Relevance of Histone Marks H3K9me3 and H4K20me3 in Cancer

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Abstract. *Background: Circulating nucleosomes are valuable biomarkers for therapy monitoring and estimation of prognosis in cancer disease. While epigenetic and genetic modifications of DNA have been reported in blood of cancer patients, little is known about modifications of histones on circulating nucleosomes. Patients and Methods: Sera of 45 cancer patients (21 colorectal, 4 pancreatic, 15 breast, 5 lung cancer), 12 patients with benign gastrointestinal and inflammatory diseases, and 28 healthy individuals were investigated. Histone modifications were detected by chromatin-immunoprecipitation (ChIP) using antibodies for triple histone methylations at sites H3K9me3 and H4K20me3 and subsequent real-time polymerase chain reaction using primers for the centromeric satellites SAT2. Additionally, the amount of circulating nucleosomes, as well as of carcino-embryonic antigen (CEA) and cancer antigen (CA) 19-9 were measured. Results: Levels of SAT2 on H3K9me3 (median 0.507 ng/ml) and on H4K20me3 (0.292 ng/ml) were elevated in sera of patients with breast cancer when compared with healthy controls (0.049 and 0.035 ng/ml), but were lower in patients with colorectal cancer (0.039 and 0.027 ng/ml). Both histone marks were correlated with each other but did not correlate with CEA or CA 19-9 in cancer patients. When H3K9me3 and H4K20me3 were normalized to nucleosome content in sera, ratios were significantly higher in all types of cancer as well as in colorectal and breast subtypes when compared with healthy controls. Best discrimination was achieved by normalized H4K20me3 reaching areas under the curves (AUC) of 79.1%, 90.4% and 81.2% in receiver operating characteristic (ROC) curves of these three comparisons. Conclusion: SAT2 levels on H3K9me3 and H4K20me3 are up-regulated in breast cancer and down-regulated in colorectal cancer. Normalization to total nucleosome content enables better discrimination between cancer and control groups.*

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Circulating nucleosomes have been reported to be elevated in sera and plasma of patients with diverse types of cancer (1, 2). However, elevated concentrations have also been found in benign disease conditions particularly after acute ischemia, trauma, during inflammation and sepsis, and various autoimmune diseases (2-5), limiting their use for diagnostic purposes. Kinetics of circulating nucleosomes have been observed to be correlated with the response to cytotoxic therapy of cancer patients (6-9). Nucleosomes have also been described as having prognostic relevance, especially in advanced cancer stages, and as indicating the response to chemo- and radiotherapy even at the initial phase of the treatment (10-12) In a comprehensive study on 311 patients with advanced non-small cell lung cancer (NSCLC), poor therapy response was predicted by nucleosomes and cytokeratin-19 fragments (CYFRA 21-1) as soon as after only one application of chemotherapy with 29% sensitivity at 100% specificity and 56% sensitivity at 90% specificity, respectively (13). Similar results were obtained for patients with recurrent NSCLC (14) and in combination with progastrin-releasing peptide (ProGRP) for patients with small cell lung cancer (SCLC) (15) and have also been confirmed by independent groups in NSCLC patients (16). If these results are confirmed in further prospective trials, they may contribute to a potentially more efficient individual management of patients with lung cancer.

Nucleosomes are the basic units of eucaryotic chromatin, and are organized as complexes of a central histone octamer

consisting of two copies of the histones H2A, H2B, H3, and H4 that are surrounded 1.65 times by 147 bp of DNA. Within the nucleosome, DNA is fixed to the histones at 14 sites (17). Nucleosomes are connected with each other by linker DNA of varying lengths forming a chain-like structure of nucleosomes. The core histones within the nucleosomes have highly dynamic N-terminal amino acid tails of 20-35 residues protruding from the surface of the nucleosome. These tails are post-translationally modified by the addition of methyl, acetyl and phosphoryl groups at basic amino acids such as lysine and arginine (17, 18). These modifications play an important role in the folding of nucleosomes into higher order chromatin and in functional regulation of transcription factor binding (19). The pattern of covalent histone modifications has been described as the "histone code" (20, 21). It contains distinct modifications at specific histone tail residues serving as domains for interaction with specific proteins that are involved in compartmentalizing chromatin into heterochromatin and euchromatin. Among the diverse modifications, there is a hierarchical order including those with stronger binding properties such as acetylation marks that regulate the conformational characteristics and those that may change according to functional conditions like methylation or phosphorylation marks (22).

