Functional, genetic and epigenetic aspects of base and nucleotide excision repair in colorectal carcinomas

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Translational relevance:
DNA repair influences cancer development and sensitivity to treatment. Categorization of tumors according to their DNA repair characteristics can be relevant for personalized therapy, but functional assays to define DNA repair status of target tissue are needed. In this respect, we optimized BER- and NER-specific assays and showed their reliability and applicability to high-throughput screening of human solid tissues. The complexity of multi-gene DNA repair processes is comprehensively reflected by functional analysis of overall DNA repair capacity and should be recommended for DNA repair investigations. Analysis of BER and NER with a functional, genetic and epigenetic approach confirmed that colorectal carcinomas are only moderately altered in these repair pathways as compared to adjacent healthy tissue. Consistency of our and previously reported observations suggests that excision repair is not a factor contributing to the malignant transformation, but might rather contribute to chemoresistance and growth advantage of tumor cells.
Abstract

**Purpose:** DNA repair capacity (DRC) is a determinant not only of cancer development but also of individual response to therapy. Previously, altered base and nucleotide excision repair (BER and NER) have been described in lymphocytes of sporadic colorectal cancer patients. We, for the first time, evaluate both excision repair capacities in human colon biopsies in order to study their participation on colorectal tumorigenesis.

**Experimental design:** Seventy pairs of tumor and adjacent healthy tissues were analyzed for BER- and NER-specific DRC by a comet repair assay. Tissue pairs were further compared for expression levels of a panel of 25 BER and NER genes complemented by their promoter methylation status.

**Results:** We observed a moderate increase of NER-DRC (p=0.019), but not BER-DRC in tumors. There was a strong correlation between both tissues for all investigated parameters (p<0.001). However, four NER (CSB, CCNH, XPA, XPD) and BER (NEIL1, APEX1, OGG1, PARP1) genes showed a 1.08-1.28-fold change difference in expression in tumors (p<0.05). Individual gene expression levels did not correlate with overall DRC and we did not detect any aberrant methylation of the investigated genes.

**Conclusions:** Our complex analysis showed that tumor cells are not deficient in BER and NER, but rather follow patterns characteristic for each individual and are comparable with adjacent tissue. Alteration of excision repair pathways is not a pronounced event in colorectal carcinogenesis. This study shows the feasibility of DRC evaluation in human solid tissues, representing a complex marker of multi-gene DNA repair processes.

**Keywords:** DNA repair capacity, base excision repair, nucleotide excision repair, colorectal cancer, *in vitro* DNA repair assay
1. Introduction

Despite long and intensive research, colorectal cancer (CRC) has at present one of the highest rates of incidence and mortality worldwide (1). With the exception of KRAS mutational status for selection of biological treatment, no predictive or prognostic biomarker has yet been validated (2, 3). Keeping in mind the importance of DNA repair in the disease development and treatment response, it seems reasonable to consider a categorization of tumors based on DNA repair characteristics. Such an approach would require a panel of functional biomarkers that can define the DNA repair status of the target tissue (4).

DNA repair is a defensive mechanism that copes with ubiquitous DNA damage, occurring as a consequence of cellular metabolism or through exogenous exposure. Moreover, a large number of antineoplastic drugs impart their effect by DNA disruption. Therefore, an effective DNA damage response is essential for the maintenance of genome stability in normal cells while in malignant cells the suppression of DNA repair would, presumably, increase the effectiveness of chemotherapy through damage accumulation and consequent apoptosis. Based on the evidence so far, mismatch repair (MMR) defines the strongest link between DNA repair and CRC. A subset of hereditary and sporadic CRC shows genetic or epigenetic defects in MMR that are manifested by microsatellite instability. The phenomenon may also be accompanied by epigenetic instability, characterized by a high degree of aberrant methylation of CpG islands. Germ-line, and not somatic inactivation of base excision repair (BER) gene MUTYH, causes polyposis which transforms almost always into carcinoma (5). No study has to date reported any defect in nucleotide excision repair (NER) in any form of CRC.

