

Epigenetics, microRNAs and cancer: an update

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ABSTRACT

Genetics alone cannot explain disease: in fact, people sharing the same DNA sequence (monozygotic twins) often present different levels of disease penetrance. The term epigenetics partially explains this phenomenon. Originally introduced to name the causal interactions between genes and their products, bringing the phenotype into being, it was subsequently used to define the heritable gene expression changes not related to any alteration in DNA sequence. Epigenetics has captured the attention of biomedical research, from immunology, neurology, cardiovascular to oncology; particularly in the fields of DNA methylation, histone modifications, and chromatin remodeling. Since the initial characterization of methylation in human tumors, the epigenetic research has gone far introducing recently also the preliminary descriptions of epigenomes of human cells. MicroRNAs are a class of small noncoding RNAs in many diseases including cancer. This chapter focuses on the link between epigenetics and miRNAs in cancer.

KEYWORDS: microRNA, gene expression, epigenetics, cancer

1. INTRODUCTION

The concept of epigenesis is very ancient: it can be attributed to the theory of development by Aristotle in his book *On the Generation of Animals*. In its traditional understanding it represents the concept that heterogeneous complex structures during development arise from less complex structures or even a homogenous state. Today, in light of more complex studies, this concept has more molecular aspects. In fact, with the advent of molecular genetics, this concept has now new meaning. The term epigenesis can be now considered as the science about what stands above the genes, and in this context this term is substituted with epigenetics. Epigenetics is the study of changes in the hereditary material not involving a change in the DNA sequence or the sequence of the proteins associated with DNA. Epigenetic regulation includes DNA methylation, and histone modifications. DNA methylation is a reversible reaction, primarily occurring by the covalent modification of cytosine residues in CpG dinucleotides. These nucleotides are concentrated in short CpG-rich DNA regions called “CpG islands” (present in >50% of human gene promoters) and regions of large repetitive sequences. DNA methylation is catalyzed by DNA methyltransferases (DNMTs), known to catalyze the transfer of a methyl group from the methyl donor *S*-adenosyl methionine onto the 5' position on the cytosine ring. Today, three DNMTs are known: DNMT1, DNMT3A and DNMT3B¹. DNMT1 acts during replication showing preference for hemimethylated DNA sequences, while DNMT3A and DNMT3B act independently of replication methylating both unmethylated and hemimethylated DNA sequences^{2,3}. Histones, the main protein components of chromatin and comprising the nucleosome core, are proteins with a globular C-terminal domain and an unstructured protruding N-terminal tail that can undergo a variety of chemical reactions (such as acetylation, methylation, phosphorylation, sumoylation and ubiquitylation), favoring the switch versus the

accessible euchromatin or the inaccessible heterochromatin. Histone modifications can lead to either transcriptional activation or repression. For example, lysine acetylation correlates with transcriptional activation ⁴ while trimethylation of lysine 4 on histone H3 (H3K4me3) is present at gene promoters that are transcriptionally active ⁵ and in euchromatin ⁶; on the other hand, trimethylation of H3K9 (H3K9me3) and H3K27 (H3K27me3) is present at transcriptionally repressed gene promoters ⁴. Histone modification patterns are regulated by enzymes that add and remove covalent modifications such as histone acetyltransferases (HATs), histone methyltransferases (HMTs), histone deacetylases (HDACs) and histone demethylases (HDMs).

