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8-hydroxy-2'-deoxyguanosine in colorectal adenocarcinoma – is it a result of oxidative stress?

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Background: 8-hydroxy-2'-deoxyguanosine (8-OHdG) is one of the most abundant oxidatively modified lesions in DNA and is a marker of the oxidative stress. 8-OHdG is a mutagenic lesion and it can mispair with adenine, causing G: C→T: A transversion. Our task was to determine the 8-OHdG level in patients with colorectal adenocarcinoma directly in tumor tissues and corresponding normal mucosa.


Material/Methods: Samples of tumor tissues and corresponding normal mucosa of 47 patients undergoing surgery for colorectal cancer were analyzed. DNA was isolated from both tumor and normal tissues. Then, DNA was hydrolyzed to nucleotides using nuclease P1 and alkaline phosphatase. The 8-OHdG and 2'-dG (2'-deoxyguanosine) were determined in hydrolysates by high-performance liquid chromatography (HPLC) with electrochemical (EC) and UV detector.

Results: The levels of 8-OHdG in colorectal adenocarcinoma tissues were higher than in corresponding normal mucosa. No significant differences were shown in 8-OHdG levels in the cancerous and cancer-free tissues between age and sex and stages A/B and C/D of Duke's classification.

Conclusions: 8-OHdG reflects the local oxidative stress in colon adenocarcinoma tissue together with ageing processes, but not the intensity of tumorigenesis itself. Because of many factors that could influence the oxidative modification of DNA bases, its role as a diagnostic and/or prognostic factor in colon adenocarcinoma seems to be limited.

Key words: 8-hydroxy-2'-deoxyguanosine • oxidative stress • colorectal adenocarcinoma

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Background

Oxidative stress is now recognized to be a prominent feature of many acute and chronic diseases and even of normal ageing processes [1–4]. DNA and nucleotides are at high risk of being oxidized by reactive oxygen species (ROS), which are generated as byproducts of oxygen respiration or molecular executors in host defence [5], or through environmental exposure [6]. Among bases, guanine is the most susceptible DNA target for oxidation reactions [7]. 8-hydroxy-2'-deoxyguanosine (8-OHdG), one of the major DNA base-modified products, may be induced by hydroxyl radicals, singlet oxygen, or photodynamic action [8]. The formation of 8-OHdG in DNA is frequently measured in serum, leukocytes, and urine to assess oxidative stress in humans [9–11]; higher amounts have been detected in smokers and people exposed to environmental tobacco smoke compared with non-smokers [12–14]. In metabolic syndrome, diabetes, and inflammatory diseases, oxidative stress and 8-OHdG level (in blood and urine) are clearly increased [15–17]. This compound is known to be mutagenic by mispairing with adenine; thus, during the next round of replication, faultily paired adenine will pair with thymidine and transversion G:C→T:A arises [5,18,19]. Increased oxidative stress is generally thought to be associated with tumorigenesis.

Many observations suggest the role of oxidative stress in colon cancer pathogenesis. Chang et al. reported that the content of 8-OHdG in serum can act as a sensitive biomarker for colorectal carcinoma [20]. Its level was also found to be elevated in DNA isolated from lymphocytes of colon carcinoma patients [21]. Sato et al. suggested that an increased plasma level of 8-OHdG is associated with development of colorectal adenoma and early cancer, but cancer tissues themselves are not always the major source [22]. Moreover, Dincer et al. concluded that low plasma levels of 8-OHdG (together with altered antioxidant activity) may implicate the defective repair of oxidative DNA damage in patients with colon and gastric cancer [23]. In contrast, a study with more than 100 patients showed no significant differences at the individual level between the level of 8-OHdG in lymphocytes and the excretion of 8-OHdG measured by chromatographic methods [24]. It is difficult to exclude all factors that could affect the status of oxidative DNA damage and contribute to the increasing level of 8-OHdG in the entire human body. The direct measurement of 8-OHdG in the appropriate tissues can give a more precise picture of what is happening directly in the tissue. Therefore, we decided to determine the 8-OHdG level directly in tumor and corresponding normal mucosa for comparison.

