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Oxidative stress and 8-oxoguanine repair are enhanced in colon adenoma and carcinoma patients

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Running title: Oxidative stress and 8-oxoGua repair in CRC patients

Key words: 8-oxoguanine, DNA repair, colon cancer, polyps, antioxidants

Abstract

Oxidative stress is involved in the pathogenesis of colon cancer. We wanted to elucidate at which stage of the disease this phenomenon occurs. In the examined groups of patients with colorectal carcinoma (CRC, n=89), benign adenoma (AD, n=77), and healthy volunteers (controls, n=99) we measured: vitamins A, C and E in blood plasma, 8-oxodG and 8-oxoGua in leukocytes and urine, leukocyte 8-oxoGua excision activity, mRNA levels of APE1, OGG1, MTH1 and *OGG1* polymorphism. The vitamin levels decreased gradually in AD and CRC patients. 8-OxodG increased in leukocytes and urine of CRC and AD patients. 8-OxoGua was higher only in the urine of CRC patients. 8-OxoGua excision was higher in CRC patients than in controls, in spite of higher frequency of the *OGG1* Cys326Cys genotype, encoding a glycosylase with decreased activity. mRNA levels of OGG1 and APE1 increased in CRC and AD patients, which could explain increased 8-oxoGua excision rate in CRC patients. MTH1 mRNA was also higher in CRC patients. The results suggest that oxidative stress occurs in CRC and AD individuals. This is accompanied by increased transcription of DNA repair genes, and increased 8-oxoGua excision rate in CRC patients, which is, however, insufficient to counteract the increased DNA damage.

Introduction

Colorectal cancer (CRC) is one of the most frequent causes of death in western countries. The most important etiological factors of sporadic colorectal tumors are inflammation, fat metabolism, tobacco smoking as well as consumption of meat and alcohol (1). Inflammation is associated with the release of large amounts of reactive oxygen species (ROS) (2), leading to oxidation of nucleic acids, proteins and lipids. Many epidemiological studies report an inverse association between vegetable and fruit consumption and occurrence of colon cancer (3). One of the possible mechanisms of the protective effect of such food constituents as vitamins A, C and E is by exerting antioxidative activities, by scavenging free radicals and preventing DNA damage. Oxidatively damaged DNA has been blamed for the physiological changes associated with degenerative diseases such as cancer (4,5). A plethora of damaged DNA bases are formed upon ROS attack on genetic material, several of them reveal strong promutagenic properties (6). One of the major and best studied is 8-oxo-7,8-dihydroguanine (8-oxoGua), a typical biomarker of oxidative stress, which may play a role in carcinogenesis (7). The presence of 8-oxo-7,8-dihydroguanosine (8-oxodG) residues in DNA leads to GC→TA transversions (8). 8-OxodG is formed in DNA either *via* direct oxidation of nucleic acids or can be incorporated from the nucleotide pool by DNA polymerases, the latter process being an important source of DNA oxidation and genome instability (9,10). Incorporation of 8-oxodGTP into DNA by DNA polymerases is limited by the activity of MTH1 phosphohydrolase, which hydrolyzes 8-oxodGTP to 8-oxodGMP (11). 8-OxodGMP is subsequently dephoshorylated by nucleotidase and removed from the cell (12). Many observations indicate a direct correlation between 8-oxodG formation and carcinogenesis *in vivo* (7,13-16).

To counteract the deleterious effect of oxidatively damaged DNA, all organisms have developed several DNA repair pathways. Excision of 8-oxoGua from DNA is accomplished

mainly by base excision repair. Several DNA glycosylases, which specifically recognize and remove 8-oxoGua in human cells have been described, the major one apparently being OGG1 DNA glycosylase (17). Human OGG1 glycosylase recognizes and excises several lesions from oligodeoxynucleotides with single DNA damages, including 8-oxoGua, FapyGua, Me-FapyGua, 8-oxoAde (18,19). The murine enzyme was also shown to excise 8-oxo-inosine, O^6-methyl-8-oxoGua and 8-amino-Gua (20). However, when γ-irradiated DNA was used as a substrate, pure human OGG1 liberated only 8-oxoGua and FapyGua, as measured by GC/IDMS (18). FapyGua, but not 8-oxoGua, are also eliminated from DNA by NEIL1 DNA glycosylase (21). Both these DNA lesions show strong mutagenic activity in mammalian cells (22). Numerous experimental data suggest a decrease in DNA repair efficiency in cancer patients (23-27). One possibility is that such a decrease may be caused by the presence of polymorphic forms of DNA repair genes, which encode proteins with reduced enzymatic activities (26). Several polymorphic changes in the *OGG1* gene have been described, with the most common being Ser326Cys (28). Polymorphic Cys326 OGG1 protein was found to have a lower enzymatic activity, both when 8-oxoGua was excised from oligodeoxynucleotides (29), and when 8-oxoGua and FapyGua were liberated from γ-irradiated DNA by pure Cys326 or Ser326 OGG1 enzyme (18,28). It was suggested that the presence of two *OGG1* 326Cys alleles may confer an increased risk of lung, prostate and nasopharyngeal cancer (30-32), but no association with the risk for colon cancer (33,34). Our and other functional studies report a decrease in 8-oxoGua excision rate in lung (23,24) and head and neck cancer patients (25). Such a decrease in repair rate and simultaneous increase in 8-oxodG and FapydG levels in cellular DNA favors a pro-oxidant state and may accelerate the acquisition of mutations in critical genes leading to cancer. Such an idea is basically derived from the observation that a pro-oxidant environment is characteristic for advanced stages of cancer. However, it is not clear whether increased oxidative stress/oxidatively damaged DNA is merely the result of the

disease or whether it plays a role in cancer development. This prompted us to investigate the broad range of biomarkers: oxidatively damaged DNA, the status of antioxidant vitamins, and the repair of 8-oxoGua in colon cancer patients, in individuals developing benign adenomatous polyps and in healthy controls. We observed an increase in oxidatively damaged DNA and decreased antioxidant defense in leukocytes of colon cancer patients but also in adenoma individuals, of which some may be at very early stages of CRC development. Increased oxidative stress stimulates the 8-oxoGua excision rate, although this stimulation is insufficient to counteract oxidative damage to DNA.

Materials and methods

Materials

T4 polynucleotide kinase and [γ-^{32}P]ATP were from GE Healthcare. Oligodeoxynucleotide (40 nt) containing a single 8-oxodG at position 20 in the sequence 5'-d(GCT ACC TAC CTA GCG ACC T**X**C GAC TGT CCC ACT GCT CGA A)-3', where **X** indicates 8-oxodG was obtained from Eurogentec Herstal, (Herstal, Belgium). A complementary oligodeoxynucleotide containing dC opposite 8-oxodG was synthesized in the Oligonucleotide Synthesis and Sequencing Laboratory, IBB PAS (Warsaw, Poland).

Study group

This case-control study was conducted in three groups. The control group consisted of 99 healthy individuals (44 male and 55 female) of median age 55 years (range 42 to 65 years). The polyp individuals group (AD, n=77, 28 serrated adenomas and 49 adenomas) comprised 38 females and 39 males with a median age of 60 years (range 32 to 83). The colorectal cancer patient group (CRC, n=89) comprised 45 males and 44 females (median age 62 years,

range 27 to 90). All participants were Caucasians and there were no relatives among them. All individuals participating in the study were recruited through the hospital (Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland) and were examined by colonoscopy. The control group was recruited from individuals undergoing routine colon cancer screening, in whom colonoscopy revealed no cancer or polyps. All the subjects, when recruited to the study, filled in a questionnaire concerning demographic data, smoking, diet and medical history. Interviewees were asked to estimate the average frequency of consumption of various dietary items in the year preceding the interview. The majority of them reportedly consumed 3 servings of fruit and vegetables and about 250 g of meat and fat per day. To make the group even more homogenous, the subjects who reported the extreme consumption, as well as those who reported supplementation within the last month were excluded from the study. The questionnaire was administered by the team physician (Dr. Banaszkiewicz). The control group was chosen to maximally match the patient groups and adenoma individuals by age, sex, diet (consumption of fat, carbohydrates and vitamin intake), body weight, and smoking status. Among healthy volunteers, AD individuals and CRC patients, two groups were distinguished in relation to their smoking status, namely those who had never smoked and smokers who consumed 20 or more cigarettes per day.

Blood and urine was drawn from CRC and AD patients at diagnosis, and from control individuals, when they were recruited to the study following colonoscopy verifying they were free of any morphological changes within the colon and rectum. Diagnoses of all polyps and adenocarcinomas were confirmed by histological examination. All the CRC patients had histologically proven adenocarcinomas at: A (n=4), B1 (n=25), B2 (n=13), C1 (n=3), C2 (n=27) and D (n=17) stage according to Duke's scale with Astler-Coller modification. In 57 cases, cancer developed in the colon, and in 20 patients, in the rectum. In the majority of cases only one tumor was identified. The information concerning tumor histology and patient

questionnaire responses were blinded to all investigators (with the exception of the team physician) until after the statistical analysis was completed.

The patients were not treated with any anticancer drugs or vitamins during the time from the diagnoses until surgery (up to 4 weeks).

The study was conducted in accordance with the Helsinki Declaration and was approved by the medical ethics committee of Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland. All participants signed informed consent.

Determination of plasma vitamins A, E, C and uric acid concentration by HPLC

Quantification of vitamin E (α-tocopherol), vitamin A (retinol), vitamin C (ascorbic acid) and uric acid by HPLC technique was as previously described (35). Briefly, for vitamin A and E measurement, freshly prepared or freshly thawed plasma samples were mixed with 200 µl of HPLC-grade water and 400 µl of ethanol in order to precipitate proteins. For vitamin extraction, 800 µl of hexane was added, and mixed for 30 min. Then, 600 µl of the upper layer (hexane) was collected, dried in the Speed-Vac system and dissolved in 150 µl of mobile phase containing acetonitrile:methanol (85:15%, v/v) for stabilization of vitamins. 20 µl of this solution was injected into the HPLC system. Standard and control serum samples, with known α-tocopherol and retinol concentrations, were purchased from Chromsystems and prepared like the plasma samples.

The HPLC system consisting of a GP 40 gradient pump, GINA 50 autosampler (both from Dionex) and Jasco FP-920 fluorimetric detector was used for α-tocopherol and retinol quantification. Samples were separated in an isocratic system C18 reversed phase column Atlantis DC 18 (3 mm x 150 mm x 5 µm) with guard column. The mobile phase, containing acetonitrile and methanol (85:15, v/v), was used at a flow rate of 1.5 ml/min. The effluent was

monitored with fluorimetric detection (ex=340 nm, em=472 nm for retinol and ex=290 nm, em=330 nm for α-tocopherol) and analyzed by Dionex Chromeleon software.

