Differences in nucleotide excision repair capacity between newly diagnosed colorectal cancer patients and healthy controls

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Alteration of DNA integrity is a potential cause of cancer and it is assumed that reduced DNA repair capacity and accumulation of DNA damage may represent intermediate markers in carcinogenesis. In this case-control study, DNA damage and nucleotide excision repair capacity (NER-DRC) were assessed in association with sporadic colorectal cancer (CRC). Both parameters were quantified by comet assay in blood cells of 70 untreated incident patients and 70 age-matched healthy controls. mRNA expression and polymorphisms in relevant NER genes were concurrently analyzed. The aim of this study was to characterize incident CRC patients for NER-DRC and to clarify possible relations between investigated variables. Comet assay and mRNA expression analysis showed that CRC patients differ in repair capacity as compared to controls. Patients had a lower NER-DRC and simultaneously they exhibited higher endogenous DNA damage (for both $P < 0.001$). Accumulation of DNA damage and decreasing NER-DRC behaved as independent modulating parameters strongly associated with CRC. Expression levels of 6 out of 9 studied genes differed between groups ($P \leq 0.001$), but none of them was related to DRC or to any of the studied NER polymorphisms. However, in patients only, *XPC* Ala499Val modulated expression levels of *XPC*, *XPB* and *XPD* gene, whereas *XPC* Lys939Gln was associated with *XPA* expression level in controls (for all $P < 0.05$). This study provides evidence on altered DRC and DNA damage levels in sporadic CRC and proposes the relevance of the NER pathway in this malignancy. Further, alterations in a complex multigene process like DNA repair may be better characterized by functional quantification of repair capacity than by quantification of individual genes transcripts or gene variants alone.

Introduction

Colorectal neoplasia is the third most common cancer worldwide and the fourth leading cause of cancer death (1). Recently, decreasing colorectal cancer (CRC) mortality rates have been recorded in developed countries, most likely due to larger efforts in cancer screening and/or improved treatment (2). Since an early diagnosis of CRC is associated with substantially better prognosis and curability, early biomarkers and intermediary end points are called for to dissect the complex process of colorectal carcinogenesis (3).

While in the etiopathogenesis of inherited CRC forms the influence of particular genetic features prevails, sporadic forms (around 80% of all cases) show a multifactorial pattern and are determined by an interplay of multiple genetic and environmental/lifestyle factors (4). Well-established risk factors for sporadic CRC are obesity, diet low in vegetables/fruits and rich in meat, physical inactivity and smoking (5,6). Concurrently, the specific nature of colon epithelium, which is a dynamically changing system, makes it prone to genotoxic attacks of external source and/or spontaneous genetic changes. The above reasons underline the importance of efficient DNA repair machinery to maintain the cellular genomic integrity. Indeed, well-supported associations exist between DNA repair insufficiency and inherited forms of CRC: hereditary nonpolyposis CRC is caused by deficient mismatch repair and MUTYH-associated polyposis is associated with deficient mutation in the base excision repair (BER) gene *MutY* (7).

In sporadic CRC, there is no single germ-line mutation causing a strong deficiency in DNA repair activity. However, alterations of the individual DNA repair capacity (DRC) may significantly modulate the susceptibility to this cancer, especially in the context of gene–environment interactions. Family-based studies have suggested that DRC is a phenotype with a strong genetic basis, estimating heritability in the range of 48–75% (8). DNA repair may be further modulated by environmental/lifestyle factors via several possible mechanisms, such as activation/inhibition of repair enzymes, different provision of building blocks (nucleotides) for the repair machinery or regulation of repair gene expression (9). Thus, DRC, comprising the effect of both hereditary components and variable environmental factors, characterizes the actual phenotype of the cells. Due to the above aspects, estimation of DRC is naturally becoming a representative biomarker and an integral part of modern molecular epidemiological studies on cancer.

Nucleotide excision DNA repair (NER), one of the major players in maintaining genomic integrity, may modulate predisposition to various cancers as well as response to subsequent treatment (10). So far, reduced NER-DRC was

Table I. Reduced NER capacity detected in blood cells of patients with various types of sporadic cancer—an overview of the literature

Cancer	Cases	Controls	Damage-inducing agent	Assay	References
Bladder	106 incident cancer, previously untreated	137 matched by age, sex and ethnicity	BPDE	Comet assay	Schabath *et al.* (11)
Breast	33 breast carcinoma patients	47 matched by age	UVC	HCR	Ramos *et al.* (12)
	69 newly diagnosed, previously untreated	79 matched by age and ethnicity	BPDE	HCR	Shi *et al.* (13)
Colorectal	40 sporadic CRC 28 hereditary predisposition to CRC	39 not specified	N-AcO-2-FAA	UDS	Pero *et al.* (14)
Cutaneous melanoma	312 newly diagnosed, previously untreated	324 matched by age, sex and ethnicity	UV	HCR	Wei *et al.* (15)
Head and neck	55 newly diagnosed, previously untreated	61 matched by age, sex, ethnicity and smoking status	BPDE	HCR	Cheng *et al.* (16)
	123 newly diagnosed, previously untreated	136 matched by age and sex	BPDE	Comet assay	Xiong *et al.* (17)
	744 newly diagnosed, previously untreated	753 matched by age, sex and ethnicity	BPDE	HCR	Wang *et al.* (18)
Non-melanoma skin	88 primary cancer	135 matched by sex and age	UV	HCR	Wei *et al.* (19)
	255 newly diagnosed, previously untreated	333 unmatched	UV	HCR	Wang *et al.* (20)
Non-small cell lung	316 newly diagnosed	316 matched by age, sex, and smoking status	BPDE	HCR	Wei *et al.* (21)
	467 newly diagnosed, previously untreated	488 matched by age, sex, ethnicity and smoking status	BPDE	HCR	Shen *et al.* (22)
	108 with second primary cancer	99 matched by age and sex	BPDE	Comet assay	Orlow *et al.* (23)
	271 lung carcinoma patients	271 matched by age and smoking	BPDE	HCR	Deng *et al.* (24)
Prostate	75 newly diagnosed, previously untreated and 65 prevalent cancer	96 matched by age and race	UV	HCR	Hu *et al.* (25)

UV, ultraviolet light; HCR, host-cell reactivation assay; N-AcO-2-FAA, N-acetoxy-N-2-fluorenylacetamide; UDS, unscheduled DNA synthesis.

associated with several types of sporadic malignancies (summarized in Table I). However, there is a lack of data on NER-DRC in association with sporadic CRC. Additionally, the characterization of individual DNA repair profiles may be of further importance for the implementation of targeted therapies. For instance, platinum-based compounds are first-line cytostatic drugs employed in CRC treatment and variability in NER pathway has been linked to the different sensitivity towards above chemotherapeutics (26,27).