MicroRNAs or miRNA are small non-coding molecules 18–25 nucleotides long functioning as negative regulators of protein encoded genes. MiRNA genes are often located at an intronic region of a protein-coding gene, but they can also be present in exons or between genes. MicroRNA is derived from a complicate process of maturation of its primary transcript named pri-miRNA ⁷ (Figure 1). Then, the pri-miRNA is endonucleolytically processed into a ~70 nucleotides hairpin-like precursor miRNA (pre-miRNA) by the RNase III Drosha ⁸. Then, the pre-miRNA is transported from nucleus to cytoplasm where the RNase III endonuclease Dicer processes pre-miRNAs into miRNA duplexes. Next, the miRNA duplex is unwound and the released mature miRNA binds to an Argonoutge protein (Ago) forming a core effector complex (miRNP/RISC) able now to interact with their mRNA targets typically in the 3' untranslated region. Since miRNAs can target different and various genes, the modulation of a single miRNA might affect many pathways at the same time. Today, different studies suggest that miRNAs can act as switch turning cell signaling pathways on/off. The deregulation (up/down) of specific miRNAs can trigger the switch of specific cellular pathways such as apoptosis, cell proliferation,

development, differentiation, metabolism and cancer. At least half of the known miRNAs are located close or inside to fragile sites and common breakpoints associated with cancer. Different studies have shown that deregulated levels of selected miRNAs are related to human cancer development and/or progression. As matter of fact, different findings have linked miRNAs with specific type of cancers such as chronic lymphocytic leukemia ⁹, Burkitt's lymphoma ¹⁰, colorectal cancer ¹¹, glioblastoma ¹², hepatocellular carcinoma ¹³, lung cancer ¹⁴, papillary thyroid cancer ¹⁵, pancreas cancer ¹⁶, prostate cancer ¹⁷, and renal carcinoma ¹⁸.

2. EPIGENETIC ALTERATIONS AND miRNAs

Even though different studies have contributed to better information regarding the biological importance of miRNAs, the regulation of miRNA expression is still not fully understood. Different studies suggest that tumor suppressor miRNAs (the ones targeting oncogene transcripts) can be activated by chromatin modifying drugs. In human cancer cells, DNA hypermethylation and chromatin structure can silence tumor suppressor miRNAs since are present around their promoter regions. Chromatin modifying drugs (DNA methylation and HDAC inhibitors) can activate transcription of pri-miRNAs that can be processed in pre-miRNAs and then in mature miRNAs. Then, the mature tumor suppressor miRNAs can translationally repress the target genes. An example of activation of a miRNA by chromatin-modifying drugs in human cancer cells is miR-127. Recently, it was demonstrated that miR-127 is located within a CpG island and highly induced by DNA demethylation agent 5-aza-2'-deoxycytidine (decreasing its expression) and histone deacetylase inhibitor 4-phenylbutyric acid (increasing its expression) in bladder cancer cells. MiR-127 is usually expressed as part of a cluster (containing miR-136, -431, -432, and -433) in normal cells but not in cancer cells; all

these findings suggest an epigenetic regulation. The silencing of miR-127 was noted only when the drugs were used in combination suggesting a role of both epigenetic processes in controlling the expression of miR-127. This epigenetic silencing of miR-127 unlocks the expression of BCL6 oncogene contributing to bladder carcinogenesis ¹⁹. In another study, Luiambio et al. analyzed miRNA expression profiling of HCT116 colon cancer cells and DNMT1^{-/-}DNMT3B^{-/-} HCT116 cells ²⁰. More than 5% of the 320 analyzed miRNAs were upregulated in DNMT1^{-/-} DNMT3B^{-/-} HCT116 cells cells. The authors found five miRNAs embedded in canonical CpG islands and methylated in HCT116 cells but only miR-124a was unmethylated in normal colon tissue and hypermethylated in most of primary colorectal tumors. The silencing of miR-124a leads to upregulation of CDK6 oncogene, known to regulate the tumor suppressor protein Rb. Very recently, it was demonstrated that miR-124a is methylated also in more than 50% of acute lymphoblastic leukemia (ALL) patients; its epigenetic silencing confers a poor prognosis to ALL patients ^{21,22} and its promoter hypermethylation is an early event in gastric cancer ²³. The oncogene CDK6 is also targeted by miR-107 that seems to be epigenetically silenced in pancreatic cancer ²⁴. In fact, miR-107 is upregulated in pancreatic cancer cell lines when treated with a combination of the demethylating agent 5-aza-2'-deoxycytidine and the histone deacetylase inhibitor trichostatin A.

