosine were measured by gas chromatography/isotope-dilution mass spectrometry and liquid chromatography/isotope-dilution tandem mass spectrometry. We found that the levels of these DNA lesions were significantly lower in cancerous colorectal tissues than those in surrounding non-cancerous tissues. In addition, the level of DNA lesions varied between colon and rectum tissues, being lower in the former than in the latter. The results strongly suggest upregulation of DNA repair in malignant colorectal tumors that may contribute to the resistance to therapeutic agents affecting the disease outcome and patient survival. The type of DNA base lesions identified in this work suggests the upregulation of both base excision and nucleotide excision pathways. Development of DNA repair inhibitors targeting both repair pathways may be considered for selective killing of malignant tumors in colorectal cancer.

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1. Introduction

Colorectal cancer is one of the principal causes of cancer-related deaths worldwide [1]. A genetic model for colorectal carcinogenesis has been developed and associated genetic events have been extensively studied [2]. It is now established that mutations in the genes controlling the cell cycle and/or in DNA repair genes lead to colorectal carcinogenesis. Defects in expression of DNA repair proteins may lead to the therapy resistance and affect overall survival in cancer patients [3,4]. Single-base substitutions such as C → T transversions dominate the majority of mutations found in colorectal cancer [5,6]. Most colorectal cancers are sporadic; however, 10–50% of incidents appear to involve an inherited predisposition [7]. Dietary habits such as high consumption of red meat, fat and alcohol may also play an important role [8]. In the past three decades, numerous studies have investigated the association of consumption of fruits and vegetables with risk of colorectal cancer. The results were often contentious and inconsistent in many cases (e.g., reviewed in [9,10]). Persistent oxidative stress has been shown in colorectal carcinoma patients [11–13]. Chronic inflammation, which is an important tumor promoter [14,15], may also contribute to colorectal carcinogenesis [16]. Oxidative stress and chronic inflammation cause oxidative damage to DNA and other biological molecules via free radicals such as the highly reactive hydroxyl radical (reviewed in [17]). Various DNA repair mechanisms exist in eukaryotes to repair oxidatively induced DNA damage, which is mainly repaired by base excision repair (BER) pathway and, to a lesser extent, by nucleotide excision repair (NER) [18]. A direct link has been discovered between colorectal cancer and mutations in the *myh* gene. The product of this gene, MYH is a DNA

Abbreviations: 8-OH-Gua, 8-hydroxyguanine; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; *R*-cdA, (5′*R*)-8,5′-cyclo-2′-deoxyadenosine; *S*-cdA, (5′*S*)-8,5′-cyclo-2′-deoxyadenosine; FapyAde, 4,6-diamino-5-formamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; OGG1, 8-hydroxyguanine DNA glycosylase; CSB, Cockayne syndrome group B protein; GC/MS, gas chromatography/mass spectrometry; LC–MS/MS, liquid chromatography/tandem mass spectrometry; •OH, hydroxyl radical.

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glycosylase and removes adenine paired with 8-hydroxyguanine (8-OH-Gua) [19]. Mice deficient in *myh* alone or in combination with the *ogg1* gene deficiency have been found to be susceptible to spontaneous and oxidative stress-induced colorectal carcinogenesis [20]. The role of other BER genes in colorectal carcinogenesis is widely unknown. However, the study of BER genes may contribute to understanding of colorectal cancer risk [16]. DNA repair pathways as a whole may be used as targets for cancer therapy [21,22]. Polymorphic forms of human repair enzyme 8-hydroxyguanine DNA glycosylase (OGG1) such as OGG1-Cys^{326} have been found in human populations and several cancers [23–27]. In contrast, the presence of OGG1-Cys^{326} did not associate with the risk of colorectal cancer [28,29].