Based on the above considerations, we have focused this case-control study on evaluating NER-DRC and endogenous DNA damage in peripheral blood mononuclear cells (PBMC) from 70 newly diagnosed CRC patients and 70 healthy age-matched controls from the Czech Republic. In this country, CRC constitutes a serious health problem as it has among the highest rates of incidence and mortality worldwide (28). Simultaneously, we have also profiled mRNA expression levels of nine genes involved in the incision phase of NER, whose rate is measured by our functional assay. Further, we have genotyped common single nucleotide polymorphisms (SNPs) in four NER genes. Additionally, the above variables were also analyzed in the context of individual biological and lifestyle factors and principal clinical characteristics.

The strength of our approach was that we included in the study only patients with newly diagnosed, histologically confirmed sporadic CRC. The homogeneity of the study group was further potentiated by the fact that all patients were sampled prior to any surgical intervention or therapy, thus eliminating the effects of these factors on the individual level of investigated parameters and minimizing the bias on the results.

Materials and methods

Study population

The study population comprised 70 patients with sporadic CRC and 70 healthy controls frequency matched for age. All participants were of Caucasian origin.

Incident cases were recruited at the time of the diagnosis among patients visiting two surgical departments (Thomayer Teaching Hospital in Prague and Teaching Hospital and Medical School in Pilsen, Czech Republic) between 2008 and 2010. Only new histologically confirmed CRC cases who did not receive any surgery or other specific treatment prior to sampling were included into the study. Collaborating clinicians provided clinical and pathological characteristics for each CRC patient: presence of inflammatory processes, number of polyps at colonoscopy, tumor localization, stage of the malignancy [tumor, nodes, metastases staging system (TNM) according to Union of International Cancer Control] and microsatellite instability (MSI) status.

Controls were selected from healthy individuals of similar age distribution who provided blood samples voluntarily. Only subjects with no previous diagnosis and without manifestation of any disease were included into the study. Controls have not been exposed to any potentially harmful chemicals except for those from environmental sources. No other selection criteria have been applied.

Participating subjects were properly informed about the aim of the research; they signed a written consent and the approval for genetic analysis, in accord with the Helsinki declaration. The Ethics Committees of the Thomayer Teaching Hospital in Prague and Teaching Hospital and Medical School in Pilsen (Czech Republic) approved the design of the study. Trained personnel

Table II. Characteristics of the study population

Characteristics	Category	Controls[a]	CRC patients	P-value[b]
Sex		n = 70	n = 70	
	Female	36	24	
	Male	34	46	**P = 0.042**
Age		n = 70	n = 70	
	Mean ± standard deviation	62.1 ± 12.7	65.4 ± 10.1	
	Median (quartiles)	60 (52–74)	66 (58–74)	
	Range	39–86	39–84	P = 0.079
Diagnosis			n = 70	
	Colon		38 (54.3%)	
	Rectum		32 (45.7%)	
TNM			n = 69	
	I		7 (10.2%)	
	II		25 (36.2%)	
	III		16 (23.2%)	
	IV		21 (30.4%)	
Smoking status		n = 69	n = 66	
	Non-smokers	54 (78.3%)	50 (75.8%)	
	Smokers	15 (21.7%)	16 (24.2%)	P = 0.732
Number of cigarettes/day		n = 14	n = 14	
	≤10	9 (64.3%)	6 (42.9%)	
	>10	5 (35.7%)	8 (57.1%)	P = 0.592
Alcohol consumption		n = 69	n = 65	
	No	26 (37.7%)	18 (27.7%)	
	Yes	43 (62.3%)	47 (72.3%)	P = 0.222
Alcohol intake (grams/day)		n = 43	n = 43	
	<25	24 (55.8%)	28 (65.1%)	
	25–49.9	12 (27.9%)	80 (18.6%)	
	50–74.9	40 (9.3%)	50 (11.6%)	
	75–100	3 (7%)	2 (4.7%)	P = 0.342
Body mass index		n = 61	n = 54	
	Mean ± standard deviation	26.1 ± 4.2	27.5 ± 4.5	
	<18.5	1 (1.7%)	2 (3.7%)	
	18.5–24.9	29 (47.5%)	14 (25.9%)	
	25.0–29.9	24 (39.3%)	23 (42.6%)	
	30.0–40.0	7 (11.5%)	15 (27.8%)	**P = 0.039**
Family history of cancer		n = 68	n = 66	
	Negative	41 (60.3%)	35 (53%)	
	Positive	27 (39.7%)	31 (47%)	P = 0.400
CRC in family		n = 61	n = 63	
	No	53 (86.9%)	56 (88.9%)	
	Yes	8 (13.1%)	7 (11.1%)	P = 0.735
Diabetes		n = 66	n = 65	
	No	59 (89.4%)	53 (81.5%)	
	Yes	7 (10.6%)	12 (18.5%)	P = 0.205

[a]Data were not available for all study participants for some parameters.
[b]One-way ANOVA for categorical variables, Kruskal–Wallis test for continuous variables. Significant P-values shown in bold.

interviewed patients and controls, using a structured questionnaire. Study subjects provided information on their lifestyle habits, body mass index (BMI), diabetes and family/personal history of cancer (Table II). Lifelong or long-term (at least six consecutive months) drug use or exposures to genotoxins were also investigated by the questionnaire.

Isolation of PBMC
Eight milliliters of peripheral venous blood were drawn from each subject into heparinized tubes, mixed 1:1 with RPMI 1640 medium (HEPES modification, containing 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 1.5% phytohemagglutinin and 0.2% penicillin/streptomycin, Sigma-Aldrich), layered over Histopaque-1077 (Sigma-Aldrich) and centrifuged at 320g for 40 min at room temperature (RT). Isolated PBMC were counted and their viability was checked by trypan blue exclusion. When viability was higher than 95%, cells were aliquoted into cultivation tubes with medium (~10^5 cells per 5-ml medium). Tubes were incubated at 37°C. After a mitogen-stimulation period of 20 h, PBMC were further processed for the challenge assay.