A potential role of both BER and NER in the pathogenesis of sporadic CRC is plausible. Colon epithelium is one of the most constantly regenerated tissues in the body. It therefore has increased vulnerability to a variety of mutagens present in the bowel contents or in the blood. Cigarette smoking, alcohol, over-cooked red meat or processed saturated fat have been established CRC risk factors through the generation of strong DNA-reactive compounds (6). Amongst other agents, benzo[a]pyrene, aromatic amines, alkylating agents or reactive...
oxygen species represent substrates for excision repair. Even detoxified carcinogens can interact with mucosal DNA as carcinogen-activating enzymes have been detected in colon epithelium and in colonic bacteria (7-9). Further, the well-known role of chronic inflammation in colon carcinogenesis is explained by enhanced epithelial cell turnover, accompanied by sustainable oxidative stress contributing to neoplastic transformation (10). Moreover, both pathways are plausibly expected to influence the effectiveness of anticancer therapy. The mainstays of CRC treatment are regimes based on 5-fluorouracil and/or oxaliplatin. The NER pathway is known to be essential for the removal of platinum adducts; BER, on the other hand, is involved in response to 5-fluorouracil (11, 12).

In general, there is limited information available on the mechanisms of BER and NER in sporadic CRC. No somatic genetic alteration of genes involved in either of the excision repair pathways has been identified. The investigations failed to prove any clear relationship between common genetic variants and the risk of sporadic CRC (13, 14). Aberrant promoter methylation of BER and NER genes have already been reported in other types of cancer, but not studied in CRC (15). An investigation of expression profiles of some BER and NER genes in tumor tissue did provide preliminary characterizations (16). Previous studies carried out on peripheral blood mononuclear cells (PBMC) showed suppressed BER and NER capacities in CRC patients compared to healthy individuals (17-19). However, the validity of blood as a surrogate for cancer tissue to estimate DNA repair capacity (DRC) remains disputable.

The aim of the present study was to compare BER- and NER-DRC in different tissue specimens – colorectal adenocarcinomas, adjacent healthy mucosas and peripheral blood cells from 70 newly diagnosed CRC patients in order to investigate: (i) tissue specificity of the DNA repair processes and (ii) possible alteration of both repair pathways in colorectal carcinogenesis. DRCs analyses were carried out by comet repair assays, modified for measurement of DRC in human solid tissues. The expression levels of a panel of 8 BER and 17 NER genes were also studied and complemented by the evaluation of aberrant promoter methylation in their promoter regions.
2. Materials and methods

Study patients and collection of biological specimen

The study included seventy sporadic CRC patients who underwent surgical resection. Patients were recruited between 2009 and 2011 at the Thomayer Hospital (Prague), the General University Hospital (Prague) and Teaching Hospital and the Medical School of Charles University (Pilsen). All patients signed informed consent. Ethics approval was granted by the appropriate committees at the three hospitals. The group of patients included 53 men and 17 women with a mean age of 66.2 (±10.6). The clinical stage of patients at diagnosis was classified according to the tumor-node-metastasis (TNM) system. Seven patients were diagnosed with pathologic stage I (10%), 29 as stage II (41.4%), 15 as stage III (21.4%) and 19 as stage IV (27.2%). All patients had adenocarcinomas; 44 patients had tumor localized in the colon (62.9%) and 26 in the rectum (37.1%). In 12 (17.2%) patients, tumors were of well differentiated grade, in 47 (67.1%) moderately differentiated and in 11 patients (15.7%) poorly differentiated. Eleven rectal cancer patients (15.7%) received neoadjuvant therapy prior to surgery. Tumor tissue and adjacent healthy colon/rectal tissue (5-10 cm distant from the tumor) were resected from all patients. Samples were deep frozen immediately after removal. A day before surgery, peripheral blood was also drawn from a sub-set of patients and was stored at 4°C no longer than 3 hours before being processed. Due to various logistical reasons, not all patients could be analyzed for all the studied parameters. Therefore, each particular analysis is further specified for actual number of cases for whom analysis was carried out.

Isolation of nucleic acids and protein extracts from blood and tissues

Extraction of nucleic acids: DNA from blood was isolated by a standard phenol/chloroform method. Prior to tissue processing, histological analysis was carried out to assess the proportion of tumor cells in tumor tissues and to rule out the presence of neoplastic cells in the normal mucosal tissues. Briefly, samples were embedded in optimal cutting temperature compound (Sakura Finetek), and cut with a Leica CM 1850 cryostat. Five µm thick serial sections were fixed in 90% ethanol on microscope slides and stained with 1% cresyl violet acetate (Sigma-Aldrich), dehydrated with ethanol, dried and inspected using a Leica DM6000
microscope. Tissue samples were subsequently homogenized in the MagNA Lyser (Hoffmann-La Roche). AllPrep DNA/RNA mini kit (Qiagen) was used to isolate nucleic acids.

