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Clinical significance of the interaction between non-coding RNAs and the epigenetics machinery

Challenges and opportunities in oncology

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Abbreviations: ncRNAs, non-coding RNAs; DNMTs, DNA methyltransferases; HDAC, histone deacetylases; HMTs, methyltransferases; rRNAs, ribosomal RNAs; tRNAs, transfer RNAs; siRNAs, short interfering RNAs; endo-siRNAs, endogenous small interfering RNAs; microRNAs, miRNAs; piRNAs, PIWI-interacting RNAs; lncRNAs, long non-coding RNAs; lincRNAs, long intergenic non-coding RNAs; NATs, natural antisense transcripts; HCC, hepatocellular carcinoma; CLL, chronic lymphocytic leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance; c-MYC, v- myc avian myelocytomatosis viral oncogene homolog; E2F3, E2F transcription factor 3; CDK6, cyclin-dependent kinase 6; TGIF2, TGFB-induced factor homeobox 2; GBM, glioblastoma multiforme; MGMT, O6-methylguanine methyltransferase; PRC, polycomb repressive complex; EZH2, enhancer of zeste homolog 2; HOTAIR, homeobox transcript antisense RNA

Non-coding RNAs and epigenetics are remarkable mechanisms of cellular control. In this review we underline the processes by which non-coding RNAs (ncRNAs), shown to be involved in various diseases, are capable of modifying and being modified by the epigenetic machinery, emphasizing the clinical importance of this network in cancer. Many ncRNAs have been described that play important roles in the establishment and maintenance of the epigenome. However, only a few studies deeply take into account the role of ncRNAs from a clinicopathological standpoint. The wide range of interactions between the non-coding RNome and the epigenome, and the roles of these networks in the pathogenesis, prognosis and early diagnosis of many diseases, present new challenges and opportunities for future studies regarding therapeutic strategies in oncology.

Introduction

For many decades, since molecular biology arose as a shedding light in the oncology field, cancer was believed to be a purely genetic disease. However, with the emergence of epigenetics, the unquestionable intricacy of this disease became even more evident. Derived from Latin (the prefix *epi* means upon, over), epigenetics is the study of alterations that are beyond those at the genetics level—Watson and Crick DNA pairing properties; the term is used to describe those events that cannot be solely explained by genetics but can be inherited by mitosis or meiosis.[1,2]

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These events are of outstanding importance, as they are known to contribute to deregulation of gene expression. Among the most important epigenetic mechanisms are DNA methylation, histone modifications and non-coding RNAs (ncRNAs) effects.

Non-coding RNAs are an extensive class of evolutionarily conserved RNAs that are not translated into proteins.[4] Their discovery was a breakthrough in science, responsible for promoting a new vision of the former biology "central dogma" (DNA→RNA→Protein) and for the "junk RNA" theory, in which the term "junk RNA" comprised all those RNAs that were not translated—about 70% of the genome is transcribed but approximately only 2% is translated—and, therefore, were believed to have no function.[5-7] By now, these molecules have been extensively shown to play roles in the regulation of the translation and transcription of both coding and non-coding genes. As a result, the cancer transcriptome is currently viewed as more complex than previously imagined.[4,8]

Many important functions are encompassed by ncRNAs, including, (1) architectural functions, such as structuring ribosomal subunits (rRNAs); (2) transportation functions during the translation of proteins, in which tRNA (tRNA) act together with RNA ligands and mRNA;[9] and (3) regulatory functions through modulation of RNA, DNA, and proteins. Depending on their sizes, regulatory ncRNAs can be classified into small ncRNAs (<200–300 bp)—including microRNAs (miRNAs), short interfering RNAs (siRNAs), endo-siRNAs and PIWI-interacting RNAs (piRNAs)—and large ncRNAs (lncRNAs; >200–300 bp).[8] **Figure 1** provides a broad overview of ncRNAs and their epigenetic levels of regulation.