In the past two decades, numerous studies have shown elevated levels of oxidatively induced DNA base lesions in cancerous tissues when compared with surrounding normal tissues [30–36]. These findings are supported by the fact that persistent oxidative stress exists in cancer [37,38]. Moreover, low OGG1 activity has been shown to be a risk factor in several cancers [39,40]. In general, variations in DNA repair capacity may have an effect on cancer susceptibility, outcomes and treatment of cancer (reviewed in [21,22]). Greater over-expression of *ogg1* mRNA and normal repair activity for the excision of 8-OH-Gua has been observed in a number of lung cancer cell lines compared to control lung cell lines [41]. Similarly, lower levels of ethano-DNA adducts have been found in cancerous colon tissues than in surrounding non-cancerous tissues of colorectal cancer patients [42,43]. These findings agreed with the significantly greater excision activities for these DNA adducts in cancerous tissues than in surrounding non-cancerous tissues [43]. Greater levels of 8-OH-Gua have been found in DNA of leukocytes and in urine of colorectal cancer patients than in healthy individuals, accompanied with increased mRNA levels of DNA repair enzymes including OGG1 and with increased excision rate for 8-OH-Gua [44]. It has been concluded that oxidative stress may stimulate the 8-OH-Gua excision rate; however, this stimulation may not be sufficient to thwart oxidatively induced damage to DNA. Single base substitution mutations in colorectal cancer have been found to be substantially different from those in breast cancer [5,6]. For example, the most common C → T transitions were much more frequent in the former than in the latter. All these findings may reflect differences in DNA repair processes in different types of cancers.

Oxidatively induced DNA lesions in cancerous tissues of colorectal cancer patients have not been investigated in detail. In view of differences in excision rates of DNA lesions and in mutations between cancer types, we hypothesized that repair of oxidatively induced DNA base damage may be increased in colorectal tumors, resulting in lower levels of DNA base lesions than in surrounding normal tissues. To test this hypothesis, we investigated the levels of several DNA bases modifications in tumors of colorectal patients and in non-cancerous tissues surrounding the tumors. We also checked whether tumor location, tumor stage and perineural invasion had an effect on the levels of DNA lesions.

2. Materials and methods

2.1. Materials

Nuclease P1 (from *Penicillium citrinum*) and alkaline phosphatase were purchased from United States Biological (Swampscott, MA) and Roche Applied Science (Indianapolis, IN), respectively. Snake venom phosphodiesterase and acetonitrile (HPLC grade) were from Sigma (St. Louis, MO). Biomax5 ultrafiltration membranes (5 kDa molecular mass cutoff) from Millipore (Bedford, MA) were used to filter hydrolyzed DNA samples. Water (HPLC-grade) for analysis by liquid chromatography/isotope-dilution tandem mass spectrometry (LC–MS/MS) was from Sigma (St. Louis, MO). Water purified through a Milli-Q system (Millipore, Bedford, MA) was used for all other applications. N,O-bis(trimethylsilyl)trifluororacetamide containing 1% trimethylcholorosilane was purchased from Pierce Chemicals (Rockford, IL).

2.2. Patients and control individuals

This study included 55 randomly selected patients with colorectal cancer (median age 57 years; range 32–78 years; 37 men, 18 women) treated surgically in the Division of Colorectal Surgery, Department of General Surgery, School of Medicine, Dokuz Eylul University. The tissue samples were collected over a period of two years. They comprised cancerous tumor tissues removed during the resection and the surrounding histologically normal mucosa. Ethical approval was granted by the School of Medicine's Ethics and Medical Research Committee of Dokuz Eylul University to conduct this study.

2.3. DNA isolation

DNA was isolated from tissues as soon as possible after collection. Colon and rectum tissue samples were minced and rinsed free of blood with 5 mL PBS buffer (pH 7.4). The tissue was then homogenized with 1 mL PBS buffer and then centrifuged at 10,000 g for 15 min. The pellet was suspended in 3 mL of lysis buffer (10 mM Tris HCl, 0.4 M NaCl and 2 mM EDTA, pH 8.2) and then incubated at 37 °C for 60 min. The sample was centrifuged and the pellet was digested with 0.2 mL of 10% SDS and 0.5 mL of protease K solution (1 mg protease K in 1% SDS and 2 mM EDTA) overnight at 37 °C. After digestion, 1 mL of saturated NaCl (6 M) was added. Tubes were shaken vigorously until foam from protein appeared. The sample was then incubated for 10 min at 56 °C followed by centrifugation at 5000 g for 30 min at room temperature. The supernatant fraction containing the DNA was transferred to a 15 mL polypropylene tube. Two volumes of absolute ethanol kept at room temperature were added. After centrifugation and removal of the supernatant fraction, the DNA pellet was washed twice with 70% ethanol and centrifuged. After the removal of ethanol, and air-drying for 1 h, DNA was dissolved in water for 24 h at 4 °C. The UV spectrum of each DNA sample was recorded between 230 nm and 350 nm by an absorption spectrophotometer. The absorbance at 260 nm was used to measure the DNA concentration of each sample (absorbance of 1 = 50 μg of DNA/mL). Aliquots (50 μg) of DNA samples were dried in a SpeedVac under vacuum.

