Increased DNA Integrity in Colorectal Cancer

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Abstract. Background: Blood-based DNA integrity, defined as the relation of long to small fragments of cell-free circulating DNA, is known to be increased in various types of cancers. Since different DNA fragments and formulae are used by different researchers, conflicting results on the relevance of this marker for cancer diagnosis have been reported. Patients and Methods: Sera from 24 patients with colorectal cancer, 11 patients with benign gastrointestinal diseases, and 24 healthy individuals were investigated. After DNA isolation, ALU repeats with 115 bp and 247 bp length were measured using quantitative polymerase chain reaction and resulting DNA integrities were calculated by the two formulae of Umetani et al. (DNA Int 1) and Wang et al. (DNA Int 2) Results: DNA integrity by both formulae correlated strongly with each other. DNA integrity was significantly higher in patients with colorectal cancer when compared with healthy controls (p=0.005 and p=0.006, respectively), while there was no significant difference from those with benign colorectal diseases. In receiver operating characteristic curve analysis, areas under the curve of 0.74 and 0.73 and sensitivities of 71% at 75% specificity (DNA Int 1 and 2, respectively) were achieved for the discrimination between patients with colorectal cancer and healthy controls. Conclusion: DNA integrity is significantly increased in patients with colorectal cancer and may be useful in prospective trials.

Cancer is one of the leading causes of death worldwide (1). Among all types of cancers in both sexes, colorectal cancer is the most frequent type and holds second place with regard to cancer mortality (2). In Europe, colorectal cancer is most deadly in the eastern part of the continent (2). As early stages of colorectal cancer usually do not lead to specific symptoms, colorectal cancer is often only diagnosed at advanced stages. From 2002 to 2008, only 39% of patients were diagnosed with colorectal cancer in an early, localized stage; 36% had regional and 20% had distant metastases (3). Therefore there is a need for new markers which will improve cancer detection. Blood-based tests are most promising, as drawing a patient’s blood is a simple and fast means of examination. Established serum tumour markers for colorectal cancer, carcinoembryonic antigen (CEA) and cancer antigen (CA) 19-9, are mainly used in cancer surveillance but have only limited use in early cancer detection and cancer screening as pointed-out by the guidelines of the European Group on Tumor Markers (4).

Cell-free DNA (cfDNA) circulating in the human blood has been suggested to be a promising tumor marker (5). However, its levels are also elevated in various other disorders such as infectious and autoimmune diseases, stroke, infarction and trauma (6). Therefore, more specific approaches such as measuring the integrity of cfDNA, which describes the relation between longer and shorter DNA fragments, have been proposed. This approach is based on the findings that cfDNA varies in length according to its mechanism of release from the cell: apoptosis, which usually takes place in normal tissues, results mainly in DNA fragments of 180 bp, whereas necrosis, which is the usual form of cell death in cancer tissues, produces longer fragments (7). In consequence, the DNA integrity as the ratio of longer to shorter fragments is purported to be increased in patients with colorectal cancer (8). Other groups failed to confirm these promising results in other cancer types (9-11). However, these studies also differed in the DNA sequences and algorithms used for the calculation of DNA integrity.

In the present study, we tested for the frequently used ALU115 and ALU247 fragments and compared the two most frequently applied formulae for DNA integrity calculation on their diagnostic relevance for discrimination of patients with colorectal cancer from healthy and benign control groups. Furthermore, other cancer groups were also included in the evaluation.
Patients. We analysed serum samples from 59 patients who were admitted to the University Hospital Munich, Grosshadern. Among them were 24 patients with colorectal cancer and, as controls, 11 patients with benign gastrointestinal diseases and 24 healthy donors. Patient characteristics are given in Table I. Blood was centrifuged within 2 h after venous puncture, and serum was stored at −80°C until measurement.

DNA isolation from serum. For DNA isolation from serum, we used the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) with 200 μl of serum following the manufacturer’s approved vacuum protocol. The isolated DNA was stored at −20°C until use.

