Review Article

Potential of circulating nucleosome-associated histone modifications in cancer

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Abstract: Posttranslational modifications of histone proteins (PTHMs) are involved in the regulation of chromatin packaging and gene expression thus affecting cellular homeostasis. Enzymatic machinery that set PTHMs is often deregulated in cancer resulting in altered global patterns of PTHMs in various cancer types. Detection of cancer-related histone marks in biological fluids such as plasma and serum could serve as useful biomarkers in cancer detection, diagnosis and management. Such biomarkers offer many advantages, such as being minimally invasive and easily accessible. Reflecting this, increasing amounts of data on the impact of circulating histone marks are being published. Here we provide a short insight into PTHMs and their potential utility as circulating cancer biomarkers.

Keywords: Cancer; circulating nucleosomes; histone modifications

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Introduction

Cancer has traditionally been considered as a genetic disease. However, accumulating evidence revealed that epigenetic mechanisms contribute substantially to the high complexity of cancer development and progression (1). Epigenetics is defined as the study of gene expression that is not encoded by the DNA sequence and mainly refers to alterations in DNA methylation, histone modifications, and non-coding RNAs (2). These mechanisms regulate the high complexity of the mammalian genome and affect cell proliferation, differentiation, and cellular homeostasis. During the past decade, the research into the role of posttranslational modifications of histone proteins (PTHMs) in chromatin organization and gene expression has expanded (3). Enzymatic machinery that establishes PTHMs is often deregulated in cancer and altered patterns of PTHMs have been defined for various cancers (4). Nucleosomes constitute complexes of histones and DNA and may be released from cells into the blood circulation during cell death processes. They could be valuable sources for the detection of cancer-related alterations of PTHMs in bodily fluids that can be utilized in cancer detection, diagnosis, treatment evaluation or prognosis (5). As such biomarkers offer many advantages including minimally invasiveness and easy accessibility (6), scientific interest in circulating nucleosomes and PTHMs as relevant parts in the field of "liquid biopsy" is growing. In this review we provide a short insight into PTHMs and outline published data revealing their potential as circulating cancer biomarkers.

Posttranslational histone modifications

Eukaryotic chromatin has a compact organization composed of DNA, histones and the non-histone proteins. Non-histone proteins are less abundant than histones and more variable between tissues and species and include mainly

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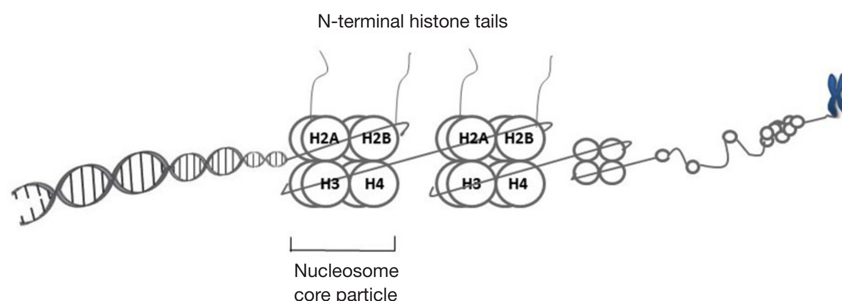

Figure 1 Chromatin organization and the nucleosome core particle.

scaffold proteins, DNA polymerase, heterochromatin protein 1 and polycomb proteins. Histones are primary protein components of chromatin and include small and highly conserved proteins H1, H2A, H2B, H3, and H4 (7). The basic structural repeating unit of chromatin is the nucleosome core particle that contains two copies of core histone proteins (H2A, H2B, H3 and H4) and 147-bp DNA wrapped around this histone octamer (*Figure 1*). H1 protein binds to the "linker DNA" region between nucleosomes.

The core histones possess highly dynamic *N*-terminal amino acid tails extending from the surface of nucleosome. These tails are subject to a variety of posttranslational modifications (8). Several different types of PTHMs have been identified in eukaryotic cells where in histone acetylation and methylation as ubiquitous marks of chromatin are key players of gene regulation. These have been mostly implicated in cancer development and progression (9). Acetylation of histone tails is typically associated with transcriptional activation of genes as this mark changes net positive charge of the histone proteins, providing access to DNA sequence information (10). The acetylation status of the histones is determined by a dynamic balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs). By removing acetyl groups, HDACs reverse histone acetylation and affect the expression of many cancer critical genes. Aberrant expression of HDACs has been linked to a variety of malignancies (11). They are supposed to be involved in multiple stages of cancer and in most cases; a high level of HDACs is associated with advanced disease and poor outcomes in patients (11). Thus HDACs represent a relevant target of cancer therapeutics.