**Extraction of proteins:** PBMC were separated on Histopaque-1077 (Sigma-Aldrich), counted and evaluated by trypan blue exclusion. Cells were suspended in freezing medium (RPMI 1640, 20% FBS, 0.2% ATB, 10% DMSO, Sigma-Aldrich) and frozen at -80°C. Tissues were weighed and ground while frozen. Further, 50µL of buffer A (45mM HEPES, 0.4M KCl, 1mM EDTA, 0.1mM DTT, 10% glycerol, pH 7.8) was added to every 50mg of ground tissue or 5x10^6 of PBMC. Samples were vortexed, snap frozen and 15µL of 1% Triton X-100 in buffer A was added per each 50µL. Protein concentration was measured by a fluorescamine assay (Sigma-Aldrich), with a NanoDrop 3300 (Thermo Scientific).

**In vitro DNA repair assay**

In vitro repair assays, adopted from Langie et al. (20, 21), were implemented using a 12-gel slide format (22). Briefly, protein extracts were incubated with two types of substrate DNA, containing artificially induced lesions known to be repaired specifically by BER or NER pathway. Levels of DNA strand breaks, generated during removal of lesions, reflect the repair activity of the extract.

**Substrate DNA:** For BER, human PBMC were treated with 2µM Ro 19-8022 (Hoffmann-La Roche) for 5 min, and irradiated by a 500W halogen lamp at a 33 cm distance to induce 8-oxoguanines. For NER, TK6 cells were irradiated with 5 Jm^-2 of UVC (50 sec at 0.1 Jm^-2s^-1) to generate cyclobutane pyrimidine dimers and 6-4 photoproducts. Untreated PBMC and TK6 cells were prepared in parallel. Cells were aliquoted at 0.5x10^6 in 1mL of freezing medium (see above) and frozen. Before each experiment, cells were thawed by adding 1mL of cold PBS, spun at 400g, 5 min, 4°C and resuspended in 1mL of PBS. Eighty µL of the cell suspension was mixed with 260µL of 1% LMP agarose to reach the desired concentration of cells. Using a multi-dispensing pipette, 12 gels per 5µL agarose were placed on each microscope slide. Cells embedded in agarose underwent lysis for 1 hour in 2.5M NaCl, 100mM EDTA, 10mM Tris, 250mM NaOH, 1% Triton X-100, pH 10. Before incubation with protein extracts, slides were washed twice for 5 min with buffer B (45mM HEPES, 0.25mM EDTA, 0.3mg/mL BSA, 2% glycerol, pH 7.8) and placed in incubation chambers (Severn Biotech) (22).
Protein extracts: Extracts were diluted into protein concentration of 3mg/mL in buffer A in the final volume of 50 µL and mixed with 4-volumes of buffer B. For the NER-specific assay, 2.5mM of adenosine-5'-triphosphate was added. Thirty µL of extract was pipetted per agarose gel.

BER-specific assay: Each extract was incubated with Ro-treated and untreated PBMC to determine nonspecific endonuclease activity of the extract. This was used for background correction for each sample. Incubation time was 20 min, at 37°C in a humid environment. Formamidopyrimidine DNA glycosylase was used as a positive control and, for a negative control, substrate gels were incubated with 1:4 buffer A + buffer B. Each experimental point was performed in duplicates. Five µM PARP inhibitor ABT-888 (Selleckchem) was added to the extract to test the effect of inhibition of post-incision phase of BER. Reproducibility of the assay was tested by independent repeat of measurement for randomly chosen 25 samples.

NER-specific assay: Each extract was in parallel incubated for 30 minutes with UV-treated and untreated TK6 cells and used for background subtraction. UV substrate incubated with T4 endonuclease V was used as positive control and 1:4 buffer A + buffer B as negative control. Aphidicolin (DNA polymerase delta inhibitor; Sigma-Aldrich) at a concentration of 2.5 µM was added to the extract to test the effect of DNA resynthesis inhibition. Reproducibility of the assay was tested by independently repeated measurement of randomly chosen 25 samples.