Challenge assay for evaluating NER capacity
NER-DRC was analyzed as a level of intermediate single-strand breaks (SSBs) in DNA of (+)-anti-Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE)-treated cells, originated during the incision of BPDE-DNA adducts by NER pathway. The increase in DNA breaks reflects the ability of NER machinery to recognize and remove corresponding adducts from DNA.

The methodology is described in detail in Slyskova *et al.* (29). Briefly, BPDE was added into the medium with mitogen-stimulated PBMC at a concentration of 1 μM for 30 min at 37°C. After challenge, old medium was replaced by a new one to remove BPDE excess. PBMC were harvested immediately after the treatment (time 0) or further cultured and harvested at 1, 2 and 4 h. Separated from medium by centrifugation, cells from each experimental point were rewashed with PBS and further processed using a standard comet assay protocol.

In parallel, culturing, harvesting and processing of PBMC of the same individual and in the same conditions but without any BPDE treatment were performed. Untreated PBMC represented the basal control DNA damage.

Comet assay
SSBs in DNA were analyzed by the alkaline comet assay based on a routinely used protocol (30). Experimental conditions for lysis, alkali treatment, electrophoresis, neutralization and scoring are presented in (29). Data are reported as tail DNA%, determined in 50 randomly selected cells from two parallel slides per experimental point.

DRC and endogenous DNA damage calculation
For each experimental point, the net DNA damage value was calculated by subtracting the basal control tail DNA% of untreated cells from the tail DNA% of treated cells. Final and reported DRC value was obtained as a difference between the net level of tail DNA% measured immediately after the treatment

with BPDE at time 0, and the net value at the maximal increase of tail DNA% detected up to 4 h of culturing.

The parameter reported as 'endogenous DNA damage' represents the mean value of all independent measurements of tail DNA% of the untreated control PBMCs.

SSBs in DNA per 10^9 dalton can be derived from tail DNA% by multiplying by a conversion factor of 0.042 based on a calibration curve (over the range of damage detected in the current study, the calibration curve is linear) as reported in (31).

Expression analysis

RNA isolation and quality control. Total RNA from 2 ml of fresh peripheral blood sampled into EDTA vacutainers was isolated using TRIzol according to the procedure supplied by manufacturer (Invitrogen, Paisley, UK) and was kept at −80°C. RNA integrity (RIN) was measured using capillary electrophoresis performed on Agilent Bioanalyzer 2100, with RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). RIN of all samples were in the range between 8.0 and 10.0. RNA quantity and purity was measured using ASP-3700 Micro-volume UV/Vis Spectrophotometer (Avans-Biotechnology, Taiwan). $OD_{260/280}$ ratios for all samples were between 1.8 and 2.0. Inhibition testing was performed for all samples by adding internal control template DNA (spike DNA), using Internal DNA extraction control kit (Primer Design, Southampton, UK) and following manufacturer's instructions.

cDNA synthesis and RT-qPCR. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA by using a RevertAid™ First strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania) with random hexamer primers in a final volume of 40 μl following manufacturer's instructions. cDNA was stored at −20°C. qPCR was performed on 7500 Real Time PCR system (Applied Biosystems, Foster City, CA) using chemicals produced by Primer Design Ltd. Precision™ 2× qPCR Mastermix and custom designed real-time PCR assays with PerfectProbe™ were used. All target genes (*ERCC1*, *RAD23B*, *RPA1*, *XPA*, *XPB*, *XPC*, *XPD*, *XPF*, *XPG*) assays were individually designed and were fully validated, with guaranteed priming specificity (BLAST screening) and > 90% of efficiency. Primer sequences are shown in Supplementary Table I, available at *Mutagenesis* Online. The PCR reactions were performed in a volume of 20 μl, containing 25 ng of cDNA for each sample. Cycling program was set at initial hold at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 32 sec and 72°C for 15 sec. Each run contained positive (interplate calibrator, 25 ng of human cDNA) and negative (no template) control. Results were analyzed using integrated 7500 System SDS Software version 1.3.1 (Applied Biosystems).

Reference genes were selected from a geNorm™ housekeeping gene selection kit of 12 genes with PerfectProbe™ and analyzed by both Genorm and Normfinder algorithms (GenEx Professional, MultiD Analyses AB, Göteborg, Sweden). Two combinations of selected reference genes (*TOP1*, *EIF4A2* and *B2M*, *CYC1*) were tested for stability in all study samples, but none of them proved to be a reliable normalization factor, and the same was observed for total RNA amount. Therefore, Cq values of target genes were normalized to mean expression of all genes, as it was shown to be the best normalization factor, applying both Genorm and Normfinder algorithms. M-value for mean expression of all genes was 0.1, when ignoring groups, or 0.02, when stratified for groups (patients versus controls). Data are expressed as relative to maximum quantities (lowest expression was considered as 1). Expression analyses were performed following MIQE guidelines (11).

Genotyping analysis

Considering the size of our study population, SNPs were chosen according to the minor allele frequency (MAF > 0.25) and according to the expected effect on DRC phenotype based on (12). All subjects were genotyped for five polymorphisms in four NER genes: *XPA*, *XPC*, *XPD* and *XPG*. For *XPD* Lys751Gln (rs28365048), *XPG* Asn1104His (rs17655) and *XPC* Lys939Gln (rs2228001) genotyping a PCR-RFLP procedure was carried out using primers and conditions previously described (13). For *XPC* Ala499Val (rs2228000), primers and conditions of reaction have been described in (15). *XPA* G23A (rs1800975) has been analyzed with TaqMan allelic discrimination assay (Applied Biosystems; Assay-on-demand, SNP genotyping products: C_482935_1). The results were regularly confirmed by random regenotyping of >10% of the samples for each polymorphism and showed concordant results. The genotypes with ambiguous and/or no results were excluded from the data set. Distribution of genotypes in the study group is shown in Supplementary Table II, available at *Mutagenesis* Online.