Unlike HATs and HDACs, histone methyltransferases (HMTs) and histone demethylases (HDMs) work in a specific manner to recognize and modify distinct basic target amino acid residues (12). Histones are methylated by addition of one to three methyl groups to the side chains of lysine or arginine residues by histone lysine methyltransferases (HKMTs) and protein arginine methyltransferases (PRMTs), respectively. HKMTs consist of two main classes, the SET domain containing family and the DOT1 family (13). The functional consequences of methylation depend mainly on the number of methyl groups and their location within the histone tail (14). For instance, histone 3 lysine 4 di- and trimethylation (H3K4me2 and H3K4me3, respectively) and histone 3 lysine 9 monomethylation (H3K9me1) are examples for modifications that are associated with open chromatin and active gene expression. In contrast, histone 3 lysine 27 di- and trimethylation (H3K27me2 and H3K27me3, respectively) are linked with gene repression. Histone 3 lysine 9 trimethylation (H3K9me3) and histone 4 lysine 20 trimethylation (H4K20me3) are two repressive epigenetic marks that are enriched in pericentric heterochromatin and important mediators of genomic stability (*Figure 2*) (15). Similar to HATs and HMTS, the dysregulation of HMTs and HDMs results in aberrant histone modification patterns in cancer cells (13). Several studies linked global changes of methylation marks to prognosis of patients with different types of cancer (16-18).

Techniques for studying histone modifications

Various techniques are available to study the function, abundance or interacting partners of PTHMs. Antibody-based approaches such as enzyme-immunoassays or Western Blots are broadly applied to detect PTHMs. One should be aware that the quality and specificity of antibodies being used is a major matter of the histone research (19). Cross-reactivity with similar PTHMs (such as double or triple methylation), with alternative histone modification sites or with other

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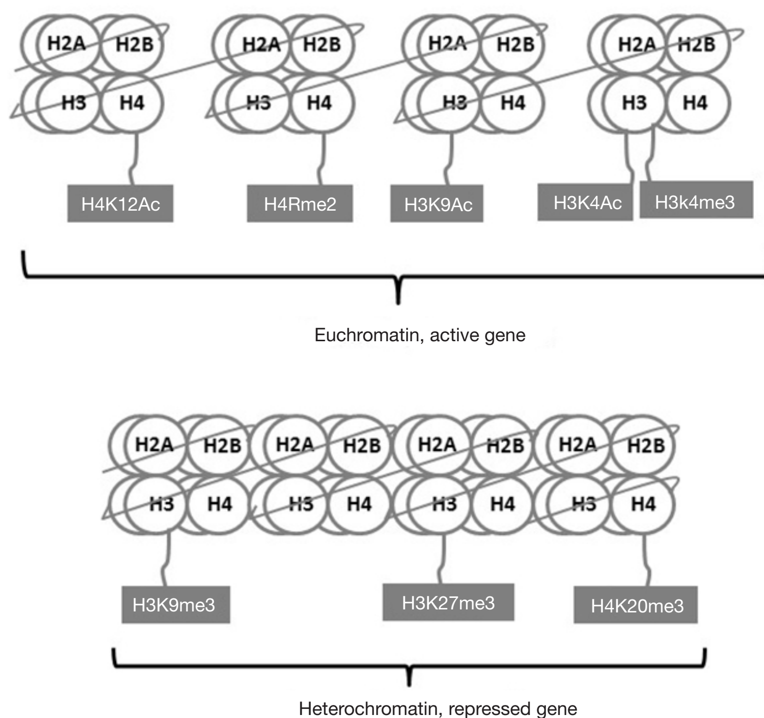

Figure 2 Examples for activating or repressing histone marks associated with euchromatin and heterochromatin, respectively.

nuclear proteins represent the most obvious obstacles in these assays. For the detection of PTHMs by Western blotting, whole-cell lysates are often used (20). For some applications, it may be necessary to enrich or purify histone proteins. This approach provides, however, no quantitative data. Enzyme linked immunosorbent assays (ELISAs) are another antibody based technique at low cost and easy to be used that allows to quantify the number of histone marks in cell lysates or bodily fluids. Current research reveals a relatively high reproducibility of PTHMs detection by this approach (21,22). The number of studies that have measured PTHMs in serum or plasma using ELISA-based assays is accumulating (22-26). Chromatin immunoprecipitation (ChIP) is another approach to employ specific antibodies. The strength of this technique is the identification of genomic sites that are enriched for a particular histone modification. For this purpose, immunoprecipitated DNA is applied in subsequent real-time PCR to determine histone marks at given loci. Large-scale enrichment analysis can also be performed using a variety of massive parallel DNA sequencing (ChIP-seq) methods to identify the distribution of PTHMs genome-wide (27). Our research revealed the detection of circulating nucleosome-associated histone marks in blood circulation by using ChIP assays (28-30). It is, however, to note that sample preparation for ChIP and ChIP-seq assays includes multiple steps which are critical to the success of the experiments and affect the reproducibility, bias, and sensitivity of the technique.