Among the most frequently investigated modified lysine residues within the N-terminal tails are lysines (K) 4, 9, 27 and 36 of histone (H) 3 as well as lysine 20 of histone 4 that can be methylated by specific histone methyl transferases to the mono-, di-, or trimethylated state (me1, me2, me3). These histone methylations may be associated both with active gene expression or repression. Trimethylated lysine residues H3K9me3 and H4K20me3, which are among the repressive histone methylation markers, are concentrated at the pericentric and centric heterochromatin (23-26). In cancer, variations for histone methyl markers have been reported: Fraga *et al*. found a loss of H4K20me3 in the vicinity of the pericentromeric repeats in cancer cell lines and tumor tissues (27). Other studies on prostate and renal cell cancer have reported cancer-specific histone methylation patterns by use of tissue microarrays (28, 29). Furthermore, H3K4 and H4K20 methylation was diminished in bladder cancer tissues and H4K20me3 was associated with bladder cancer-specific mortality (30). Similarly, low expression levels of H3K27 methylation in renal cell cancer have been found to be associated with poor prognosis, suggesting that decreased H3K27 methylation indicates transcriptional activation (31).

In contrast to the plentitude of studies on histone modifications in cell lines and tumor tissues and their relevance for gene regulation and nucleosome remodelling, there are only few studies dealing with the diagnostic or prognostic relevance of histone modifications on circulating nucleosomes in serum and plasma. However, this would be highly attractive and could potentially improve the diagnostic specificity of circulating nucleosomes and their prognostic value in cancer disease considerably.

Table I. *Characteristics of patients*.

	N	Gender (female/ male)	Age (years)	
			Median	Range
Healthy controls	28	23/5	33.8	23-72
Benign disease	12	5/7	61.5	21-69
Benign gastrointestinal disease	5	4/1	62.2	21-68
Inflammatory disease	7	1/6	60.7	35-69
Cancer	45	24/21	61.2	36-86
Colorectal cancer	21	5/16	65.8	38-75
Breast cancer	15	15/0	56.4	36-85
Lung cancer	5	2/3	60.5	42-70
Pancreatic cancer	4	2/2	74.5	39-86

In 2008, a method was described for the detection of methylated histones circulating in plasma and their association with pericentric DNA repeats (32). Using this method, different patterns of H3K9me3 and H4K20me3 were reported in diverse types of cancer with lower proportions of both marks in colorectal cancer and higher values in patients with myeloma (33). Similarly, lower values of H3K27me3 were found in metastatic prostate cancer (34).

In the present study, we investigated the histone marks H3K9me3 and H4K20me3 and total nucleosome concentration in an independent set of patients with colorectal and breast cancer and correlated them with established biomarkers.

Materials and Methods

Patients. In total, we analyzed serum samples from 85 patients who were admitted to the University Hospital Munich-Grosshadern. Among them were 45 patients with cancer (21 colorectal cancer, 4 pancreatic cancer, 15 breast cancer, 5 lung cancer) and, as controls, 12 patients with benign gastrointestinal and inflammatory diseases, and 28 healthy donors. Patient characteristics are given in Table I. Blood was centrifuged within 1 to 2 hours after venous puncture, and serum was stored at –80˚C until measurement.

Chromatin immunoprecipitation (ChIP) from serum. For chromatin immunoprecipitation from serum, a modified version of the ChIP protocol reported previously (32) was used. Briefly, agarose beads were blocked with 2% bovine serum albumin (BSA) in freshly prepared ChIP buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100) for 30 min at room temperature on a rocking platform. Following washing, beads were pre-incubated with antibodies against the H3K9me3 and H4K20me3 (1 μl, Millipore, Temecula, CA, USA) in ChIP buffer for 4 h at 4˚C on a rocking platform. Subsequently, 200 μl plasma were diluted to 800 μl with the ChIP dilution buffer (25 mM Tris, 190 mM NaCl, 1.2 mM EDTA, 0.12% SDS, 1.2% Triton X-100, and protease

inhibitors) and were added to pelleted agarose beads pre-incubated with antibodies. After overnight incubation at 4˚C, beads were washed for 20 min each with low salt (150 mM NaCl), high salt (500 mM NaCl), LiCl (0.25 M) and Tris/EDTA buffers. Finally, chromatin was eluted by incubating the beads at 65˚C twice for 15 min in elution buffer (100 mM $NaHCO_3$ and 1% SDS) followed by centrifugation. Supernatants of both elution steps were mixed, and the proteins were digested by adding proteinase K (0.2 mg/ml). ChIP DNA was purified using an appropriate purification kit (Roche Diagnostics, Mannheim, Germany) and stored at –20˚C until use.