2.4. Analysis of DNA samples

LC–MS/MS was used to identify and quantify (5′*R*)-8,5′cyclo-2′-deoxyadenosine (*R*-cdA) and (5′*S*)-8,5′cyclo-2′-deoxyadenosines (*S*-cdA). Internal standards *R*-cdA-$^{15}N_5$ and *S*-cdA-$^{15}N_5$ were prepared using dATP-$^{15}N_5$ [Medical Isotopes, Inc. (Pelham, NH)] as described [45]. Aliquots (50 μg) of DNA samples were supplemented with aliquots of *R*-cdA-$^{15}N_5$ and *S*-cdA-$^{15}N_5$, and hydrolyzed with nuclease P1, snake venom phosphodiesterase and alkaline phosphatase and then analyzed by LC–MS/MS [46]. 4,6-Diamino-5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 8-OH-Gua were identified and quantified using gas chromatography/isotope-dilution mass spectrometry (GC/MS), following hydrolysis of DNA samples with *E. coli* Fpg protein and using FapyAde-$^{13}C,^{15}N_2$, FapyGua-$^{13}C,^{15}N_2$ and 8-OH-Gua-$^{15}N_5$ as internal standards [47].

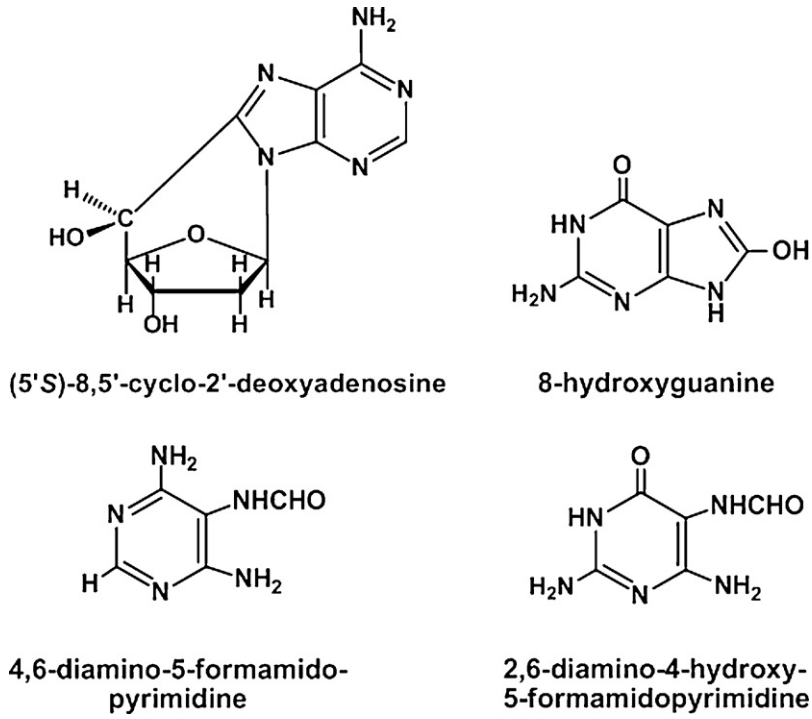

Fig. 1. Structures of the DNA lesions identified in this work.