For determination of plasma vitamin C and uric acid a standard stock solution (1 mM uric acid) was made by dissolving uric acid in deionized water. Working standards (in the range 10-500 μM in 5% metaphosphoric acid, MPA) were freshly prepared for each analysis. All solutions were carefully protected from light during preparation and analyzes. Standard and control serum samples, with known ascorbic acid concentrations, were purchased from Chromsystems and prepared like the plasma samples. Aliquots (200 μl) of freshly prepared or freshly thawed plasma samples were mixed with 200 μl of 20% MPA for protein precipitation and ascorbic acid stabilization. After centrifugation (10 min, 3000×*g*, 4°C), the supernatants were collected and filtered through Millipore microcentrifuge filters (NMWL 5000). Aliquots of 20 μl from these filtrates were injected into the HPLC system. The HPLC system consisted of HPLC 515 pump and 717 PLUS autosampler (both from Waters), and Photodiode Array Detector 2996 (Waters) was used for ascorbic acid quantification. Samples were separated in the isocratic system C18 reversed-phase column Spherisorb 5 μm ODS2 250 mm × 4.6 mm with C18 guard column, at a flow rate of 1 ml/min. The mobile phase containing 5 mM KH_2PO_4, 1 mM Na_2EDTA, adjusted to pH 3.0 with phosphoric acid, was prepared from deionised water and filtered through a 0.22 μm membrane before use. The effluent was monitored with a UV detector at 245 nm (ascorbic acid detection), 280 nm (uric acid detection) and analyzed by Empower software.

Isolation of leukocytes from venous blood

Blood samples were withdrawn from patients and controls in the morning before breakfast in Clinical Units of Collegium Medicum Nicolaus Copernicus University in Bydgoszcz. Blood samples (18 ml) were carefully applied on top of Histopaque 1119 solution (Sigma-Aldrich

Inc.; St. Louis, MO, USA) and leukocytes were isolated by centrifugation according to the manufacturer's procedure.

DNA isolation and 8-oxodG determination in DNA isolates

DNA from leukocytes was isolated using the method described earlier (35). Briefly; the pellet of cells was dispersed by vortexing in ice-cold buffer B (10 mM Tris, 5 mM Na_2EDTA, 0.15 mM deferoxamine mesylate, pH 8.0). A solution of SDS was added (to the final concentration of 0.5%), and vortexing was repeated; RNase in 10 mM Tris pH 8.0 was added, and the mixture was gently vortexed. After incubation for 30 min at 37°C, the protease was added; the mixture was gently vortexed and incubated at 37°C for 1 h. The mixture was cooled to 4°C and transferred to a centrifuge tube containing chloroform/3-methyl-1-butanol and vortexed vigorously. After centrifugation, the supernatant containing DNA was treated with 2 volumes of cold absolute ethanol in order to precipitate high molecular weight DNA. The precipitate was removed with a plastic spatula, washed with 70% ethanol, and after centrifugation dissolved in nuclease P1 buffer (40 mM sodium acetate, 0.1 mM $ZnCl_2$, pH 5.1).

Determination of 8-oxodG by means of the HPLC/EC technique was described previously (36). For genotyping, DNA was extracted from frozen leukocytes using Genomic Mini Kit (A&A Biotechnology; Gdansk, Poland).

Urine analysis

Overnight spot urine samples were collected. 0.5 nmol of [$^{15}N_3$, ^{13}C] 8-oxoGua, 0.05 nmol of [$^{15}N_5$] 8-oxodG and 10 µl of acetic acid (Sigma, HPLC grade) were added to 2 ml of urine. Isotopic purity of the applied standards was 97.6 and 99.7%, respectively. After centrifugation

(2000×*g*, 10 min), the supernatant was filtered through a Millipore GV13 0.22 μm syringe filter, and 500 μl of this solution was injected onto the HPLC system.

Urine HPLC purification of 8-oxoGua and 8-oxodG was performed according to Gackowski et al. (37).

GC/MS analysis was performed according to the method described by Dizdaroglu (38), adapted for additional [$^{15}N_5$] 8-oxoGua analyses (m/z 445 and 460 ions were monitored).

Cases and controls were interspersed throughout the batches to reduce the possibility of batch effects. The samples were run randomly in duplicate. Intrasample coefficient of variation (cv) for the measurements of 8-oxoGua in urine was 0.9% and intersample measurements differed by 6%. Intersample cv for 8-oxodG was 7% and intrasample 3%.

Preparation of tissue extracts

Blood leukocytes were homogenized with four volumes of 50 mM Tris-HCl, pH 7.5 buffer containing 1 mM EDTA, and proteases inhibitor cocktail (Sigma). Cells were disrupted by sonication (three 15 s pulses and 30 s intervals), centrifuged (7000x*g*, 4°C, 30 min), and the supernatant was collected. Protein concentration was determined by the Bradford method (39).

8-OxoGua excision activity assay

8-OxoGua excision activity was measured by the nicking assay (23,40) using ^{32}P-labelled 40 nt oligodeoxynucleotide duplex containing a single 8-oxodG, as described previously (10). The nicking assay allows simultaneous measurements of the glycosylase and AP endonuclease activities of the extract. The amount of product was quantified in Molecular

Dynamics Storm 820 PhosphorImager using Image Quant software (Molecular Dynamics, version 5.2). Cases and controls were interspersed throughout the batches to reduce the possibility of batch effects.

OGG1 genotyping by the Multitemperature PCR-Single Strand Conformation Polymorphism (MSSCP) method

OGG1 Ser326Cys polymorphism was investigated by the MSSCP method (41). A pair of intron based primers of the sequence: forward 5' ACT GTC ACT AGT CTC ACC AG 3', reverse 5' TGA ATT CGG AAG GTG CTT GGG GAA T 3' (42) was used to PCR amplify exon 7 of the *OGG1* gene. Cycling conditions for *OGG1* PCR-MSSCP were: 95°C for 3 min, 35 cycles of 1 min at 95°C, 1 min at 55°C, 2 min at 72°C, followed by an extension step of 7 min at 72°C. PCR products (4 μl) were denatured and separated by electrophoresis on native 10% polyacrylamide gel in 0.5 x TBE buffer in DNA Pointer System Plus (BioVectis, Warsaw, Poland). Initial electrophoresis was performed at 100V for 10 min at 35°C, and subsequently at three temperatures of 35-15-5°C for 30 min each at 40W. DNA bands were visualized by silver staining (BioVectis). Abnormally migrating conformers were sequenced in the Oligonucleotide Synthesis and Sequencing Laboratory, IBB PAS.

RNA extraction and cDNA synthesis

Total RNA was isolated from frozen leukocytes using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality of total RNA was checked by formaldehyde-agarose gel electrophoresis, and for further analyses, only RNA samples with clearly distinguished 18S and 28S ribosomal RNAs and no visible RNA degradation were used. Total RNA (1 μg) from each sample was used to generate cDNA using the Advantage RT-for-PCR cDNA synthesis kit (Clontech; Mountain View, CA, USA) with oligo(dT) primers.

Real-time PCR using SYBR-Green chemistry

Real-time PCR assays were carried out on an Applied Biosystems 7500 apparatus. Each reaction was carried out in 25 µl mixture containing: 1x Taq polymerase buffer (without $MgCl_2$), 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 5% DMSO, 0.5 ng/µl acetylated BSA, dATP, dCTP, dGTP and dTTP – 400 µM each, 1x concentrated reference dye ROX, 1:40000 diluted SYBR Green, 0.625 U of Taq polymerase, forward and reverse primers, 400 µM each, and cDNA template. Time-temperature program was as follows: 95ºC for 3 min as initial denaturation step followed by 45 cycles consisting of a denaturation step at 95ºC for 15 s, primer annealing at 60ºC for 15 s and an extension step at 72ºC for 1 min. Fluorescence was read during the extension step of each cycle. Melting-point temperature analysis was performed in the range of 60 to 95ºC, with temperature increments of 0.33ºC. Background range and threshold for C_t evaluation in each experiment were adjusted manually.

The following primers designed using the Primer Express program (Applied Biosystems; Foster City, CA, USA) were used: 5'-ATTCGAACGTCTGCCCTATCA-3' and 5'-TGCCTTCCTTGGATGTGGTAG-3' for the *18S rRNA* gene, 5'-GCCTTTCGCAAGTTCCTGA-3' and 5'-GCGTGAAGCCAGCATTCTTT-3' for *APE1,* 5'-CCCCACGTCTCATGTTG-3' and 5'-CCATCCTTAGCGCTGTCTCC-3' for the *OGG1* gene. Annealing temperatures for these primers were from 58 to 60ºC. The product from each pair was 131-132 bp long.

Before use, the primers were tested for equal efficiency of the PCR reaction. To ensure that, the $2^{-\Delta\Delta Ct}$ method validation was applied (43), each experiment involved measurement of C_t values for four or five amounts of the template, each in duplicate. The template amounts per sample were as follows: for *18S rRNA* – 10, 20, 40, 80 and 160 ng, for *APE1* – 40, 80, 160, 320 and 640 ng, for *OGG1* – 80, 160, 320 and 640 ng. The efficiency (which may have a

value between 0 and 1) of the QPCR reaction with each primer pair was calculated, and subsequently used to calculate the ratio of each studied gene to the reference gene. Only efficiencies of values ≥ 0.95 were accepted.

For each cDNA sample four reactions were carried out using two template amounts of 10 and 40 ng, each in duplicate. The quality of results was evaluated based on expected C_t differences between the two cDNA amounts as well as product melting curves. Few rare outlying results were omitted in the calculations. For each gene the amounts of cDNA were chosen individually (if possible, the same for all genes) to obtain C_t values in the range between 14 and 34 cycles.

The results were calculated with normalization of C_t values to mean C_t value for the 18S rRNA reference gene as described (44).

Statistical analysis

Data are presented as median and interquatrile range. The statistical differences of the results were analyzed by Mann-Whitney *U* test and Mann-Whitney *U* test with Bonferroni correction after the Kruskal-Wallis ANOVA. Associations between different variables were calculated using Spearman's correlation analysis. All statistical analyses were performed using STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK). *P* values less than 0.05 were considered statistically significant.

Results

Oxidative status

In order to investigate the difference in oxidative status between patients with CRC and adenoma in relation to healthy volunteers, we measured antioxidant vitamins in blood plasma and the level of oxidized nucleotides in urine of these three groups. CRC patients had

significantly lower levels of ascorbic acid (34.76 μM), α-tocopherol (23.92 μM) and retinol (1.13 μM) as well as uric acid (277.79 μM) in blood plasma than healthy controls (56.70 μM, 35.71 μM, 1.99 μM, and 312.25 μM, *P*=0.000000, 0.00001, 0.00001 and 0.00011, respectively; Table I). In adenoma individuals the level of ascorbic acid (49.79 μM) as well as of uric acid (325.23 μM), which is one of the main ROS scavengers in the human body, was similar to that in control individuals (*P*=0.058 and 0.70, respectively, Table I). The levels of α-tocopherol and retinol in plasma of adenoma individuals (30.15 μM and 1.70 μM, respectively) were intermediate between those of CRC patients and healthy volunteers, and differences between all groups were statistically significant (*P*=0.000000, C *vs.* A and 0.0083, A *vs*. H, for α-tocopherol, and *P*=0.00001, C *vs.* A and 0.00015, A *vs*. H for retinol; Table I).

