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(Article begins on next page)
Post-Treatment Recovery of Suboptimal DNA Repair Capacity and Gene Expression Levels in Colorectal Cancer Patients

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DNA repair in blood cells was observed to be suboptimal in cancer patients at diagnosis, including colorectal cancer (CRC). To explore the causality of this phenomenon, we studied the dynamics of DNA repair from diagnosis to 1 yr follow-up, and with respect to CRC treatment. Systemic CRC therapy is targeted to DNA damage induction and DNA repair is thus of interest. CRC patients were blood-sampled three times in 6-mo intervals, starting at the diagnosis, and compared to healthy controls. DNA repair was characterized by mRNA levels of 40 repair genes, by capacity of nucleotide excision repair (NER), and by levels of DNA strand breaks (SBs). NER and base excision repair genes were significantly under-expressed \( P < 0.016 \) in patients at diagnosis compared to controls, in accordance with reduced NER function \( P = 0.008 \) and increased SBs \( P = 0.015 \). Six months later, there was an increase of NER capacity, but not of gene expression levels, in treated patients only. A year from diagnosis, gene expression profiles and NER capacity were significantly modified in all patients and were no longer different from those measured in controls. All patients were free of relapse at the last sampling, so we were unable to clarify the impact of DNA repair parameters on treatment response. However, we identified a panel of blood DNA repair-related markers discerning acute stage of the disease from the remission period. In conclusion, our results support a model in which DNA repair is altered as a result of cancer. © 2014 Wiley Periodicals, Inc.

Key words: Colorectal cancer; DNA instability; DNA repair; biomarker; anti-cancer therapy; follow-up study

INTRODUCTION

Colorectal cancer (CRC) is one of the most frequently occurring malignancies in Western countries. The genetic basis of hereditary forms (≈6% of all cases) is relatively well explored while for sporadic forms (≈75% of all cases) there is still a lack of knowledge about the genetic-epigenetic-environmental triggers of the disease. More importantly, CRC has high mortality worldwide due to the insufficient treatment efficacy and a lack of predictive markers [1]. Only approximately half of CRC patients will be cured using currently available therapies [2]. A commonly used conventional regimen for CRC treatment is based on the synergistic action of two DNA-damaging agents: 5-fluorouracil (5FU) and oxaliplatin. In response to this treatment, cells activate a range of resistance-promoting mechanisms including the DNA repair pathways [3]. Some of the optimal biomarkers might be thus found within the DNA repair network.

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The present study represents a continuation of our previous report, where we showed that peripheral blood mononuclear cells (PBMCs) collected from CRC patients at the time of diagnosis exhibited elevated genetic instability. Specifically, they had increased levels of DNA strand breaks (SBs) and low DNA repair capacity accompanied by altered expression of repair genes [4]. The suboptimal DNA repair capacity in PBMCs of cancer patients is now well documented and observed in multiple cancer types [4]. Thus, there is a large body of evidence obtained in case-control studies supporting the association between cancer and a decrease in the ability of blood cells to protect against DNA damage. However, a sampling of the patients at one time point does not bear information about the causality of this phenomenon and so it remains to be established whether low DNA repair is one of the susceptibility factors for sporadic CRC, or it is a consequential effect of the disease. On the other hand, it is known that DNA repair capacity detectable in PBMCs is significantly related to the repair capacity of colorectal tissue [5,6]. These observations give more confidence for using blood as a surrogate for cancer-target tissue to study DNA repair involvement in CRC. Subsequently, a few recent studies provided evidence that tumor tissue maintains comparable base excision repair (BER) capacity and slightly elevated capacity of nucleotide excision repair (NER) as compared to adjacent healthy colorectal mucosa, although there is a differential mRNA expression of DNA repair genes [6–8]. Impaired excision repair, thus, may not contribute to the malignant transformation of the colon, but rather might be involved in the treatment response of the patients.