2.5. Statistical analysis

Statistical analyses of the data were performed using the GraphPad Prism 5.04 software (La Jolla, CA, USA) and two-tailed nonparametric Mann Whitney test with Gaussian approximation and confidence interval of 95%. A p-value < 0.05 was assumed to correspond to statistical significance.

3. Results

We investigated some typical oxidatively induced DNA lesions in malignant tumors and their surrounding normal tissues to test the hypothesis that repair of DNA lesions may be enhanced in colorectal cancer tissues when compared to normal counterparts. If this were the case, it would have consequences concerning therapeutic approaches used for the treatment of colorectal cancer. We were able to accurately measure four DNA lesions using two mass spectrometric techniques with isotope-dilution for quantification. The structures of these lesions are shown in Fig. 1. We also attempted to measure *R*-cdA by LC–MS/MS; however, it was not possible to accurately measure this lesion in all tissue samples to obtain statistically significant results. Fig. 2 illustrates the levels of *S*-cdA in colorectal cancerous tissues and the surrounding non-cancerous tissues. The scattered dot plots of all the values are shown, which were obtained with normal and cancerous colorectal tissues from 18 patients. The horizontal lines in these plots represent the mean value of all the measured data points in each group. The graph clearly shows that the level of *S*-cdA was statistically lower in cancerous tissues than in surrounding normal tissues with a p-value of 0.0004. The levels of FapyAde in both tissues types are shown in Fig. 3. This lesion could only be accurately measured in 11 normal and 8 cancerous tissues. A statistically significant difference with a p-value of 0.0073 between the level of FapyAde in normal tissues and that in cancerous tissues was observed, with the latter being lower than the former. The levels of FapyGua and 8-OH-Gua are illustrated in Figs. 4 and 5, respectively. We were able to measure the levels of these lesions in tissues of up to 55 patients. The level of FapyGua was significantly lower in cancerous tissues than those in normal tissues with a p-value of 0.0018. A statistically significant difference ($p = 0.0302$) between the values of 8-OH-Gua in two different tissues was also observed, with the level in cancerous tissues being lower than that in normal tissues. We also checked whether the tumor location, i.e., colon vs. rectum, makes a difference in the levels of DNA lesions. Fig. 6 illustrates the levels of FapyGua and 8-OH-Gua in cancerous tissues of colon and rectum. Significantly greater levels of these lesions were observed in rectum than in colon tissues. Other parameters analyzed were perineural invasion and tumor stage. No statistically significant difference between tumor stages, and between presence and absence of perineural invasion was observed, although there was a trend for greater levels in tumors stage 4 and in those with perineural invasion (data not shown).

We also wished to measure the activities of the DNA repair enzymes such as NEIL1 and OGG1 in the collected clinical sam-

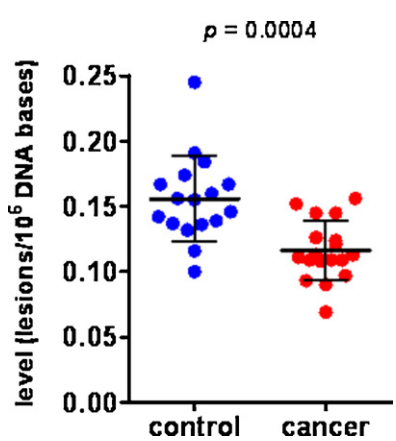

Fig. 2. The level of *S*-cdA in normal ($n = 17$) and cancerous ($n = 18$) colorectal tissues. Thick horizontal lines represent the means. Uncertainties are the standard deviations (thin lines).

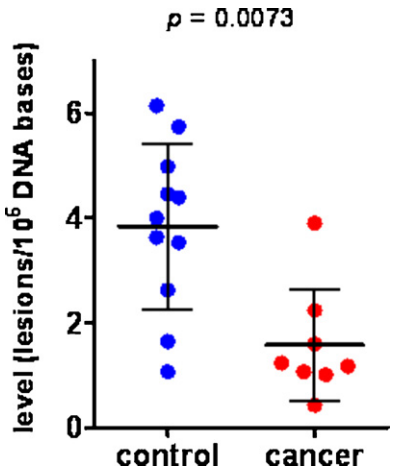

Fig. 3. The level of FapyAde in normal ($n = 11$) and cancerous ($n = 8$) colorectal tissues. Other details as in Fig. 2.

