

Novel Serum Nucleosomics Biomarkers for the Detection of Colorectal Cancer

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Abstract. *Background:* To improve prognosis of patients with colorectal cancer, powerful blood-based biomarkers enabling for early detection are needed. As genome-wide DNA hypomethylation is associated with carcinogenesis, and cell-free DNA, thought to be of tumor origin, is found in the circulation of patients with cancer, we investigated the relevance of 5-methylcytosine-modified DNA present in cell-free circulating nucleosomes as a serum biomarker using a convenient enzyme-linked immunosorbent assay (ELISA) technique. *Materials and Methods:* Serum samples from 90 individuals [24 with colorectal cancer (CRC), 10 with benign colorectal diseases (BCD) and 56 healthy controls (HC)] were tested for the differential diagnostic performance of a novel ELISA for nucleosome-bound methylated DNA. *Methodical features, including intra- and interassay imprecision, were tested using serum pools. To minimize interassay variability, values were transformed to adjusted optical densities and robust statistics were applied for clinical evaluation. Findings were later re-evaluated on a set of 113 patients (49 CRC, 26 BCD and 38 HC). Results:* Intra- and interassay reproducibility were 3.4% and 15.3%, respectively. Levels of circulating methylated DNA were significantly decreased in CRC and BCD when compared to HC ($p < 0.05$), although there was no difference between BCD

and CRC. For discrimination of CRC from HC, the area under the curve in receiver operating characteristic curve was 0.78 and sensitivities were 33% at 95% specificity and 75% at 70% specificity, respectively. The findings were generally confirmed when validated in the second set of patients. *Conclusion:* Reduced methylation of DNA on circulating nucleosomes detected by ELISA can potentially serve as a diagnostic tool in patients with CRC.

Colorectal cancer is a major challenge for many health systems worldwide due to its continuing high incidence and mortality rate (1). While screening programs, such as fecal occult blood testing or colonoscopy, have led to improvements in terms of earlier colorectal tumor detection (2, 3), there remains a clinical need for sensitive and specific blood-based biomarkers for better and earlier detection of colorectal cancer, and for therapy stratification and monitoring (3, 4).

Immunohistological and mRNA tissue studies have shown that chromatin and nucleosome composition is altered in a diverse range of cancer indications. This includes genetic changes such as mutations of driver genes *p53*, Kirsten rat sarcoma viral oncogene homolog (*KRas*) and epidermal growth factor receptor (*EGFR*), chromosomal instability, microsatellite instability, as well as epigenetic changes such as genome-wide alterations of histone modifications, histone variants and DNA methylation patterns (5-9). These epigenetic changes occur early during the transformation process, and may have functional roles in tumor growth, invasiveness and development of metastases, and thus provide prognostic information supporting their potential application as oncological biomarkers (5-7). Clinical application of such methods, however, would involve invasive sample collection procedures.

Circulating cell-free DNA (ccfDNA) is known to be present in the circulation of cancer patients bound to mono-

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Key Words: Colorectal cancer, nucleosomics, DNA methylation, serum, diagnosis.

and oligo-nucleosomes (10-11). Whilst the origin of ccfDNA is not established, a tumor origin for some fractions is suggested by reports that matched blood sample ccfDNA and solid tumor tissue samples DNA from patients with cancer to contain the same genetic and epigenetic changes (11, 12). Furthermore, cell-free nucleosomes are markers of cell death and circulating levels in patients with cancer have been reported to rise transiently following radiotherapy or chemotherapy in a manner consistent with the tumor origin (13). Earlier serum studies showed that levels of circulating cell-free nucleosomes are elevated in patients with cancer and that serial measurements correlate with disease progression (13). However, clinical use of measurements of circulating nucleosomes has been limited by their non-specific release during acute and chronic non-malignant conditions (13).

The most studied potential epigenetic ccfDNA biomarkers in cancer are the methylation levels of a variety of tumor-suppressor genes, particularly O-6-methylguanine-DNA methyltransferase and septin-9 genes that show promising results in CRC (8, 16, 17).