Real-time quantitative polymerase chain reaction (PCR). Five microlitres of isolated DNA were each subjected to two different real-time quantitative PCRs to amplify ALU repeats of two different lengths. We used primer sets described by Umetani et al. (12) to produce amplicons of 247 bp and 115 bp. For the PCR, we used prepared water and Mastermix by Roche already containing a Taq DNA polymerase, adjusted reaction buffer, dNTP mix (with dUTP instead of dTTP) and SYBR Green I dye (LightCycler 480 SYBR Green I Master; Roche Diagnostics, Mannheim, Germany). We also added a heat-labile uracil–DNA glycosylase (UNG; Roche Diagnostics) to prevent carry-over contamination. Each final reaction mixture consisted of 5 μl template, 10 μl Mastermix, 0.5 U UNG and 2 μM of each primer (forward and reverse), and was filled with water to a total volume of 20 μl. We performed the PCR in a LightCycler® 480 Instrument II (Roche Diagnostics). The PCR program consisted of an UNG pre-cleaning step (pre-incubation at 40°C for 10 min and inactivation of UNG at 95°C for 10 min) and 45 cycles of amplification including denaturation at 95°C for 10 s, annealing at 62°C for 15 s and the extension at 72°C for 15 s. All experiments were performed at least twice and every plate contained internal controls and a serum pool (containing human genomic DNA). We focused on DNA integrity, which has already been reported for people in Europe and worldwide (2, 3). Established serum tumor markers such as carcinoembryonic antigen and CA19-9 are of limited use in cancer detection and surveillance (4).

Real-time quantitative PCR. Five microlitres of isolated DNA were each subjected to two different real-time quantitative PCRs to amplify ALU repeats of two different lengths. We used primer sets described by Umetani et al. (12) to produce amplicons of 247 bp and 115 bp. For the PCR, we used prepared water and Mastermix by Roche already containing a Taq DNA polymerase, adjusted reaction buffer, dNTP mix (with dUTP instead of dTTP) and SYBR Green I dye (LightCycler 480 SYBR Green I Master; Roche Diagnostics, Mannheim, Germany). We also added a heat-labile uracil–DNA glycosylase (UNG; Roche Diagnostics) to prevent carry-over contamination. Each final reaction mixture consisted of 5 μl template, 10 μl Mastermix, 0.5 U UNG and 2 μM of each primer (forward and reverse), and was filled with water to a total volume of 20 μl. We performed the PCR in a LightCycler® 480 Instrument II (Roche Diagnostics). The PCR program consisted of an UNG pre-cleaning step (pre-incubation at 40°C for 10 min and inactivation of UNG at 95°C for 10 min) and 45 cycles of amplification including denaturation at 95°C for 10 s, annealing at 62°C for 15 s and the extension at 72°C for 15 s. All experiments were performed at least twice and every plate contained internal controls and a serum pool (containing human genomic DNA). A linear standard curve generated through serial dilutions of human genomic DNA was used to quantify the amounts of ALU115 and ALU247. We focused on DNA integrity, which has already been reported for people in Europe and worldwide (2, 3). Established serum tumor markers such as carcinoembryonic antigen and CA19-9 are of limited use in cancer detection and surveillance (4).

Statistics. Results are presented as the median, interquartile range and whiskers representing total ranges except outliers. DNA integrity was calculated on one hand as the ratio of the concentration of ALU247 over ALU115 as described by Umetani et al. [DNA Int 1; (12)] and on the other hand using the formula of Wang et al. [DNA Int 2; (13)] in order to compare both methods. For DNA Int 2, ΔCp115 and ΔCp247 were calculated by subtracting the Cp value of ALU115 or ALU247 from the Cp value of the serum pool (which was run with every plate), then a ΔΔCp value was calculated by subtracting the ΔCp247 from ΔCp115. Finally, this ΔΔCp was inserted into the formula:

\[ \text{DNA Int 2} = e^{-\Delta\Delta C_p \times \ln(2)} \]

Correlations between the parameters were calculated by the Spearman test. Discrimination between two groups was calculated by Mann–Whitney test. Receiver operating characteristic (ROC) curves were established and the area under the curves (AUC) were calculated. A p-value of less than 0.05 was considered statistically significant. Calculations were carried out using SPSS statistics software (IBM, Armonk, NY, USA, version 21.0).

Results

When the group of patients with colorectal cancer was compared against healthy controls, there was a significant difference in the levels of both DNA integrity indices \((p=0.005 \text{ for DNA Int 1 and } p=0.006 \text{ for DNA Int 2, respectively})\). Healthy controls had lower median values (1.07 for DNA Int 1 and 0.85 for DNA Int 2) than patients with colorectal cancer (1.31 for DNA Int 1 and 1.29 for DNA Int 2), while indices for patients with benign colorectal diseases were intermediate (median 1.16 and 1.03, respectively). In consequence, there was no significant difference of indices for benign colorectal diseases from colorectal cancer \((p=0.14 \text{ and } p=0.30)\) nor from healthy controls \((p=0.41 \text{ and } 0.11)\) (Figure 1).

As expected, DNA Int 1 correlated strongly with DNA Int 2 \((r=0.80, p<0.001)\).

ROC curve analyses for distinguishing healthy patients from patients with colorectal cancer showed an AUC of 0.738 for DNA Int 1 and 0.729 for DNA Int 2, and sensitivity at 75% specificity was 70.8% for both types of DNA integrity. AUCs for the discrimination between benign colorectal disease and colorectal cancer were lower, with 0.659 for DNA Int 1 and 0.614 for DNA Int 2 (Figure 2).