Since their discovery,[10,11] much has been said about miRNAs. These small ncRNAs are approximately 22 nucleotides long, single-stranded RNAs that are involved in the pathogenesis of all tumor types studied so far, acting in a very complex

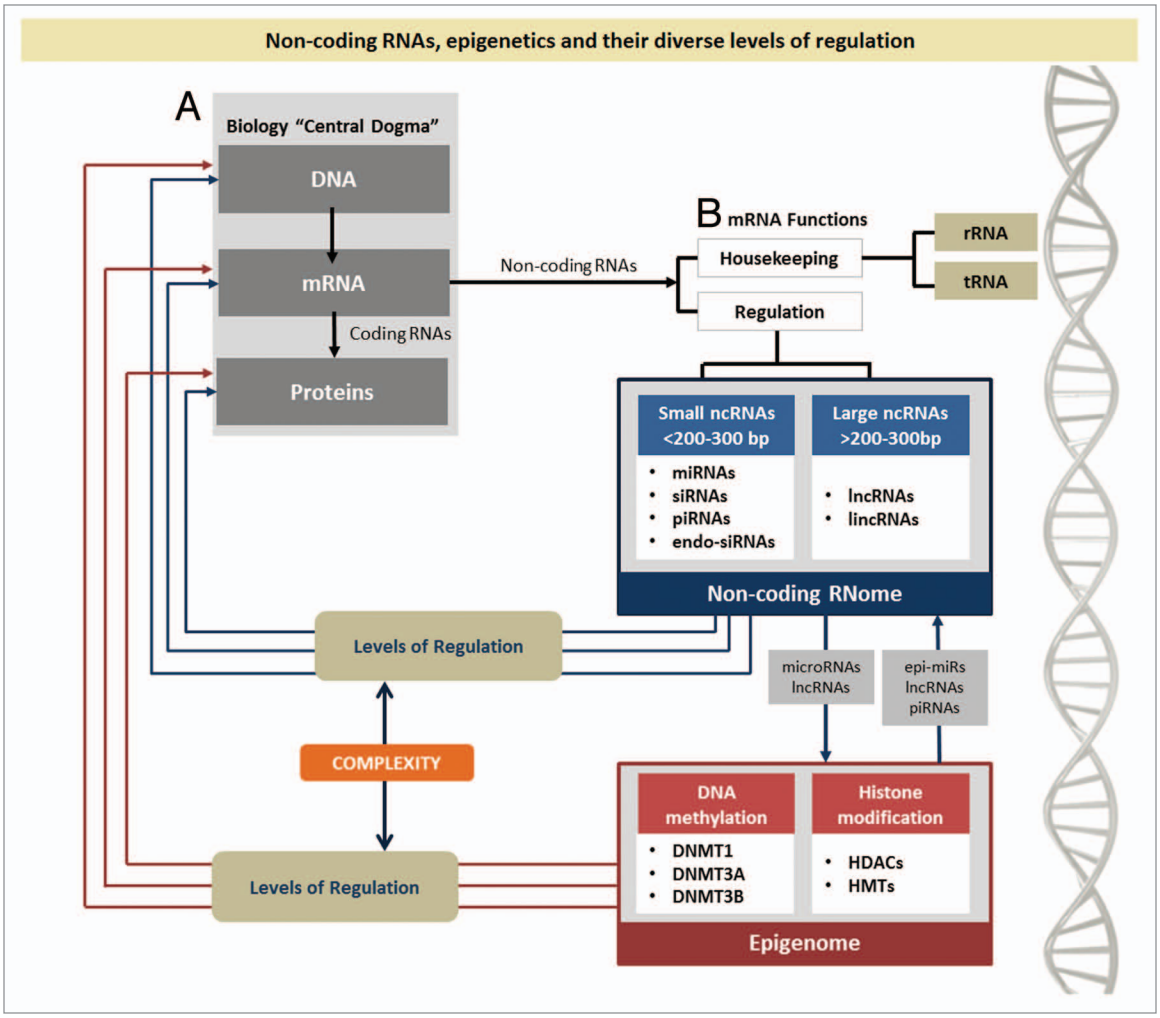

Figure 1. Non-coding RNAs, epigenetics and their diverse levels of regulation. (**A**) The gray box illustrates the classical Biology "Central Dogma" and the emergence of a new class of RNAs, the ncRNAs, and their main functions (**B**). The non-coding RNome is shown in the blue box, comprised by short and long ncRNAs and the epigenome is shown as a red box. Their levels of regulation are shown by arrows. The complexity of the interactions between the miRNome and the epigenome can be easily visualized.

manner. Their regulatory networks involve, among others, the translational repression and/or cleavage of target complementary mRNA, the targeting of mRNA-binding proteins, and the binding of promoters and regions other than 3′ UTRs, such as genic regions.[12]

Piwi-interacting RNAs (piRNAs) represent a set of small ncRNAs that are 26–31 nucleotides long and were discovered by their association Piwi, a protein from the Argonaute subfamily restricted to germline. piRNAs possess a 5′ uridine and target antisense transposon sequences.[4,8] They are produced from the cleavage of selfish and repetitive genomic element transcripts.[13]