Conventional therapy of CRC, based on the mutagenic properties of anticancer drugs, is expected to be more harmful to fast growing tumor cells than to normal cells. However, details of the effects of radiotherapy and chemotherapy on the cellular and molecular functions of the tumor and normal cells are still unexplored. Almost all DNA repair pathways are presumably involved in the cellular response to CRC treatment. SPU-mediated DNA lesions are recognized by the BER and the mismatch repair (MMR) systems [9]. Oxaliplatin binds to nucleobases forming intra- and inter-strand crosslinks. The former are eliminated mainly by the NER pathway while the latter require NER together with cross-link repair activity, translesion synthesis, and homologous recombination repair (HR) [10]. Understanding the involvement of DNA repair processes in the response of cancer cells to antineoplastic drugs is crucial for the design of improved therapy regimens and for the prediction of therapeutic response in CRC.

The present exploratory study was aimed to address several questions: (i) Do variations in DNA repair contribute to the risk of developing sporadic CRC, or are they rather a consequence of the systemic disease? (ii) Is the DNA repair response to CRC treatment measurable at the mRNA/functional level? (iii) Is it induced or suppressed by the systemic genotoxic exposure mediated by chemotherapy? In an attempt to answer these questions, we have designed a prospective study in which sporadic CRC patients were blood-sampled at the diagnosis (i.e., active disease), 6 mo, and 1 yr later (i.e., covering the tumor resection, administration of chemotherapy, either neoadjuvant or adjuvant, and remission) and were compared with an healthy population. The dynamics of DNA repair over a 1 yr period and with respect to ongoing CRC treatment were analyzed. All main repair pathways including excision repair (BER, NER, and MMR), repair of double-strand breaks (HR and non-homologous end joining), and DNA damage response (DDR) were characterized at gene expression levels. NER, the most deregulated pathway in patients observed by us, was also studied using a functional assay and through measuring SBs accumulation.

MATERIALS AND METHODS

Study Population

Incident CRC cases were recruited in the Czech Republic between 2008 and 2010. Patients were newly diagnosed and histologically confirmed for CRC. Eighty-three patients were initially recruited for the study but only 39 (47%) attended all the three planned blood samplings. Reasons for dropout were: (i) 7 (8%) patients died before third sampling, (ii) 10 (12%) patients moved and were treated in other hospitals or interrupted therapy for unspecified reasons, (iii) 19 (23%) patients missed one out of the three samplings or did not attend regular hospital follow-up at agreed times, or the material provided did not meet the quality standards required, and (iv) 8 (10%) patients were excluded because they were found not to be primary cancer cases. No other selection of patients was performed. Forty-seven controls were recruited among individuals who had never been diagnosed with any cancer and did not manifest any relevant systemic disease, nor had any known significant exposure to potentially harmful chemicals. Study subjects were all of Caucasian origin. They signed a written informed consent with the study in accordance with the Helsinki declaration. The Ethics committees of participating hospitals approved the study. Trained personnel interviewed the study subjects using a structured questionnaire for lifestyle habits, body mass index, diabetes, and family/personal history of cancer. Expression analysis and functional assays could not be performed on all patients, due to various reasons, that is, viability of PBMCs or quality of RNA. For each analysis, the actual number of examined cases is therefore specified.

Study Design

Blood samples were collected from patients three times: at diagnosis, that is, before tumor resection and
administering any therapy (T0); 6 mo after tumor resection, that is, approximately 6 mo from administration of chemotherapy (T1); and after 1 yr, during the regular follow-up appointment (T2). At T1 all patients had completed the planned chemotherapy, except for seven patients who received chemotherapy for additional 1 or 2 mo. In eight patients, no therapy was administered. At T2, all patients were free of relapse.

Blood Processing

Blood samples were drawn into heparin and EDTA vacutainers, and kept at 4 °C until processed within 3 h. PBMCs from EDTA tubes were isolated on Ficoll–Paque PLUS (GE Healthcare Life Sciences, Prague, Czech Republic) and stored in TRIzol (Invitrogen, Carlsbad, CA) at −80 °C until RNA extraction. PBMCs from heparin tubes were isolated on Histopaque-1077 (Sigma–Aldrich, St. Louis, MO), counted, suspended in full medium (RPMI 1640, 2 mM l-glutamine, 10% FBS, 0.2% penicillin/streptomycin, 1.5% phytohemagglutinin) and incubated at 37 °C for 20 h to stimulate mitosis. PBMCs were then processed for DNA damage and DNA repair analysis.