Single cell gel electrophoresis: After the incubation period, the protocol followed was the same as previously described for the comet assay (23). In brief: slides were treated for 20 min under alkaline conditions (300mM NaOH, 1mM EDTA, pH 12) to allow DNA denaturation and subsequently electrophoresed for 20 min, at 25V and 300mA. Washing with PBS, H₂O and ethanol followed, each for 10 min.Slides were stained with SybrGold (Invitrogen) and evaluated with a Nikon fluorescence microscope. Comets were scored exclusively by one person. DRC data were evaluated as tail DNA% (%T).
Gene expression profiling

Gene selection: A panel of BER and NER genes (supplementary Table 1) was selected from the list of all DNA repair genes organized according to pathways, which are available online (24).

Sample preparation: Total RNA was measured on ASP-3700 Spectrophotometer (Avans-Biotechnology) for quantity and OD<sub>260/280</sub> ratio, which was between 1.8 and 2.0. RNA integrity number (RIN) was checked using Agilent Bioanalyzer 2100, with RNA 6000 Nano Assay (Agilent Technologies). Each pair of tumor/healthy tissue did not differ by more than ±2 RIN units. cDNA was synthesized from 1 µg of RNA using a RevertAid™ First strand cDNA synthesis kit (MBI Fermentas) using random hexamers and following the manufacturer’s instructions. All samples were tested to exclude possible inhibition of the qPCR reaction by spiking with DNA from an extraction control kit (Primer Design). cDNA was diluted to 10 ng/µL and preamplified for 18 cycles on a Bio-Rad CFX96 Real Time PCR Instrument (Biorad) with TaqMan Preamp Master Mix (Applied Biosystems) according to the manufacturer’s protocol.

High-throughput real-time PCR: qPCR was performed using the high-throughput platform BioMark™ HD System (Fluidigm). Five µL of Fluidigm sample premix consisted of 1 µL of 20x diluted preamplified cDNA, 0.25 µL of 20x GE sample loading reagent (Fluidigm), 2.5 µL of Taqman universal mastermix II without UNG (Life Technologies) and 1.25 µL of RNase/DNase-free water. Each sample premix was combined with 5 µL FAM-MGB assays (Primer Design) at a final concentration of 300 nM and 2.5 µL 2x Assay loading reagent (Fluidigm). The reaction volume for a single qPCR reaction was 6.7 nL. Thermal conditions for qPCR were: 95°C for 10 min, 45 cycles of 95°C for 15 s and 50°C for 60 s. TOP1 and 18S rRNA were reference genes selected from a geNorm™ reference genes selection kit (Primer Design) by Normfinder (GenEx Enterprise).

qPCR data processing: Data were collected from two GE Dynamic Arrays 96.96 (Fluidigm) and preprocessed in GenEx Enterprise software (MultiD). Inter-plate calibration was performed and the technical replicates were averaged. Cut off value for Cq was set at 25 and values higher than that were replaced by the Cq value of 25. The Cq 25 measured in BioMark system would approximately correspond to Cq 35 at the conventional qPCR cyclers (25). When more than 12% of the data were missing for each sample/gene due to a very low
expression and low fluorescence signal, the particular sample/gene was removed from the
dataset. Due to this selection, 6 repair genes (CSA, MMS19L, POLB, UNG, XPG and XRCC1)
were excluded from analyses. The rest of the missing data was replaced through an
interpolation, i.e. the missing data were replaced with values calculated from the other
genes in the group of selected samples by GenEx algorithm. Data were normalized to
reference genes, recalculated to relative quantities with the lowest expression set to 1 and
transformed to log2 scale. This format was used for visualization of data in plots and for
further statistical analysis.

Promoter CpG islands methylation profiling

Methylation specific PCR (MSP): A Prediction of CpG island site within the promoter region of
target genes was carried out by screening with CpG Islands Searcher (26). Genomic DNA was
treated with sodium bisulfite using the Epitect Whole Bisulfitome Kit (Qiagen). MSP analysis
of bisulfite converted (BC) DNA was performed using the Epitect MSP kit, following the
producer’s instructions. Primers specific to methylated and unmethylated BC DNA for OGG1,
ERCC1 and XRCC1 genes were designed by applying MethPrimer algorithm (27) and
produced by Sigma-Aldrich. Previously described primers were used for XPA, XPC, XPD and
XPG genes (28) (supplementary Table 2).