Long ncRNAs are RNAs ranging from 200 bp to multiple kilobases in size. In several pathological states, including cancer, they have been verified as major players in gene expression regulation, representing the largest class of ncRNAs and accounting for a great part of the transcribed genome.[5] However, in spite of their magnitude, they are the least comprehended entity generated by the eukaryotic genome.[4] Similarly to RNAs that encode for proteins, lncRNAs are 5′ capped, spliced, and polyadenylated; the absence of a protein-coding open reading frame is what makes them different from mRNAs. Long ncRNAs can be divided into several major subtypes, the long intergenic ncRNAs (lincRNAs) and the natural antisense transcripts (NATs) being the most well-known so far. LincRNAs are located between protein-coding loci or in introns; NATs are RNAs with sequence complementarity to another RNA transcript.[5,14]

Non-coding RNAs and epigenetics are remarkable mechanisms of cellular control. In this review, we attempted to provide a better understanding of how the epigenome regulates and is regulated by ncRNAs and what clinical importance these interactions may have in cancer. A summary of the most relevant findings in regard to epigenetics and ncRNAs is provided in **Table 1**.

Epigenetics and Non-Coding RNAs: Clinical Aspects

Epigenetic regulation of miRNAs

Throughout the last decade, many studies have contributed to a better understanding concerning the role of epigenetics in

Table 1. Summary of the most relevant findings in regard to epigenetics and non-coding RNAs

Type of lncRNA	Molecule	Cancer type	Clinical significance	Reference
Epigenetic regulation of non-coding RNAs				
microRNAs	miR-129–2	Hepatocellular carcinoma	miR-129–2 methylation was specifically detected in plasma samples. Potential marker for early diagnosis.	15
	miR-129–2	Hematological malignancies	miR-129–2 methylation was found as adversely impacting survival in chronic lymphocytic leukemia (CLL)	16
	miR-124–3	Renal carcinoma	Increased methylation of miR-124–3 CpG islands associated with unfavorable prognosis such as tumor grade and diameter, metastasis and advanced disease.	18
	miR-124	Acute lymphoblastic leukemia	Hypermethylation of this locus was found as an independent prognostic factor for overall and disease-free survival and associated with higher relapse and mortality rates.	19
	miR-34a	Colon cancer	Methylation of miR-34 associated with metastasis to liver and lymph nodes	20
	miR-212	Lung cancer	Silencing of miR-212 was correlated with later stages of the disease – staging T3/T4	22
lncRNAs	H19	Hepatocellular carcinoma	H19 involved disease progression	25
	H19	Hepatocellular carcinoma	Epigenetic activation of miR-200 family promotes H19 downregulation, driving metastasis through EMT induction. Low T/R ratio was associated with poor prognosis.	26
	KIAA0495, PART1, MGC21881, MIAT, GAS5 and PAR5	Glioblastoma multiforme	The six-lncRNA signature was linked with overall survival, alluding that this signature may possibly supply new markers for prognosis treatment and outcome prediction.	27
	ANRIL	Cancer in general	Epigenetic modifications contribute to its silencing, which may bring both diagnostic and prognostic perspectives in the future.	30
Non-coding RNAs as regulators of epigenetics				
epi-miRs	miR-29 family	Lung cancer	Lower survival rates in patients with high levels of DNMT3A	31
	miR-22	Hepatocellular carcinoma	Decreased survival and worse prognosis when miR-22 was downregulated.	32
piRNAs	Hiwi	Esophageal squamous cell carcinoma	Hiwi (Piwi human ortholog) associated with poor prognosis	46
lncRNAs	XIST	Testicular germ cells tumor	Methylation status as a biomarker in plasma for early detection and prognosis.	43
	HOTAIR	Laryngeal squamous cell carcinoma	Associated with poor tumor differentiation, lymph node metastasis and advanced clinical stages	44
	HOTAIR	Breast cancer (primary and metastasis)	Predictor of metastasis and survival	45

miRNA expression.[15] Furthermore, ever since the detection of DNA methylation evolved as a promising biomarker in cancer, many studies have anticipated that epigenetically regulated miRNAs could have new roles in prognosis, early detection, and predictive values in cancer.[16,17]