Some researchers believe that transcriptional regulation of microRNA expression might be achieved by epigenetic alterations of target gene regulatory elements distant from the microRNA locus. In fact, it is known that many microRNAs, are encoded in introns of host genes ^{25,26} explaining why they might be susceptible to transcriptional repression by aberrant methylation of CpG island(s) located in the 5'UTR of the target gene. An example of human intronic microRNA is miR-342. This miRNA is embedded, on the plus strand, in the center of a 25.9 kb intron

between the third and fourth exons of the *EVL* gene on chromosome 14. A recent study reported that the expression of hsa-miR-342 is commonly suppressed in human colorectal cancer, the expression of *EVL* and hsa-miR-342 is coordinately suppressed in colorectal cancer and that the repression of hsa-miR-342 and *EVL* is associated with CpG island methylation upstream of *EVL*²⁷.

Another example of intronic miRNA epigenetically regulated is miR-126. The tumor suppressor miR-126 is located within intron 7 of *EGFL7*, an epidermal growth factor-domain gene frequently downregulated in several cancer cell lines. Saito et al. demonstrated that miR-126 is downregulated in human cancer cell lines and bladder and prostate tumors, but is upregulated together with gene *EGFL7* by epigenetic treatment. In fact, miR-126 is activated by inhibitors of DNA methylation (5-aza-2'-deoxycytidine) and histone deacetylation (4-phenylbutyric acid). Interestingly, treatment of cancer cell lines with the 4-phenylbutyric acid alone was not able to activate miR-126 expression²⁸.

Another miRNA epigenetically regulated is miR-1. Datta et al. analyzed the microRNA expression profile in hepatocarcinoma (HCC) cell lines HepG2 and Hep3B treated with a DNA hypomethylating agent (5-azacytidine) and/or a histone deacetylase inhibitor (trichostatin A)²⁹. Among the analyzed microRNAs, miR-1 was found significantly upregulated ($p \leq 0.0001$) in both cell lines upon treatment with 5-azacytidine alone or in combination with trichostatin A. More, miR-1, coded by an intron 1 of the putative ORF166 is embedded in CpG islands of which the one located upstream of miR-1 is methylated in both HCC cell lines and primary hepatocellular carcinomas.

Recently, Lujambio et al. identified miRNAs undergoing transcriptional silencing in lymph node metastatic cancer cells from colon, melanoma, and head and neck by miRNA expression

microarray analysis upon DNA-demethylating agent 5-aza-2'-deoxycytidine treatment ³⁰. The authors identified miR-148a, miR-34b/c, and miR-9 undergoing specific hypermethylation-associated silencing in cancer cells compared with normal tissues. The epigenetic inactivation of these three miRNAs contributed to tumor dissemination “*in vitro*” and “*in vivo*” and the epigenetic silencing of miR-148a and miR-34b/c mediates the activation of oncogenic and metastasis target genes such as *c-MYC*, *E2F3*, *CDK6*, and *TGIF2*.

Epigenetic regulation is a mechanism for miRNA inactivation also in human breast cancer. In fact, an aberrant hypermethylation was shown for miR-9-1, miR-124a3, miR-148, miR-152, and miR-663 in 34-86% of cases in a series of 71 primary human breast cancer specimens ³¹. Also, the authors demonstrated a reactivation of miR-9-1 in breast cancer cell lines treated with 5-aza-2'-deoxycytidine and hypermethylation of microRNA genes in human breast cancer, suggesting that miRNA gene methylation might serve as a sensitive marker for epigenetic instability.

Another known CpG island embedded microRNA is miR-370. Meng et al. reported that miR-370 showed IL-6-driven methylation regulation in cholangiocarcinoma cells. In this study, IL-6 was found to enhance the growth of cholangiocarcinoma cells by repressing the expression of this miRNA epigenetically ³².