Methylation-sensitive high resolution melting (MS-HRM): MS-HRM was conducted to verify
MSP-positive samples. Primers specific for BC DNA (supplementary Table 3) were designed
using Methyl Primer Express Software v1.0 (Applied Biosystems). Real-time PCR followed by
HRM was carried out with the high-performance Eco Real-Time PCR system (Illumina). The
reaction in a final volume of 10 µl consisted of 10 ng of template, 1x EpiTect HRM Master
Mix (Qiagen) and 300 nM of each primer. PCR conditions were: 95°C for 5 min, 50 cycles at
95°C for 10 s, 57°C for ERCC1, 56°C for XPC and 58°C for OGG1 for 20 s and 72°C for 10 s.
HRM thermal profile was set up according to the manufacturer’s recommendations
(Qiagen).

Statistical analysis

Statistical analysis was performed by IBM SPSS Statistics 18, GenEx Enterprise and SAS 9.2
software. All investigated parameters showed normal distribution in the study population
(Kolmogorov-Smirnov test), with the exception of expression data, which were logarithmically transformed. Principal component analysis of the expression analysis indicated that data from different hospitals needed to be adjusted by the vector error correction model according to the Granger representation theorem. After processing, two-tailed T-test or ANOVA for differences between groups were employed. Correlations were determined by a Pearson test. All statistical tests were performed at a 95% confidence level; expression data were corrected for multiple testing analyses according to the method of Dunn-Bonferroni (significant p-value after correction was 0.0021).
3. Results

**BER-specific DNA repair capacity (BER-DRC)**

DRCs were measured in matched pairs of tumor and adjacent healthy tissue of all 70 patients. For 28 individuals, DRC were simultaneously assessed in PBMC.

The BER-specific DRC assay showed a high degree of reproducibility, as repeated analysis in 25 samples showed Pearson's correlation coefficient of $R=0.75$. The presence of PARP inhibitor in the reaction did not influence the incision activity of the 8 analyzed extracts (with or without inhibitor: $R=0.92$).

The difference in BER-DRCs between tumor and healthy tissues was not statistically significant (mean±SD; 17.7±8.3 vs. 15.7±9.6 %T; $p=0.22$). However, we observed that PBMC showed significantly decreased ability to repair oxidative damage compared to healthy or tumor tissues (8.4±6.3 vs. 16.2±10.4 and vs. 17.1±8.9 %T respectively, $p=0.001$). There was a strong correlation in BER-DRCs between tumor and healthy epithelium ($R=0.57$, $p<0.001$). Similarly, a significant correlation was observed for BER-DRCs between PBMC and healthy epithelium ($R=0.48$, $p=0.009$), but not between PBMC and tumor tissue ($R=0.26$, $p=0.16$).

Results for BER-DRC are presented in Figure 1.

Sex and age did not influence BER-DRC, and nor did the localization of tumors; DRC values were 18.4±9.0 %T for colon and 16.7±9.2 %T for rectum, $p=0.45$. No statistical significance was observed in BER-DRC based on pathological stage of the tumors. For TNM 1 and 2 combined, we observed BER-DRC of 16.2±10.2 %T (N=36), while for diffused stages 3 and 4 it was 19.4±7.4 %T (N=34), $p=0.14$. Distribution of the BER-DRC in different grades of the tumors was 16.2±9.3 %T for grade 1, 17.7±9.1 %T for grade 2, and 19.6±8.8 %T for grade 3, $p=0.66$.

**NER-specific DNA repair capacity (NER-DRC)**

We also observed high reproducibility in DRC assay for NER (N=25, $R=0.62$), and the incision activity of the extracts did not change in the presence of the DNA polymerase inhibitor (N=8, $R=0.84$).

Tumor tissues exhibited significantly higher NER-DRC than healthy epithelia (20.2±11.6 vs. 15.4±10.8 %T, $p=0.019$). The lowest NER-DRC was detected in PBMC compared to both healthy and tumor tissues (6.1±5.0 vs. 17.7±14.2 vs. 24.3±13.0 %T, $p<0.001$). Similarly to
BER-DRC, a correlation between tumor and healthy tissues in NER-DRC was observed (R=0.58, p<0.001), and also between PBMC and healthy tissues (R=0.51, p=0.006), and PBMC and tumor tissues (R=0.47, p=0.011). Results for NER-DRC are presented in Figure 2. Interestingly, BER-DRC and NER-DRC showed a mutual correlation in healthy epithelium (R=0.32, p=0.007; Figure 3).