Statistical analysis

Investigated parameters were normalized by logarithmic transformation due to their asymmetric distribution in the study population. The relationships

between variables of interest at the bivariate level were studied by means of *T*-test, ANOVA and Pearson correlation. The strength of associations between CRC occurrence and categorical variables at binary level were tested by Chi-square test. The binary logistic regression was employed to study the simultaneous association of the DNA damage, NER-DRC and gene expression with CRC, adjusted for age, sex, smoking habit, alcohol consumption, BMI and family history of cancer. Genotype frequencies for each polymorphism were tested for compliance with Hardy–Weinberg equilibrium. All statistical tests were performed at 5% level of statistical significance; for expression data, correction for multiple testing analyses (significant *P*-value after correction being 0.005) was applied. The SPSS analytical package version 16.0 (Chicago, IL, USA) was employed for all statistical analyses.

Results

Study population

The study was carried out on 70 incident CRC patients and 70 healthy controls (mean age ± SD 65.4 ± 10.1 and 62.1 ± 12.7 years, respectively). In patients, malignancy in colon accounted for 54.3% of cases, whereas the rest were diagnosed for rectal cancer. The TNM staging was available for 69 patients: 7 were classified as stage I, 25 belonged to the stage II, 16 to stage III and stage IV was assigned for 21 individuals. In one patient, the pathologist failed to determine TNM. MSI status was available for 41 cases and 7 out of them (17.1%) were MSI unstable. The distribution of all clinical, biological or lifestyle characteristics is reported in Table II. No differences were observed between patients and controls, except for sex distribution (males prevailed among patients, $P = 0.042$) and for BMI (lower in controls, $P = 0.039$).

Endogenous DNA damage

Significantly higher endogenous DNA damage was observed in CRC patients, with median 25.9 (interquartile range 4.0–43.0) tail DNA% as compared to controls, median 9.3 (interquartile range 2.4–21.5) tail DNA%, ($P < 0.001$; Figure 1). After categorizing DNA damage into quartiles, we observed that incident CRC patients were over-represented in the fourth quartile category with the highest DNA damage (odds ratio [OR] 11.49, 95% confidence interval [CI] 3.36–39.34, $P < 0.001$), while the controls prevailed among those with the lowest level of DNA damage (first quartile). Investigated biological or lifestyle factors (age, sex, smoking status, alcohol consumption, BMI, family history of cancer and diabetes),
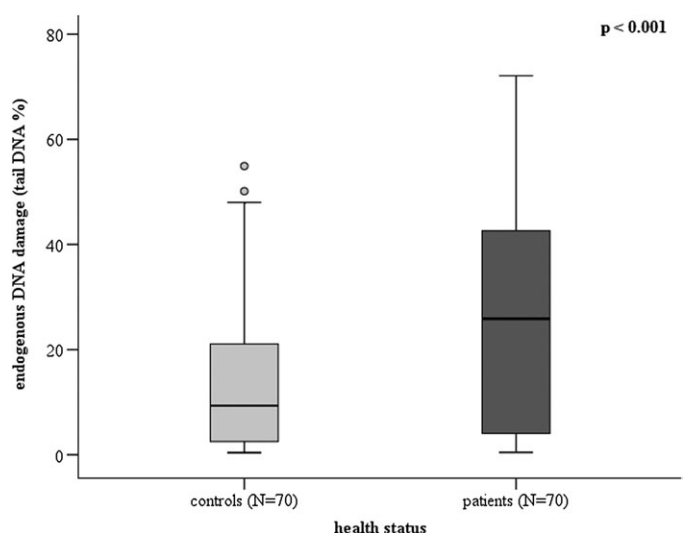

Fig. 1. Endogenous DNA damage level in CRC patients and healthy controls.

when included into the binary logistic regression model, did not significantly affect the value of regression coefficients.
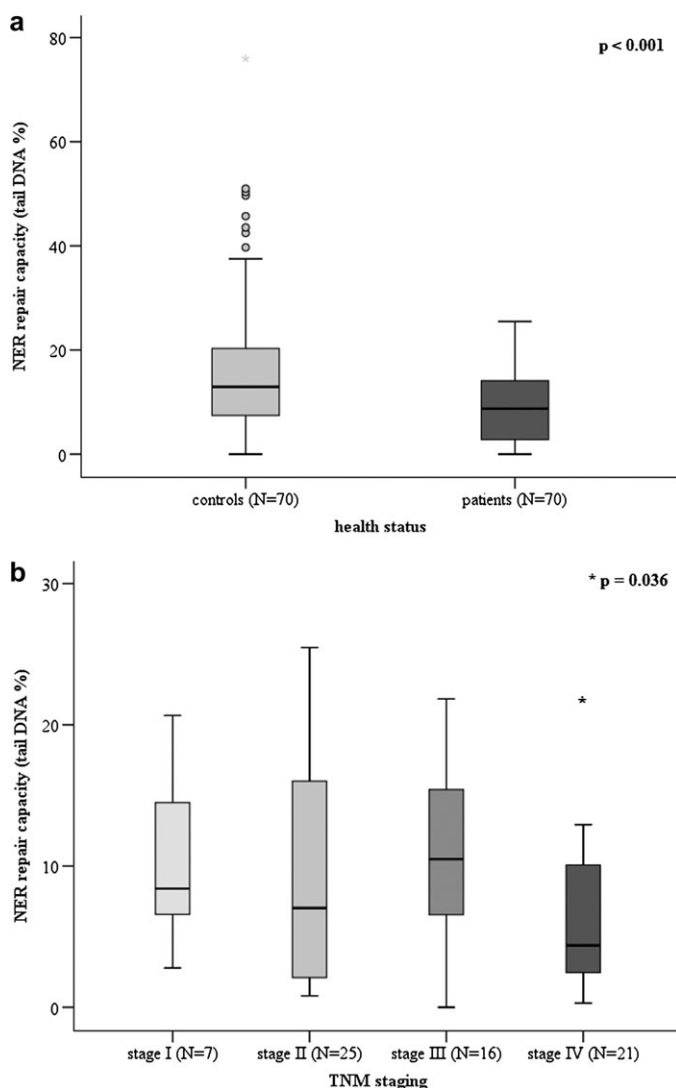

Fig. 2. (a) NER-DRC in CRC patients and healthy controls. (b) NER-DRC in CRC patients stratified according to TNM staging.

There was no association between DNA damage and TNM stage or localization of the tumor.

