Epigenetic regulation by DNA methylation and miRNA molecules in cancer

“DNA methylation pattern and miRNA expression profile allow characterization of tumor tissue origination and metastatic potential, and are suitable diagnostic and prognostic markers that might be observed in early stages of cancer.”

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The leading causes of death across the world are cancer, cardiovascular diseases and chronic lower respiratory diseases, cerebrovascular diseases, Alzheimer’s disease, diabetes and other [1]. All of these conditions still appear due to the immense scope of research and management at basic molecular or targeted clinical level. In normal eukaryotic organisms, the key process in embryonic development, differentiation, imprinting, chromosome stability and inactivation of large chromosomal domains – for example, X-chromosome is epigenetic silencing. One well-studied epigenetic modification is DNA methylation that represents a heritable state without altered nucleotide sequence; moreover, modified patterns in DNA methylation are predominantly found in many cancers [2]. This commentary covers actual information about epigenetic regulation of gene expression by DNA methylation and miRNA molecules. Recent findings about DNA methylation may also have an influence on miRNA promoter region and may regulate miRNA expression in normal and cancer tissue.

Recent studies focused on DNA methylation

DNA methylation occurs as a covalent addition of a methyl group generally in cytosine within CpG dinucleotides that are concentrated in CpG islands (CGIs). CGIs are GC-rich, CpG-rich, mostly unmethylated when localized at transcription start sites of genes or predominantly methylated when scattered across the genome (within gene bodies – intragenic or between genes – intergenic). CpGs outside of transcription start sites are also sites of transcriptional initiation during development and become methylated hence lose these properties. Silencing of CGI promoters by methylation and demethylation reactions is differentially sensitive to the distance between interacting CpGs (CpG clustering), [3] and accomplished through dense CpG methylation or polycomb recruitment. CGIs are therefore equipped to influence local chromatin structure and simplify regulation of gene activity. CGIs have also been found in tissue-specific gene promoters and are known as tissue-specific differentially methylated regions (T-DMRs), [4]. Tissue-specific DNA methylation pattern is important for tissue-specific gene regulation, tissue-specific genes are not expressed in...
tissues which are methylated and are expressed in tissues which are unmethylated. However, a significant portion of T-DMRs have been found positively correlated with gene expression considering that they are more enriched in the promoter regions and bind negative regulators, such as transcriptional repressors. For positive and negative T-DMRs, two distinct sets of DNA sequence motifs exist, suggesting that two distinct sets of transcription factors (TFs) are involved in positive and negative regulation mediated by DNA methylation. Cytosine methylation is mediated through a family of DNMTs that transfer a methyl group to DNA. DNMT3a and DNMT3b can establish a new methylation pattern to unmodified DNA and DNMT1 copies the DNA methylation pattern from the parental DNA strand onto the newly synthesized daughter strand during cell replication and differentiation.

Widespread DNA methylation has a general inhibitory effect on gene expression, as confirmed in a recent study. Gene expression is regulated by recruiting proteins involved in gene repression or by inhibiting the binding of TFs to DNA. Using new techniques such as whole genome sequencing or microarray, it is possible to quantify DNA methylation in normal and cancer tissues to clarify the role of methylation in gene regulation and cancer. There is a correlation between differentially methylated regions and gene expression changes. Changes in gene expression and a function of demethylating agents like azacitidine, decitabine and zebularine were studied on cell cultures. The agents have two mechanisms of antitumor activity, especially cytotoxicity due to incorporation into DNA and RNA for azacitidine) leading to induction of DNA damage response and DNA hypomethylation through inhibition of DNA methyltransferase, enabling restoration of normal growth and differentiation. Low doses of demethylating agents are therefore used for treatment of leukemias, myelodysplastic syndromes (MDS) and in other hematologic and solid cancers for 10 years after approval by the US FDA. Despite significant improvement in treatment of cancers, patients have still overall poor prognosis. It is necessary to better understand how these inhibitors act and to identify and validate biomarkers for prediction of treatment response and to find effective combination of demethylation agents with other drugs to improve treatment success and its durability for patients.