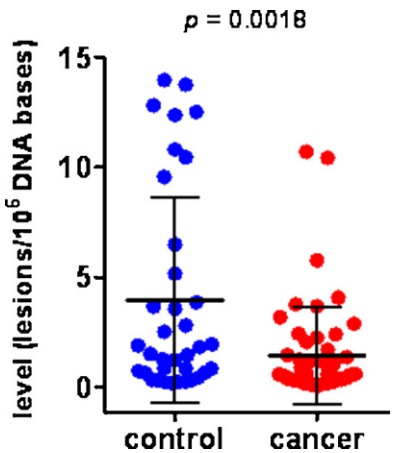

Fig. 4. The level of FapyGua in normal ($n = 36$) and cancerous ($n = 53$) colorectal tissues. Other details as in Fig. 2.

ples. However, the availability of the human tissues was extremely limited. We just had a sufficient number of collected tissues for the isolation of adequate amounts of DNA for mass spectrometric analyses. Consequently, no sufficient amounts of tissues were left for any kind of measurement of enzyme activities in most cases. In future, we hope to perform such measurements using mass

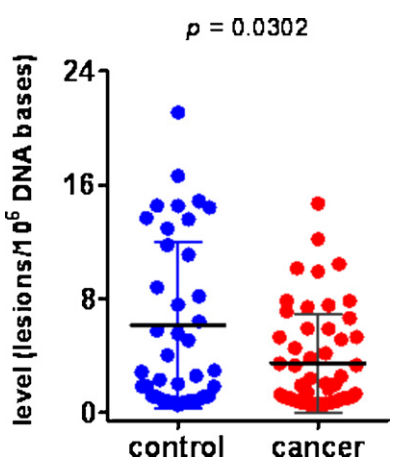

Fig. 5. The level of 8-OH-Gua in normal ($n = 39$) and cancerous ($n = 55$) colorectal tissues. Other details as in Fig. 2.

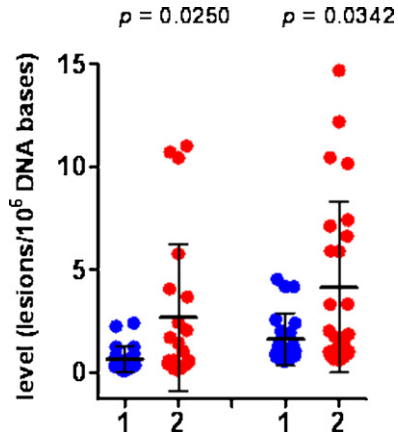

Fig. 6. The levels of FapyGua (left) and 8-OH-Gua (right) in cancerous colon ($n = 22$) (1) and rectum ($n = 25$) (2) tissues. Other details as in Fig. 2.

spectrometric methodologies developed in our laboratory as described in our just recently published two papers [48,49].