Sex and age did not significantly influence NER-DRC, which was also very similar irrespective of tumor localization, with values of 20.0±12.9 %T in the colon and 20.5±11.4 %T in the rectum, p=0.89. Tumors in non-invasive stages TNM 1 and 2 exhibited 22.0±12.2 %T, while 18.3±12.3 %T was detected in more diffused stages TNM 3 and 4, p=0.22. We did not observe any apparent effect of tumour differentiation on NER-DRC. Well differentiated tumor tissues had NER-DRC of 20.3±12.6 %T, tumors with moderate differentiation 18.9±11.2 %T and those with poor differentiation 25.5±15.9 %T, p=0.29.

Gene expression profiling
Successful expression analyses were performed in 53 pairs of tumor/healthy tissue. In total expression levels were determined for 8 BER and 17 NER genes (listed in Table 1). We observed a statistically significant correlation in levels of expression of all genes between paired tumors and healthy tissues (overall p<0.001). We found decreased transcription levels of BER genes NEIL1 and OGG1 and NER genes CSB, CCNH and XPA in tumor tissues compared to controls. In contrast, APEX1 and PARP1 (BER) and XPD (NER) showed higher expression in tumor tissues than healthy tissues. Although changes in expression of DNA repair genes between the healthy mucosa and tumor tissue were small (1.08-1.28-fold), they were significant (p<0.05). Individual gene p-values and fold changes of transcript levels in tumors relative to healthy tissues are reported in Table 1. No correlation was detected between DRC and individual gene expression levels, either for BER or for NER. Expression of studied genes was not influenced by any recorded clinic pathological parameter. The expression pattern of excision repair genes was similar irrespectively of tumor localization in colon or rectum and no modifying effect was exerted by TNM stages and tumor differentiation.
Promoter CpG islands methylation profiling

CpG promoter methylation status of OGG1, ERCC1, XPA, XPC, XPD, XPG and XRCC1 was evaluated in DNA from 70 tumor samples. MSP showed aberrant methylation of XPC, ERCC1 and OGG1 in 24, 56 and 51% of tumors, respectively. However, these findings were not confirmed by MS-HRM analysis, which clearly showed only non-methylated cytosines in the analyzed promoter sequences for all three genes (Figure 4).
4. Discussion

In this study, we investigated the possible involvement of the BER and NER alterations in the sporadic colorectal carcinogenesis by comparing tumors with adjacent healthy tissues in 70 patients. Both repair pathways are fundamental for the removal of a vast spectrum of DNA lesions and they process DNA damage in a broadly similar way by cleaving the damaged site and leaving DNA breaks behind. These breaks reflect the excision phase of the repair process that has been recognized as the rate-limiting step (29), and is measurable by the well-established comet assay technique. In order to assess DRC, diversely modified comet-based assays have been developed (30). Based on recently published methodological reports on BER- (20) and NER-specific assays (21), in this study we employed an in vitro repair assay, adapted for the evaluation of DRC in solid tissues. Langie et al. developed in vitro assays to measure DRC in animal tissues. We, for the first time, applied that approach to investigate DRC in human colon biopsies after carrying out the necessary optimization and validation experiments. Reproducibility was tested by repeating the assay on 25 duplicate samples at different times. Both BER- and NER-specific DRC assays showed high degrees of reproducibility. We also tested whether the results truly reflect the excision process of DNA repair and are not influenced by ongoing resynthesis and ligation. There was no detectable difference between DRCs of extracts with or without addition of inhibitors of polymerization; ABT-888, specific for BER, and aphidicolin for NER. In order to process a large number of samples, we utilized a medium-throughput 12-gel format (22) which proved to be efficient and gave consistent and reliable results. The assay is versatile and suitable for application in large molecular epidemiological studies. Additionally, the assay appears to be sensitive enough to detect the substantial inter-individual variability of both DNA repair capacities, with yet unknown biological relevance as this is one of a few pilot reports on this topic.