**Brief summary of miRNA biogenesis & function**

In addition to previous epigenetic modifications, a great importance has been attributed to the RNA interference phenomena as another epigenetic phenomena which has proven to be implicated in transcriptional silencing through small duplex RNA. Currently, the research of small noncoding molecules – microRNAs (miRNAs) – is successful through modern technologies. In the miRbase database version 21, nearly 2000 sequence precursors and 2588 mature miRNAs have been identified in humans. Aberrant expression profiles of miRNAs have been found directly associated with human diseases, such as autoimmune and cardiac disorders, and schizophrenia, and are highly dysregulated in cancer. A different miRNA expression has been found between normal and benign or malignant tissue including leukemias, lymphomas; lung, breast, colorectal cancers (CRCs), papillary thyroid carcinomas, glioblastomas and other brain tumors, hepatocellular carcinomas, pancreatic tumors, cervical cancers, prostate cancers, kidney and bladder cancers or pituitary adenomas. MiRNAs are a family of small noncoding RNAs, 21–25 nucleotides (nt) in length that are transcribed by RNA polymerase II as long primary transcripts (pri-miRNAs) in nucleus. Primary transcripts are processed into stem-loop precursors of approximately 70 nt miRNAs (pre-miRNAs) by the microprocessor complex consisting of RNase III enzyme – DROSHA. Pre-miRNAs are then exported to the cytoplasm where the RNase III endonuclease – Dicer – cleaves the pre-miRNAs to produce two mature miRNAs, of which one is usually degraded. Mature miRNAs are thought to be extremely stable molecules with little evidence for active degradation. MiRNA-mediated regulation of gene expression acts primarily by imperfect base pairing with the 3′-untranslated region (3′UTR) of target messenger RNA (mRNA) and repression of protein production. Shortening of mRNA 3′UTR causes loss of miRNA target sites and post-transcriptional upregulation of critical oncopgenes in cancer cells. On the other hand, lengthening of 3′UTRs and increasing the number of potential miRNA binding sites in mRNAs has been observed during mouse embryonic development, indicating that alterations in miRNA regulation can lead to pathological processes.

Whole genome studies have also found miRNAs that are cancer specific and not...
expressed in normal tissue. MiRNA profiling allows accurate classification of malignancies and altered expression of miRNA genes between tumor and normal cells allows sorting of specific miRNAs such as tumor suppressors and oncogenes. The well-known tumor-suppressor miRNA is miR-34 cluster (miR-34a, miR-34b and miR-34c) that target many oncogenes related to proliferation, apoptosis and invasion. miR-34 is silenced by hypermethylation in cancer and reintroduction of miR-34a inhibits cancer cell growth and shows important role in tumorigenesis [13]. An opposite example that acts as an miRNA oncogene is the miR-17–92 cluster, which comprises miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a, and has been found to promote proliferation, angiogenesis, invasion and metastasis in a wide array of human disease, including hematopoietic and solid cancers [14]. However, this specific classification as oncogenes and tumor suppressors is inconsistent in some molecules. Specific miRNA have been reported to be oncogenic in one context and tumor-suppressive in another which may depend on tissue and/or differentiation specificity. A good example is miR-125b that acts as an oncomiR in the vast majority of hematologic malignancies and as a tumor-suppressor in many solid tumors. These opposite roles could be explained by the fact that miR-125b targets multiple mRNAs, it suppresses the hematopoietic differentiation factors in hematological malignancies that have a weak importance in a majority of solid tumors. MiRNA molecules are also involved in the interactions with cancer-immune system and other tumor-modifying extrinsic factors on which their expression in oncogenic or tumor-suppressive pathways depends [15].

**Regulation of miRNA expression & its epigenetic silencing**

Over the last few years it has been shown that changes in the expression of miRNA genes are involved in the pathogenesis of most if not all human cancers. Regulation of miRNA gene expression is taking place on many levels. First, it is controlled during miRNA transcription in the nucleus and processing of miRNA molecules by DROSHA and Dicer enzymes in the cytoplasm. Second, it is modified by RNA editing, uridylation, adenylation and RNA methylation and lastly, loading of Argonaute proteins and RNA disintegration is equally important to cooperation with RNA-binding proteins and other miRNAs. Synchronously, the result of cooperation between these regulatory mechanisms is an advanced molecular reply and appropriate cellular function and differentiation [16].