Studies on the global epigenetic markers carried on circulating nucleosomes in cancer are still rare. There are some preliminary reports on H3K9me3, H3K9me27 and H4K20me3 blood-based chromatin-immunoprecipitation polymerase chain reaction techniques showing significant epigenetic de-regulation in colorectal, breast and prostate cancer that may be useful for diagnostic purposes (18, 19). Furthermore, parallel sequencing of DNA attached to circulating nucleosomes containing H3K9me3 and H4K20me3 modifications indicated that long interspersed nuclear elements 1 (LINE-1) were most frequent and distinguished best between cancer cases and controls (20).

Some studies indicate global hypomethylation of repetitive *LINE1* retrotransposons may be a meaningful serum biomarker in CRC (21, 22). The global DNA methylation status of leukocyte DNA has also been investigated in the blood of patients with CRC (23, 24). However, the global DNA methylation status of ccfDNA in patients with CRC has not been so far addressed.

As a new approach, we studied the relevance of a convenient enzyme-linked immunosorbent assay (ELISA) technique measuring the levels of circulating nucleosomes carrying 5-methylcytosine in serum samples of patients with CRC, diverse organ-related benign diseases, and healthy individuals. We tested the differential diagnostic relevance of this novel biomarker in patients with CRC and performed a subsequent validation study.

Materials and Methods

Patients. The present study comprised of 90 serum samples from 24 patients with CRC), 10 with benign colorectal diseases (BCD; mainly diverticulitis, polyps and inflammatory bowel disease) and 56 healthy controls (HC). The cancer group included 10 patients

with colonic, nine with rectal and five with sigmoidal cancer; at least 15 had metastatic disease. Findings were later re-evaluated on a set of 113 patients including 49 CRC, 26 BCD and 38 HC.

Samples were collected from patients admitted to the University Hospital Bonn between January 2011 and August 2012 at the time of active disease. Patients gave informed consent for blood collection prior to venepuncture. The study was approved by the Ethics Committee of the Medical Faculty of the University Bonn (Nr. 319/12).

Methods. Serum samples from patients with cancer were collected at Clinics prior to treatment or surgery. Sera were collected, centrifuged, aliquoted, pseudoanonymized, and clinically annotated according to the Standard Operating Procedures of the Biofluid Biobank of the University Hospital Bonn at the Institute of Clinical Chemistry and Clinical Pharmacology. Whole blood samples were collected by venipuncture using gel-separator tubes (Fa. Sarstedt, Nürnberg, Germany), and sent to the Biobank where they were centrifuged within two hours of venipuncture and the serum fraction was frozen at -80°C .

Nucleosome-bound methylated DNA was measured by use of the NuQ-X ELISA from Belgian Volition SA (Namur, Belgium) performed according to the manufacturer's instructions. Briefly, serum samples (10 μl) were diluted with 50 μl 0.05 M TRIS/HCl buffer pH 7.5 and incubated overnight at $4-8^{\circ}\text{C}$ in microtiter wells coated with a monoclonal mouse antibody to nucleosome. The wells were washed with 0.05 M TRIS/HCl buffer pH 7.5 containing 0.1% Tween 20 and 50 μl mouse monoclonal anti-5-methylcytosine antibody-biotin conjugate was added. Wells were incubated 90 min at room temperature, washed again and 50 μl of streptavidin-horse radish peroxidase was added. Wells were incubated for a further 30 min at room temperature, washed again and the bound enzyme was determined using a 2,2'-acino-di-(3-ethylbenzthiazoline-6-sulfonic acid)-substrate reaction. The quantity of nucleosomes in serum test samples was determined according to a calibration curve as a percentage of the highest standard, established as previously described (13), after adjustment of optical densities over all microtiter plates using parameters of the calibration curve.