Material and Methods

Study group

The patients included in the study underwent surgery at the Maria Skłodowska-Curie, Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Poland, during 2005–2007. The study group consisted of 47 patients (27 men and 20 women) diagnosed with sporadic colorectal adenocarcinoma. In this group the cancer was located in the distal part of the colon. In each case the cancerous tissues and corresponding normal mucosa (taken from at least 2 cm, mean 4.5 cm, distance from the tumor) were resected. The age of the patients ranged from 26 to 82 years (mean age: 65 ± 11 years). These patients had not been exposed to radio- or chemotherapy before the surgery. None of them had a history of colorectal cancer diagnosed in the family. The coincidence of diabetes, lipid disorders, chronic alimentary tract diseases, and cigarette smoking were also exclusion criteria. The tumors were staged using conventional Duke's classification [25]. Twenty-eight patients (60%) were diagnosed as A or B Duke's stage and 19 (40%) as C or D Duke's stage.

Ethical approval for this study was received from the Ethics Committee of the Center of Oncology, Maria Skłodowska-Curie Memorial Institute of Oncology in Gliwice (No. D0/DGP/493-10/05) and (No. KB/493-54/07). Written informed consent was obtained from all patients who participated in this study.

Tissue samples

Cancerous tissues and corresponding normal mucosa were dissected separately, immediately after surgical resection. The tumor and corresponding normal mucosa samples were frozen on dry ice and stored at -20°C until needed for further investigation.

DNA extraction and preparation for high-performance liquid chromatography

The frozen tissue samples were homogenized with the FastPrep-24 System (MP Biomedicals, USA) using Lysing Matrix D (MP Biomedicals, USA). DNA was isolated using the DNeasy® Tissue Kit (Qiagen, Germany) according to the provided protocol. DNA was dissolved in 100 μl AE buffer. Then DNA was hydrolyzed to nucleotides: DNA samples were mixed with 40 mM sodium acetate (POCH, Poland), 0.1 mM ZnCl_2 (Merck, Germany), pH 5.1, and nuclease P1 (Sigma-Aldrich, USA) solution (20 μg protein per sample). Samples were incubated at 37°C for 1 h. Thereafter, 1 M Tris-HCl buffer (Fluka, USA), pH=7.4 and alkaline phosphatase (Sigma-Aldrich, USA) solution containing 1.5 units of the enzyme were added to the samples for phosphate residue removal, and the samples were incubated

Table 1. The comparison of 8-OHdG levels in cancer and corresponding normal mucosa in patients with colorectal adenocarcinoma (Wilcoxon's matched pairs test).

Group of patients	N	Tissue	Me [#] (Q1–Q3) ^{##} [8-OHdG/10 ⁶ 2'-dG]	p value
Study group	47	Normal mucosa	21 (9–39)	0.012*
		Cancer	49 (23–114)	
Women	20	Normal mucosa	19 (4–840)	0.905 ^{####}
		Cancer	25 (16–41)	
Men	27	Normal mucosa	21 (9–39)	0.0051 ^{#####}
		Cancer	89 (34–143)	
<65 years	21	Normal mucosa	36 (9–62)	0.609 ^{####}
		Cancer	41 (17–172)	
≥65 years	26	Normal mucosa	20 (9–38)	0.0081 ^{#####}
		Cancer	55 (23–111)	
AB ^{###}	28	Normal mucosa	17 (9–38)	0.0258 ^{####}
		Cancer	53 (16–105)	
CD ^{###}	19	Normal mucosa	22 (17–45)	0.33 ^{####}
		Cancer	38 (27–115)	

Me – median; ## (Q1–Q3) – lower quartile – higher quartile; ### Duke's classification with modifications: A/B, tumor confined to the mucosa (A) / tumor penetrates into, but not through the muscularis propria of the bowel wall (B1) or tumor penetrates into and through the muscularis propria of the bowel wall (B2), C/D, tumor penetrates into, but not through the muscularis propria of the bowel wall and tumor spread into regional lymph nodes (C1) or tumor penetrates into and through the muscularis propria of the bowel wall and tumor spread into regional lymph nodes (C2) / distant metastases (D); * statistical significance, #### After Bonferroni correction, p value less than 0.025.