Antioxidant vitamin deficit in individuals, who may be at early or are at later stages of neoplastic transformation (AD and CRC) was accompanied by an increased level of oxidatively damaged DNA. 8-OxodG level in DNA of blood leukocytes was elevated both in CRC patients (6.31 8-oxodG/10^6dG) and AD individuals (5.89 8-oxodG/10^6dG) in relation to healthy volunteers (4.41 8-oxodG/10^6dG, *P*=0.000000, C *vs.* H and 0.000004, A *vs*. H, Table II). Urinary excretion of 8-oxoGua was higher for CRC patients than for healthy controls and adenoma individuals (10.07 nmols/mmol creatinine for CRC, *vs.* 7.68 nmols/mmol creatinine for healthy individuals, *P*=0.000075 and *vs*. 7.55 nmols/mmol creatinine for adenoma, *P*=0.0079, Table II). In urine of AD individuals the 8-oxoGua level (7.55 nmols/mmol creatinine) was similar to that of healthy volunteers (7.68 nmols/mmol creatinine, *P*=0.51). The level of 8-oxodG was higher in urine of both CRC patients (1.74 nmols/mmol creatinine) and AD individuals (1.70 nmols/mmol creatinine) in comparison to control individuals (1.38 nmols/mmol creatinine, *P*=0.00026 and 0.000008, respectively, Table II). There was no correlation between age and antioxidant vitamin levels in colon cancer patients.

8-OxoGua repair capacity and OGG1 gene polymorphism

CRC patients revealed significantly higher 8-oxoGua excision activity in leukocytes (40.10 pmols/h/mg protein) than healthy controls (22.50 pmols/h/mg protein, *P*=0.000000; Table III). Due to insufficient quantity of clinical material we were unable to measure 8-oxoGua repair in leukocytes of AD subjects.

Differences in 8-oxoGua repair capacity between CRC patients and controls might be caused by different frequencies of OGG1 protein Ser326Cys polymorphism in both groups. We identified *OGG1* genotypes of CRC patients and AD individuals, as well as of controls. We found that the population of healthy individuals does not confirm Hardy-Weinberg equilibrium very well. In the healthy control group the frequency of the Ser326Cys genotype is higher and the frequency of Ser326Ser genotype is lower than expected (34% *vs.* 18% and 65% *vs*. 81%, respectively; Table IV). In all the studied groups from 35% to 48.7% of individuals carried at least one 326Cys allele. However, among CRC patients there was a significantly higher number of Cys326Cys homozygotes (23%) than in the remaining two groups (1.3% and 1% in AD and controls, respectively, Table IV). This is inconsistent with the expected repair activity distribution among cancer patients and controls in relation to known properties of pure variant proteins (18,29). Although 8-oxoGua excision rate was lower in leukocytes of CRC patients bearing the Cys326Cys genotype (24.5 pmols/h/mg protein) than in those bearing the Ser326Ser genotype (42 pmols/h/mg protein, respectively, Table V), CRC patients with homozygous *OGG1* Cys variant had similar 8-oxoGua repair capacity to that of healthy Ser326Ser control individuals. This may suggest that although *OGG1* polymorphism may have an impact on the rate of 8-oxoGua excision in human tissues, the effective excision activity is also influenced by other factors.

In CRC patients increased levels of 8-oxoGua were observed in urine of both *OGG1* 326 heterozygotes (*P*=0.011) and *OGG1* Ser326Ser homozygotes (*P*=0.049). However, when

comparing 8-oxoGua urinary excretion within the groups of CRC patients and controls, no significant differences were found in relation to the *OGG1* Ser326Cys polymorphism (Table V).

The level of 8-oxodG in leukocytes was higher in homozygous Ser/Ser colon cancer patients than in healthy Ser/Ser individuals (P=0.000001). However, heterozygous CRC patients and heterozygous healthy volunteers had comparable amounts of 8-oxodG in their leukocytes (P=0.1; Table V). When comparing the 8-oxodG level in leukocytes within the groups of healthy individuals in relation to the *OGG1* polymorphism, no differences were found between Ser326Ser homozygotes and Ser326Cys heterozygotes. Among CRC patients the 8-oxodG level in leukocytes was lower in Ser326Cys heterozygotes than in Cys326Cys and Ser326Ser homozygotes (P=0.003 and P=0.016, respectively; Table V).

Interestingly, we did not find any correlation between 8-oxoGua repair capacity and 8-oxodG level in blood leukocytes and urine, either in the group of healthy controls or in CRC patients, both for the whole groups and the *OGG1* variants.

mRNA level of OGG1, APE1 and MTH1

In order to obtain an insight into the mechanism of increased 8-oxoGua repair in CRC patients, we investigated the mRNA level of OGG1 glycosylase and the next enzyme in the BER pathway – AP endonuclease, APE1, as well as 8-oxodGTP phosphohydrolase, MTH1. OGG1 and APE1 mRNA levels were about an order of magnitude higher in leukocytes of CRC patients (1.28 for OGG1 and 88.25 for APE1) and AD individuals (0.99 for OGG1 and 113.83 for APE1) in comparison to healthy volunteers (0.19 for OGG1, P=0.000000 C *vs*. H and 0.000002 A *vs*. H, and 13.87 for APE1, P=0.000000 C *vs*. H and 0.000009 A *vs*. H, Table VI). There was no difference in OGG1 and APE1 mRNA levels between CRC patients and AD individuals (P=0.46 and 0.5, respectively, Table VI). Also, no statistically significant

differences in mRNA levels of *OGG1* and *APE1* were found among CRC patients and adenoma individuals in relation to the *OGG1* genotype. However, in healthy controls OGG1 expression in individuals bearing the Ser/Ser genotype was lower than in those with the Ser/Cys genotype (*P*=0.008, Table VI). For APE1 a strong, but statistically insignificant, tendency of decrease in the APE1 mRNA level was also observed in healthy volunteers bearing the Ser/Ser rather than the Ser/Cys genotype (*P*=0.057, Table VI). Similarly, the *MTH1* mRNA level was significantly higher in leukocytes of CRC patients (86.82) than of healthy individuals (5.26, *P*=0.0.0012; Table VI). Interestingly, an increased *MTH1* mRNA level was observed only in CRC patients bearing the *OGG1 326Cys* allele (Table VI).

We found a strong positive correlation between OGG1 and APE1 mRNA levels in both healthy controls (ρ=0.77, *P*=0.000000, n=39) and adenoma individuals and colon cancer patients (ρ=0.9, *P*=0.000000, n=38 and ρ=0.68, *P*=0.000000, n=45, respectively). In the group of healthy controls, but not in the group of colon cancer patients and adenoma individuals, there was a positive correlation between OGG1 and APE1 mRNA levels and the amount of 8-oxoGua in urine (ρ=0.41, *P*=0.04, n=24 and ρ=0.42, *P*=0.04, n=24, respectively). However, we did not observe any association between OGG1, APE1 and MTH1 mRNA levels and the amount of 8-oxodG in blood leukocyte DNA in all the studied groups.

Effect of tobacco smoking, sex, age and cancer stage

No effect of tobacco smoking, sex and age on 8-oxoGua excision rate as well as mRNA level of repair enzymes was observed in leukocytes of CRC patients and controls. Moreover, no differences in leukocyte 8-oxodG level and 8-oxoGua urinary excretion were observed in relation to tumor localization within the colon or rectum. In the majority of adenoma individuals only one polyp was found, and if more were present there was no correlation between measured parameters of oxidative stress and multiplicity of polyps. A weak positive

correlation was found between 8-oxoGua level in urine and the stage of disease (ρ=0.22, *P*=0.05, n=57).

Discussion

We measured the oxidative status and 8-oxoGua repair in leukocytes of healthy individuals, patients bearing benign adenomas (AD), and colon cancer (CRC) patients. CRC usually develops by neoplastic transformation of a colon epithelial cell, giving rise to a benign polyp, which subsequently may progress to invasive carcinoma. The group of AD patients might thus represent individuals at an early stage of colon cancer development, and give an insight to the contribution of oxidative processes in colon carcinogenesis in humans. However, it is necessary to bear in mind that probably not all of AD individuals will develop cancer in the future. We used the most extensively applied method of exploring the level of oxidatively damaged DNA in the whole organism, namely determination of 8-oxodG in DNA of surrogate tissues such as white blood cells or urinary excretion of 8-oxoGua and/or 8-oxodG. 8-OxoGua may reflect the rate of base excision repair, and 8-oxodG sanitation of the cellular nucleotide pool by the MTH1 directed pathway (12) or less likely nucleotide excision repair (45).

We observed increased oxidative stress in CRC patients, but also in AD individuals in comparison to healthy controls. This manifested as an elevation of the 8-oxodG level in leukocytes and in urine, as well as depletion of antioxidant vitamins in blood plasma. Moreover, increased mRNA levels of repair enzymes, OGG1, APE1 and MTH1 were found in leukocytes of CRC and AD individuals, and an increased 8-oxoGua excision rate in leukocytes of CRC patients with an increased frequency of the *OGG1* Cys326Cys genotype.

An increase in oxidative stress during colon carcinogenesis was demonstrated by several groups. Goodman et al. (46) showed an inverse association between oxidative balance score

(which characterizes pro-oxidant and anti-oxidant exposures) and colorectal adenoma. Leung et al. (47) further demonstrated that oxidative stress increases during CRC progression from operable CRC to non-operable liver metastasis, as observed by depletion of antioxidant vitamins and increase in lipid peroxidation. Several observations also suggest increase in oxidative processes in cancer tissues (48). The suggested mechanisms responsible for the oxidative stress in cancer patients include (4): i/ granulocyte activation with release of ROS; ii/ stimulation of cytokines, of which some, e.g. tumor necrosis factor, produce large amounts of ROS; iii/ production of hydrogen peroxide by malignant cells, which in advanced stages of cancer may be released into the blood stream and penetrate into other tissues (2).