NER-DRC capacity

The CRC patients, with median 8.7 (interquartile range 2.7–14.3) tail DNA%, exhibited significantly lower NER-DRC than the controls with median 12.9 (interquartile range 7.4–20.6) tail DNA% ($P < 0.001$; Figure 2a). After categorizing NER-DRC into quartiles, the subjects with the lowest NER-DRC (the first quartile) comprised mainly CRC patients, whereas control subjects were the majority in the fourth quartile with the highest NER-DRC (OR 0.1, CI 0.03–0.32, $P < 0.001$). Similar to DNA damage, none of the investigated biological or lifestyle factors were associated with NER-DRC and their inclusion into the binary logistic model did not affect the value of regression coefficient. DNA damage and NER-DRC did not significantly correlate, either in the pooled study population or according to the diagnosis. Despite only a moderate decrease of NER-DRC in patients with TNM from I to III, a most pronounced reduction in NER-DRC was observed in patients with stage IV ($P = 0.036$, Figure 2b).

Expression and genotyping analyses

Expression profile was analyzed in a subgroup of 66 patients and 42 controls, for which RNA material was available and which pass the selection criteria for RNA purity and quality and control of PCR inhibition (as described in Materials and methods). Out of nine studied genes, mRNA levels of six of them significantly differed between patients and controls, also after applying correction for multiple testing analysis (significant P-value after correction being 0.005). Expression levels of *XPB* and *XPF* genes were higher in control group, while higher expression levels of *XPA*, *XPG*, *ERCC1* and *RAD23B* were detected in patients (for all $P \leq 0.001$, Figure 3). A strong relationship between expression levels of *RAD23B*, *XPG* and *ERCC1* genes was observed in the whole study group ($R = 0.98$, $P < 0.001$) and after stratification for patients and controls. Expression levels of any studied repair gene did not correlate with DNA damage or DRC and were not modulated by any of clinical, biological or lifestyle factors.

Distribution of the analyzed genotypes was in agreement with the Hardy–Weinberg equilibrium. None of the studied
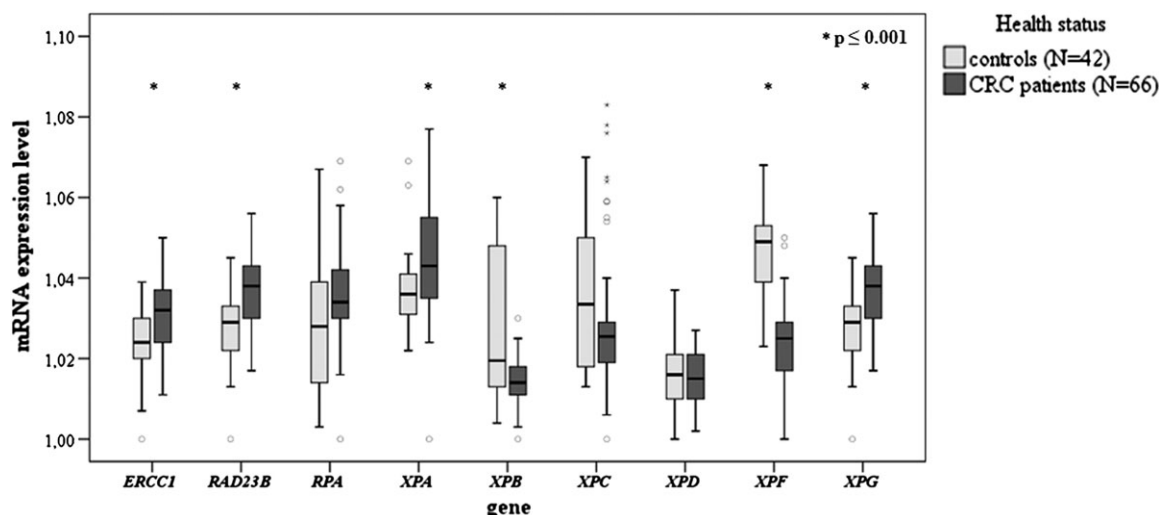
Fig. 3. mRNA expression levels of nine NER genes in CRC patients and healthy controls, expressed as quantities relative to the lowest detected expression assigned as value 1.

SNPs were significantly associated with DNA damage levels or DRC, either in patients or in controls. Expression levels were only moderately modulated by some SNPs. In cases only, variant allele of *XPC* Ala499Val was associated with lower *XPC* and higher *XPB* and *XPD* expression levels (for all $P <$ 0.05). In controls, variant allele of *XPC* Lys939Gln was associated with higher *XPA* expression level ($P < 0.05$; data not shown).

Discussion

DRC reflects the actual capacity of the organism to maintain DNA integrity and constitutes an informative biomarker of intermediate cancer phenotype (19). Our study represents an investigation on basal DNA damage and DRC in relation to sporadic CRC. In particular, we have focused our interest on evaluation of individual DRC characterizing NER activity. To approach this, we have challenged PBMC of study subjects by BPDE and quantified the removal of BPDE adducts from DNA (which reflects rate-limiting incision step of NER pathway) by a modified version of comet assay. BPDE was chosen as a model compound for two main reasons. First, it is a metabolite of carcinogen benzo(a)pyrene (BaP), to which an organism is commonly exposed from various sources. Along with environmental or occupational pollution, BaP is generated also by pyrosynthesis in burning tobacco or in meat prepared at high temperatures. Second, BPDE binds to DNA, forming predominantly N^2-deoxyguanosine bulky adducts, which have been detected in colonic mucosa (20) and are specifically removed by NER (16). Generally, NER recognizes a wide spectrum of bulky DNA lesions induced by UV light and a variety of helix-distorting agents (17). A reduced NER capacity may consequently enhance the CRC risk due to a diminished protection of intestinal epithelium against genotoxic compounds, present in the lumen or transported by the blood.