for 1 h at 37°C. All DNA hydrolysates were ultrafiltered using Amicon® Ultra-4 (Millipore, USA) with a speed of 8000 rpm for 40 min at a temperature of 22°C. Isolated DNA was prepared for high-performance liquid chromatography according to the procedure described by Foksinski et al. [26], calculating the reagents volume for the amount of DNA.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) with electrochemical (EC) and UV detectors was used for 8-OHdG and 2'-dG (2'-deoxyguanosine) estimation. Detection of dG was performed by UV detector (Dione, Germany) at 260 nm and 8-OHdG was determined by electrochemical detector (Gilson, France), for which an oxidation potential of +700 mV was used (an electrode set to sensitivity of 10 nA/V). The mobile phase consisted of 50 mM KH₂PO₄ (POCH, Poland) buffer (pH 5.5) and methanol (POCH, Poland). Those components were well-proportioned in 9:1 ratio, based on the procedure elaborated by Shigenaga et al. [27]. The separations were performed at a flow rate of 0.7 ml/min, and the volume of loop equalled 20 µl. 8-hydroxy-2'-deoxyguanosine (Sigma-Aldrich, USA) and 2'-deoxyguanosine (Fluka, USA) were used as a standard for HPLC. The amount of 8-OHdG in DNA was calculated as the number of 8-OHdG molecules/10⁶ unmodified 2'-dG molecules.

Statistical analysis

Our data had a non-normal distribution, so we used a non-parametric test.

For comparisons of 8-OHdG level in cancer tissue and corresponding mucosa samples, Wilcoxon's matched pairs test was used. Analyses of this parameter according to age, sex, and Duke's staging were conducted with the Mann-Whitney U test. A p value of less than 0.05 was considered significant. The Bonferroni correction for multiple comparisons was applied, and the corrected p value of less than 0.025 (for 2 comparisons) and less than 0.01 (for 3 comparisons) was considered statistically significant. The statistical software package STATISTICA 8.0 PL was used for data management and analysis.

Results

The 8-OHdG level in the cancer tissues was significantly higher than in corresponding normal mucosa (Table 1). More detailed analysis revealed that these differences were significant only in men and in patients over 65 years old. They were not documented for women and for patients under 65 years old (Table 1). The differences between the 2 subgroups (patients

below 65 and over 65 years old) were not significant within the normal mucosa ($p=0.77$) or within the cancer tissues ($p=0.78$). Analysis of 8-OHdG levels within normal mucosa and within cancer between women and men did not reveal significant differences ($p=0.283$ and $p=0.026$, respectively).

A comparison between subgroups according to age and sex showed that 8-OHdG level was not significantly different in normal colonic mucosa or in cancer. Patients with A and B colon adenocarcinoma stages were brought together, as well as C and D, and 2 subgroups (A/B and C/D) were analyzed between themselves in relation to 8-OHdG level. No statistical differences between normal mucosa and cancer tissue were confirmed in the A/B subgroup or in the C/D subgroup (Table 1).

The comparison of 8-OHdG level within cancer and corresponding normal mucosa in the cases of A/B and C/D stages showed no significant differences ($p=0.452$ and $p=0.871$, respectively).

Discussion

In recent studies, 8-OHdG has been widely used, not only as a biomarker indicating the level of endogenous oxidative DNA damage, but also as a risk factor for many diseases, including cancer [30]. A variety of analytical techniques used in assessing damage of oxidative DNA exist. The high-performance liquid chromatography with electrochemical detection is one of the most widely used methods for quantitative analysis [28,29]. In our study we used it for 8-OHdG estimation in tumor tissues and normal/adjacent mucosa in patients with colon adenocarcinoma. Generally, we found that 8-OHdG levels were significantly higher in cancer tissue than in normal mucosa. Kondo et al. and Park et al. reported high levels of 8-OHdG in colorectal tumour cells obtained by surgical resection [30,31]. Moreover, the imbalance of colon adenocarcinoma cancer tissue antioxidant potential compared with tissue without histopathologically detected neoplastic changes was previously described [31,32]. Our results also confirmed higher oxidative stress/inadequate activity of DNA repair systems in colorectal cancer tissue, leading to an increase of local 8-OHdG concentrations.

Confusing results were obtained from data showing differences between 8-OHdG level in cancer tissue and normal mucosa in men and lack of significance in women and in patients over 65 years old, but not in younger patients. Shiota et al. reported that androgen may increase oxidative stress [33]; however, Kominea et al. found no difference in androgen receptor expression in gastric cancer of male and female subjects [34]. Another study confirmed that alternations in the androgen receptor signalling axis are central to castration-recurrent prostate cancer [35]. Oxidative processes and the antioxidant