Control of methodical quality. Analytical quality of the assay was tested extensively by use of serum pools distributed over the whole plates to determine the intra- and inter-assay imprecision. The influence of inter-assay variability on clinical discrimination was minimized by including mixtures of cancer samples, as well as BCD and HC in all assay batches. All samples were tested in duplicate and only results with a coefficient of variation (CV) $<20\%$ and of plates with a median CV $<7\%$ were accepted. While the assay was improved in the meantime, the measurements were repeated nine months later on an alternative set of patient samples.

Robustness regarding pre-analytical influences. The robustness of the nucleosomics biomarkers regarding variation in pre-analytical sample handling factors was investigated prior to commencement of the study. We tested the effect of time and ambient temperature on marker concentrations during delay prior to sample centrifugation, as well as of delay in freezing after centrifugation. Sera were obtained from six donors and i) stored for 3 or 6 h at 4°C or 25°C prior to centrifugation and freezing; ii) stored for 2, 4, 6, 24 or 48 h at 4°C or 25°C after centrifugation before freezing; and were compared against samples that were centrifuged and frozen immediately.

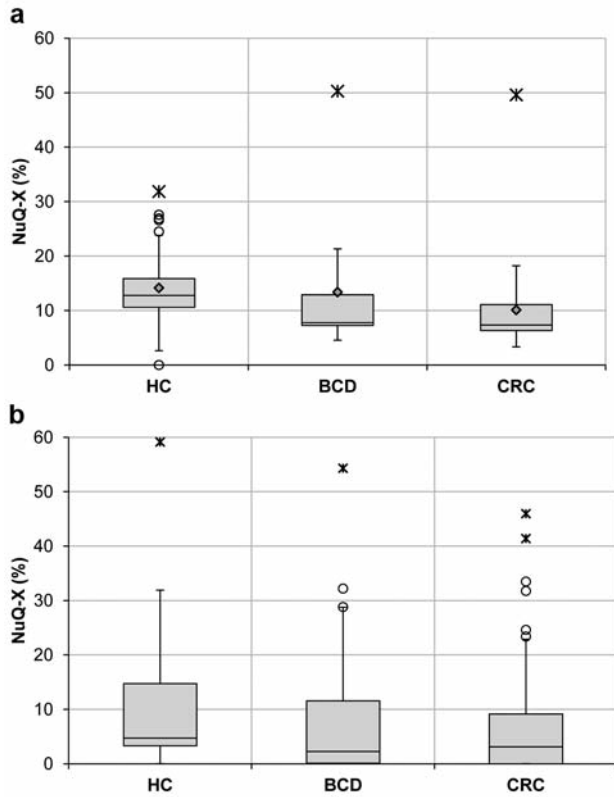


Figure 1. Distribution of serum levels of 5-methylated cytosines (NuQ-X), indicating means (rhombes), medians (lines), interquartile (boxes), 1.5-times interquartile ranges (whiskers), outliers (circles), and extreme values (crosses) in healthy controls (HC), patients with benign colorectal diseases (BCD) and patients with colorectal cancer (CRC) in the first (a) and second (b) set of patients.

Statistics. Imprecision of the assays is indicated as percentage coefficients of variation (%CV). Influence of pre-analytical factors was calculated by the percentage recovery with respect to standard sample treatment.

For the different clinical groups, the distribution of marker concentrations is given as medians, interquartile and total ranges. *p*-Values of less than 0.05 indicate statistically significant differences between the groups. For significant comparisons, areas under the curve (AUC) of receiver operating characteristic (ROC) curves and sensitivities at defined specificities were calculated to test for the performance of the biomarkers for differential diagnosis of cancer.

Results

Imprecision. Intra- and interassay reproducibility testing using a serum pool in the measuring range of the assay showed an imprecision of 3.4% in within-assay comparisons and 15.3% in between-assay comparisons, respectively.