MiRNAs also undergo the same epigenetic regulation as many other protein coding genes. According to data from whole genome sequencing, the position of miRNA genes in the genome may contribute in overall miRNA expression. MiRNA genes have been found to be located in intergenic and intragenic regions within introns or exons and in either sense or antisense orientation. Some of the specific miRNAs are located in tumor-associated gene regions or in fragile sites and these regions are frequently deleted, amplified or mutated in miRNA loci. Other factors influencing miRNA gene expression are epigenetic silencing or the dysregulation of TFs that target specific miRNAs [17]. Most of the miRNA genes were found in CpG-rich regions and therefore DNA methylation may play an important role in altered miRNA expression. In addition to CGIs, promoters of miRNA genes frequently contain TATA boxes, TFIIIB recognition sites and histone modifications as well. MiRNA promoters are transcribed independently on or within the host gene. It was found that nucleosomes occupy the body of mature miRNA coding regions and may serve to mark miRNA regions for binding of factors participating in pre-miRNA processing [16].

DNA hypermethylation/hypomethylation and histone modifications directly regulate expression of miRNA promoters. However, DNA methylation could also appear beyond the promoter region. A comprehensive study has shown that 63 miRNA genes were epigenetically regulated in connection with 21 diseases, including tumor diseases, like melanoma, leukemias (acute myeloid leukemia, chronic lymphocytic leukemia) and solid cancers (lung, gastric, colorectal, prostate, bladder, breast, ovarian and cervical cancer) [18]. A previous study identified miRNA genes regulated via DNA methylation and authors found overall 6.9% of miRNAs to be epigenetically regulated by DNA methylation, including 45.5% of miRNA genes methylated in at least two cancer types. Other 54.4% of miRNA genes regulated by DNA methylation are specific for a certain type of cancer and suggesting it for a cancer-specific biomarker. The most frequently reported epigenetically dysregulated miRNAs in different cancer types were miR-34a, miR-34b and miR-34c following...
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Detection of cancer biomarkers in body fluids

In the last years, detection of circulating nucleic acids, such as cell-free DNA, mRNA and miRNA is getting ahead and taking a place in noninvasive diagnostics of cancer. Body fluids such as venous peripheral blood, saliva or buccal epithelium, bronchial aspirates, urine and stools are a great source of molecular markers for the early detection of cancer, monitoring of the disease, prognosis prediction and early detection of recurrence. The precise mechanism of the release is not completely known, some particles are presumably released as a consequence of normal cell death or cell death associated with a pathological process and are spread to circulation after being released from apoptotic and necrotic cells, although circulating tumor cells may not be present [22].

Several studies reported cancer-specific DNA methylation in body fluids around the primary tumor – for example, stool of patients with CRC, urine of patients with bladder or prostate cancer, among others. [22]. Cancer-specific DNA methylation detectable in plasma of a patient is a feasible approach in the improvement of a blood-based test for cancer diagnostics. They are the first blood tests for CRC that are based on the detection of SEPT9 promoter methylation in circulating DNA. The test has been applied to lung cancer (LC) detection; however, the problem in the identification of LC is correct control group. Some difficulties arise in the development of a blood test for the detection of breast cancer, where the highest sensitivity and specificity was obtained using a panel of eight genes, which accurately classified 91.7% of cancer samples and 90% of cancer-negative samples. Development of the test for pancreatic cancer detection is problematic, the best results have been given by microarray test of 56 methylated genes. Blood cancer screening tests also have challenges, such as low concentration of circulating DNA in plasma, the amount of DNA shedding into the blood is influenced by the size of early stage tumors and DNA is fragmented in 180–360 bp. High-sensitive methods are necessary for optimal detection of methylation which is about 1–2 copies of DNA sequence per assay. For sample collection, standardized protocols are required to minimalize discrepancy in sample handling between laboratories [23].