The conditions outlined above may not be relevant for patients with benign colon tumors. However, our results demonstrate that the 8-oxodG level in leukocyte DNA and in urine is significantly higher in individuals with polyps in comparison with the control group (Table II). In contrast, urinary excretion of 8-oxoGua, which may reflect excision by DNA glycosylases, was not elevated in AD individuals (Table II). The nucleotide pool is much more susceptible to oxidation than dsDNA; hence, 8-oxodG in urine may be a more sensitive indicator of oxidative processes in the body, and shows that even in AD individuals the oxidative processes are enhanced. This is confirmed by the observed decrease of antioxidant vitamins, α-tocopherol and retinol in blood plasma of AD individuals. Further decrease of these antioxidants, as well as ascorbic acid was observed in CRC patients (Table 1). This may suggest that antioxidant defense gradually decreases in the course of disease development. Other studies show a similar depletion of antioxidant vitamins in cancer patients in comparison to controls (49). The mechanism of such depletion may involve either insufficient vitamin intake, or malabsorption in the intestine or accelerated usage during disease progression. Vitamins E and A may also participate in regulation of cell proliferation, *e.g.*, α-tocopherol inhibits protein kinase C activity and in effect cell proliferation. Supplementation

of animals with vitamin E prevents chemically induced colon cancer (50). Vitamin A deficiency is responsible for decreased mucosa production and expansion of proliferation zones (51). Absorption of vitamins C and E occurs *via* intestinal transporters (52,53). Moreover, cytochrom P450-catalyzed catabolism of tocopherols may be a decisive factor responsible for the bioavailability of vitamin E (54).

Since in our study the subjects had similar dietary habits, the differences in antioxidant vitamin levels might be at least partially genetically determined.

The second line of defense against oxidative stress is DNA repair. 8-OxoGua excision activity was significantly higher in leukocytes of CRC patients in comparison to healthy volunteers (Table III). The increase in 8-oxoGua repair capacity might be due to increased transcription from *OGG1* and *APE1* genes, which was observed in AD and CRC individuals (Table VI). Thus, induction of DNA repair genes seems to occur at an early stage of the carcinogenic pathway, and may be caused by increased oxidative stress. Expression of *APE1* and *OGG1* is induced by ROS (26), and we found that oxidative stress is enhanced both in the CRC and AD group. Our results corroborate the findings of Winnepenninckx and coworkers (55) that induction of most DNA repair genes occurs very early in the carcinogenic process, *e.g.*, four years before clinical manifestation of malignant skin melanoma. This may be a part of a carcinogenic program, which might decrease apoptosis in aberrant cells and/or favor genomic instability. In yeast and human cells, overexpression of some repair enzymes, namely methylpurine DNA glycosylase and/or APE1, was associated with frameshift mutations and microsatellite instability (56).

Increase in 8-oxoGua excision in blood leukocytes of CRC patients was in contrast to studies of other cancer types, which showed that 8-oxoGua repair capacity was decreased in lung (23,24) and in head and neck (25) cancer patients. Such a decrease was frequently linked to the *OGG1* Ser326Cys polymorphism. Pure 326Cys OGG1 variant excises 8-oxoGua and

FapyGua from DNA at lower rates than the wild-type enzyme (28,29). However, controversial results showing no association between *OGG1* Ser326Cys polymorphism and 8-oxoGua excision rate have also been reported (26). Recent work of Bravard and coworkers (57) shows that the OGG1 Cys variant is more sensitive to oxidative inactivation than the Ser variant, so different degrees of oxidative stress in different studies might partially explain the controversies in the literature.

This study shows a higher frequency of Cys326Cys homozygotes among CRC patients than among AD individuals or the control group (Table IV). Our results in contrast to the observations of Hansen and coworkers (33), who found a higher frequency of Cys allele among the controls than CRC patients. Allelic distribution may depend on genetic background of the local population, specifically if the population is isolated, like it was in Poland after the Second World War till the nineteen nineties. Another study performed on the Polish population did not find an association between the *OGG1* polymorphism and CRC risk, but a higher, although statistically insignificant, frequency of the Ser326Cys genotype was found in CRC patients in comparison to controls (P=0.07) (34). Similarly to our results, Moreno and coworkers (58) showed an association between *OGG1* Cys/Cys genotype and CRC risk in the Spanish population. These inconsistent results regarding the association of the *OGG1* genotype and CRC suggest the influence of other factors.

In our study the effect of the *OGG1* Ser326Cys polymorphism on 8-oxoGua excision rate was clearly seen in the CRC patient group, in which Cys homozygotes were found to have a decreased 8-oxoGua excision rate in comparison with Ser homozygotes. Interestingly, the *OGG1* genotype exerted a limited effect on 8-oxodG level in leukocytes and urinary excretion of 8-oxoGua (Table V). The only difference found was the decreased level of 8-oxodG in leukocytes of CRC patients with the *OGG1* Ser326Cys heterozygous genotype in comparison to both Cys/Cys and Ser/Ser homozygotes. This is difficult to explain, since

leukocytes of CRC patients with at least one Cys allele exhibited an increased mRNA level of MTH1 phosphohydrolase (Table VI). MTH1 activity prevents incorporation of 8-oxodGTP to DNA and decreases the level of 8-oxodG in DNA. Thus, several pathways are engaged in elimination of 8-oxoGua from DNA, for example nucleotide pool sanitation and base excision repair (10).

In the group of healthy subjects there was significant positive correlation between mRNA levels of the main enzymes involved in 8-oxoGua removal (OGG1 and APE1), and the amount of 8-oxoGua in urine. Therefore, our finding is the first experimental evidence which suggests that urinary 8-oxoGua measurements may be attributed to DNA repair. However, there was no such correlation in the groups of adenoma individuals and carcinoma patients. The main reason for this inconsistency may be aberrant DNA oxidation in cancerous and precancerous conditions which may overshadow the subtle relationship observed in healthy subjects.

This study shows that oxidative stress and antioxidant vitamin deficiency are increased in individuals developing colon adenomas and carcinomas, and may suggest that they contribute to the development of colon cancer. At the early stage of colon carcinogenesis, a defense pathway for elimination from DNA of 8-oxoGua and FapyGua is induced. This induction may, nevertheless, be insufficient to counteract the increased DNA damage.

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Author disclosure statement

No competing financial interests exist.

Abbreviations

8-oxodG - 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGua – 8-oxo-7,8-dihydroguanine; AD – adenoma; CRC – colorectal cancer; cv – coefficient of variation; FapyGua – 2,6-diamino-4-hydroxy-5-formamidopyrimidine; MPA – metaphosphoric acid; MSSCP - Multitemperature PCR-Single Strand Conformation Polymorphism; ROS – reactive oxygen species; SDS – sodium dodecyl sulfate

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Oxidative stress and 8-oxoguanine repair are enhanced in colon adenoma and carcinoma patients

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Running title: Oxidative stress and 8-oxoGua repair in CRC patients

Key words: 8-oxoguanine, DNA repair, colon cancer, polyps, antioxidants

Abstract

Oxidative stress is involved in the pathogenesis of colon cancer. We wanted to elucidate at which stage of the disease this phenomenon occurs. In the examined groups of patients with colorectal carcinoma (CRC, n=89), benign adenoma (AD, n=77), and healthy volunteers (controls, n=99) we measured: vitamins A, C and E in blood plasma, 8-oxodG and 8-oxoGua in leukocytes and urine, leukocyte 8-oxoGua excision activity, mRNA levels of APE1, OGG1, MTH1 and *OGG1* polymorphism. The vitamin levels decreased gradually in AD and CRC patients. 8-OxodG increased in leukocytes and urine of CRC and AD patients. 8-OxoGua was higher only in the urine of CRC patients. 8-OxoGua excision was higher in CRC patients than in controls, in spite of higher frequency of the *OGG1* Cys326Cys genotype, encoding a glycosylase with decreased activity. mRNA levels of OGG1 and APE1 increased in CRC and AD patients, which could explain increased 8-oxoGua excision rate in CRC patients. MTH1 mRNA was also higher in CRC patients. The results suggest that oxidative stress occurs in CRC and AD individuals. This is accompanied by increased transcription of DNA repair genes, and increased 8-oxoGua excision rate in CRC patients, which is, however, insufficient to counteract the increased DNA damage.

Introduction

Colorectal cancer (CRC) is one of the most frequent causes of death in western countries. The most important etiological factors of sporadic colorectal tumors are inflammation, fat metabolism, tobacco smoking as well as consumption of meat and alcohol (1). Inflammation is associated with the release of large amounts of reactive oxygen species (ROS) (2), leading to oxidation of nucleic acids, proteins and lipids. Many epidemiological studies report an inverse association between vegetable and fruit consumption and occurrence of colon cancer (3). One of the possible mechanisms of the protective effect of such food constituents as vitamins A, C and E is by exerting antioxidative activities, by scavenging free radicals and preventing DNA damage. Oxidatively damaged DNA has been blamed for the physiological changes associated with degenerative diseases such as cancer (4,5). A plethora of damaged DNA bases are formed upon ROS attack on genetic material, several of them reveal strong promutagenic properties (6). One of the major and best studied is 8-oxo-7,8-dihydroguanine (8-oxoGua), a typical biomarker of oxidative stress, which may play a role in carcinogenesis (7). The presence of 8-oxo-7,8-dihydroguanosine (8-oxodG) residues in DNA leads to GC→TA transversions (8). 8-OxodG is formed in DNA either *via* direct oxidation of nucleic acids or can be incorporated from the nucleotide pool by DNA polymerases, the latter process being an important source of DNA oxidation and genome instability (9,10). Incorporation of 8-oxodGTP into DNA by DNA polymerases is limited by the activity of MTH1 phosphohydrolase, which hydrolyzes 8-oxodGTP to 8-oxodGMP (11). 8-OxodGMP is subsequently dephoshorylated by nucleotidase and removed from the cell (12). Many observations indicate a direct correlation between 8-oxodG formation and carcinogenesis *in vivo* (7,13-16).