Pre-analytic testing. Rough estimates of the influence of pre-analytic factors in six donor serum samples did not reveal major influences due to delay in centrifugation for up to 24 h

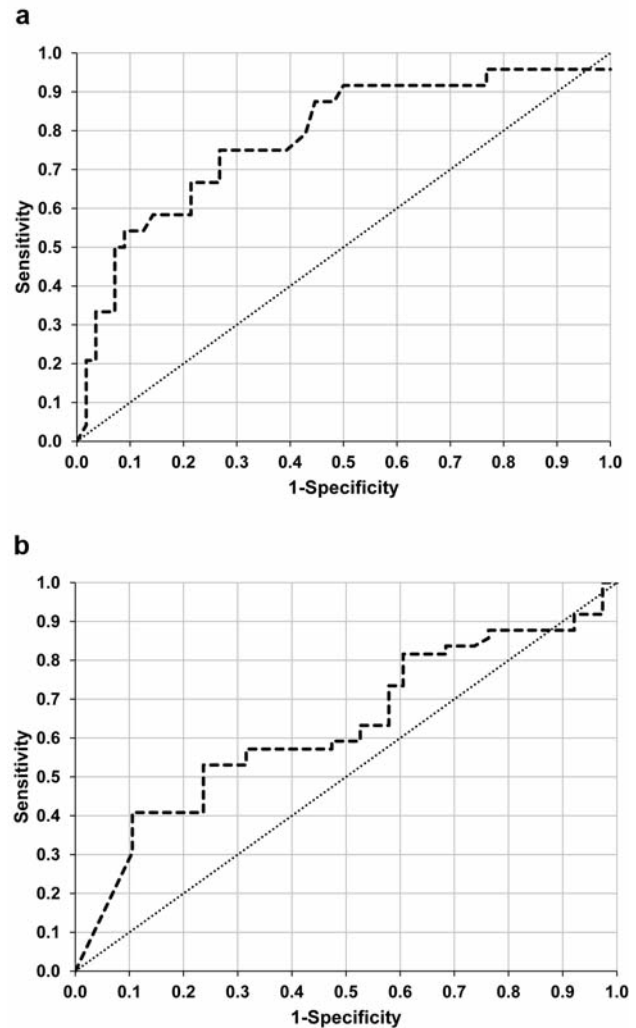


Figure 2. Receiver operating characteristic curves of 5-methylated cytosine (NuQ-X) for the differentiation of patients with colorectal cancer from healthy controls in the first (a) and second (b) set of patients.

at 4°C or 25°C (median recoveries between 92% and 101%), nor to delay of storage after centrifugation prior to freezing for up to 48 h at 4°C or 25°C (median recoveries between 95% and 110%).

Clinical testing. Levels of nucleosome-bound methylated DNA were significantly decreased in CRC and BCD when compared to HC ($p < 0.05$ for both comparisons), although there was no difference between BCD and CRC (Figure 1a).

In ROC curves, AUCs for the discrimination of CRC from HC reached 0.78; sensitivities were 33% at 95% specificity, and 75% at 70% specificity, respectively (Figure 2a). For the discrimination of BCD from HC, an AUC of 0.69 was obtained; sensitivities were 20% at 95% specificity, and 70% at 70% specificity, respectively. These findings were generally

confirmed when validated in a second set of patients, although the same levels of AUC (0.62 and 0.63, respectively) and sensitivity were not reached (Figure 1b and 2b)

Discussion

The involvement of methylated DNA in cancer was reported as early as 1983 and DNA methylation patterns observed in cancer cells differ from those of healthy cells (25). It is known that regulatory CpG islands in promoters of numerous tumor-suppressor and DNA-repair genes are hypermethylated and transcriptionally-silenced in CRC (9). However, only 6% of DNA methylation occurs at CpG island sites and 76% are reported to occur at island 'shores' located 200-2000 bp from CpG islands. These island shores may be hypermethylated (56% of sites) or hypomethylated (44% of sites) in CRC (26, 27). In addition to altered methylation patterns in CpG islands and island shores, extended hypomethylation occurs in repetitive DNA sequences, such as *LINE1* retrotransposons. While heavy methylation in normal cells is known to suppress transposon activity and to maintain genomic stability, de-methylation of *LINE1* is associated with chromatin instability and poor prognosis in CRC (28). Similar observations have been made in other types of cancers (21). The balance of these effects results in global DNA hypomethylation in cancer cells (29, 30).