enzymes activity, as well as efficiency of DNA repair systems, are age-dependent parameters. Cavallini et al., using animal model experiment, provided indirect evidence that 8-OHdG might accumulate in a small pool of mitochondria, proportionally with increasing age [36]. Cancer is attributed to DNA degeneration increasing exponentially with age and accumulating in the genome [37]. It is assumed that 8-OHdG level in cancer tissue of older people reflects a cumulative effect of oxidative damage of DNA as the result of both ageing and tumorigenesis. However, Tsurudome et al. concluded that 8-OHdG level generally increase with age, and a similar effect is observed in colorectal cancer tissue, but without significant differences between patients with cancer and those with benign colorectal polyps [38]. Gackowski et al. revealed higher 8-OHdG level in lymphocytes of patients with colorectal carcinoma compared with the healthy control group, but comparison by sex showed no differences [21]. Rainis et al. demonstrated an enhancement of oxidative stress in the neoplastic tissues of the colon, together with higher leukocyte activation within the carcinogenic tissue, suggesting a possible contribution of these cells to further oxidative stress-derived injury [39]. Unfortunately, these results were not analyzed in relation to age and sex. The 8-OHdG levels were also measured in lymphocytes, used as surrogate cells. Data that should inform about oxidative stress in other tissues, in healthy men and women from 5 European countries (France, Ireland, The Netherlands, Spain, and the U.K.) revealed a lower level in healthy women, stressing that mostly premenopausal women were examined [40]. A comparison between subgroups according to age and sex in our study showed that 8-OHdG levels were no significantly different in normal colonic mucosa or in cancer. In both subgroups of patients, analysis of 8-OHdG level according to clinical staging estimated with Duke's scale revealed the lack of differences between cancer and adjacent mucosa. No differences were found between A/B and C/D stages within normal colonic mucosa or within tumorous tissues. This is in contrast to Kondo et al., who concluded that colorectal carcinoma cells are exposed to higher oxidative stress than corresponding non-tumorous epithelial cells, regardless of clinical stage [41]. However, Sato et al. showed that the plasma 8-OHdG level was not as high in patients with advanced cancer in comparison with patients with early cancer [22].

We found that 8-OHdG levels were not related with age, sex, or tumor stage, possibly suggesting that higher 8-OHdG levels in colon adenocarcinoma tissue are not age, sex, or tumor stage dependent, but could be connected with a molecular mechanism within tumorous and corresponding normal mucosa. According to our best knowledge, similar results have not been reported so far, and some of our observations are difficult to explain. It was postulated that understanding the regulation of free-radical formation and its consequences regarding the oxidative repair phenotype and its relation to basic human differences

like age and sex need further exploration [42]. Moreover, there are many factors (dietary and non-dietary) that influence DNA oxidative damage and many of them could interfere with cancer tissue metabolism. And, last but not least, the effectiveness of several techniques measuring oxidative DNA damage in various biological samples with likely spurious oxidation of DNA during collection, assay, and/or storage of samples represents a real challenge [43,44]. Studies in the past 20 years have improved the quantitative estimation of 8-OHdG by various analytical techniques and have established it as a very important biomarker for carcinogenesis, aging, and degenerative diseases [28]. Sova et al. showed that low 8-oxodG level in serum and in breast cancer cells strongly indicate a more aggressive disease and concluded that negative 8-oxodG immunohistochemical staining is a powerful prognostic factor in breast carcinoma patients [45].

In DNA (nuclear and mitochondrial), 8-OHdG is one of the predominant agents of free-radical-induced oxidative lesions. This is the reason why 8-OHdG has been used in many studies as a biomarker for the measurement of endogenous oxidative DNA damage, and as a risk factor for many diseases such as cancer or degenerative diseases [46]. The idea of using oxidative

DNA damage, like 8-OHdG, as a biomarker of oxidative stress, chronic inflammation, and susceptibility to cancer, gives a new perspective. There are many documented cases of higher level DNA damage in malignant cells and tissues compared to non-malignant controls, but reactive oxygen species, produced either directly by tumors or indirectly via inflammatory responses, can cause DNA damage in healthy neighboring cells as well as distant sites [47].

Therefore, 8-OHdG as a marker of oxidative DNA damage and/or imbalance in antioxidant processes could be a useful parameter, but its role as a diagnostic and prognostic factor in colon adenocarcinoma seems to be limited.

Conclusions

8-OHdG reflects the local oxidative stress in colon adenocarcinoma tissue together with the ageing processes more than the intensity of tumorigenesis itself. Because many factors could have an effect on oxidative modification of DNA bases, its role as a diagnostic and/or prognostic factor in colon adenocarcinoma seems to be limited.

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