Real-time PCR. Three microliters of ChIP-DNA were subjected to real-time PCR to amplify the pericentric heterchromatin-specific satellite SAT2 repeats. The primer sequences were 5-CATCGAAT GGAAATGAAAGGAGTC-3 (F) and 5-ACCATTGGAT GATTGC AGTCAA-3 (R) (35), with SYBR Green (Roche Diagnostics) as the fluorescent dye in a LightCycler 1.2 instrument (Roche Diagnostics). The PCR program consisted of an initial denaturation at 95˚C for 10 min and 45 cycles of amplification including denaturation at 95˚C for 10 s, annealing at 60˚C for 10 s and extension at 72˚C for 10 s. All experiments were performed at least twice. A linear standard curve generated through serial dilutions of human genomic DNA was used to quantify the H3K9me3 and H4K20me3 at the pericentric heterochromatin. The transcriptionally active cyclin-dependent kinase 2A (CDKN2A) and human MutL homologue-1 (hMLH-1) genes were used as controls. Samples in which the amount of the SAT2 repeats were above the threshold value were considered positive for histone methylation. Both immunoprecipitation and real-time PCR were performed at the Istanbul Laboratory.

Quantification of circulating nucleosomes. The concentration of circulating nucleosomes was determined at the Laboratory in Munich by use of the Cell Death Detection ELISA plus (Roche Diagnostics), as described earlier (36). All patient samples were measured in one run to avoid interassay variation.

Quantification of cancer biomarkers. Cancer biomarkers were measured according the recommendations for the diverse types of cancer at the Munich Laboratory using automated and quality controlled ECLIA technique (ElecSys 2010, Roche Diagnostics): carcino-embryonic antigen (CEA) in colorectal cancer, cancer antigen (CA) 15-3 and CEA in breast cancer, CA 19-9 in pancreatic cancer as well as CEA, CYFRA 21-1, neuron-specific enolase (NSE) and ProGRP in lung cancer.

Statistics. Results are presented as median, interquartile and total ranges. Ratios were calculated for both histone marks and for the marks divided by nucleosome values in order to normalize them. Correlations between the parameters were calculated by Spearman test. Discrimination between two groups were calculated by Mann-Whitney test. Receiver operating characteristic (ROC) curves were established and the area under the curves (AUC) were calculated. A p-value <0.05 was considered statistically significant. Calculations were done using Graph-Pad-Prism5 software (GraphPad Software, La Jolla, CA, USA).

Results

When the all-cancer group was compared with healthy and with benign controls, no differences in median levels of SAT2 repeats on H3K9me3 and H4K20me3 were found, while there was a considerable scatter within the single groups. However, patients with breast cancer had significantly higher levels of both marks (median, H3K9me3=0.507 ng/ml; H4K20me3= 0.292 ng/ml) when compared with healthy controls (H3K9me3=0.049 ng/ml, p=0.0003; H4K20me3=0.035 ng/ml, p=0.0007, respectively). In contrast, patients with colorectal cancer had significantly lower values (H3K9me3=0.039 ng/ml; H4K20me3=0.027 ng/ml) than patients with benign gastrointestinal and inflammatory diseases (H3K9me3=0.122 ng/ml, p=0.0246; H4K20me3=0.099 ng/ml, p=0.0160, respectively). Ratios of H3K9me3 and H4K20me3 did not discriminate between the various groups.

While in this setting, nucleosome values were lower in the cancer group than in controls, H3K9me3 and H4K20me3 values normalized to nucleosome contents (ratio of histones to nucleosomes) were significantly higher in the all-cancer group (p=0.0012 and p<0.0001), in colorectal cancer (p=0.0034 and p=0.0003), and in breast cancer (p=0.0002 and p=0.0001) as compared with healthy controls (Figure 1).

Correlations were observed between H3K9me3 and H4K20me3 (R=0.651; p<0.0001) and between H4K20me3 and nucleosome values (R=0.301; p=0.0066), but neither between H3K9me3 and nucleosome values nor between any of the three markers with CEA or CA 19-9 levels in patients with colorectal cancer.