Another elegant technique applied in histone research is mass spectrometry that proved to be effective in identifying and quantifying PTHMs and their binding proteins, beyond the limitations of antibody use (31,32). A major advantage of mass spectrometry-based methods is their capability to characterize combinatorial histone PTHMs simultaneously occurring on the same molecule (33). However, higher workload and sensitivity issues for the detection of specific PTHMs have to be considered. Nevertheless, mass spectrometry may be superior to immunoassays in order to provide the specific and comprehensive quantification of PTHM panels. In a first report employing mass spectrometry Fraga *et al.* (34) have described global loss of histone H4 trimethylation and acetylation as a common hallmark of human cancer cells. Since then numerous studies have applied this technique to characterize PTHMs in clinical samples (34,35).

Sample preparation for liquid biopsy

Pre-analytical variables may affect the specificity and

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sensitivity of detection of circulating genetic and epigenetic markers (36). A crucial step is blood processing and storage temperature that may influence marker stability and concentration (37). Ethylenediaminetetraacetic acid (EDTA) is the most used anticoagulant to stabilize blood during the time between sample drawn and processing. In order to get rid of contaminating cells, both filtration and repeated centrifugations of plasma or serum were found to be useful (38). It has been reported that circulating nucleosomes are relatively stable on long-term storage of sera at −70 ℃ (39). Similarly, pre-analytical variables such as contaminating cells, within-day variation, varying time before centrifugation had no significant influence on the level of histone methylation in circulating nucleosomes measured by an ELISA-based assay (25).

Clinical impact of circulating nucleosomes

Cells dying from necrosis or apoptosis are considered to be the main sources of extracellular nucleosomes (40,41). It is also assumed that active release from living cells contributes to some part of circulating nucleosomes (42). Circulating nucleosomes are shown predominantly as mono- or oligonucleosome (43). By deep sequencing cell-free DNA (cfDNA) from blood plasma, it was found out in a recent study that the cfDNA nucleosome occupancies correlate well with the nuclear architecture, gene structure, and expression observed in cells, suggesting that they could inform the cell type of origin (44). Nucleosomes are stable structures in circulation (39) and can be detected by ELISA-based measurement in serum and plasma. In the case of cancer increased amounts of nucleosomes enter blood circulation due to a higher cellular turnover (45) and impaired clearance by macrophages (41). In a number of studies circulating nucleosomes have been investigated as a diagnostic marker. A first study measuring circulating nucleosomes was conducted in breast cancer and described elevated levels of circulating nucleosomes in patients compared with those of healthy controls (46). In a subsequent larger study including 418 patients with malignant tumors, 109 patients with benign diseases and 63 healthy individuals, it was reported that sera of patients with malignant tumors contained considerably higher amounts of nucleosomes compared with those of healthy individuals (47). However, comparing these results to levels in patients with many benign diseases, the difference was not statistically significant, reducing their clinical utility for detecting cancer.

As circulating nucleosomes are released in response to chemotherapeutic agents, few studies have utilized the quantification of circulating nucleosomes levels to predict tumor responses. Detection of patients not responding to therapy before or at an early phase of treatment regime would enable to modify the treatment regime and ultimately save patients from the systemic side-effects of ineffective chemotherapy (48). In patients with advanced lung cancer, pre-therapeutic levels of circulating nucleosomes were significantly lower in patients who responded to chemotherapy (49,50). Furthermore, these patients experienced a smaller increase and greater decrease in circulating nucleosomes following the start of treatment. Similar results were obtained by Fahmueller *et al.* when they analyzed the nucleosome levels in sera of patients with metastasized colorectal cancer (CRC) undergoing selective internal radiation therapy (SIRT). They found that high increases 24 hours after application of this therapy, indicated poor therapy response and reduced survival time (51). Also Yörüker *et al.* (52) reported that the patients with CRC who had distant metastasis have higher nucleosome levels supporting the hypothesis that circulating nucleosomes provide predictive and prognostic information in diverse cancer types.