DNA Strand Breaks

The level of SBs in DNA was evaluated by the alkaline comet assay [11]. Experimental conditions were as follows: lysis (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% TritonX-100, 10% DMSO, pH 10, >1 h, 4 °C), alkali treatment (300 mM NaOH, 1 mM EDTA, pH 13, 20 min, 4 °C), electrophoresis (300 mM NaOH, 1 mM EDTA, pH 13, 20 min, 4 °C, 1.3 V/cm), and neutralization (0.4 M Tris–HCl, pH 7.5, 2 × 10 min). Data are reported as tail DNA%, determined immediately after the BPDE treatment, and the highest level of SBs detected within 4 h of culturing. Data were pre-processed with GenEx Enterprise. DNA damage and DNA repair capacity were consistent with the Gaussian distribution, and so were the gene expression data when log transformed. Student's t-test, paired t-test, ANOVA and Pearson's rank correlation coefficient were used to compare means and calculate bivariate correlations. Statistical tests were performed at 5% level of statistical significance.

Gene Expressions

A panel of 40 genes (Supplementary Table S1) were selected from the list of all known DNA repair genes [12]. Total RNA was isolated using TRIzol (Invitrogen). RNA integrity was between 8.0 and 10.0 units. cDNA was synthesized from 0.5 μg of RNA using the RevertAid™ First strand cDNA synthesis kit (Fermentas, Ontario, Canada). cDNA was preamplified and qPCR was performed using the BioMark™ HD System (Fluidigm) and using FAM-MGB assays (Primer Design, Southampton, UK) as described previously [6]. TOP1 was the reference gene selected by Normfinder using GenEx Enterprise software (MultiD, Goteborg, Sweden). Data were converted to relative quantities and transformed to log2 scale. The repeatability of gene expression assays was calculated by mixed ANOVA, comparing the values obtained in two different experiments in 2 days for each assay. All assays showed high degree of precision. The results are reported in Supplementary Table S1 for each assay separately.

Data Analysis

Statistical analysis was conducted using R environment version 2.15 (open tool). Gene expression data were pre-processed with GenEx Enterprise. RNA integrity was between 8.0 and 10.0 units. cDNA was synthesized from 0.5 μg of RNA using the RevertAid™ First strand cDNA synthesis kit (Fermentas, Ontario, Canada). cDNA was preamplified and qPCR was performed using the BioMark™ HD System (Fluidigm) and using FAM-MGB assays (Primer Design, Southampton, UK) as described previously [6]. TOP1 was the reference gene selected by Normfinder using GenEx Enterprise software (MultiD, Goteborg, Sweden). Data were converted to relative quantities and transformed to log2 scale. The repeatability of gene expression assays was calculated by mixed ANOVA, comparing the values obtained in two different experiments in 2 days for each assay. All assays showed high degree of precision. The results are reported in Supplementary Table S1 for each assay separately.

RESULTS

Study Population

Patients and controls did not differ significantly for any recorded confounders, except for family history of cancer, which was more prevalent among patients (P = 0.01). The clinical, biological, and lifestyle
characteristics of the study population, as well as an overview of the administered therapies, are summarized in Table 1. All patients underwent surgical resection of the tumor. Eighteen patients (46%) received neo-adjuvant therapy prior to resection. Neoadjuvant therapy included the following regimens: two patients received radiotherapy (6 MeV X-rays, 45–46 Gy total dose in 23–25 fractions), three patients received pre-operative chemotheraphy without radiotherapy (FOLFOX or capecitabine), and 13 patients received a combined regimen. Twenty-two patients (56%) received adjuvant systemic therapy; nine of them were given 5FU-based therapy, while 13 received a combination of 5FU and oxaliplatin. Adjuvant therapy was given over a period of 1–8 mo. Tumor relapse after the treatment was not detected in any of the patients. Eight patients did not receive any systemic treatment.