In a comparison of 70 matched sets of tumor/normal tissues, we found a significant increase of 24% in NER-DRC in tumors. The differences in BER-DRC between tumor and healthy tissues were not significant. Interestingly, we observed significant correlations of both DRCs as well as comparable expression profiles of all analyzed genes in healthy and tumor tissues of the investigated CRC patients, indicating distinct individual traits of excision repairs that are not driven by malignant transformation. We are aware of the likelihood of healthy tissue
phenotype being influenced by the tumor tissue, considering the relatively short distance between the origins of two specimens. However, the distance between healthy and tumor tissues was limited by the conventional surgical practices and requirement to have both specimens from the same bowel segment. The histological examination excluded any contamination of the healthy tissues by the tumor cells. To the best of our knowledge, there is only one study with design similar to ours that investigated 23 pairs of tumor/adjacent healthy tissues of CRC patients for NER-DRC only. The authors reported, in agreement with our findings, a strong correlation between DRCs in two tissues; however, NER activity in tumor tissue was increased ($p=0.015$) (31). Several studies have inferred higher BER or NER capability in tumors via an indirect approach of measuring the steady state level of DNA damage, assuming that a low damage level reflects a high repair rate. All those studies reported a significantly lower level of specific damage in DNA from tumors, presumably explained by up-regulation of repair (32-34). No study has found evidence for deficiency of excision repair pathways in tumors. The consistency of the listed observations might lead to the conclusion that excision repair is not a factor contributing to malignant transformation, but most likely it is contributing to the growth advantage of existing tumor mass by decreasing the vulnerability to DNA damage accumulation normally followed by cell death. In this regard, Sarasin and Kauffmann hypothesized that relative genetic stability given by upregulation of DNA repair might be associated with the higher ability of cells to metastasize (35). The observed positive correlation between BER and NER in the healthy tissue, although disrupted in the tumors, is not so surprising in the light of still growing evidence of functional cross-talk between BER and NER factors. It has been documented that the XPC-HR23B complex regulates the loading and turnover of OGG1 (36). It also appears that, in the case of OGG-/- knockouts, 8-oxoG repair has a backup mechanism involving NER proteins (37).

In our previous case-control study, we reported a reduced NER-DRC in PBMC of incident CRC patients with no family history of this disease as compared to a healthy population (19). This is consistent with many other investigations on various cancers. But are PBMC a valid cell type to study in relation to CRC? There is a belief that PBMC may represent the general condition of the organism and specifically reflect individual DRC. Accordingly, in the current study we attempted to test that hypothesis by comparing DRC in PBMC, tumor and normal
colon epithelium. To ensure the validity of the comparison, we assayed all studied tissues at equal protein concentration. For both excision repair pathways, PBMC exhibited on average approximately 3-fold lower DRC than either healthy or tumor tissue. This finding is somehow understandable considering that PBMC, if un-stimulated, do not divide and therefore do not require the pool of repair proteins in contrast to constantly reproducing cells, such as colon epithelial cells (38). Furthermore, in non-replicating cells, NER is carried out predominantly by transcription-coupled repair with suppressed global genome repair (39). Interestingly, despite the difference in repair capacities between colorectal epithelial cells and blood cells, there was a clear positive correlation between their repair capacities. This shows that DRC measured in blood cells does indeed reflect the repair potential of the cancer target tissue (40). PBMC, technically easy to obtain, might thus provide a useful index of individual DRC in comparative population studies.

Expression levels of the analyzed genes correlated positively between the normal and tumor tissues. Although some genes within both pathways were observed to be up or down-regulated in tumors, this difference was relatively modest, never exceeding 1.3-fold. It is questionable whether these nuances in gene expressions might have any fundamental functional consequences. In fact, many potential biomarkers have failed because they showed only a slight change in expression in cancer compared to normal tissue or their cognate protein levels did not correlate with transcript levels (41). In our study, the transcript level of the major BER player, 8-oxoguanine DNA glycosylase (OGG1) was observed to be significantly lower in tumors compared to normal tissues. However, a 1.2-fold change in expression did not cause any difference in its activity (BER-DRC, measured with substrate DNA presenting 8-oxoguanines, reflects predominantly the activity of OGG1 protein). Moreover, no correlation between mRNA level of OGG1 and its enzymatic activity was observed. On the other hand, overall NER-DRC comprises the joint performance of many genes. In this case, the repair process relies on the formation of protein complexes that assemble at the site of the DNA lesions and facilitate their removal in a coordinated fashion. The expression level of none of the NER genes showed any correlation with NER-DRC. Moreover, according to the expression profiling, there were several genes up- or down-regulated in tumor cells while the final repair ability was enhanced. Thus, individual gene
expression levels did not prove to be sufficiently informative about the overall DRC and measurement of enzymatic activity which can presumably give more relevant and interpretable information than can individual transcript measurement. In this respect, several other studies have reported an inconsistency between transcript level and respective protein quantity (42), or actual protein/pathway activity (19, 43-45).