Similar situation is seen in the detection of circulating cell-free miRNAs that may be found in three forms; miRNAs enclosed in small membranous vesicles – microvesicles or exosomes; miRNAs with RNA-binding proteins like agronaute proteins or high density lipoproteins; and miRNAs released to circulation from apoptotic or necrotic cells, or during tumorigenesis and inflammation. The first circulating miRNA molecules were discovered in the plasma of pregnant woman and in serum of patients with B-cell lymphoma. Cancer cells deliver lipid vesicles comprising angiogenic factors to induce angiogenesis and thus circulating lipid vesicles can play an important role in intercellular communication. Lipid vesicles can be gathered by cells through endocytosis or fusion of membranes and consequently, circulating miRNAs can play an important role in the disease progression. These molecules are promising biomarkers in the field of early diagnosis of the different cancer types, progression and cancer therapy. The levels of circulating miRNAs in body fluids can be determined by techniques as qRT-PCR, miRNA microarrays and deep sequencing (detailed information is reviewed in [24]). Circulating miRNA expression patterns are used for LC detection and prognosis of patients. Several upregulated and
downregulated miRNA molecules were identified and therefore, authors developed panels of circulating miRNAs for clinical purpose, such as early detection, prognosis and therapy monitoring of LC. Unfortunately, the specificity and accuracy of these biomarkers are not adequate. Overall performance might be improved by combination of biomarkers within different molecular classes including cell-free DNA/RNA, DNA methylation and many other [25]. Another described function of miRNAs is that they may act similar to hormones after being released from donor cells and are delivered from body fluids into receptor cells located in other areas [20]. However, a study analyzing circulating miRNAs from blood samples of healthy people argues against the hormone-like impact on target tissue, mainly due to extremely low concentration of circulating miRNAs when compared with plasma concentration of steroid hormones. Further, common hormones bind receptors amplifying their signals, miRNAs act with their mRNA targets in a ratio of 1:1 and show measurable activity in the intracellular levels greater than 1000 copies per cell. It has been confirmed that the majority of circulating miRNAs have an origin from endothelial cells and blood components. Tissue-specific miRNA molecules derived from liver and gut were present as well, illustrating a wide source of tissue contribution in total miRNA circulation. On the contrary, miRNAs specific for brain, heart and skeletal muscles were present at very low levels, providing a broad dynamic range for applications of potential biomarkers [26]. Overall, circulating miRNA findings require further steps of validation and a standardization of all procedures, in order to control for all potential technical biases. In addition, other external factors and lifestyle that could contribute to affect miRNA levels in the circulation should also be considered in the evaluation of circulating miRNA profiles [27].

Conclusion

Epigenetic silencing is a major player in regulation of gene expression during embryonal development and tissue differentiation, and is important in most cellular processes. Moreover, miRNA expression has an influence on DNA methylation pattern and on the contrary, DNA methylation of miRNA promoter loci seems to control its corresponding gene expression. In recent years, tissue-specific DNA methylation profile has been identified by novel techniques, such as whole genome sequencing and microarray, and its effect on gene expression of mRNA or miRNA molecules. Obtained data shed light on many questions related to crucial cellular processes involved in normal and tumor tissue development, however, answering one question leads to many more questions. MiRNA profiling also uncovered diverse functions of miRNA molecules in normal and cancer tissues and classified specific miRNAs as onco-genes and tumor suppressors, similarly as DNA methylation works. Although, the function is recently discovered so miRNAs are not fully understood, the information about miRNA function, regulation and aberrant expression in cancer is continually expanding. DNA methylation pattern and miRNA expression profile allow characterization of tumor tissue origination and metastatic potential, and are suitable diagnostic and prognostic markers that might be observed in early stages of cancer. Epigenetic changes also appear as interesting biomarkers in the development of targeted therapies. Some markers are already used in therapy approaches but it is necessary to consider the side effects on normal tissue and the effective delivery of drugs to cancer cells. The discovery of circulating DNA and miRNA biomarkers has a great potential in the early diagnosis of cancer, especially in disease progression, monitoring of cancer therapy and prognosis of the disease. However, validation and standardization of all procedures are essential for evaluation of results from studies dealing with circulating miRNA molecules. More attention should be paid to obtain the regulatory associations in key pathways of cancer development and there is a need for more comprehensive data about mechanism of carcinogenesis and associated therapy.

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