

Review

Post-translational Histone Modifications in Circulating Nucleosomes as New Biomarkers in Colorectal Cancer

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Abstract. *Although many potential biomarkers for colorectal cancer (CRC) have been detected, rarely do they have usage in clinical practice. New biomarkers are needed to detect CRC at an early phase, to detect recurrence and monitor therapeutic response. It has become increasingly evident that altered epigenetic control of gene expression plays an important role in carcinogenesis. In addition to DNA methylation and non-coding RNAs, post-translational histone modifications play an important role in gene regulation and carcinogenesis. Knowledge regarding the patterns of histone modifications in CRC is accumulating. Additionally, histone modification alterations can be detected in nucleosomes circulating in the blood of patients with cancer offering the possibility to use them as biomarkers in CRC and other types of cancer. In the present review, we discuss the potential clinical utility of histone modifications in circulating nucleosomes for the diagnosis and estimation of prognosis in patients with CRC.*

The Need for Biomarkers for Colorectal Cancer (CRC) Detection

Despite intensive research, CRC represents a serious public health issue as it is still one of the leading types of cancer with respect to incidence and mortality (1). Over the past two decades, advances in screening programs, surgical techniques, adjuvant chemotherapy and surveillance programs have

improved the survival rates of patients with CRC (2). Overall 5-year survival rates for disease localized to the colon (stage I-II) is high (about 80%) but decreases with spread of the disease to regional lymph nodes (about 60%) or distant organs (only 8%) (3). Metastasis occurs most commonly to the liver (4). The majority of patients are diagnosed with stage III or IV disease for whom several chemotherapy regimens are available, while advances in molecular diagnostics have allowed the development of targeted therapeutic agents (*e.g.* inhibitors of vascular endothelial growth factor or its receptors) to reduce the risk of metastasis or to treat it.

At the molecular level, CRC is a heterogeneous disease and this heterogeneity is associated with differences in disease progression, survival and response to chemotherapeutic agents (5). Three molecular pathways, chromosomal instability, microsatellite instability and CpG island methylator phenotype, have been proposed to contribute to colorectal carcinogenesis. The initiation of cancer through these mechanisms is followed by pathways with distinct clinical, pathological, and genetic characteristics. Recent data have demonstrated that several genetic and epigenetic changes are important in determining patient prognosis and survival (6).

Despite advancements in treatment options, improvements in patient survival for CRC have been limited due to lack of early detection (7). Even if many potential biomarkers have been detected, the number of biomarkers that have been incorporated into clinical practice is surprisingly small (8). Biomarkers to improve CRC diagnosis, prognosis and prediction of treatment response, therefore, represent opportunities to improve patient outcome. Carcinoembryonic antigen (CEA) is the most widely used tumor marker for CRC in clinical practice, with its main utility in indicating disease recurrence in the liver. Tumor biomarkers identified in CRC tissues have been used to guide chemotherapy regimens and include the Kirsten rat sarcoma viral oncogene homolog (*KRAS*), v-raf murine sarcoma viral oncogene homolog B (*BRAF*), microsatellite instability (MSI) and smad and dad related family member 4 (*SMAD4*) (8). Reliable

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biomarkers are needed to detect CRC in an early phase, to guide treatment, as well as for surveillance to detect recurrence and monitor therapeutic response.

Cancer Epigenetics

Traditionally, cancer development has been attributed to purely genetic mechanisms, however, accumulating evidence suggests that much of its complexity can be directly attributed to epigenetics (9). The term epigenetics is defined as the study of inherited phenotypes which are not encoded by the DNA sequence (10) and commonly refers to changes in DNA methylation, microRNAs, histone modifications, and other chromatin elements that can alter gene expression. Together these form the epigenome and regulate the high complexity of the mammalian genome, providing the basis of differentiation, development and cellular homeostasis. These mechanisms are interrelated and stably maintained during cell divisions and enable the cell to react and adapt to altered environmental conditions (11).