In our study group, newly diagnosed CRC patients had significantly lower NER-DRC and higher levels of SSBs in DNA as compared to the age-matched healthy subjects. This was clearly documented after categorization of the above parameters into quartiles, where accumulation of SSBs and decreasing DRC were characteristic for CRC patients. Interestingly, the two parameters behaved as factors independently associated with sporadic CRC. Furthermore, when the cases were stratified according to the TNM staging, significantly lower NER-DRC was observed in patients with the stage IV in comparison to those with less severe/invasive stages of the disease. A gradual decrease of DRC with increasing TNM stage in a surrogate tissue possibly reflects important biological phenomenon in relation to progression of the disease. Deficient or reduced NER-DRC was already reported as a risk factor for several different cancers, including bladder (18), breast (21,22), skin (23–25), head and neck (14,32,33), lung (34–37) and prostate cancer (38), as summarized in Table I. Our results contribute to the list of evidences on the importance of NER-DRC in carcinogenesis, showing the same relevance also for sporadic CRC. To our knowledge, the only study investigating specifically NER capacity in sporadic CRC patients was performed almost 30 years ago by unscheduled DNA synthesis in smaller study group (39).

Simultaneously with reduced NER-DRC, CRC patients exhibited elevated endogenous DNA damage. The arbitrary unit of tail DNA% may be converted to SSBs/10^6 nucleotides (31,40). By expressing our data in this latter unit, we obtain 0.18 versus 0.37 SSBs/10^6 nucleotides in studied controls and patients, respectively which means ∼540 versus 1113 breaks/cell. Pooling together data from 119 publications reporting endogenous DNA damage extent in cancer-free populations (41), a clear positive correlation was observed between level of SSBs and age. For individuals belonging to the 50- to 64-year-old group, an average SSB level of 510 breaks/cell was calculated, which is in full agreement with our data. This demonstrates that CRC patients bear >2-fold higher level of strand breaks in DNA than the reference value for healthy population of the same age. The enhanced levels of DNA damage represent an additional suggestion for a generally altered status of the DNA repair machinery among cancer patients. In agreement with our findings, several studies have shown that basal DNA damage is indeed increased in leukocytes of patients suffering from a variety of different forms of cancer, as reviewed by (42). On the other hand, we cannot rule out that the level of endogenous DNA damage may comprise various alkali-labile DNA lesions, converted during comet assay into SSBs. These lesions are a target for BER pathway, which was also reported to be deficient in cancer patients (43,44).

Additionally, mRNA quantity of nine genes involved in the recognition/incision step of NER was studied. Expression levels of individual genes were not significantly related to either NER-DRC or the extent of DNA damage. However, a coordinated expression of *RAD23B*, *XPG* and *ERCC1* genes was observed. This is an interesting finding considering that XPC-RAD23B complex acts in DNA damage recognition and subsequently recruits the XPG and ERCC1/XPF to the site of damage directly or via strong interactions with TFIIH nine subunits complex (45,46). Six NER genes were found to be differently transcribed between patients and controls. *XPB* and *XPF* had higher expression in controls, while *XPA*, *XPG*, *ERCC*1 and *RAD23B* were more expressed in patients. Such a finding is somehow surprising, as total DRC was shown to be lower in patients. Observed expression profiles and the lack of correlation between quantity of mRNA and DRC may be in concordance with previous observations showing that mRNA quantity does not necessarily reflect the activity of protein (47,48) or overall repair capacity (49,50). In some cases, it is the absence rather than the relative content of a functional protein that is important for cellular activity. Moreover, genes act in interactive networks and alterations of each of them might have different impact on the overall cell function (51). Communication between the NER system and DNA damage signaling may also play a critical role (52).

In the present study, we have also attempted to relate outcomes of functional tests and expression levels to relevant variation in some NER genes. The individual differences in DNA damage levels and DRC as well as expression levels have been hypothesized for many years to be associated to individual genetic background in DNA repair genes (13,29). In our hands, investigated SNPs were not significantly associated with either DNA damage or DRC after stratification for health status. However, there were some associations with NER gene expression, the most interesting being a modulation of expression levels of *XPC*, *XPB* and *XPD* by *XPC* Ala499Val variant allele in CRC patients. A potential functional effect of this SNP is supported by previous studies on susceptibility to cancer (53,54). A role of SNPs in NER pathway on sporadic CRC risk has been recently postulated (55,56), but the limited size of our currently studied population precludes evaluation of the association of individual SNPs with CRC risk. The actual

association of variation in DNA repair genes and CRC risk has not been clearly disclosed either in the context of recent genome-wide association studies (57).

In conclusion, our study provides evidence on alterations of cellular DRC among sporadic CRC patients and suggests the role of NER in its etiology. However, one of the main future challenges with intermediate biomarkers, like DNA damage and DRC, is to understand whether they belong to the causal pathway of a disease, whether they are simply a side effect of a disease or whether their measurement may be confounded by some other factors.

Genes involved in DNA damage recognition/incision phase of NER act in an interplay and in a synchronized way (45,58). Based on our findings, this process is more comprehensively characterized by functional quantification of repair capacity than by quantification of individual gene transcripts or gene variants. Overall, our observation points out the usefulness of DRC analyses, which measure the real outcome of a complex multigene process, as also recently concluded by Collins and Azqueta (59). Comet assay is a convenient methodology for DRC evaluation and high-throughput versions of it are currently under development. This will allow the simultaneous determination of DRC in multiple samples and thus making this assay suitable for large population screening minimizing inter-experimental variation (60).

Supplementary data

Supplementary Tables I and II are available at *Mutagenesis* Online.