Case–Control Study

DNA repair genes expression

Twenty-seven CRC cases and 38 controls were analyzed for the expression of 40 DNA repair genes, sampled at diagnosis and before any treatment intervention (T0). Patients had different expression levels of BER and NER genes compared to controls: four out of nine BER genes (APEX1, NTHL1, PARP1, and MPG) and 10 out of 19 NER genes (RPA1, RPA2, RPA3, CDK7, DDB1, DDB2, XPA, XPD, ERCC8, and RAD23B) had significantly decreased levels in patients. Only LIG3 (BER) was increased in patients as compared to controls. Fold-changes and P values are reported in Supplementary Table S2. The dendogram in Figure 1, performed by HCL, discriminates between patients and controls by expression profiles of all 40 repair genes. The same discrimination was observed for the NER genes only (data not shown).

NER capacity and SBs

Functional assays were performed for 31 CRC cases and 38 controls. NER capacity was significantly lower in the CRC patients analyzed at T0 compared to the controls (mean ± SD; 9.9 ± 7.5 and 15.5 ± 13.6, respectively; t-test P = 0.008; Figure 2A), and the patients had significantly higher levels of SBs than

Table 1. Study Population Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Category</th>
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<th>Controls (n = 47)</th>
<th>P-value</th>
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<tr>
<td></td>
<td>Male</td>
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<tr>
<td>Age (years)</td>
<td>Mean ± SD</td>
<td>64.5 ± 10.5</td>
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<td>Median</td>
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<td></td>
<td>Range</td>
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<td>33–87</td>
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<td>Body mass index</td>
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<td>Range</td>
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<tr>
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<td>III</td>
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<td></td>
<td>5FU + oxaliplatin</td>
<td>13</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Significant differences are shown in bold.

Molecular Carcinogenesis
the controls (25.6 ± 21.4 and 13.9 ± 13.8, \( t \)-test \( P = 0.015; \) Figure 2B).

Longitudinal Study

Changes in DNA repair genes expression during 1 yr of follow-up

Twenty-seven patients analyzed at T0 were sampled two more times with at 6-mo intervals (T1 and T2). Expression of the majority of the studied genes did not change appreciably between T0 and T1 but substantially changed at T2. PCA of all 40 studied genes revealed similar expression patterns of samples obtained at T0 and T1 while the expression at T2 had a significantly different pattern and the samples were organized in a distinct cluster together with controls (Figure 3). To identify differentially expressed genes that best discriminate both clusters, dynamic PCA in combination with Kohenen SOM was used. Six genes (BER: LIAG3, NER: RPA3, CDK7, DDB2, HR: NBN, and DDR: CHEK2) were found to be responsible for the aggregation of patients into two distinct clusters (Figure 4).

DNA repair genes expression over time in relation to therapy

The \( k \)-means algorithm was used to correlate the gene expression levels over time to treatment (untreated vs. treated patients) and to the different regimens of adjuvant therapy (5FU vs. 5FU + oxaliplatin). Four main clusters were generated based on the \( C_q \) delta values representing the differences between T1–T0 and T2–T1 (Supplementary Figure 1). A trend line for the four clusters was constructed and each cluster was also represented by a heatmap showing for each gene the expression values over time. The heatmap revealed great variability in the expression dynamics of the studied genes, particularly in the T1–T2 interval. The expression of the majority of the genes did not differ between treated and untreated patients, except for three genes. The BER gene MUTYH mRNA levels increased in adjuvantly treated patients but was reduced in untreated. POLB (BER) and XPB (NER) had constant expression over time in treated patients, but were upregulated from T0 to T2 in untreated patients (Supplementary Figure 1). No differences were observed between therapy regimens of 5FU + oxaliplatin versus 5FU only.

Changes in NER capacity and SBs during 1 yr of follow-up

NER capacity and SBs were compared between the three samplings in a group of 27 patients. NER capacity increased significantly between T0 and T1, and did not change further between T1 and T2 (T0: 9.9 ± 7.5, T1: 15.8 ± 13.2, T2: 11.2 ± 6.9, ANOVA \( P = 0.002; \) Figure 2A). NER capacity at T1 and T2 did
not differ from that in the controls (t-test $P = 0.87$ and $P = 0.10$, respectively). SBs increased gradually, with the largest rise being observed between T1 and T2 (T0: 25.6 ± 21.4, T1: 29.2 ± 17.1, T2: 41.8 ± 12.8, ANOVA $P < 0.001$; Figure 2B). At all the three time points, SBs level was significantly higher in cases than in the controls (t-test $P < 0.001$). $P$ values for comparisons between each sampling in patients computed by paired t-test are reported in Supplementary Table S3.