It has become increasingly evident that altered epigenetic control of gene expression plays an important role in carcinogenesis (12). Many epigenetic changes, such as hypomethylation of oncogenes, hypermethylation of tumor-suppressor genes, depletion of hydroxymethylation, changes of histone acetylation and methylation patterns, and variations in microRNA expression level, are known to be associated with many types of cancers (13). With the advancement of new technologies, it becomes clear that altered epigenetic patterns occur in different types of cancer (11). In the 1990s, DNA methylation was the main focus of epigenetic cancer studies (14). However, during the past decade, this focus has been broadened by research into the role of covalent chromatin modifications in gene regulation, carcinogenesis, and cancer prognosis (15). In the present review, we discuss the potential utility of histone modifications in CRC and their detection in blood circulation.

Post-translational Modifications of Histones

The coiling of DNA around core histone proteins (H2A, H2B, H3 and H4) forms nucleosomes that are the basic units of eucaryotic chromatin packaging. The core histones display highly dynamic *N*-terminal amino acid tails of 20-35 residues in length extending from the surface of nucleosome. Given that histone residues on these tails can become post-translationally methylated, phosphorylated, acetylated, sumoylated, ubiquitinated, and ADP-ribosylated. Lysine residues (K) can either be mono-, di-, or trimethylated, while arginine residues (R) can be monomethylated and symmetrically or asymmetrically dimethylated. The addition or removal of post-translational modifications from histone

tails is dynamic and achieved by a number of different histone-modifying enzymes. These include histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), histone demethylases (HDMs), histone ubiquitinating enzymes as well as de-ubiquitinating enzymes. They can be either specific (*i.e.* HMTs and HDMs) or general (*i.e.* HATs and HDACs) in their ability to recognize and alter the amino acid residues of histone tails (16, 17) (Figure 1).

Nearly a dozen different histone modifications have been detected which can modify more than 150 conserved residues within histone proteins (18). Post-translational modifications of histones can regulate the accessibility of chromatin. Generally, acetylation and phosphorylation are thought to change chromatin structure by altering the net positive charge of the histone proteins, thereby rendering DNA sequence information more accessible (19). Acetylation of histone tails is typically associated with transcriptional activation of genes, while the functional consequences of methylation depend mainly on the number of methyl groups and their location within the histone tail (20). Examples for modifications that are associated with open chromatin and active gene expression include histone 3 lysine 4 di- and trimethylation (H3K4me2 and H3K4me3, respectively) and histone 3 lysine 9 monomethylation (H3K9me1). Histone 3 lysine 27 di- and trimethylation (H3K27me2 and H3K27me3, respectively) and histone 3 lysine 9 di- and trimethylation (H3K9me2 and H3K9me3, respectively) are associated with inactive chromatin and repression of gene expression (21).

The high combinatorial potential of different modifications has been described as the 'histone code' (20, 22) and a multitude of different post-translational modifications play an important role in eukaryotic gene regulation and in fine-folding of nucleosomes into higher-order chromatin (23). Distinct modifications at specific histone tail residues serve as domains for interaction with specific proteins, and such interactions compartmentalize chromatin into heterochromatin and euchromatin as illustrated by recent genome-wide chromatin modification mapping studies (24). Distinct histone modifications correlate with distinct genomic regions; for example, H3K4me3 with promoters; H3K39me1 with enhancers; H3K9 and H3K27 acetylation (H3K9ac, H3K27ac) with active regulatory regions; H3K36me3, H3K79me2 and H4K20me1 with transcribed regions and intron/exon usage; H3K27me3 with polycomb-repressed regions; and H3K9me3 with pericentromeric heterochromatin (24, 25).

As histone modifications play fundamental roles in gene regulation and expression, it is not surprising that aberrant patterns of histone marks are found in cancer. De-regulation of histone-modifying enzymes, such as HDACs, HATs, HMTs and HDMs, is often responsible for these aberrant histone modifications. Genetic, cytogenetic and molecular approaches have identified many chromosomal translocations, deletions,