To counteract the deleterious effect of oxidatively damaged DNA, all organisms have developed several DNA repair pathways. Excision of 8-oxoGua from DNA is accomplished

mainly by base excision repair. Several DNA glycosylases, which specifically recognize and remove 8-oxoGua in human cells have been described, the major one apparently being OGG1 DNA glycosylase (17). Human OGG1 glycosylase recognizes and excises several lesions from oligodeoxynucleotides with single DNA damages, including 8-oxoGua, FapyGua, Me-FapyGua, 8-oxoAde (18,19). The murine enzyme was also shown to excise 8-oxo-inosine, O^6-methyl-8-oxoGua and 8-amino-Gua (20). However, when γ-irradiated DNA was used as a substrate, pure human OGG1 liberated only 8-oxoGua and FapyGua, as measured by GC/IDMS (18). FapyGua, but not 8-oxoGua, are also eliminated from DNA by NEIL1 DNA glycosylase (21). Both these DNA lesions show strong mutagenic activity in mammalian cells (22). Numerous experimental data suggest a decrease in DNA repair efficiency in cancer patients (23-27). One possibility is that such a decrease may be caused by the presence of polymorphic forms of DNA repair genes, which encode proteins with reduced enzymatic activities (26). Several polymorphic changes in the *OGG1* gene have been described, with the most common being Ser326Cys (28). Polymorphic Cys326 OGG1 protein was found to have a lower enzymatic activity, both when 8-oxoGua was excised from oligodeoxynucleotides (29), and when 8-oxoGua and FapyGua were liberated from γ-irradiated DNA by pure Cys326 or Ser326 OGG1 enzyme (18,28). It was suggested that the presence of two *OGG1* 326Cys alleles may confer an increased risk of lung, prostate and nasopharyngeal cancer (30-32), but no association with the risk for colon cancer (33,34). Our and other functional studies report a decrease in 8-oxoGua excision rate in lung (23,24) and head and neck cancer patients (25). Such a decrease in repair rate and simultaneous increase in 8-oxodG and FapydG levels in cellular DNA favors a pro-oxidant state and may accelerate the acquisition of mutations in critical genes leading to cancer. Such an idea is basically derived from the observation that a pro-oxidant environment is characteristic for advanced stages of cancer. However, it is not clear whether increased oxidative stress/oxidatively damaged DNA is merely the result of the

disease or whether it plays a role in cancer development. This prompted us to investigate the broad range of biomarkers: oxidatively damaged DNA, the status of antioxidant vitamins, and the repair of 8-oxoGua in colon cancer patients, in individuals developing benign adenomatous polyps and in healthy controls. We observed an increase in oxidatively damaged DNA and decreased antioxidant defense in leukocytes of colon cancer patients but also in adenoma individuals, of which some may be at very early stages of CRC development. Increased oxidative stress stimulates the 8-oxoGua excision rate, although this stimulation is insufficient to counteract oxidative damage to DNA.

Materials and methods

Materials

T4 polynucleotide kinase and [γ-^{32}P]ATP were from GE Healthcare. Oligodeoxynucleotide (40 nt) containing a single 8-oxodG at position 20 in the sequence 5'-d(GCT ACC TAC CTA GCG ACC T**X**C GAC TGT CCC ACT GCT CGA A)-3', where **X** indicates 8-oxodG was obtained from Eurogentec Herstal, (Herstal, Belgium). A complementary oligodeoxynucleotide containing dC opposite 8-oxodG was synthesized in the Oligonucleotide Synthesis and Sequencing Laboratory, IBB PAS (Warsaw, Poland).

Study group

This case-control study was conducted in three groups. The control group consisted of 99 healthy individuals (44 male and 55 female) of median age 55 years (range 42 to 65 years). The polyp individuals group (AD, n=77, 28 serrated adenomas and 49 adenomas) comprised 38 females and 39 males with a median age of 60 years (range 32 to 83). The colorectal cancer patient group (CRC, n=89) comprised 45 males and 44 females (median age 62 years,

range 27 to 90). All participants were Caucasians and there were no relatives among them. All individuals participating in the study were recruited through the hospital (Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland) and were examined by colonoscopy. The control group was recruited from individuals undergoing routine colon cancer screening, in whom colonoscopy revealed no cancer or polyps. All the subjects, when recruited to the study, filled in a questionnaire concerning demographic data, smoking, diet and medical history. Interviewees were asked to estimate the average frequency of consumption of various dietary items in the year preceding the interview. The majority of them reportedly consumed 3 servings of fruit and vegetables and about 250 g of meat and fat per day. To make the group even more homogenous, the subjects who reported the extreme consumption, as well as those who reported supplementation within the last month were excluded from the study. The questionnaire was administered by the team physician (Dr. Banaszkiewicz). The control group was chosen to maximally match the patient groups and adenoma individuals by age, sex, diet (consumption of fat, carbohydrates and vitamin intake), body weight, and smoking status. Among healthy volunteers, AD individuals and CRC patients, two groups were distinguished in relation to their smoking status, namely those who had never smoked and smokers who consumed 20 or more cigarettes per day.

Blood and urine was drawn from CRC and AD patients at diagnosis, and from control individuals, when they were recruited to the study following colonoscopy verifying they were free of any morphological changes within the colon and rectum. Diagnoses of all polyps and adenocarcinomas were confirmed by histological examination. All the CRC patients had histologically proven adenocarcinomas at: A (n=4), B1 (n=25), B2 (n=13), C1 (n=3), C2 (n=27) and D (n=17) stage according to Duke's scale with Astler-Coller modification. In 57 cases, cancer developed in the colon, and in 20 patients, in the rectum. In the majority of cases only one tumor was identified. The information concerning tumor histology and patient

questionnaire responses were blinded to all investigators (with the exception of the team physician) until after the statistical analysis was completed.

The patients were not treated with any anticancer drugs or vitamins during the time from the diagnoses until surgery (up to 4 weeks).

The study was conducted in accordance with the Helsinki Declaration and was approved by the medical ethics committee of Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland. All participants signed informed consent.

Determination of plasma vitamins A, E, C and uric acid concentration by HPLC

Quantification of vitamin E (α-tocopherol), vitamin A (retinol), vitamin C (ascorbic acid) and uric acid by HPLC technique was as previously described (35). Briefly, for vitamin A and E measurement, freshly prepared or freshly thawed plasma samples were mixed with 200 µl of HPLC-grade water and 400 µl of ethanol in order to precipitate proteins. For vitamin extraction, 800 µl of hexane was added, and mixed for 30 min. Then, 600 µl of the upper layer (hexane) was collected, dried in the Speed-Vac system and dissolved in 150 µl of mobile phase containing acetonitrile:methanol (85:15%, v/v) for stabilization of vitamins. 20 µl of this solution was injected into the HPLC system. Standard and control serum samples, with known α-tocopherol and retinol concentrations, were purchased from Chromsystems and prepared like the plasma samples.

The HPLC system consisting of a GP 40 gradient pump, GINA 50 autosampler (both from Dionex) and Jasco FP-920 fluorimetric detector was used for α-tocopherol and retinol quantification. Samples were separated in an isocratic system C18 reversed phase column Atlantis DC 18 (3 mm x 150 mm x 5 µm) with guard column. The mobile phase, containing acetonitrile and methanol (85:15, v/v), was used at a flow rate of 1.5 ml/min. The effluent was

monitored with fluorimetric detection (ex=340 nm, em=472 nm for retinol and ex=290 nm, em=330 nm for α-tocopherol) and analyzed by Dionex Chromeleon software.

For determination of plasma vitamin C and uric acid a standard stock solution (1 mM uric acid) was made by dissolving uric acid in deionized water. Working standards (in the range 10-500 μM in 5% metaphosphoric acid, MPA) were freshly prepared for each analysis. All solutions were carefully protected from light during preparation and analyzes. Standard and control serum samples, with known ascorbic acid concentrations, were purchased from Chromsystems and prepared like the plasma samples. Aliquots (200 μl) of freshly prepared or freshly thawed plasma samples were mixed with 200 μl of 20% MPA for protein precipitation and ascorbic acid stabilization. After centrifugation (10 min, 3000×*g*, 4°C), the supernatants were collected and filtered through Millipore microcentrifuge filters (NMWL 5000). Aliquots of 20 μl from these filtrates were injected into the HPLC system. The HPLC system consisted of HPLC 515 pump and 717 PLUS autosampler (both from Waters), and Photodiode Array Detector 2996 (Waters) was used for ascorbic acid quantification. Samples were separated in the isocratic system C18 reversed-phase column Spherisorb 5 μm ODS2 250 mm × 4.6 mm with C18 guard column, at a flow rate of 1 ml/min. The mobile phase containing 5 mM KH_2PO_4, 1 mM Na_2EDTA, adjusted to pH 3.0 with phosphoric acid, was prepared from deionised water and filtered through a 0.22 μm membrane before use. The effluent was monitored with a UV detector at 245 nm (ascorbic acid detection), 280 nm (uric acid detection) and analyzed by Empower software.

Isolation of leukocytes from venous blood

Blood samples were withdrawn from patients and controls in the morning before breakfast in Clinical Units of Collegium Medicum Nicolaus Copernicus University in Bydgoszcz. Blood samples (18 ml) were carefully applied on top of Histopaque 1119 solution (Sigma-Aldrich

Inc.; St. Louis, MO, USA) and leukocytes were isolated by centrifugation according to the manufacturer's procedure.

DNA isolation and 8-oxodG determination in DNA isolates

DNA from leukocytes was isolated using the method described earlier (35). Briefly; the pellet of cells was dispersed by vortexing in ice-cold buffer B (10 mM Tris, 5 mM Na_2EDTA, 0.15 mM deferoxamine mesylate, pH 8.0). A solution of SDS was added (to the final concentration of 0.5%), and vortexing was repeated; RNase in 10 mM Tris pH 8.0 was added, and the mixture was gently vortexed. After incubation for 30 min at 37°C, the protease was added; the mixture was gently vortexed and incubated at 37°C for 1 h. The mixture was cooled to 4°C and transferred to a centrifuge tube containing chloroform/3-methyl-1-butanol and vortexed vigorously. After centrifugation, the supernatant containing DNA was treated with 2 volumes of cold absolute ethanol in order to precipitate high molecular weight DNA. The precipitate was removed with a plastic spatula, washed with 70% ethanol, and after centrifugation dissolved in nuclease P1 buffer (40 mM sodium acetate, 0.1 mM $ZnCl_2$, pH 5.1).

Determination of 8-oxodG by means of the HPLC/EC technique was described previously (36). For genotyping, DNA was extracted from frozen leukocytes using Genomic Mini Kit (A&A Biotechnology; Gdansk, Poland).

Urine analysis

Overnight spot urine samples were collected. 0.5 nmol of [$^{15}N_3$, ^{13}C] 8-oxoGua, 0.05 nmol of [$^{15}N_5$] 8-oxodG and 10 µl of acetic acid (Sigma, HPLC grade) were added to 2 ml of urine. Isotopic purity of the applied standards was 97.6 and 99.7%, respectively. After centrifugation

(2000×*g*, 10 min), the supernatant was filtered through a Millipore GV13 0.22 μm syringe filter, and 500 μl of this solution was injected onto the HPLC system.

Urine HPLC purification of 8-oxoGua and 8-oxodG was performed according to Gackowski et al. (37).

GC/MS analysis was performed according to the method described by Dizdaroglu (38), adapted for additional [$^{15}N_5$] 8-oxoGua analyses (m/z 445 and 460 ions were monitored).

Cases and controls were interspersed throughout the batches to reduce the possibility of batch effects. The samples were run randomly in duplicate. Intrasample coefficient of variation (cv) for the measurements of 8-oxoGua in urine was 0.9% and intersample measurements differed by 6%. Intersample cv for 8-oxodG was 7% and intrasample 3%.