4. Discussion

The aim of this study was to investigate oxidatively induced DNA base damage in cancerous tissues and surrounding normal mucosa in colorectal cancer patients. Previous studies demonstrated enhanced repair of ethano-DNA adducts and oxidatively induced 8-OH-Gua in cancerous colon tissues when compared to normal colon tissues [42–44], in contrast to DNA repair deficiency for 8-OH-Gua observed in other types of cancers [39,50]. We used two mass spectrometric techniques for positive identification and accurate quantification of four typical oxidatively induced DNA lesions in colon tissues of colorectal cancer patients. These were *S*-cdA, FapyAde, FapyGua and 8-OH-Gua. We observed statistically significant lower levels of these lesions in cancerous tissues than in surrounding normal tissues. These findings suggest enhanced repair of these lesions in cancerous tissues. DNA repair mechanisms exist in mammalian cells to repair oxidatively induced DNA damage (reviewed in [18]). FapyAde, FapyGua and 8-OH-Gua are removed from DNA by base excision repair (BER) pathway (reviewed in [51]). OGG1 is specific for the removal of FapyGua and 8-OH-Gua from DNA with multiple lesions in the first step of BER; however, it does not act on FapyAde [52,53]. FapyAde and FapyGua are the primary substrates of mammalian DNA glycosylase NEIL1 *in vitro* and *in vivo*, which in turn exhibits no activity for 8-OH-Gua [54–58]. *Neil1*$^{-/-}$ mice accumulated FapyAde and FapyGua, but not 8-OH-Gua, in different organs and developed several types of cancers in the second half of their lives, strongly suggesting a role for NEIL1 in cancer prevention and a possible involvement of FapyAde and FapyGua in carcinogenesis [58]. Recently, Cockayne syndrome group B protein (CSB), which participates in NER, has been shown to stimulate the repair by NEIL1 of FapyAde and FapyGua *in vitro* [59]. In addition, these lesions accumulated in organs of *csb*$^{-/-}$ mice, suggesting a functional and physical interaction between CSB and NEIL1 not only *in vitro*, but also *in vivo*, in recognizing and removing them. Among the DNA lesions identified in the present work, *S*-cdA is unique in that it is repaired by NER rather than by BER, because the 8,5′-covalent bond in the molecule prevents the repair by BER [60–62]. At present, little is known about NER mechanisms for *in vivo* repair of this lesion. Recent studies shed some light on the understanding of the repair of such lesions by NER. Thus, *neil1*$^{-/-}$ mice accumulated in *R*-cdA and *S*-cdA, providing evidence for a role of NEIL1 in *in vivo* repair of these lesions [63]. This may also mean the involvement of NEIL1 in NER in general, in addition to its function as a DNA

glycosylase in BER. Similarly, *csb*$^{-/-}$ mice accumulated significant amounts of *S*-cdA in three different organs [64]. Moreover, accumulation of *S*-cdA has been observed in Cockayne syndrome group A protein (CSA)-deficient keratinocytes from Cockayne syndrome-A patients when exposed to oxidative stress followed by a repair period [65]. These results clearly indicate the role of NER proteins in *in vivo* repair of *S*-cdA. Since OGG1 and NEIL1 are the main enzymes that remove FapyGua, 8-OH-Gua and FapyAde from DNA *in vivo*, the results of the present work suggest that OGG1 and NEIL1 may be over-expressed in cancerous colorectal tissues. Moreover, the lower level of *S*-cdA observed in cancerous colorectal tissues suggests over-expression of not only BER enzymes, but also NER proteins such as CSA and CSB in these tissues when compared with normal colon tissues.

There is a possibility that the reduced levels of DNA lesions in malignant colorectal tissues may be due to diluting out the damage because cancerous cells are rapidly dividing. However, it is unlikely that multiplying cells would generate cells with DNA containing less damage. This is because DNA in new cancerous cells produced by replication would be exposed to the same damage and repair conditions as parent cells, leading to similar steady state levels of damage as in parent cells. Furthermore, previous research showed increased levels of oxidatively induced DNA lesions in other types of cancers, strongly suggesting that no dilution of DNA damage exists in cancerous cells [30–36]. Increased levels of DNA damage is likely due to persistent oxidative stress in cancerous cells [37,38]. Persistent oxidative stress is also a hallmark of colorectal cancer [11–13]. The present work, however, shows decreased levels of DNA lesions in cancerous colorectal tissues. Therefore, the dilution of DNA damage may not exist in colorectal cancer, either. These facts lend credence to our hypothesis supported by the data of this work that DNA repair may be upregulated in colorectal cancer. Furthermore, one should take into consideration the fact that differences in DNA repair processes may exist in different types of cancers as outlined in Section 1.