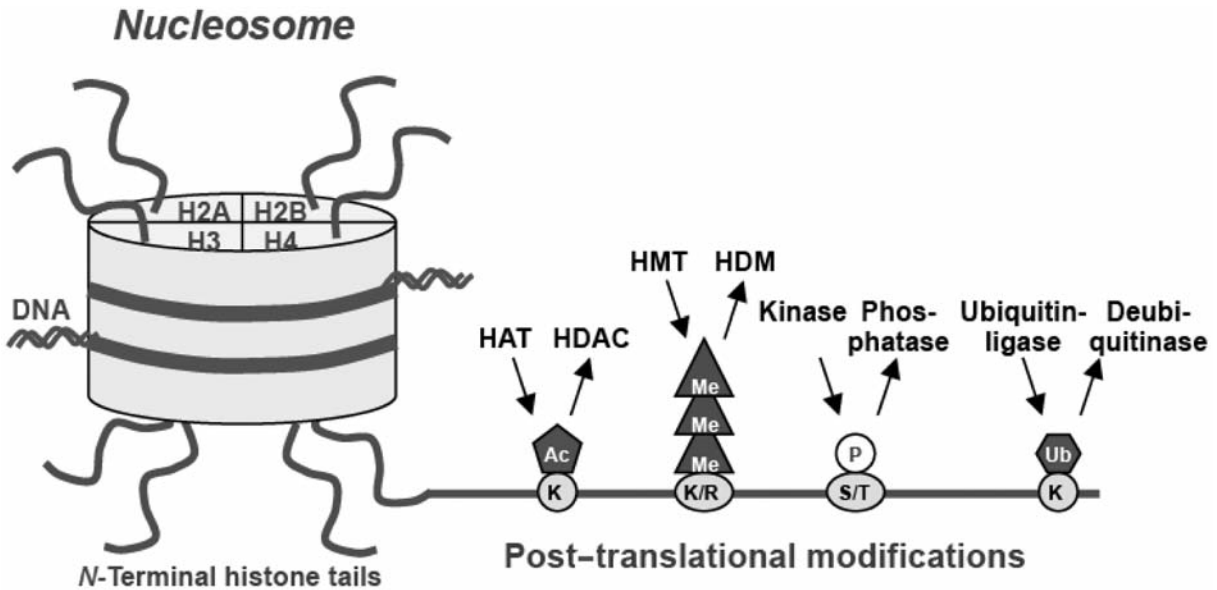


Figure 1. Chromatin is formed by nucleosomal units consisting of a central histone octamer with double-represented histones H2A, H2B, H3 and H4 and 147 bp double-stranded DNA. N-Terminal histone tails protruding from the nucleosomes can be post-translationally modified by acetyl (Ac), methyl (Me), phosphate (P), ubiquitin (Ub) and other groups at the basic amino acids lysine (K) and arginine (R), as well as at serine (S) and threonine (T). Various enzymes are involved in these processes, such as histone acetyltransferases (HAT) and deacetylases (HDAC), histone methyltransferases (HMT) and demethylases (HDM).

and amplification events that link histone-modifying enzymes to cancer (26). HDACs, for example, are often found to be overexpressed in multiple types of cancer (27). Dysregulation of HMTs and HDMs in cancer cells also contributes to aberrant histone modification patterns (28). Advances in high-throughput techniques enable genome-wide mapping of chromatin changes that occur during carcinogenesis (29). Several studies linked global changes of PTMs to prognosis of patients with different types of cancer (reviewed in reference 30).

Histone Modifications in Prognosis of CRC

Knowledge regarding the patterns of histone modification alterations in CRC is accumulating. The first report on a global change of histone modification in CRC was reported by Fraga *et al.* in 2005 (31). By immunodetection, high-performance capillary electrophoresis and mass spectrometry, they found global loss of H4K16ac and H4K20me3 in cancer cells and primary tumors, including colonic tumors. Subsequent studies investigated the global pattern of individual histone marks, mainly by immunohistochemistry. Two different studies reported that global levels of H4K12ac and H3K18ac increased in adenocarcinomas in respect to normal tissue or adenoma and (32, 33). A recent report from Stypula-Cyrus *et al.* (34)

found up-regulation of HDACs (HDAC1, HDAC2, HDAC3, HDAC5, and HDAC7) in human CRC.