NER capacity and SBs over time in relation to therapy

NER capacity increased over time in adjuvantly treated patients (ANOVA $P = 0.01$), while the trend in untreated patients was not significant (ANOVA $P = 0.08$; Figure 5A). This trend was significant also for patients receiving neoadjuvant treatment (ANOVA $P = 0.02$). No differences in NER capacity were seen after stratification for treatment regimen: SFU (ANOVA $P = 0.07$) and 5FU + oxaliplatin (ANOVA $P = 0.19$). SBs increased over time in all patients irrespectively of the treatment. Figure 5B displays SBs values over time in untreated (ANOVA $P < 0.001$) and adjuvantly treated patients (ANOVA $P = 0.05$), and the same trend of increasing SBs was observed for neoadjuvantly treated patients (ANOVA $P = 0.04$). By stratifying adjuvantly treated patients, it was found that the degree of SBs was not affected by SFU treatment (ANOVA $P = 0.78$), but increased with the SFU + oxaliplatin regimen (T0: 19.0 ± 22.4, T1: 28.6 ± 21.6, T2: 42.4 ± 14.1, ANOVA $P = 0.047$). Paired t-test $P$ values for comparisons between each sampling in patients stratified for different treatments are reported in Supplementary Table S3.

DISCUSSION

The present study was focused on characterizing differences in DNA repair between CRC patients and cancer-free population. Subsequently, defined impairment of DNA repair in cancer patients was
followed-up during a post-treatment period, with samplings at 6 mo after tumor resection and chemotherapy administration, and an additional sample 6 mo later. This study should reflect changes in the DNA repair proficiency in patients under the genotoxic stress of the anti-cancer treatment. We have characterized DNA repair processes in blood samples of CRC patients. PBMCs are considered to be representatives of the general condition of the organism and we have shown that their DNA repair capacity reflects that of colonic mucosa [6]. Therefore, PBMCs represent potential surrogates of CRC target tissue, with the great advantage that if repeated biopsies are difficult to obtain, repeated blood samplings are feasible and much better suited for therapy monitoring.

DNA Repair in Patients Versus Controls

In the present case–control study, we have identified genes that were differentially expressed between CRC patients at diagnosis and controls. Several BER and the majority of the studied NER genes were underexpressed in CRC patients. We evaluated NER also on functional level to determine if the reduced mRNA expression levels were concomitant with reduced functional capacity of the pathway. Decreased NER capacity in patients was confirmed and was accompanied by increased levels of SBs. These findings are consistent with our previous observations of CRC patients sampled at diagnosis having lower NER capacity and higher DNA damage than controls [4].

A large body of evidence is now available on impaired NER in patients diagnosed with different types of malignancies, as reviewed by us [4] and by [13]. However, these are case-control studies with no further indication on causality of this phenomenon in relation to cancer onset.

DNA Repair in Patients’ Follow-Up

We designed a longitudinal study to follow up the CRC patients from diagnosis to 1 yr in three different...
samplings obtained 6 mo apart. Interestingly, while expression profiles at diagnosis and 6 mo later were substantially similar for the 40 analyzed genes, significant changes in mRNA levels were observed at the third sampling obtained 12 mo after diagnosis. At that time point, patients were considered “cured” and there were no cases in the study group that relapsed. We can hypothesize that the expression profiles of DNA repair genes reflected the disease activity. Indeed, patients’ expression profiles a year from diagnosis were comparable to those of the healthy subjects. NER capacity showed a similar tendency. From reduced level in patients at the time of diagnosis, NER capacity increased during the post-treatment period between diagnosis and 1 yr later, eventually matching the level of the healthy subjects. Different results were obtained for the comparison of SBs levels overtime in patients. SBs were twofold higher in patients at diagnosis compared to controls, and their level further increased reaching threefold difference a year from diagnosis. This accumulation of SBs was independent of the presence of the tumor in the body as well as treatment with DNA-damage inducing drugs. Possibly some other clinical or biological parameters that we have not controlled for may be responsible for this late effect.