References

1. Ferlay, J., Shin, H. R., Bray, F., Forman, D., Mathers, C. and Parkin, D. M. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer*, **127**, 2893–2917.
2. Center, M. M., Jemal, A., Smith, R. A. and Ward, E. (2009) Worldwide variations in colorectal cancer. *CA Cancer J. Clin.*, **59**, 366–378.
3. Pritchard, C. C. and Grady, W. M. (2011) Colorectal cancer molecular biology moves into clinical practice. *Gut*, **60**, 116–129.
4. de la Chapelle, A. (2004) Genetic predisposition to colorectal cancer. *Nat. Rev. Cancer*, **4**, 769–780.
5. Giovannucci, E. and Michaud, D. (2007) The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. *Gastroenterology*, **132**, 2208–2225.
6. Botteri, E., Iodice, S., Bagnardi, V., Raimondi, S., Lowenfels, A. B. and Maisonneuve, P. (2008) Smoking and colorectal cancer: a meta-analysis. *JAMA*, **300**, 2765–2778.
7. Tops, C. M., Wijnen, J. T. and Hes, F. J. (2009) Introduction to molecular and clinical genetics of colorectal cancer syndromes. *Best Pract. Res. Clin. Gastroenterol*, **23**, 127–146.
8. Wu, X., Spitz, M. R., Amos, C. I. *et al.* (2006) Mutagen sensitivity has high heritability: evidence from a twin study. *Cancer Res.*, **66**, 5993–5996.
9. Mathers, J. C., Coxhead, J. M. and Tyson, J. (2007) Nutrition and DNA repair—potential molecular mechanisms of action. *Curr. Cancer Drug Targets*, **7**, 425–431.
10. Friedberg, E. C. (2001) How nucleotide excision repair protects against cancer. *Nat. Rev. Cancer*, **1**, 22–33.
11. Schabath, M. B., Spitz, M. R., Grossman, H. B., Zhang, K., Dinney, C. P., Zheng, P. J. and Wu, X. (2003) Genetic instability in bladder cancer assessed by the comet assay. *J. Natl. Cancer Inst.*, **95**, 540–547.
12. Ramos, J. M., Ruiz, A., Colen, R., Lopez, I. D., Grossman, L. and Matta, J. L. (2004) DNA repair and breast carcinoma susceptibility in women. *Cancer*, **100**, 1352–1357.
13. Shi, Q., Wang, L. E., Bondy, M. L., Brewster, A., Singletary, S. E. and Wei, Q. (2004) Reduced DNA repair of benzo[a]pyrene diol epoxide-induced adducts and common XPD polymorphisms in breast cancer patients. *Carcinogenesis*, **25**, 1695–1700.
14. Pero, R. W., Miller, D. G., Lipkin, M., Markowitz, M., Gupta, S., Winawer, S. J., Enker, W. and Good, R. (1983) Reduced capacity for DNA repair synthesis in patients with or genetically predisposed to colorectal cancer. *J. Natl. Cancer Inst.*, **70**, 867–875.
15. Wei, Q., Lee, J. E., Gershenwald, J. E. *et al.* (2003) Repair of UV light-induced DNA damage and risk of cutaneous malignant melanoma. *J. Natl. Cancer Inst.*, **95**, 308–315.
16. Cheng, L., Eicher, S. A., Guo, Z., Hong, W. K., Spitz, M. R. and Wei, Q. (1998) Reduced DNA repair capacity in head and neck cancer patients. *Cancer Epidemiol. Biomarkers Prev.*, **7**, 465–468.
17. Xiong, P., Hu, Z., Li, C., Wang, L. E., El-Naggar, A. K., Sturgis, E. M. and Wei, Q. (2007) In vitro benzo[a]pyrene diol epoxide-induced DNA damage and chromosomal aberrations in primary lymphocytes, smoking, and risk of squamous cell carcinoma of the head and neck. *Int. J. Cancer*, **121**, 2735–2740.
18. Wang, L. E., Hu, Z., Sturgis, E. M. *et al.* (2010) Reduced DNA repair capacity for removing tobacco carcinogen-induced DNA adducts contributes to risk of head and neck cancer but not tumor characteristics. *Clin. Cancer Res.*, **16**, 764–774.
19. Wei, Q., Matanoski, G. M., Farmer, E. R., Hedayati, M. A. and Grossman, L. (1995) DNA repair capacity for ultraviolet light-induced damage is reduced in peripheral lymphocytes from patients with basal cell carcinoma. *J. Invest. Dermatol.*, **104**, 933–936.
20. Wang, L. E., Li, C., Strom, S. S. *et al.* (2007) Repair capacity for UV light induced DNA damage associated with risk of nonmelanoma skin cancer and tumor progression. *Clin. Cancer Res.*, **13**, 6532–6539.
21. Wei, Q., Cheng, L., Amos, C. I., Wang, L. E., Guo, Z., Hong, W. K. and Spitz, M. R. (2000) Repair of tobacco carcinogen-induced DNA adducts and lung cancer risk: a molecular epidemiologic study. *J. Natl. Cancer Inst.*, **92**, 1764–1772.
22. Shen, H., Spitz, M. R., Qiao, Y., Guo, Z., Wang, L. E., Bosken, C. H., Amos, C. I. and Wei, Q. (2003) Smoking, DNA repair capacity and risk of nonsmall cell lung cancer. *Int. J. Cancer*, **107**, 84–88.
23. Orlow, I., Park, B. J., Mujumdar, U. *et al.* (2008) DNA damage and repair capacity in patients with lung cancer: prediction of multiple primary tumors. *J. Clin. Oncol.*, **26**, 3560–3566.
24. Deng, L., Kimmel, M., Foy, M., Spitz, M., Wei, Q. and Gorlova, O. (2009) Estimation of the effects of smoking and DNA repair capacity on coefficients of a carcinogenesis model for lung cancer. *Int. J. Cancer*, **124**, 2152–2158.
25. Hu, J. J., Hall, M. C., Grossman, L., Hedayati, M., McCullough, D. L., Lohman, K. and Case, L. D. (2004) Deficient nucleotide excision repair capacity enhances human prostate cancer risk. *Cancer Res.*, **64**, 1197–1201.
26. Bustin, S. A., Benes, V., Garson, J. A. *et al.* (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.*, **55**, 611–622.
27. Friedberg, E. C. (2006) *DNA Repair and Mutagenesis*. 2nd edn. ASM Press, Washington, DC.
28. Vodicka, P., Stetina, R., Polakova, V. *et al.* (2007) Association of DNA repair polymorphisms with DNA repair functional outcomes in healthy human subjects. *Carcinogenesis*, **28**, 657–664.
29. Hu, Z., Wang, Y., Wang, X. *et al.* (2005) DNA repair gene XPC genotypes/haplotypes and risk of lung cancer in a Chinese population. *Int. J. Cancer*, **115**, 478–483.
30. Jernstrom, B. and Graslund, A. (1994) Covalent binding of benzo[a]pyrene 7,8-dihydrodiol 9,10-epoxides to DNA: molecular structures, induced mutations and biological consequences. *Biophys. Chem.*, **49**, 185–199.

31. Gillet, L. C. and Scharer, O. D. (2006) Molecular mechanisms of mammalian global genome nucleotide excision repair. *Chem. Rev.*, **106**, 253–276.

32. Carvajal-Carmona, L. G. (2010) Genetic dissection of intermediate phenotypes as a way to discover novel cancer susceptibility alleles. *Curr. Opin. Genet. Dev.*, **20**, 308–314.