Use of circulating histone modifications in cancer patients

While the total amount of circulating nucleosomes is not cancer-specific, PTHMs in circulating nucleosomes could mirror cell-specific and disease-related processes and therefore could be a valuable source for novel biomarkers in cancer diagnostics. Initial studies of our group on this subject employed ChIP-based qPCR assays to detect and quantify PTHMs in blood circulation. In a first report, H3K9me3 and H4K20me3, hallmarks of pericentric heterochromatin (53), were investigated in blood plasma of patients with CRC, multiple myeloma and healthy controls using pericentric heterochromatin specific satellite 2 (29). H3K9me3 levels were found to be significantly decreased in patients with CRC whereas the decline for H4K20me3 was statistically not significant. The same approach was employed on a second set of cancer samples including CRC, breast and lung cancer as well as respective benign diseases and healthy individuals as controls. Both marks were found to be significantly decreased in CRC samples while increased in sera of breast cancer patients (30). In a subsequent study, deep sequencing of H4K20m3- and H3K9me3-related immunoprecipitated nucleosomal DNA was performed confirming the decrease of both markers in plasma of CRC patients as compared with healthy controls (54). Thereby, line-1 was found to be an abundant

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genetic marker that would be useful for further research. Given potential of these markers, we investigated H4K20me3 and H3K9me3 and another suppressive mark, H3K27me3, in CRC patients using an ELISA-based assay. Supporting previous results, we found reduced levels of H4K20me3 and H3K27me3 in CRC patients, in comparison to colonoscopy-verified cancer-free controls. However, unlike previous reports the levels of H3K9me3 were similar between the two groups. Encouragingly, when H3K27me3 and H4K20me3 were combined the results showed greater area under the curve (0.769), and sensitivity of 49.2% at 90% specificity for CRC (22). In another report with ELISA-based approach, it has also been demonstrated that H3K27 methylation levels can distinguish metastatic prostate cancer from organ confined, locally controlled disease (23).

Apart from our work, evidence for the clinical relevance of PTHMs in blood circulation is accumulating. An immunoassay for 5-methylated cytosines on circulating nucleosomes (5mc) reached 75% sensitivity at 70% specificity for detection of CRC versus healthy controls (55). In a Swedish study a panel of five epigenetic biomarkers (5mc, H2A.Z, H2A.A, H3K4me2 and H2AK119Ub) on circulating nucleosomes was superior to CA19-9 in patients with resectable pancreatic cancer in comparison to benign pancreatic disease and healthy controls (24). Combining CA19-9 with four of these epigenetic biomarkers increased the sensitivity in diagnosing pancreatic cancer. As an important precondition for retrospective analyses, the effects of pre-analytical variables were investigated recently to determine the stability of circulating nucleosomes under variable conditions. Thereby, parameters such as stasis, contamination with white cells, within-day variation, varying time before centrifugation, performance of colonoscopy and presence of a surgical trauma had no significant influence on the level of 5-methylcytosine DNA (5mc) or H3K9me3 in circulating nucleosomes (25). Furthermore, 5mc and H3K9me3 levels were significantly lower in cancer patients compared to healthy individuals. In a further study conducted in individuals referred to endoscopic screening for CRC, a combination of 12 different epigenetic marks were measured by ELISA assay and a panel of four markers provided an AUC of 0.97 enabling the discrimination of CRC from healthy controls with high sensitivity at early stages (sensitivity of 75 and 86 at 90% specificity for stages I and II, respectively) (26). Although numbers of investigated samples in these studies were limited, the findings confirm the potential of circulating nucleosome-associated epigenetic markers as a promising approach for cancer detection and differential diagnosis.

Conclusions and future perspectives

In recent years, "liquid biopsy" has gained considerable attention as a novel source of biomarkers and refers to the use of blood-based biomarkers in cancer detection and management. In addition to methylated circulating DNA and circulating non-coding RNAs, histone modifications in circulating nucleosomes generated a novel and promising class of epigenetic biomarkers in cancer. Several studies have provided proof-of-principle data on the potential of circulating histone marks such as methylation and acetylation for cancer detection. The combination of several histone marks rather than single histone marks could be utilized to enhance sensitivity and specificity of cancer detection. On the other side, different techniques including ChIP-PCR, ChIP-sequencing, ELISA-based assays or mass spectrometry were employed to detect and quantify PTHMs in serum or plasma. Sensitivity and specificity of PTHM detection using different techniques were not compared to each other. Furthermore, the reproducibility of results using different analytical methods across independent laboratories should be ensured before extensive, prospective trials are conducted to depict the clinical impact of PTHMs in cancer detection and diagnosis.

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Footnote

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appropriately investigated and resolved.

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