Preparation of tissue extracts

Blood leukocytes were homogenized with four volumes of 50 mM Tris-HCl, pH 7.5 buffer containing 1 mM EDTA, and proteases inhibitor cocktail (Sigma). Cells were disrupted by sonication (three 15 s pulses and 30 s intervals), centrifuged (7000x*g*, 4°C, 30 min), and the supernatant was collected. Protein concentration was determined by the Bradford method (39).

8-OxoGua excision activity assay

8-OxoGua excision activity was measured by the nicking assay (23,40) using ^{32}P-labelled 40 nt oligodeoxynucleotide duplex containing a single 8-oxodG, as described previously (10). The nicking assay allows simultaneous measurements of the glycosylase and AP endonuclease activities of the extract. The amount of product was quantified in Molecular

Dynamics Storm 820 PhosphorImager using Image Quant software (Molecular Dynamics, version 5.2). Cases and controls were interspersed throughout the batches to reduce the possibility of batch effects.

OGG1 genotyping by the Multitemperature PCR-Single Strand Conformation Polymorphism (MSSCP) method

OGG1 Ser326Cys polymorphism was investigated by the MSSCP method (41). A pair of intron based primers of the sequence: forward 5' ACT GTC ACT AGT CTC ACC AG 3', reverse 5' TGA ATT CGG AAG GTG CTT GGG GAA T 3' (42) was used to PCR amplify exon 7 of the *OGG1* gene. Cycling conditions for *OGG1* PCR-MSSCP were: 95°C for 3 min, 35 cycles of 1 min at 95°C, 1 min at 55°C, 2 min at 72°C, followed by an extension step of 7 min at 72°C. PCR products (4 µl) were denatured and separated by electrophoresis on native 10% polyacrylamide gel in 0.5 x TBE buffer in DNA Pointer System Plus (BioVectis, Warsaw, Poland). Initial electrophoresis was performed at 100V for 10 min at 35°C, and subsequently at three temperatures of 35-15-5°C for 30 min each at 40W. DNA bands were visualized by silver staining (BioVectis). Abnormally migrating conformers were sequenced in the Oligonucleotide Synthesis and Sequencing Laboratory, IBB PAS.

RNA extraction and cDNA synthesis

Total RNA was isolated from frozen leukocytes using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality of total RNA was checked by formaldehyde-agarose gel electrophoresis, and for further analyses, only RNA samples with clearly distinguished 18S and 28S ribosomal RNAs and no visible RNA degradation were used. Total RNA (1 µg) from each sample was used to generate cDNA using the Advantage RT-for-PCR cDNA synthesis kit (Clontech; Mountain View, CA, USA) with oligo(dT) primers.

Real-time PCR using SYBR-Green chemistry

Real-time PCR assays were carried out on an Applied Biosystems 7500 apparatus. Each reaction was carried out in 25 µl mixture containing: 1x Taq polymerase buffer (without $MgCl_2$), 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 5% DMSO, 0.5 ng/µl acetylated BSA, dATP, dCTP, dGTP and dTTP – 400 µM each, 1x concentrated reference dye ROX, 1:40000 diluted SYBR Green, 0.625 U of Taq polymerase, forward and reverse primers, 400 µM each, and cDNA template. Time-temperature program was as follows: 95ºC for 3 min as initial denaturation step followed by 45 cycles consisting of a denaturation step at 95ºC for 15 s, primer annealing at 60ºC for 15 s and an extension step at 72ºC for 1 min. Fluorescence was read during the extension step of each cycle. Melting-point temperature analysis was performed in the range of 60 to 95ºC, with temperature increments of 0.33ºC. Background range and threshold for C_t evaluation in each experiment were adjusted manually.

The following primers designed using the Primer Express program (Applied Biosystems; Foster City, CA, USA) were used: 5'-ATTCGAACGTCTGCCCTATCA-3' and 5'-TGCCTTCCTTGGATGTGGTAG-3' for the *18S rRNA* gene, 5'-GCCTTTCGCAAGTTCCTGA-3' and 5'-GCGTGAAGCCAGCATTCTTT-3' for *APE1,* 5'-CCCCACGTCTCATGTTG-3' and 5'-CCATCCTTAGCGCTGTCTCC-3' for the *OGG1* gene. Annealing temperatures for these primers were from 58 to 60ºC. The product from each pair was 131-132 bp long.

Before use, the primers were tested for equal efficiency of the PCR reaction. To ensure that, the $2^{-\Delta\Delta Ct}$ method validation was applied (43), each experiment involved measurement of C_t values for four or five amounts of the template, each in duplicate. The template amounts per sample were as follows: for *18S rRNA* – 10, 20, 40, 80 and 160 ng, for *APE1* – 40, 80, 160, 320 and 640 ng, for *OGG1* – 80, 160, 320 and 640 ng. The efficiency (which may have a

value between 0 and 1) of the QPCR reaction with each primer pair was calculated, and subsequently used to calculate the ratio of each studied gene to the reference gene. Only efficiencies of values ≥ 0.95 were accepted.

For each cDNA sample four reactions were carried out using two template amounts of 10 and 40 ng, each in duplicate. The quality of results was evaluated based on expected C_t differences between the two cDNA amounts as well as product melting curves. Few rare outlying results were omitted in the calculations. For each gene the amounts of cDNA were chosen individually (if possible, the same for all genes) to obtain C_t values in the range between 14 and 34 cycles.

The results were calculated with normalization of C_t values to mean C_t value for the 18S rRNA reference gene as described (44).

Statistical analysis

Data are presented as median and interquatrile range. The statistical differences of the results were analyzed by Mann-Whitney *U* test and Mann-Whitney *U* test with Bonferroni correction after the Kruskal-Wallis ANOVA. Associations between different variables were calculated using Spearman's correlation analysis. All statistical analyses were performed using STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK). *P* values less than 0.05 were considered statistically significant.

Results

Oxidative status

In order to investigate the difference in oxidative status between patients with CRC and adenoma in relation to healthy volunteers, we measured antioxidant vitamins in blood plasma and the level of oxidized nucleotides in urine of these three groups. CRC patients had

significantly lower levels of ascorbic acid (34.76 μM), α-tocopherol (23.92 μM) and retinol (1.13 μM) as well as uric acid (277.79 μM) in blood plasma than healthy controls (56.70 μM, 35.71 μM, 1.99 μM, and 312.25 μM, *P*=0.000000, 0.00001, 0.00001 and 0.00011, respectively; Table I). In adenoma individuals the level of ascorbic acid (49.79 μM) as well as of uric acid (325.23 μM), which is one of the main ROS scavengers in the human body, was similar to that in control individuals (*P*=0.058 and 0.70, respectively, Table I). The levels of α-tocopherol and retinol in plasma of adenoma individuals (30.15 μM and 1.70 μM, respectively) were intermediate between those of CRC patients and healthy volunteers, and differences between all groups were statistically significant (*P*=0.000000, C *vs.* A and 0.0083, A *vs*. H, for α-tocopherol, and *P*=0.00001, C *vs.* A and 0.00015, A *vs*. H for retinol; Table I).

Antioxidant vitamin deficit in individuals, who may be at early or are at later stages of neoplastic transformation (AD and CRC) was accompanied by an increased level of oxidatively damaged DNA. 8-OxodG level in DNA of blood leukocytes was elevated both in CRC patients (6.31 8-oxodG/10^6dG) and AD individuals (5.89 8-oxodG/10^6dG) in relation to healthy volunteers (4.41 8-oxodG/10^6dG, *P*=0.000000, C *vs.* H and 0.000004, A *vs*. H, Table II). Urinary excretion of 8-oxoGua was higher for CRC patients than for healthy controls and adenoma individuals (10.07 nmols/mmol creatinine for CRC, *vs.* 7.68 nmols/mmol creatinine for healthy individuals, *P*=0.000075 and *vs*. 7.55 nmols/mmol creatinine for adenoma, *P*=0.0079, Table II). In urine of AD individuals the 8-oxoGua level (7.55 nmols/mmol creatinine) was similar to that of healthy volunteers (7.68 nmols/mmol creatinine, *P*=0.51). The level of 8-oxodG was higher in urine of both CRC patients (1.74 nmols/mmol creatinine) and AD individuals (1.70 nmols/mmol creatinine) in comparison to control individuals (1.38 nmols/mmol creatinine, *P*=0.00026 and 0.000008, respectively, Table II). There was no correlation between age and antioxidant vitamin levels in colon cancer patients.

8-OxoGua repair capacity and OGG1 gene polymorphism

CRC patients revealed significantly higher 8-oxoGua excision activity in leukocytes (40.10 pmols/h/mg protein) than healthy controls (22.50 pmols/h/mg protein, *P*=0.000000; Table III). Due to insufficient quantity of clinical material we were unable to measure 8-oxoGua repair in leukocytes of AD subjects.

Differences in 8-oxoGua repair capacity between CRC patients and controls might be caused by different frequencies of OGG1 protein Ser326Cys polymorphism in both groups. We identified *OGG1* genotypes of CRC patients and AD individuals, as well as of controls. We found that the population of healthy individuals does not confirm Hardy-Weinberg equilibrium very well. In the healthy control group the frequency of the Ser326Cys genotype is higher and the frequency of Ser326Ser genotype is lower than expected (34% *vs.* 18% and 65% *vs*. 81%, respectively; Table IV). In all the studied groups from 35% to 48.7% of individuals carried at least one 326Cys allele. However, among CRC patients there was a significantly higher number of Cys326Cys homozygotes (23%) than in the remaining two groups (1.3% and 1% in AD and controls, respectively, Table IV). This is inconsistent with the expected repair activity distribution among cancer patients and controls in relation to known properties of pure variant proteins (18,29). Although 8-oxoGua excision rate was lower in leukocytes of CRC patients bearing the Cys326Cys genotype (24.5 pmols/h/mg protein) than in those bearing the Ser326Ser genotype (42 pmols/h/mg protein, respectively, Table V), CRC patients with homozygous *OGG1* Cys variant had similar 8-oxoGua repair capacity to that of healthy Ser326Ser control individuals. This may suggest that although *OGG1* polymorphism may have an impact on the rate of 8-oxoGua excision in human tissues, the effective excision activity is also influenced by other factors.

In CRC patients increased levels of 8-oxoGua were observed in urine of both *OGG1* 326 heterozygotes (*P*=0.011) and *OGG1* Ser326Ser homozygotes (*P*=0.049). However, when

comparing 8-oxoGua urinary excretion within the groups of CRC patients and controls, no significant differences were found in relation to the *OGG1* Ser326Cys polymorphism (Table V).