33. Alexandrov, K., Rojas, M., Kadlubar, F. F., Lang, N. P. and Bartsch, H. (1996) Evidence of anti-benzo[a]pyrene diol epoxide-DNA adduct formation in human colon mucosa. *Carcinogenesis*, **17**, 2081–2083.

34. Helleday, T., Petermann, E., Lundin, C., Hodgson, B. and Sharma, R. A. (2008) DNA repair pathways as targets for cancer therapy. *Nat. Rev. Cancer*, **8**, 193–204.

35. Martin, L. P., Hamilton, T. C. and Schilder, R. J. (2008) Platinum resistance: the role of DNA repair pathways. *Clin. Cancer Res.*, **14**, 1291–1295.

36. Center, M. M., Jemal, A. and Ward, E. (2009) International trends in colorectal cancer incidence rates. *Cancer Epidemiol. Biomarkers Prev.*, **18**, 1688–1694.

37. Slyskova, J., Naccarati, A., Polakova, V. *et al.* (2011) DNA damage and nucleotide excision repair capacity in healthy individuals. *Environ. Mol. Mutagen*, **52**, 511–517.

38. Collins, A. R. (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol. Biotechnol.*, **26**, 249–261.

39. Collins, A. R., Dusinska, M., Gedik, C. M. and Stetina, R. (1996) Oxidative damage to DNA: do we have a reliable biomarker? *Environ. Health Perspect.*, **104** (Suppl. 3), 465–469.

40. Collins, A. R., Oscoz, A. A., Brunborg, G., Gaivao, I., Giovannelli, L., Kruszewski, M., Smith, C. C. and Stetina, R. (2008) The comet assay: topical issues. *Mutagenesis*, **23**, 143–151.

41. Moller, P. (2006) Assessment of reference values for DNA damage detected by the comet assay in human blood cell DNA. *Mutat. Res.*, **612**, 84–104.

42. McKenna, D. J., McKeown, S. R. and McKelvey-Martin, V. J. (2008) Potential use of the comet assay in the clinical management of cancer. *Mutagenesis*, **23**, 183–190.

43. Paz-Elizur, T., Sevilya, Z., Leitner-Dagan, Y., Elinger, D., Roisman, L. C. and Livneh, Z. (2008) DNA repair of oxidative DNA damage in human carcinogenesis: potential application for cancer risk assessment and prevention. *Cancer Lett.*, **266**, 60–72.

44. Collins, A. R. and Gaivao, I. (2007) DNA base excision repair as a biomarker in molecular epidemiology studies. *Mol. Aspects Med.*, **28**, 307–322.

45. Park, C. J. and Choi, B. S. (2006) The protein shuffle. Sequential interactions among components of the human nucleotide excision repair pathway. *FEBS J.*, **273**, 1600–1608.

46. Araujo, S. J., Nigg, E. A. and Wood, R. D. (2001) Strong functional interactions of TFIIH with XPC and XPG in human DNA nucleotide excision repair, without a preassembled repairosome. *Mol. Cell Biol.*, **21**, 2281–2291.

47. Vogel, U., Dybdahl, M., Frentz, G. and Nexo, B. A. (2000) DNA repair capacity: inconsistency between effect of over-expression of five NER genes and the correlation to mRNA levels in primary lymphocytes. *Mutat. Res.*, **461**, 197–210.

48. Stevens, E. V., Nishizuka, S., Antony, S. *et al.* (2008) Predicting cisplatin and trabectedin drug sensitivity in ovarian and colon cancers. *Mol. Cancer Ther.*, **7**, 10–18.

49. Damia, G., Guidi, G. and D'Incalci, M. (1998) Expression of genes involved in nucleotide excision repair and sensitivity to cisplatin and melphalan in human cancer cell lines. *Eur. J. Cancer*, **34**, 1783–1788.

50. Hanova, M., Stetina, R., Vodickova, L. *et al.* (2011) Modulation of DNA repair capacity and mRNA expression levels of XRCC1, hOGG1 and XPC genes in styrene-exposed workers. *Toxicol. Appl. Pharmacol.*, **248**, 194–200.

51. Cheung, V. G. and Spielman, R. S. (2009) Genetics of human gene expression: mapping DNA variants that influence gene expression. *Nat. Rev. Genet.*, **10**, 595–604.

52. Chang, Y. C., Jan, K. Y., Cheng, C. A., Liao, C. B. and Liu, Y. C. (2008) Direct involvement of the tumor suppressor p53 in nucleotide excision repair. *DNA Repair (Amst)*, **7**, 751–761.

53. Francisco, G., Menezes, P. R., Eluf-Neto, J. and Chammas, R. (2008) XPC polymorphisms play a role in tissue-specific carcinogenesis: a meta-analysis. *Eur. J. Hum. Genet.*, **16**, 724–734.

54. Qiu, L., Wang, Z. and Shi, X. (2008) Associations between XPC polymorphisms and risk of cancers: a meta-analysis. *Eur. J. Cancer*, **44**, 2241–2253.

55. Naccarati, A., Pardini, B., Hemminki, K. and Vodicka, P. (2007) Sporadic colorectal cancer and individual susceptibility: a review of the association studies investigating the role of DNA repair genetic polymorphisms. *Mutat. Res.*, **635**, 118–145.

56. Pardini, B., Naccarati, A., Novotny, J. *et al.* (2008) DNA repair genetic polymorphisms and risk of colorectal cancer in the Czech Republic. *Mutat. Res.*, **638**, 146–153.

57. Tomlinson, I., Houlston, R., Montgomery, G., Sieber, O. and Dunlop, M. (2012) Investigation of the effects of DNA repair gene polymorphisms on the risk of colorectal cancer. *Mutagenesis*, **27**, 219–223.

58. Friedberg, E. C. (1987) The molecular biology of nucleotide excision repair of DNA: recent progress. *J. Cell. Sci. Suppl.*, **6**, 1–23.

59. Collins, A. R. and Azqueta, A. (2011) DNA repair as a biomarker in human biomonitoring studies; further applications of the comet assay. *Mutat. Res. Fundam. Mol. Mech. Mugag.* Apr 1 2011 [Epub ahead of print].

60. Dusinska, M. and Collins, A. R. (2008) The comet assay in human biomonitoring: gene-environment interactions. *Mutagenesis*, **23**, 191–205.