In CRC, epigenetic alteration of gene expression, the so-called CpG island methylator phenotype, is known to affect several DNA repair genes (*MLH1*, *MSH2*, *MGMT*) (46). DNA hypermethylation is often observed as a targeted event in tumor cells, resulting in loss of gene expression. BER and NER genes have been described to be aberrantly methylated in a variety of cancers, such as *OGG1* in thyroid, *XPC* in bladder, *XPG* in ovarian or *ERCC1* in glioma as well as in some CRC cell lines (15). In the present study, we investigated the CpG island methylation status of core BER and NER genes in colorectal carcinomas. Neither BER genes (*OGG1* and *XRCC1*) nor NER genes (*XPA, XPC, XPD, XPG* and *ERCC1*) were hypermethylated in tumor cells. In the light of gene expression data that showed fairly similar levels of gene transcripts between tumor and control tissues, these findings are coherent. Nevertheless, we have observed a relatively high frequency of false-positive signals provided by MSP analysis that have not been confirmed by subsequent verification by MS-HRM. Inaccuracy of MSP outcome might be for various reasons such as an incomplete bisulfide conversion which might result in an overestimation of DNA methylation (47). Another possible source of discrepancies may be allelic dropout or semi-allelic methylation (48). Therefore, MSP does not seem to be reliable enough for methylation studies and a subsequent verification of its results by supplementary techniques is desirable (49).

**Conclusion**

A complex analysis of BER and NER processes by a functional, genetic and epigenetic approaches demonstrated that colorectal carcinomas are only moderately altered in these repair pathways. BER-DRC did not differ from adjacent healthy epithelium, while NER-DRC showed moderate up-regulation in tumors. Thus, alterations of excision repair capacities may not be the major driving events in the malignant transformation of human colon or rectum, but they might influence chemical sensitivity of the tumor cells to antineoplastic
drugs. From a methodological point of view, DRC represents a complex marker for functional evaluation of multi-gene DNA repair processes. In particular, the BER- and NER-specific in vitro repair assays employed in this study have proven to be highly informative and applicable for high-throughput screening in molecular epidemiological investigations.

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Conflicts of interest
No conflicts of interest are disclosed.
References

36. D'Errico M, Parlanti E, Teson M, de Jesus BM, Degan P, Calcagnile A, et al. New functions of XPC in the protection of human skin cells from oxidative damage. EMBO J. 2006;25:4305-15.
Table 1. Fold-change differences in expression level of BER and NER genes in tumor relative to healthy epithelium. Significant differences are in bold, differences significant after Dunn-Bonferroni correction (p <0.0021) are underlined.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pathway</th>
<th>Fold change</th>
<th>p-value</th>
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<tr>
<td>NEIL1</td>
<td>BER</td>
<td>-1.26</td>
<td>0.0004</td>
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<td>APEX1</td>
<td>BER</td>
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Legend to figures:

**Figure 1.** Differences in BER-DRC of tumor and healthy tissue (A); correlation of BER-DRC between tumor and healthy tissue (B); differences in BER-DRC between tissues and PBMC (C); correlation of BER-DRC between healthy tissue and PBMC (D).

**Figure 2.** Difference in NER-DRC of tumor and healthy tissue (A); correlation of NER-DRC between tumor and healthy tissue (B); differences in NER-DRC between tissues and PBMC (C); correlation of NER-DRC between healthy tissue and PBMC (D).

**Figure 3.** Correlation between BER-DRC and NER-DRC in 70 healthy colorectal tissues.

**Figure 4.** Example of 6 samples analyzes for ERCC1 promoter methylation by MSP (A) and by MS-HRM (B). Positive signals from MSP (arrows) have not been confirmed in MS-HRM.

NOTE: (A) Pc, positive control of fully methylated DNA; Nc, negative control of fully unmethylated DNA; Bc, nonbisulfite converted DNA; U, primers specific to unmethylated sequence; M, methylation specific primers. (B) Fluorescence of each sample was normalized against 100% methylated DNA control. Percentage shows positive controls of 100, 75, 50, 25 and 0% methylated DNA. For all 6 samples, 0% methylation was detected.
Figure 1

A

B

C

D

Base excision repair (tail DNA%)

Tumour tissue

Healthy tissue

Healthy tissue (tail DNA%)

Tumour tissue (tail DNA%)

N=70
p=0.22

R=0.57
p<0.001
R²=0.33

N=28
p=0.001

R=0.48
p=0.009
R²=0.23

Base excision repair (tail DNA%)

Tumour tissue

Healthy tissue

PBMC

Healthy tissue (tail DNA%)

Blood mononuclear cells (tail DNA%)
Clinical Cancer Research

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