The level of 8-oxodG in leukocytes was higher in homozygous Ser/Ser colon cancer patients than in healthy Ser/Ser individuals (P=0.000001). However, heterozygous CRC patients and heterozygous healthy volunteers had comparable amounts of 8-oxodG in their leukocytes (P=0.1; Table V). When comparing the 8-oxodG level in leukocytes within the groups of healthy individuals in relation to the *OGG1* polymorphism, no differences were found between Ser326Ser homozygotes and Ser326Cys heterozygotes. Among CRC patients the 8-oxodG level in leukocytes was lower in Ser326Cys heterozygotes than in Cys326Cys and Ser326Ser homozygotes (P=0.003 and P=0.016, respectively; Table V).

Interestingly, we did not find any correlation between 8-oxoGua repair capacity and 8-oxodG level in blood leukocytes and urine, either in the group of healthy controls or in CRC patients, both for the whole groups and the *OGG1* variants.

mRNA level of OGG1, APE1 and MTH1

In order to obtain an insight into the mechanism of increased 8-oxoGua repair in CRC patients, we investigated the mRNA level of OGG1 glycosylase and the next enzyme in the BER pathway – AP endonuclease, APE1, as well as 8-oxodGTP phosphohydrolase, MTH1. OGG1 and APE1 mRNA levels were about an order of magnitude higher in leukocytes of CRC patients (1.28 for OGG1 and 88.25 for APE1) and AD individuals (0.99 for OGG1 and 113.83 for APE1) in comparison to healthy volunteers (0.19 for OGG1, P=0.000000 C *vs*. H and 0.000002 A *vs*. H, and 13.87 for APE1, P=0.000000 C *vs*. H and 0.000009 A *vs*. H, Table VI). There was no difference in OGG1 and APE1 mRNA levels between CRC patients and AD individuals (P=0.46 and 0.5, respectively, Table VI). Also, no statistically significant

differences in mRNA levels of *OGG1* and *APE1* were found among CRC patients and adenoma individuals in relation to the *OGG1* genotype. However, in healthy controls OGG1 expression in individuals bearing the Ser/Ser genotype was lower than in those with the Ser/Cys genotype (P=0.008, Table VI). For APE1 a strong, but statistically insignificant, tendency of decrease in the APE1 mRNA level was also observed in healthy volunteers bearing the Ser/Ser rather than the Ser/Cys genotype (P=0.057, Table VI). Similarly, the *MTH1* mRNA level was significantly higher in leukocytes of CRC patients (86.82) than of healthy individuals (5.26, P=0.0.0012; Table VI). Interestingly, an increased *MTH1* mRNA level was observed only in CRC patients bearing the *OGG1 326Cys* allele (Table VI).

We found a strong positive correlation between OGG1 and APE1 mRNA levels in both healthy controls (ρ=0.77, P=0.000000, n=39) and adenoma individuals and colon cancer patients (ρ=0.9, P=0.000000, n=38 and ρ=0.68, P=0.000000, n=45, respectively). In the group of healthy controls, but not in the group of colon cancer patients and adenoma individuals, there was a positive correlation between OGG1 and APE1 mRNA levels and the amount of 8-oxoGua in urine (ρ=0.41, P=0.04, n=24 and ρ=0.42, P=0.04, n=24, respectively). However, we did not observe any association between OGG1, APE1 and MTH1 mRNA levels and the amount of 8-oxodG in blood leukocyte DNA in all the studied groups.

Effect of tobacco smoking, sex, age and cancer stage

No effect of tobacco smoking, sex and age on 8-oxoGua excision rate as well as mRNA level of repair enzymes was observed in leukocytes of CRC patients and controls. Moreover, no differences in leukocyte 8-oxodG level and 8-oxoGua urinary excretion were observed in relation to tumor localization within the colon or rectum. In the majority of adenoma individuals only one polyp was found, and if more were present there was no correlation between measured parameters of oxidative stress and multiplicity of polyps. A weak positive

correlation was found between 8-oxoGua level in urine and the stage of disease (ρ=0.22, *P*=0.05, n=57).

Discussion

We measured the oxidative status and 8-oxoGua repair in leukocytes of healthy individuals, patients bearing benign adenomas (AD), and colon cancer (CRC) patients. CRC usually develops by neoplastic transformation of a colon epithelial cell, giving rise to a benign polyp, which subsequently may progress to invasive carcinoma. The group of AD patients might thus represent individuals at an early stage of colon cancer development, and give an insight to the contribution of oxidative processes in colon carcinogenesis in humans. However, it is necessary to bear in mind that probably not all of AD individuals will develop cancer in the future. We used the most extensively applied method of exploring the level of oxidatively damaged DNA in the whole organism, namely determination of 8-oxodG in DNA of surrogate tissues such as white blood cells or urinary excretion of 8-oxoGua and/or 8-oxodG. 8-OxoGua may reflect the rate of base excision repair, and 8-oxodG sanitation of the cellular nucleotide pool by the MTH1 directed pathway (12) or less likely nucleotide excision repair (45).

We observed increased oxidative stress in CRC patients, but also in AD individuals in comparison to healthy controls. This manifested as an elevation of the 8-oxodG level in leukocytes and in urine, as well as depletion of antioxidant vitamins in blood plasma. Moreover, increased mRNA levels of repair enzymes, OGG1, APE1 and MTH1 were found in leukocytes of CRC and AD individuals, and an increased 8-oxoGua excision rate in leukocytes of CRC patients with an increased frequency of the *OGG1* Cys326Cys genotype.

An increase in oxidative stress during colon carcinogenesis was demonstrated by several groups. Goodman et al. (46) showed an inverse association between oxidative balance score

(which characterizes pro-oxidant and anti-oxidant exposures) and colorectal adenoma. Leung et al. (47) further demonstrated that oxidative stress increases during CRC progression from operable CRC to non-operable liver metastasis, as observed by depletion of antioxidant vitamins and increase in lipid peroxidation. Several observations also suggest increase in oxidative processes in cancer tissues (48). The suggested mechanisms responsible for the oxidative stress in cancer patients include (4): i/ granulocyte activation with release of ROS; ii/ stimulation of cytokines, of which some, e.g. tumor necrosis factor, produce large amounts of ROS; iii/ production of hydrogen peroxide by malignant cells, which in advanced stages of cancer may be released into the blood stream and penetrate into other tissues (2).

The conditions outlined above may not be relevant for patients with benign colon tumors. However, our results demonstrate that the 8-oxodG level in leukocyte DNA and in urine is significantly higher in individuals with polyps in comparison with the control group (Table II). In contrast, urinary excretion of 8-oxoGua, which may reflect excision by DNA glycosylases, was not elevated in AD individuals (Table II). The nucleotide pool is much more susceptible to oxidation than dsDNA; hence, 8-oxodG in urine may be a more sensitive indicator of oxidative processes in the body, and shows that even in AD individuals the oxidative processes are enhanced. This is confirmed by the observed decrease of antioxidant vitamins, α-tocopherol and retinol in blood plasma of AD individuals. Further decrease of these antioxidants, as well as ascorbic acid was observed in CRC patients (Table 1). This may suggest that antioxidant defense gradually decreases in the course of disease development. Other studies show a similar depletion of antioxidant vitamins in cancer patients in comparison to controls (49). The mechanism of such depletion may involve either insufficient vitamin intake, or malabsorption in the intestine or accelerated usage during disease progression. Vitamins E and A may also participate in regulation of cell proliferation, *e.g.*, α-tocopherol inhibits protein kinase C activity and in effect cell proliferation. Supplementation

of animals with vitamin E prevents chemically induced colon cancer (50). Vitamin A deficiency is responsible for decreased mucosa production and expansion of proliferation zones (51). Absorption of vitamins C and E occurs *via* intestinal transporters (52,53). Moreover, cytochrom P450-catalyzed catabolism of tocopherols may be a decisive factor responsible for the bioavailability of vitamin E (54).

Since in our study the subjects had similar dietary habits, the differences in antioxidant vitamin levels might be at least partially genetically determined.

The second line of defense against oxidative stress is DNA repair. 8-OxoGua excision activity was significantly higher in leukocytes of CRC patients in comparison to healthy volunteers (Table III). The increase in 8-oxoGua repair capacity might be due to increased transcription from *OGG1* and *APE1* genes, which was observed in AD and CRC individuals (Table VI). Thus, induction of DNA repair genes seems to occur at an early stage of the carcinogenic pathway, and may be caused by increased oxidative stress. Expression of *APE1* and *OGG1* is induced by ROS (26), and we found that oxidative stress is enhanced both in the CRC and AD group. Our results corroborate the findings of Winnepenninckx and coworkers (55) that induction of most DNA repair genes occurs very early in the carcinogenic process, *e.g.*, four years before clinical manifestation of malignant skin melanoma. This may be a part of a carcinogenic program, which might decrease apoptosis in aberrant cells and/or favor genomic instability. In yeast and human cells, overexpression of some repair enzymes, namely methylpurine DNA glycosylase and/or APE1, was associated with frameshift mutations and microsatellite instability (56).

Increase in 8-oxoGua excision in blood leukocytes of CRC patients was in contrast to studies of other cancer types, which showed that 8-oxoGua repair capacity was decreased in lung (23,24) and in head and neck (25) cancer patients. Such a decrease was frequently linked to the *OGG1* Ser326Cys polymorphism. Pure 326Cys OGG1 variant excises 8-oxoGua and

FapyGua from DNA at lower rates than the wild-type enzyme (28,29). However, controversial results showing no association between *OGG1* Ser326Cys polymorphism and 8-oxoGua excision rate have also been reported (26). Recent work of Bravard and coworkers (57) shows that the OGG1 Cys variant is more sensitive to oxidative inactivation than the Ser variant, so different degrees of oxidative stress in different studies might partially explain the controversies in the literature.

This study shows a higher frequency of Cys326Cys homozygotes among CRC patients than among AD individuals or the control group (Table IV). Our results in contrast to the observations of Hansen and coworkers (33), who found a higher frequency of Cys allele among the controls than CRC patients. Allelic distribution may depend on genetic background of the local population, specifically if the population is isolated, like it was in Poland after the Second World War till the nineteen nineties. Another study performed on the Polish population did not find an association between the *OGG1* polymorphism and CRC risk, but a higher, although statistically insignificant, frequency of the Ser326Cys genotype was found in CRC patients in comparison to controls (P=0.07) (34). Similarly to our results, Moreno and coworkers (58) showed an association between *OGG1* Cys/Cys genotype and CRC risk in the Spanish population. These inconsistent results regarding the association of the *OGG1* genotype and CRC suggest the influence of other factors.

In our study the effect of the *OGG1* Ser326Cys polymorphism on 8-oxoGua excision rate was clearly seen in the CRC patient group, in which Cys homozygotes were found to have a decreased 8-oxoGua excision rate in comparison with Ser homozygotes. Interestingly, the *OGG1* genotype exerted a limited effect on 8-oxodG level in leukocytes and urinary excretion of 8-oxoGua (Table V). The only difference found was the decreased level of 8-oxodG in leukocytes of CRC patients with the *OGG1* Ser326Cys heterozygous genotype in comparison to both Cys/Cys and Ser/Ser homozygotes. This is difficult to explain, since

leukocytes of CRC patients with at least one Cys allele exhibited an increased mRNA level of MTH1 phosphohydrolase (Table VI). MTH1 activity prevents incorporation of 8-oxodGTP to DNA and decreases the level of 8-oxodG in DNA. Thus, several pathways are engaged in elimination of 8-oxoGua from DNA, for example nucleotide pool sanitation and base excision repair (10).