Lysine methylation is one of the most prominent post-translational histone modifications that regulate chromatin structure. Changes in histone lysine methylation status have been observed during cancer formation, which is thought to be a consequence of dysregulation of histone lysine methyltransferases or the opposing demethylases (28). KDM4/JMJD2 proteins which are demethylases targeting histone H3K9 and H3K36 and histone H1.4K26 were found overexpressed in CRC (35). H3K9me3 was specifically increased in invasive regions of CRC tissues (36). Moreover, the presence of H3K9me3 positively correlated with lymph node metastasis in patients with CRC. H3K9me2 expression was also associated with the progression of adenoma to adenocarcinoma (32, 33). Dimethylation of H3K4 (H3K4me2) and acetylation of H3K9 (H3K9ac) correlated with the tumor histological type. In addition, lower levels of H3K4me2 correlated with a poor survival rate. The multivariate survival analysis showed that H3K4me2 status is an independent prognostic factor for patients with CRC (37). In addition, it has been found that the methylation level of H3K27me2 detected with immunohistochemistry is an independent prognostic factor for metachronous liver metastasis of colorectal carcinomas (38). The global level of H3K9me2 was distinctly higher in neoplastic cells (adenoma

and adenocarcinoma) than in normal glandular cells; in addition, it was significantly higher in adenocarcinoma than in adenoma. Aberration of the global H3K9me2 level is an important epigenetic event in colorectal tumorigenesis and carcinogenesis involving gene regulation in neoplastic cells through chromatin remodeling (39).

Detection of Histone Methylations in Circulating Nucleosomes and Its Biomarker Potential in CRC

Nucleosomes are released by apoptotic and necrotic cells into the blood circulation. Although macrophages efficiently clear dead cells by phagocytosis (40), nucleosomes can enter the circulation in certain diseases, reflecting either increased production or impaired clearance. In addition to apoptotic and necrotic processes, the active release of DNA from all living normal and diseased cells into the bloodstream has also been described (41). In patient with cancer, the release of nucleosomes and DNA is elevated due to the increasing cell turnover (42). Enzyme-linked immunosorbent assays have been developed to quantify circulating nucleosomes, which can be used as markers for cell death. Many studies have investigated circulating nucleosomes for their potential as diagnostic and prognostic biomarkers or their usefulness in therapy monitoring (reviewed in 43). The results of these studies have revealed that although patients with cancer have a generally higher level of circulating nucleosomes compared to healthy individuals, the diagnostic value is limited as various benign diseases were also often associated with an elevated serum level of nucleosomes. The prognostic value of the pre-therapeutic nucleosome concentration has been demonstrated in different types of cancer (44).

As nucleosomes are stable structures in the circulation (45), they could be a valuable source of novel biomarkers. Based on our work, data on the utility of detecting of methylation marks on circulating nucleosomes as a novel cancer biomarker is accumulating. Through chromatin immunoprecipitation-associated quantitative PCR, in 2008, the detection of histone methylation in blood circulation was carried out (46). Two histone methylation marks, H3K9me3 and H4K20me3, the hallmarks of pericentric heterochromatin (47), were investigated in circulating nucleosomes by subsequent studies. H3K9me3 and H4K20me3 have been found to be lower at the pericentromeric satellite II repeat in patients with CRC when compared with healthy controls or patients with multiple myeloma (48). In a further study including patients with CRC, breast, lung and benign gastrointestinal disease, similar results were obtained (49). Recently, through next-generation sequencing of immunoprecipitated plasma DNA, we confirmed reduced levels of H3K9me3 and H4K20me3-related repetitive sequences in circulation of patients with CRC (50). All these data suggest the biomarker potential of H3K9me3 and H4K20me3-related nucleosomes in CRC. As a further

approach, methylation levels of DNA on circulating nucleosomes in sera of patients with CRC was quantified by enzyme-linked immunosorbent assay. Thereby, general hypomethylation was reported in two settings of patients with CRC as compared with healthy controls (51).

Conclusion and Future Perspectives

Cell-free circulating DNA carries not only tumor-specific changes in its sequence but also distinctive epigenetic marks. The methylation pattern of circulating DNA is well-established. In addition to this, histone alterations, such as methylation and acetylation, were detected in circulating nucleosomes in blood of patients with cancer, showing considerable potential as biomarker. The combination of several histone marks rather than single histone marks are believed to further enhance sensitivity and specificity of cancer detection. Multiplex approaches could be utilized to assess the clinical relevance of such modification patterns. Large, prospective trials are needed to show their superiority or additive value to already existing protein tumor markers.

Conflicts of Interest

The Authors declare to have no conflicts of interest.

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