ROC curve analyses showed SAT2 levels on H3K9me3 and H4K20me3 normalized to nucleosome content as being superior for most comparisons than absolute SAT2 levels on H3K9me3 and H4K20me3 (Figure 2). For discrimination of all types of cancer from healthy controls, normalized H4K20me3 as best marker achieved an AUC of 79.1% [95%-confidence interval (CI)=67.9-90.3%]. For discrimination of breast cancer from healthy controls, the same markers even reached an AUC of 90.4% [CI=78.1-100%]. For discrimination of colorectal cancer from healthy controls, an AUC of 81.2% [CI=68.1-94.2%] was observed for normalized H4K20me3 as best marker, while absolute H3K9me3 was the best discriminator between colorectal cancer and benign gastrointestinal diseases, with an AUC of 71.4% [CI=51.8-91.0%].

Discussion

Histones are frequently modified post-translationally along their N-terminal tails protruding from the nucleosomes. Those modifications imply the attachment or removal of methyl, acetyl, phospho, ubiquitin, SUMO and poly-ADP-ribose groups that is orchestrated by a group of enzymes (19-21). At present, the focus of interest is mainly in the functional relevance of single, double and triple methylations and acetylation at specific basic amino acids, such as lysine and arginine at the histones H3 and H4 that are modified by