In the group of healthy subjects there was significant positive correlation between mRNA levels of the main enzymes involved in 8-oxoGua removal (OGG1 and APE1), and the amount of 8-oxoGua in urine. Therefore, our finding is the first experimental evidence which suggests that urinary 8-oxoGua measurements may be attributed to DNA repair. However, there was no such correlation in the groups of adenoma individuals and carcinoma patients. The main reason for this inconsistency may be aberrant DNA oxidation in cancerous and precancerous conditions which may overshadow the subtle relationship observed in healthy subjects.

This study shows that oxidative stress and antioxidant vitamin deficiency are increased in individuals developing colon adenomas and carcinomas, and may suggest that they contribute to the development of colon cancer. At the early stage of colon carcinogenesis, a defense pathway for elimination from DNA of 8-oxoGua and FapyGua is induced. This induction may, nevertheless, be insufficient to counteract the increased DNA damage.

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Author disclosure statement

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Abbreviations

8-oxodG - 8-oxo-7,8-dihydro-2'-deoxyguanosine; 8-oxoGua – 8-oxo-7,8-dihydroguanine; AD – adenoma; CRC – colorectal cancer; cv – coefficient of variation; FapyGua – 2,6-diamino-4-hydroxy-5-formamidopyrimidine; MPA – metaphosphoric acid; MSSCP - Multitemperature PCR-Single Strand Conformation Polymorphism; ROS – reactive oxygen species; SDS – sodium dodecyl sulfate

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Table I. The concentrations of antioxidants in the plasma of colon cancer patients, individuals with adenomas and healthy volunteers

	Carcinoma patients (C)[a]	Adenoma patients (A)[a]	Healthy volunteers (H)[a]	P^b
Ascorbic acid (μM)	34.76 (20.01–47.71) n = 89	49.79 (30.13–68.19) n = 77	56.70 (40.68–73.09) n = 99	0.000000 (C *vs.* H) 0.058 (A *vs.* H) 0.000032 (C *vs.* A)
α-Tocopherol (μM)	23.92 (17.87–27.77) n = 86	30.15 (24.95–37.69) n = 71	35.71 (28.71–41.43) n = 101	0.00001 (C *vs.* H) 0.0083 (A *vs.* H) 0.000000 (C *vs.* A)
Retinol (μM)	1.13 (0.85–1.49) n = 85	1.70 (1.39–2.14) n = 71	1.99 (1.69–2.39) n = 101	0.00001 (C *vs.* H) 0.00015 (A *vs.* H) 0.00001 (C *vs.* A)
Uric acid (μM)	277.79 (207.74–351.41) n = 89	325.23 (276.14–393.13) n = 77	312.25 (271.75–381.08) n = 99	0.00011 (C *vs.* H) 0.70 (A *vs.* H) 0.00011 (C *vs.* A)

[a] Comparison of antioxidants, expressed as median and interquartile range, between patients groups and healthy volunteers (Mann-Whitney *U* test).
[b] All *P* values are after Bonferroni correction.

Table II. The level of oxidized nucleosides in DNA of leukocytes and urine of colon cancer patients, individuals with adenomas and healthy volunteers

	Carcinoma patients (C)[a]	Adenoma patients (A)[a]	Healthy volunteers (H)[a]	P [b]
8-OxodG/10^6dG in leukocyte DNA	6.31 (4.91–8.27) n = 75	5.89 (4.56–8.57) n = 70	4.41 (3.62–5.71) n = 99	0.000000 (C *vs.* H) 0.000004 (A *vs.* H) 0.37 (C *vs.* A)
8-OxoGua in urine (nmols/mmol creatinine)	10.07 (7.37–15.20) n = 59	7.55 (5.16–11.15) n = 45	7.68 (4.61–10.13) n = 84	0.000075 (C *vs.* H) 0.51 (A *vs.* H) 0.0079 (C *vs.* A)
8-OxodG in urine (nmols/mmol creatinine)	1.74 (1.31–2.99) n = 54	1.70 (1.25–2.95) n = 44	1.38 (1.09–1.73) n = 82	0.00026 (C *vs.* H) 0.000008 (A *vs.* H) 0.54 (C *vs.* A)

[a] Comparison of the level of oxidized nucleosides, expressed as median and interquartile range, between patient group and healthy volunteers (Mann-Whitney *U* test).
[b] All *P* values are after Bonferroni correction.

Table III. 8-OxoGua repair activity in leukocytes of colon cancer patients and healthy volunteers

8-OxoGua excision activity (pmols/h/mg protein)		*P*
Carcinoma patients[a]	Healthy volunteers[a]	
40.1 (29.43–59.69) n = 68	22.50 (15.30–29.40) n = 90	0.000000

[a] Comparison of 8-OxoGua repair activity, expressed as median and interquartile range, between carcinoma patients and healthy volunteers (Mann-Whitney *U* test).

Table IV. Genotypes and allelic frequencies of the *OGG1* Ser326Cys polymorphism among colon cancer patients, individuals with adenomas and healthy volunteers

OGG1 genotype	Carcinoma patients n = 74 n (%)	Adenoma patients n = 76 n (%)	Healthy volunteers n = 97 n (%), (%)*
Cys/Cys	17 (23.0)	1 (1.3)	1 (1), (1)[a]
Ser/Cys	19 (25.7)	29 (38.2)	33 (34), (18)[b]
Ser/Ser	38 (51.3)	46 (60.5)	63 (65), (81)[c]
Allelic frequency			
C	0.52	0.89	0.9
G	0.48	0.11	0.1

* Results from Hardy-Weinberg equilibrium.
[a] Homozygous *OGG1* Cys326Cys individuals
[b] Heterozygous *OGG1* Ser326Cys individuals
[c] Homozygous *OGG1* Ser326Ser individuals

Table V. Comparison of *OGG1* Ser326Cys polymorphism and 8-oxoGua excision capacity of blood leukocytes of CRC patients and healthy volunteers as well as 8-oxodG level in blood leukocytes and urine of CRC patients

OGG1 genotype	Carcinoma patients[a]	Healthy volunteers[a]	*P*
	Repair capacity (8-oxoGua pmols/h/mg protein)		
Cys/Cys	24.50 (15.15–42.70) n = 13	–	
Ser/Cys	35.65 (25.50–50.95) n = 14	19.25 (12.4–23.45) n = 32	0.0016
Ser/Ser	42.00 (33.39–86.18) n = 34	24.20 (18.50–31.59) n = 53	0.000000
	8-OxodG in blood leukocytes DNA (8-oxodG/10^6dG)		
Cys/Cys	8.02 (5.86–11.80) n = 10	–	
Ser/Cys	5.42 (4.22–8.74) n = 19	4.72 (4.04–6.03) n = 35	0.1
Ser/Ser	6.91 (5.86–8.52) n = 29	4.29 (3.55–5.12) n = 61	0.000001
	8-OxoGua in urine (nmols/mmol creatinine)		
Cys/Cys	12.83 (7.76–19.14) n = 15	–	
Ser/Cys	10.2 (7.6–13.38) n = 18	8.05 (4–10) n = 36	0.011
Ser/Ser	9.31 (6.35–13.57) n = 27	7.6 (4.95–10.49) n = 47	0.049

[a] Comparison of *OGG1* Ser326Cys polymorphism and 8-oxoGua excision capacity as well as 8-oxodG level in carcinoma patients and healthy volunteers, expressed as median and interquartile range (Mann-Whitney *U* test).

Table VI. mRNA level of OGG1 glycosylase, APE1 endonuclease and MTH phosphohydrolase in leukocytes of colon cancer and adenoma patients and healthy volunteers

Genotype	mRNA level (mRNA of gene X/mRNA of 18S rRNA x 10^{-6}) [a]			*P*
	Carcinoma patients (C)	Adenoma patients (A)	Healthy volunteers (H)	
		OGG1		
All	1.28 (0.71–5.58) n = 44	0.99 (0.45–4.91) n = 38	0.19 (0.06–0.54) n = 40	0.000000 (C *vs.* H) [b] 0.000002 (A *vs.* H) [b] 0.46 (C *vs.* A) [b]
Cys/Cys	0.75 (0.26–3.1) n = 8	–	–	–
Ser/Cys	1.26 (0.99–2.87) n = 4	1.01 (0.6–7.16) n = 19	0.48 (0.074–0.63) n = 19	0.021 (C *vs.* H) [b] (NS) 0.003 (A *vs.* H) [b] 0.78 (C *vs.* A) [b]
Ser/Ser	1.99 (0.87–9.59) n = 23	0.97 (0.38–4.77) n = 19	0.1 (0.02–0.23) n = 18	0.000000 (C *vs.* H) [b] 0.000009 (A *vs.* H) [b] 0.11 (C *vs.* A) [b]
		APE1		
All	88.25 (42.88–362.51) n = 44	113.83 (48.74–292.37) n = 38	13.87 (2.32–43.04) n = 40	0.000000 (C *vs.* H) [b] 0.000000 (A *vs.* H) [b] 0.5 (C *vs.* A) [b]
Cys/Cys	54.35 (12.36–400.5) n = 8	–	–	–
Ser/Cys	120.6 (61.76–359.7) n = 4	129.54 (72.69–449.52) n = 19	17.67 (3.43–86.3) n = 19	0.021 (C *vs.* H) [b] (NS) 0.00003 (A *vs.* H) [b] 0.67 (C *vs.* A) [b]
Ser/Ser	78.02 (39.56–470.19) n = 23	90.51 (27.46–240.1) n = 19	3.47 (1.07–16..21) n = 18	0.000003 (C *vs.* H) [b] 0.000005 (A *vs.* H) [b] 1.00 (C *vs.* A) [b]
		MTH1		
All	86.82 (5.19–728.53) n = 37	–	5.26 (0.33–11.69) n = 19	0.0012
Cys/Cys	96.73 (5.34–295.62) n = 7	–	–	–
Ser/Cys	273.1 (86.82–459.37) n = 2	–	3.7 (0.36–9.88) n = 9	0.036
Ser/Ser	10.84 (1.76–114.65) n = 20	–	8.96 (3.94–26.59) n = 8	0.8

[a] Comparison of mRNA levels, expressed as median and interquartile range, between colon carcinoma patients, adenoma patients and healthy volunteers (Mann-Whitney *U* test).
[b] *P* values are after Bonferroni correction.
NS, not significant