Discussion

Colorectal cancer remains a major disease and cause of death for people in Europe and worldwide (2, 3). Established serum tumor markers such as carcinoembryonic antigen and CA19-9 are of limited use in cancer detection and surveillance (4). Therefore, more accurate tumor markers are required for better diagnosis and characterization of colorectal cancer.

cfDNA is substantially released from tumor cells into circulating blood and is therefore considered a promising novel oncological biomarker. In the present study, we focused on DNA integrity, which is the relation of longer to shorter DNA fragments and which has already been reported to be increased in cancer. This is believed to be due to cancer-associated cell necrosis that produces longer DNA fragments than apoptosis in normal cells does (7). Increased
DNA integrity has been described for various types of cancers, such as hepatocellular carcinoma (14), esophageal cancer (15), and acute leukemia (16). On the other hand, there have also been several reports on the lack of significant differences for DNA integrity between neoplastic and non-neoplastic diseases, e.g. lung cancer (9), glioma (10), and prostate cancer (11). Furthermore, some studies repeated inconsistent results within a given cancer entity: while Umetani et al. found a significant increase in DNA integrity in patients with breast cancer compared to healthy individuals (12), our group was unable to confirm these results (17, 18).

Figure 1. Value distribution of DNA integrity levels in the different groups for DNA Int 1 (A) and DNA Int 2 (B). Bars show the median, boxes the interquartile range, and whiskers total ranges except outliers for healthy persons, patients with benign gastrointestinal diseases and those with colorectal cancer. *Significantly different versus healthy controls.

Figure 2. Receiver operating characteristic (ROC) curves for the discrimination of patients with colorectal cancer from healthy controls (A), and from those with benign gastrointestinal disease (B).
Concerning colorectal cancer, as far as we are aware, there are only two published studies on DNA integrity measured in peripheral blood. Umetani et al. showed a significant increase in DNA integrity in patients with colorectal cancer (and periampullary cancer) compared to healthy controls (8). Agostini et al. reported that for a group of patients with rectal cancer undergoing chemotherapy, there was a significant decrease in DNA integrity in patients with tumor response versus those without response to chemotherapy (19). As the DNA integrity has led to promising results concerning patients with colorectal cancer, we aimed to verify these findings and investigated whether DNA integrity also has the potential or discriminate between cancer and benign gastrointestinal diseases.

With respect to calculation of DNA integrity, most groups simply used the ratio of the concentrations of a long to a short cfDNA fragment (11, 12, 14, 20). Among them, some used single genes with different sequences (9, 14, 16), while others used DNA repeats as the basis of their calculation (10, 12, 19). Alternatively, the delta-delta-formula of Wang et al. was applied in one study, in which significantly higher DNA integrity levels were found in patients with gynecological and breast cancer compared with patients with benign diseases (13). Under this approach, we calculated the DNA integrity by both formulae and compared their clinical impact.

As expected, DNA integrity by both formulae was found to strongly correlate with each other. Furthermore, our study confirmed earlier results from Umetani et al. (8). We measured significantly increased DNA integrity in the colorectal-cancer group compared-healthy controls. However, it should be pointed out that values for patients with benign colorectal diseases ranged between these two groups and could not be diagnostically separated from the cancer group. This is a remarkable fact as benign disorders are considerably relevant for the differential diagnosis of cancer. In ROC analyses, DNA integrity using both formulae showed comparable diagnostic utility, reaching AUCs of 0.73 and 0.74, respectively, as well as 71% sensitivity at 75% specificity.

As already reported by others (20, 21), the levels of DNA integrity surpassed the theoretically possible level of 1.0 in several cases, although the annealing sites of ALU 115 were within those of ALU 247 (12). Some potential causes such as impaired primer binding were ruled-out by the use of different additives such as dimethyl sulfoxide (DMSO) and bovine serum albumin (BSA). The high quality of PCR conditions was shown by PCR efficiencies of 1.96 for ALU 115 and 1.85 for ALU 247 and was confirmed by constant levels of two serum pools, with high inter-assay comparability. As mentioned above, similar effects were observed in previous studies on patients with renal cell cancer (20), testicular germ cell cancer (21), and melanoma (22).

Although the number of patients investigated was limited in the present pilot study, our results indicate that measurement of serum DNA integrity is potentially useful for the detection of colorectal cancer. However, it is clear that larger studies including more patients with cancer at all stages, together with a representative number of patients with respective organ-related benign diseases, are needed to confirm the preliminary results presented here.

**Conclusion**

Blood-based measurement of DNA integrity is a promising oncological biomarker for colorectal cancer that warrants further validation in future prospective trials.

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