The dynamics of DNA repair capacity were previously studied in healthy individuals by comparing BER and NER activities in six repeated samplings over a 5-mo period [14]. The values positively correlated and the coefficient of variation (CV) was 27% for BER and 49% for NER, respectively. Intra-individual variability of NER capacity was evaluated also by us in a pilot study of 16 healthy subjects sampled twice 6 mo apart. Values of two independent samplings significantly positively correlated and the CV of 20% was much lower than the inter-individual variability observed in our recent study on 340 healthy individuals (CV 84% for BER and 90% for NER; unpublished data). Similar observations were also reported in the study by Vogel et al [15]. DNA repair capacity thus seems to be a characteristic parameter for each individual (but having a rather high variability across the general population). Here, we showed that this hypothesis based on healthy population is not the general population). Here, we showed that this hypothesis based on healthy population is not.

NER capacity was increased upon tumor resection in all patients, but this trend was statistically significant only in patients administered to chemotherapy. In this context it is noteworthy that the P values observed for the increase in DNA repair in untreated patients were of borderline significance. An increase in the probability of these findings by enlarging the study group cannot be ruled out. Therefore, it is difficult at present to clearly distinguish whether the enhancement of NER capacity is a consequence of surgical tumor elimination, a defense reaction of normal cells against a systemic treatment, or both. Mutagenic activity of radio- and chemotherapeutics poses a challenge to not only the tumor, but also normal cells, that need to adjust their cellular functions in order to protect their genetic integrity. Indeed, several studies have shown that DNA repair processes are induced by genotoxic stress [20–23]. In our study group, an increase in the NER capacity at the end of treatment was not accompanied by an increase in the NER genes transcription, except for XPF and XPG. These two might be the main activators of NER function. In fact, endonucleases are critical components of NER and their knockdown dramatically reduces NER activity [24]. In this context, we cannot neglect the role of post-transcriptional [25,26] and post-translational [27–30] modifications in DNA repair regulation. At the mRNA level, only 3 out of 40 genes showed differential behavior between treated and untreated subjects, and those were involved again in the BER and NER pathways. BER gene MUTYH (MutY Homolog E. coli) increased its...
expression over time in treated patients and remained stable in untreated patients. On the contrary, POLB (Polymerase Beta) involved in BER and NER gene XPB (Xeroderma Pigmentosum B) showed increased expression over time in untreated patients only. Although these differences did not seem to have any effect on the immediate response to therapy, it cannot be excluded that they may play some role in long-term survival. Above genes have been observed to be implicated in CRC development. Aberrant MUTYH glycosylase has been linked to one type of CRC [31], and POLB is mutated in about 50% of human cancers [32], including CRC [33]. Also, therapeutic downregulation of POLB activity was recently considered in order to meet better treatment response [34], and its mRNA levels have been proposed to be a prognostic indicator in CRC treatment [35].

So far, few studies have examined DNA repair in relation to anti-cancer therapy response and survival, but they usually sampled patients only once before therapy. Jewell et al. studied melanoma patients and reported that higher mRNA levels of DNA repair genes in biopsies were associated with a higher risk of relapse [36]. Wang et al. [37] found that elevated DNA repair activity in peripheral lymphocytes correlated with shorter survival in lung cancer. Similar tendencies were reported by Asakawa et al. [38] in breast cancer biopsies in which high DNA damage response was linked with poor response to neoadjuvant therapy. We were unable to analyze DNA repair parameters in relation to the patients’ response to therapy at endpoint since no post-treatment tumor relapse was observed in the study group. Considering the small size of the study population, further stratifications for specific treatments would not reach sufficient statistical power to draw strong conclusions.

In summary, we described the dynamics of DNA repair in blood cells of CRC patients in a time frame of 1 yr. DNA repair downregulation in the presence of active disease, as reflected by quantitative PCR and functional assays, was centered on two excision repair pathways—BER and NER. One year after the diagnosis and successful treatment, the downregulation was not detectable anymore, and the patients exhibited a molecular pattern of DNA repair similar to that of healthy controls. DNA repair markers evaluated in blood cells can be used to distinguish between an acute and a post-treatment cancer-free condition, thus reflecting the disease activity in CRC patients.

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REFERENCES


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