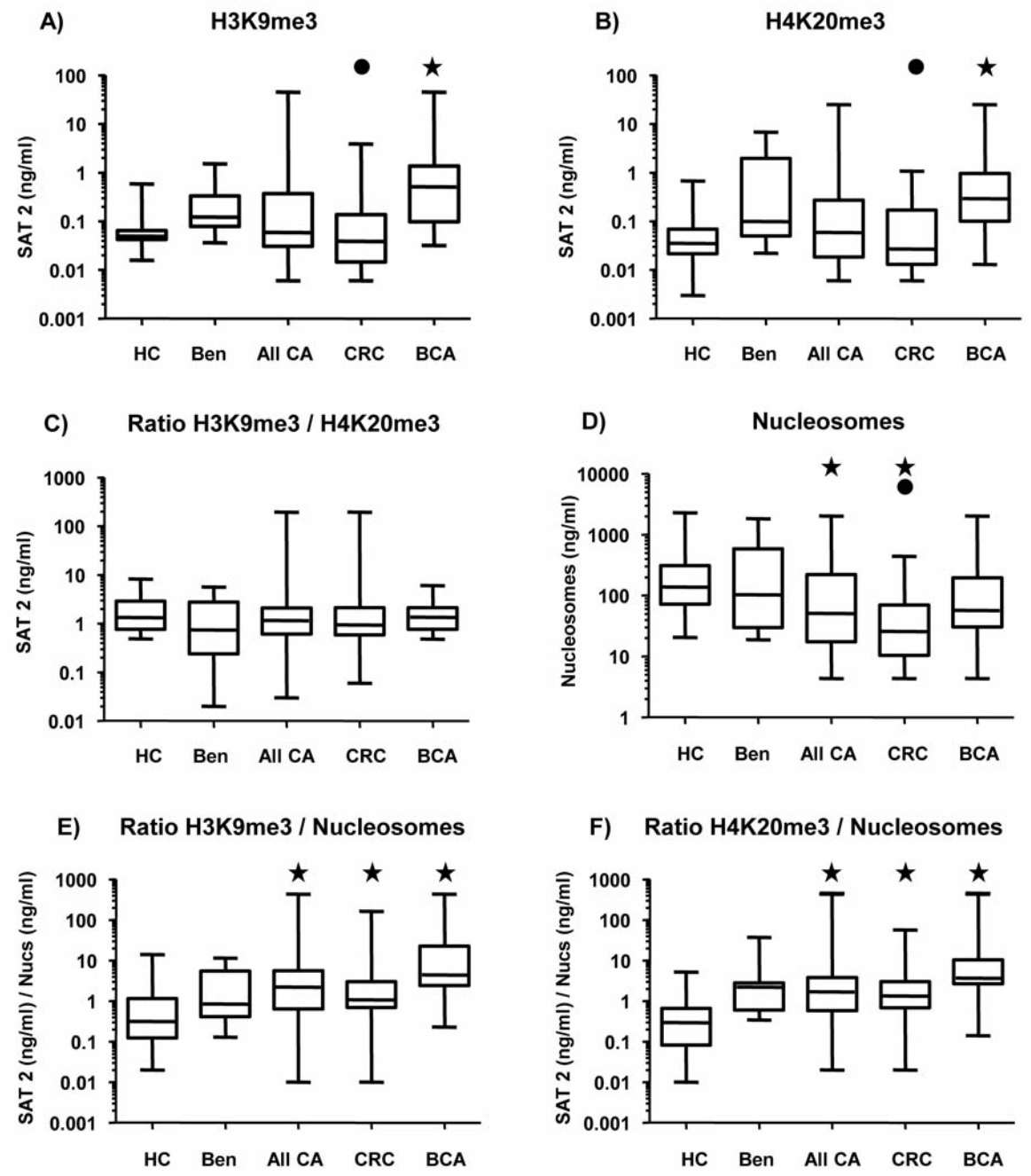

Figure 1. *Value distribution of SAT2 levels on H3K9me3 (A) and H4K20me3 (B), the ratio of H3K9me3 to H4K20me3 (C), nucleosome levels (D), and normalized levels of H3K9me3 (E) and H4K20me3 related to nucleosome content (F). Bars show medians, interquartile and total ranges for healthy controls (HC), benign diseases (Ben), all cancers (All CA), colorectal cancer (CRC) and breast cancer (BCA). Asterixes indicate significant differences to healthy controls, closed circles significant differences to benign controls.*

histone methyltransferases (HDMTs) and demethylases (HDMs) or by histone acetyltransferases (HATs) and deacetylases (HDACs and sirtuins), and their association with suppression or activation of genes involved in development of cancer or degenerative diseases (37, 38). While the open or closed state of the chromatin in a specific gene region, which enables or disables transcription processes, is believed to be controlled by the combination of several histone modifications, the so-called "histone code", single histone marks have been found to be associated with