There are a number of studies reporting *LINE1* hypomethylation of CRC cancer tissues relative to normal mucosa tissues from the same patient and that the degree of hypomethylation has a prognostic value (31-35). However the inter-individual heterogeneity is such that the range of values encountered in healthy and cancer tissues overlap considerably so that DNA methylation-alone is not diagnostic (21). Furthermore, the level of methylation observed in healthy cells from a range of different tissues also varied markedly (21). Observations for DNA in serum follow a similar pattern, with a clear trend for hypomethylation in stomach cancer but with overlapping inter-individual methylation level ranges (21).

Cell-free DNA circulates in a nucleosome-bound form and is thought to be partially tumor derived (10-13). We have investigated the global methylation level of nucleosome-associated DNA in the serum of healthy individuals, as well as those diagnosed with CRC using a simple ELISA test to quantify intact circulating nucleosomes containing 5-methylcytosine. The ELISA comprised of one antibody directed to binding the histone component of nucleosomes, together with a second antibody directed to binding to 5-methylcytosine. A significant reduction in the level of circulating nucleosome-associated 5-methylcytosine was observed in CRC relative to healthy persons. The inter-individual heterogeneity of results was such that considerable overlapping between normal and cancer methylation levels was

recorded. These results are in line with previous studies of serum ccfDNA methylation (21). For discrimination of patients with CRC from healthy individuals, sensitivities were 33% at 95% specificity and 75% at 70% specificity, respectively.

Our results suggest that the structure of tumor-derived mono- and oligo-nucleosomes in serum differ in healthy individuals and in patients with cancer. As these epigenetic changes occur early in the neoplastic transformation process and precede pre-neoplastic lesions including atypical hyperplasia (36), altered circulating cell-free nucleosomal structures represent possible biomarkers for the early detection of cancer. Although our findings of decreased levels in sera of patients with BCD limit the power for differential diagnosis of CRC, they may be advantageous as benign polyps and adenomas are detected more easily.

Furthermore, the numerous reports of other epigenetic alterations to chromatin and nucleosomal structure in cancer, including altered histone modification and histone variant composition (5-7), raise the possibility of tumor detection using ELISA panels for a number of circulating nucleosomal structures. Such panels may provide greater clinical sensitivity and specificity than a single test.

CRC is a common type of cancer, with better patient outcomes when detected and treated early, and screening tests have been implemented in many countries. Screening is recommended by colonoscopy; however, it is more commonly performed using conventional or immunological fecal occult blood testing. Whilst these tests have clinical sensitivities better than 70% when performed well, patient compliance is below 50% (37) so that overall CRC detection rates are poor. Low-cost, non-invasive and easily-performed blood tests with high clinical sensitivities would have a better compliance and may lead to enhanced overall detection rates.

A particular strength of the study presented is the control of all pre-analytic steps in the laboratory, including standardized sample processing and well-defined storage and multiple quality checks during sample analysis to minimize potential inter-assay variations. Laboratory testing was conducted independently of any clinical data collection. Statistical evaluation was carried-out independently of both laboratory testing and clinical data collection by experienced QuoData statisticians. Due to the limited number of samples, sub-type analysis with regard to stage and clinical characteristics was not performed and will be the subject of future comprehensive validation studies on the assay.

Conclusion

This is a first approach to establishing an ELISA-based assay for methylation of ccfDNA in serum for diagnostic purposes that needs to be validated in further clinical trials. However, the present results suggest that nucleosomic serum biomarkers may be useful as novel diagnostic tools in CRC.

Conflicts of Interest

SH and JM are consultants of Belgian Volition SA. MH is an employee. JM and MH have a financial interest in Belgian Volition SA.

Acknowledgements

Samples for the clinical study came from the Bonn Biofluids Biobank. Nucleosomic assays were provided by Belgian Volition SA. This work is part of the doctoral studies of YD.

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Received January 23, 2014

Revised March 22, 2014

Accepted March 24, 2014