MiR-137 is closely associated with a large CpG island and together with miR-124 might be activated in glioblastoma multiforme cell lines following treatment with a DNA methylation inhibitor (5-aza-2'-deoxycytidine) and/or a histone deacetylase inhibitor (trichostatin A). Expression of both miRNAs did not relatively change in cells treated with trichostatin A alone ³³. In epithelial ovarian cancer cell lines, miR-34b, miR-372, miR-516, miR-518a, miR-519d, miR-519e, and miR-520e are reported up-regulated by treatment with the DNA demethylating agent

5-aza-2'-deoxycytidine and the histone deacetylase inhibitor 4-phenylbutyric acid ³⁴. Interestingly, miR-34b, is reported to be epigenetically regulated also in other cancers ^{30,35,36}.

In 2008, Kozaki et al. demonstrated that the expression of miR-34b, miR-137, miR-193a, and miR-203, four miRNAs located close to CpG islands, was restored by treatment with 5-aza-2'-deoxycytidine in the oral squamous cell carcinoma cells lacking their expression ³⁷. The expression levels of the four miRNAs were inversely correlated with their DNA methylation status in the oral squamous cell carcinoma cells. MiR-137 and miR-193a are most likely miRNAs frequently silenced in oral squamous cell carcinoma and they both have tumor-suppressive effects on the growth of in oral squamous cell carcinoma cell lines.

Brueckner et al. noticed that the human let-7a-3 miRNA gene on chromosome 22q13.31 was associated with a CpG island heavily methylated in normal human tissues but hypomethylated in some human lung adenocarcinomas ³⁸. Lung cancer cells combinatorially treated with 5-aza-2'-deoxycytidine and the histone deacetylase inhibitor valproic acid showed a clear demethylation and transcriptional upregulation of let-7a-3. The epigenetic reactivation of let-7a-3 by hypometylation induced tumor phenotypes and oncogenic changes in transcription profiles. These results suggest that let-7a-3 is an onco-miRNA promoting human lung carcinogenesis.

In certain cases, the histone modification alone may regulate the miRNA expression. Scott et al. reported rapid alteration of miRNA levels by the potent hydroxamic acid histone deacetylase inhibitor LAQ824 in the breast cancer cell line SKBr3 ³⁹. The miRNA profiling by miRNA microarray analysis revealed significant changes in 40% of the 67 different miRNAs expressed in SKBr3 cells, with 5 miRNAs upregulated and 22 miRNAs downregulated.

The epigenetic regulation of miRNAs, might be also cell-type specific. For example, miR-127 expression can be significantly upregulated by 5-aza-deoxycytidine and phenylbutyrate treatment

in several cell lines, including CFPAC-1 pancreatic carcinoma, HCT116, HeLa, NCCIT embryonic carcinoma and Ramos lymphoma¹⁹ but not in CALU-1 lung carcinoma cells and MCF7 breast carcinoma. DNA demethylation or histone deacetylase inhibition can also have no effect on miRNA in lung cancer cells^{14,40}. MiRNA methylation patterns between different cell lines can be distinct and sometimes variable and this might be explained at least in part to their differences in tissue origins and differentiation states. An example is human miR-200c, recently described unmethylated in HCT116 colon carcinoma cells and HES7 embryonic stem cells but partially methylated in PHF primary fibroblast cells and HeLa cervical carcinoma cells⁴¹.

At least 29,000 CpG islands are predicted to be present in the human genome^{42,43}, and many located at the 5' of the genes. A recent study reported that 70% of promoters in the human genome are associated with CpG islands⁴⁴. Also, several miRNAs linked to epigenetic regulation are closely associated with CpG islands. In fact, it was reported that at least >40% of human miRNAs genes are associated with CpG islands⁴¹, indicating that several miRNAs can be considered candidate DNA methylation targets.