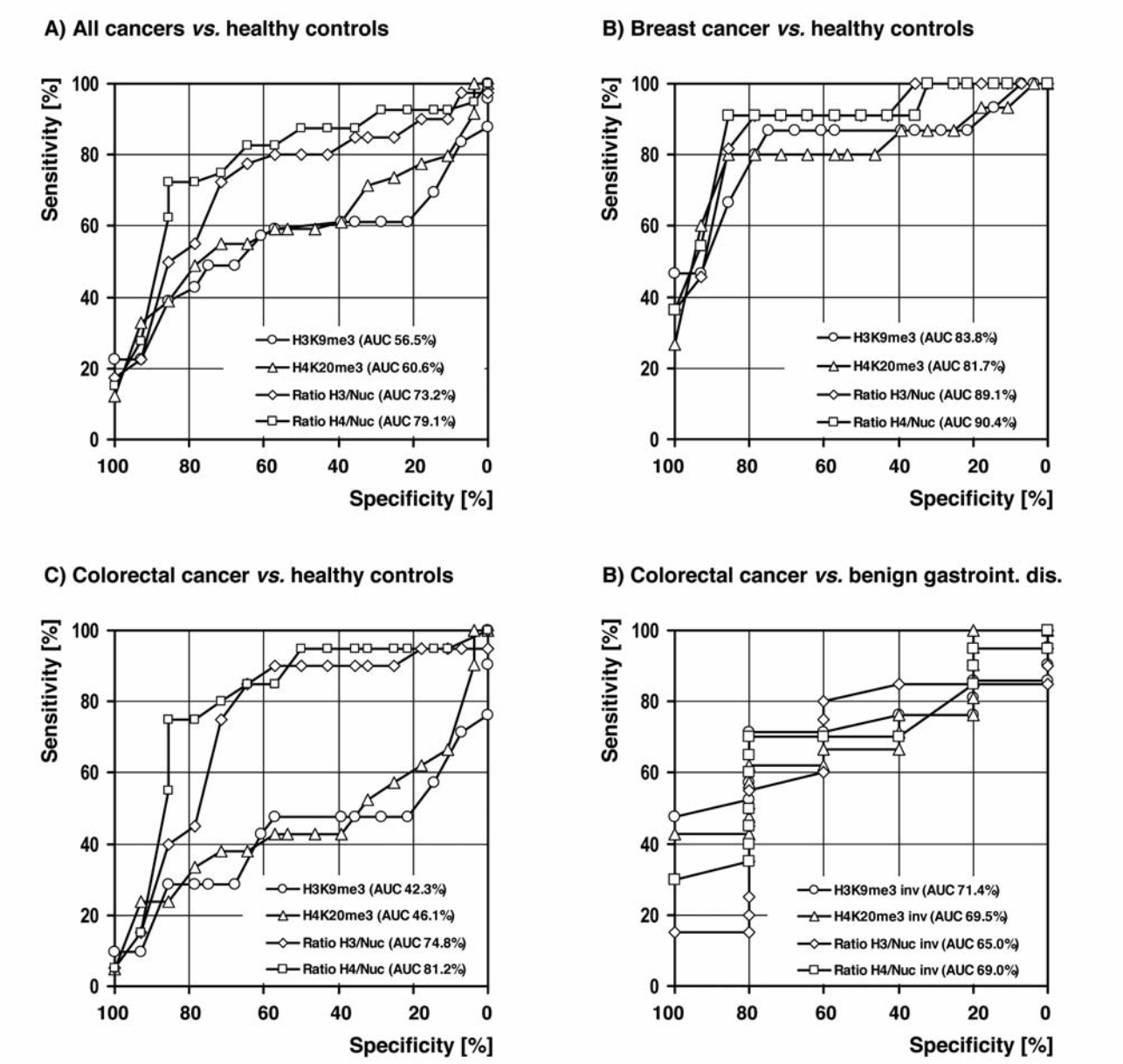

Figure 2. *Receiver operating characteristic (ROC) curves for the discrimination between all cancers from healthy controls (A), breast cancer from healthy controls (B), colorectal cancer from healthy controls (C), and colorectal cancer from benign gastrointestinal diseases (D).*

gene activation and repression in cancer. Among others, trimethylation of lysines (K) 4, 36, and 79 of histone H3 (H3K4me3, H3K36me3, H3K79me3), acetylation of H3K9 and H3K14 (H3K9ac, H3K14ac) and monomethylation of H3K20 and H2BK4 (H3K20me, H2BK4me) are known to lead to gene activation, while trimethylation of H3K9 and H3K27 (H3K9me3, H3K27me3) results in gene repression (20, 21, 37). Further hallmarks of cancer were found to be a global reduction of the trimethylation of H4K20 (H4K20me3) and of acetylation of H4K16 (H4K16ac), particularly at the pericentric repeat sequences, along with global DNA hypomethylation (27).

Clinical studies on tumor tissues reported on specific histone methylation patterns in prostate and renal cell cancer by use of microarrays (28, 29), on diminished H3K4 and H4K20 methylation in bladder cancer tissues (30) and on low H3K27 methylation expression levels in renal cell cancer being associated with poor prognosis (31). In human blood,

H3K9me3 and H4K20me3 levels associated with pericentric DNA repeats were found to be lower in patients with colorectal cancer as compared with controls (33). Even better discrimination between cancer patients and controls was achieved when H3K9me3 was normalized to H4K20me3, suggesting the ratio of these histone marks as a potential biomarker (33). Similarly, lower values of H3K27me3 were found in metastatic prostate cancer (34). In contrast, H3K9me3 and H4K20me3 levels were elevated in patients with myeloma (33).

Our results in an independent patient sample confirmed earlier results with respect to colorectal cancer. We found lower levels of SAT2 repeats on both histone marks H3K9me3 and H4K20me3 than in benign controls. This finding agrees with previous reports describing the distribution of repressive markers on repetitive sequences (39). In contrast, H3K9me3 and H4K20me3 levels were higher in breast cancer than in controls, which would suggest different distribution patterns of histone marks in diverse tumor types. As both histone marks strongly correlated with each other, their ratio obviously would not differentiate between diagnostic groups. However, we found significant differences when these histone marks were normalized to the whole serum content of nucleosomes. Then the ratios of H3K9me3 and H4K20me3 to nucleosomes, respectively, were considerably higher in all cancer patients than in controls irrespective of the subtype. Most interestingly, normalized H4K20me3 showed best diagnostic performance in ROC curves when cancer patients were compared with healthy controls, while absolute H3K9me3 values were the best discriminator between colorectal cancer and benign gastrointestinal diseases.

Although the number of patients investigated was limited in this pilot study, our results indicate that the measurement of modified histone marks may add valuable information to the more nonspecific nature of circulating nucleosomes (40). As these histone marks did not correlate with established tumor markers CEA and CA 19-9 in colorectal cancer, it can be speculated that there might be an additive value of these new markers. However, it is evident that larger studies including more cancer patients at all stages, together with a representative number of patients with the respective organ-related benign disease, and comparing them with already established tumor markers are warranted to confirm the early results presented here.

Conclusion

Blood-based detection of histone marks H3K9me3 and H4K20me3 in pericentric heterochromatin-specific circulating nucleosomes are promising new cancer biomarker candidates that warrant further validation in future prospective trials.

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