The great majority of somatic mutations in colorectal cancer has been reported to be single base substitutions, with the C → T transition being the dominant mutation especially at 5′-CpG-3′ sites [5,6]. Of the DNA lesions measured in this study, 8-OH-Gua is highly mutagenic leading to G → T transversion mutations (reviewed in [66]). These mutations are the second most common somatic mutations found in human cancers, with 14.6% of all mutations in the tumor suppressor gene *TP53* following C → T transition mutations (44.2%) [67] (also see http://www-p53.iarc.fr/). *TP53* mutations constitute 43.2% of all mutations in sporadic colorectal cancers [67]. The other major DNA lesion FapyGua has been determined to cause G → T transversion mutations just like 8-OH-Gua [68,69]. In simian kidney cells, FapyGua was even 25–35% more mutagenic than 8-OH-Gua. FapyAde is weakly mutagenic and leads to A → T and A → C transversion mutations [69,70]. Little is known about mutagenic properties of *S*-cdA. As a consequence of the 8,5′-covalent bond, 8,5′-cyclopurine-2′-deoxynucleosides such as *S*-cdA cause distortion in the DNA helix [71,72]. *S*-cdA has been shown to cause transcriptional mutagenesis and mutant transcripts resulting from its bypass by RNA polymerase II have been characterized [73]. This type of bypass misincorporates an adenosine opposite to the next nucleotide 5′- to *S*-cdA, causing multiple nucleotide deletions. In addition, *S*-cdA is a strong block to transcription and DNA polymerases, and reduces transcription by blocking transcription binding factor [61,62,74,75]. Furthermore, *S*-cdA may cause neuronal death in a number of diseases with defective NER by blocking neuronal gene expression [76]. A more recent work showed that the analogous lesion (5′*S*)-8,5′-cyclo-2′-deoxyguanosine is a strong block to replication and a highly mutagenic lesion leading to G → A transitions with G → T transversions to a lesser extent, and is inefficiently repaired in *E. coli* [77]. By inference, *S*-cdA may also be a strongly mutagenic lesion.

It is obvious that the DNA lesions identified in this work possess adverse biological effects that may be relevant to colorectal carcinogenesis, especially in view of G → T mutations caused by FapyGua and 8-OH-Gua. Accumulated DNA lesions may have detrimental consequences to a living cell. Mutator phenotype, i.e., increase in mutation rate due to accumulation of DNA lesions, leading to greater genetic instability, is a hallmark of cancer cells [78]. Genetic instability may affect protein expressions involved in DNA replication, DNA repair, chromosomal stability, apoptosis, cell cycle regulation among others [79]. Altered DNA repair gene expression may affect DNA repair status of tumors. For example, 60% of cancer cell lines have somatic mutations in DNA repair genes (see http://www.sanger.ac.uk.genetics/CGP). During evolution of cancer, genetic instability may be essential and mutations may provide cells with survival advantage; however, increased mutations due to excessive DNA damage may be detrimental to cells late in tumor evolution, leading to cell death. On the other hand, natural selection may favor those tumor cells that over-expressed DNA repair genes to become potentially capable of surviving. Enough time given, this would make tumors possess greater DNA repair capacity than non-cancerous tissues. In this respect, non-small-cell lung cancer has been shown to exhibit resistance to chemotherapy, and this resistance was associated with elevated NER in tumors [3,4].

In conclusion, we show significantly lower levels of some major products of oxidatively induced damage to DNA bases in malignant colorectal tumors than those in surrounding non-cancerous tissues. These results strongly suggest that cancer cells in colorectal tissues upregulate DNA repair of oxidatively induced DNA damage. The type of DNA base lesions identified in this work suggests the upregulation of both BER and NER. We also show that DNA repair may be greater in cancerous colon tissues than that in cancerous rectum tissues. This shows that DNA repair capacity varies between these two tissue types. However, no correlation was found concerning tumor stage and perineural invasion. Upregulation of DNA repair in cancerous colorectal tissues may have adverse consequences for the DNA damage-based treatment of colorectal cancer by causing resistance to therapeutic agents thus affecting patient survival. DNA repair pathways are promising drug targets for cancer treatments and a number of small-molecule DNA repair inhibitors are being investigated in the clinical trials for their efficacy as sensitizers of tumor cells to chemotherapy (reviewed in [21,22]). Development of DNA repair inhibitors for combination therapy or as single agents for monotherapy targeting BER as well as NER should be considered for selective killing of tumors in colorectal cancer. Understanding of all DNA repair pathways will be of fundamental importance for this purpose.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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