3. A NEW GROUP OF miRNAs: epi-miRNAs

Today, miRNA can be considered an indirect mechanism through which epigenetic mechanisms regulate expression genes involved in human cancer development and/or progression. There is increasing evidence that miRNA-encoding genes are not only targets but also regulators of methylation and acetylation processes; in other terms miRNAs might act as epigenetic players. In fact, miRNAs can target genes coding for enzymes responsible for histone modifications (*EZH2*) and DNA methylation (*DNMT3A and DNMT3B*)^{45,46}. A perfect example is miR-101 and *EZH2* gene (Polycomb group protein (PcG) enhancer of zeste homologue 2). *EZH2* is the catalytic subunit of the Polycomb Repressive Complex 2 (PRC2), and it is able to trimethylate lysine 27

of histone H3 (H3K27me3). This trimethylation act as a molecular mark and is recognized and bound by the Polycomb Repressive Complex 1, causing gene repression by a still unclear mechanisms in which histone modifications, recruitment of chromatin binding proteins such as heterochromatin binding protein 1, and chromatin compaction are involved ⁴⁷. Abnormal high levels of EZH2 lead to “de novo” silencing of genes, contributing to epigenetic reprogramming in cancer ⁴⁸. In normal cells, miR-101 is expressed and targets *EZH2* 3’UTR mRNA in a sequence dependent manner causing gene expression repression and/or transcript destabilization of this gene. Normal levels of EZH2 create normal epigenetic modifications and gene expression. In cancer cells, miR-101 expression is decreased, causing abnormal high levels of EZH2 and aberrant tumor suppressor and pro-differentiation gene silencing via H3K27me3. *EZH2* is also downregulated by miR-26a during myogenesis ⁴⁹.

Histone deacetylases are also targeted from epi-miRNAs: *HDAC1* 3’UTR is targeted from miR-449a which re-expression leads to its reduction ⁵⁰; miR-1 and miR-140 target directly *HDAC4* gene ^{51,52}.

Another example is miR-29 family and *DNMT3A* and *DNMT3B*. In a recent study, it was demonstrated that the expression of miRNA-29 family (29a, 29b and 29c) is inversely correlated to *DNMT3A* and *DNMT3B* in lung cancer and that miRNA-29 family directly target the 3’UTR of *DNMT3A* and *DNMT3B* genes ⁴⁵. MiR-29b is not only able to target both *DNMT3A* and *DNMT3B* genes, but also can indirectly target *DNMT1* gene via SP1 ⁵³.

Certain splice variants of *DNMT3B* gene are targeted by miR-148a and miR-148b. This miRNA family binds with high homology within the coding sequence of *DNMT3B* gene but the mechanism of DNMT3B repression is still unknown ⁵⁴. The fact that miR-148a is also

epigenetically regulated ³⁰, suggests a self epigenetic regulation loop for this epi-miRNA, but more studies are needed.

In 2008, two independent studies demonstrated that miR-290 cluster directly targets *Rb2/p130* gene in mouse embryonic stem cells ^{55,56}. In *Dicer*^{-/-} mouse ES cells, miR-290 cluster is not expressed leading to downregulation of *DNMT3* genes and disruption of DNA methylation pattern. These effects were reversed by reintroduction of miR-290 cluster ^{55,56}.

4. CONCLUDING REMARKS

Cancer is a multifactorial and epigenetic disease. MicroRNAs, frequently deregulated in cancer, can be controlled by epigenetics alterations but can also function as epigenetic players, suggesting that epigenetic mechanisms and miRNAs can interact on a bidirectional level. Interestingly, epigenetic changes can be reversed by certain drugs, and because there is a tight link between epigenetics and miRNAs, it is conceivable to assess the therapeutic targeting of epigenetic miRNA regulation mechanisms in cancer. The studies described here provide an update regarding miRNA and epigenetics in cancer. However, more studies are needed to characterize all the interactions between miRNAs and epigenetics to better develop “ad hoc” cancer biomarkers and/or to identify new therapeutic targets.

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FIGURE LEGENDS

Figure 1. Synthesis and processing of microRNA. The figure was prepared with ScienceSlides 2008 software (Visiscience, NC, USA).