A recent study by Lu et al.[18] employing a whole-genome approach demonstrated that miR-129-2 was frequently methylated in hepatocellular carcinoma cells (HCC) but not in normal liver cells and tissues. miRNAs are well known for their ability to posttranscriptionally regulate the expression of target mRNAs. Deregulation of these small molecules contributes to carcinogenesis through affecting major hallmarks of cancer.[12,19] Similarly, in samples from patients, methylation levels of miR-129-2 were increased in tumors when compared with normal samples. Moreover, miR-129-2 methylation was detected in plasma samples from patients with tumors but not in healthy individuals or patients with cirrhosis, suggesting miR-129-2 methylation as a specific, potential marker for early diagnosis in HCC. Moreover, miR-129-2 methylation was found in lymphoid malignancies as adversely impacting survival in chronic lymphocytic leukemia (CLL). In myeloid malignancies—acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL)—miR-129-2 was not methylated but, in multiple myeloma (MM), this miRNA was associated with the progression from monoclonal

gammopathy of undetermined significance (MGUS) to symptomatic MM.[20] For a review of epigenetics and miRNAs in acute leukemia see reference 21.

Increased methylation of miR-124-3 CpG islands in renal carcinomas (clear cell histology) was associated with unfavorable clinicopathological aspects, such as tumor grade and diameter, metastasis, and advanced disease. This hypermethylation was tumor-specific and related to poor recurrence-free survival, implying that it could be involved in cancer progression, making miR-124-3 a putative biomarker for patients that can benefit from targeted therapy by means of small molecule effectors.[22] Similarly, in ALL, hypermethylation of this microRNA locus was found as an independent prognostic factor for overall and disease-free survival and associated with higher relapse and mortality rates. Epigenetic regulation of miR-124a was shown to mediate increased expression of CDK6, which contributed to abnormal proliferation profiles of ALL cells in vitro and in vivo.[23]

Another miRNA described as epigenetically regulated is miR-34a, which, when hypermethylated, was associated with metastasis to liver and lymph nodes in colon cancer. Repressed expression of miR-34a due to methylation was probably the trigger to the metastatic phenotype obtained by subsequent upregulation of *c-Met*, *Snail*, and *b-catenin*.[24] By treating lymph node metastatic cells with a DNA demethylating agent (5-aza-2′-deoxycytidine), Lujambio et al.[25] have associated particular DNA methylation signatures of miRNAs with the metastatic behavior of tumors. This was valid for lymph node metastasis cancer cells derived from colon, melanoma and head and neck. Epigenetic silencing of miR-148a and miR-34b/c was shown to mediate the activation of oncogenic and metastasis target genes as *c-MYC, E2F3, CDK6,* and *TGIF2*.

Histone modifications, rather than DNA methylation, can also drive miRNAs suppression. A study by Incoronato et al.[26] showed silencing of miR-212 in lung cancer and correlation to later stages of the disease (T3/T4). The authors found that the promoter element of this miRNA is inserted in a CpG island, but its inactivation in lung cancer is linked to alterations in the methylation status of histone tails and not to DNA methylation status.

The wide range of interactions between the miRNome and the epigenome provide new challenges and opportunities for future studies regarding therapeutic strategies in oncology.

Regulation of lncRNAs by epigenetic mechanisms

Long ncRNAs have been shown to exhibit patterns of expression much more specific to each tissue than those exhibited by protein-coding genes,[27] offering a variety of benefits in clinical applications,[28] both for early diagnosis and disease prognosis. In hepatocellular carcinomas, the lncRNA H19 has been found to be involved in disease progression, behaving as an oncogene.[29] Zhang and colleagues[30] showed that epigenetic activation of miR-200 family promotes H19 downregulation, driving metastasis through EMT induction. Also, H19 was found to be underexpressed in intratumoral tissues (T) when compared with peritumoral tissues (R), and low T/R ratio was associated with shorter disease-free survival, more aggressive tumors with metastatic properties and, therefore, worse prognosis. Moreover, in the same study, restoration of H19 levels was capable of inhibiting the invasion capacity of hepatocellular carcinoma cells.

A lncRNA signature was obtained in glioblastoma multiforme (GBM) and a prognostic value, independently of O6-methylguanine methyltransferase (*MGMT*) promoter methylation status, was found. The lncRNAs KIAA0495, PART1, MGC21881, MIAT, GAS5, and PAR5 expression—named the six-lncRNA signature—were linked with overall survival, alluding that this signature may supply new markers for prognosis treatment and outcome prediction in GBM.[31]

Epigenetic regulation has effects on lincRNAs as well. ANRIL, also known as CDKN2b antisense or CDKN2BAS, is a lincRNA mapped in the *INK4b-ARF-INK4a* locus, which was found to be regulated by the Polycomb repressive complex (PRC). This locus has been shown to be one of the most recurrently altered in tumors[32,33] and, in a subset of tumors, epigenetic modifications contribute to its silencing, which may bring both diagnostic and prognostic perspectives in the future. For a deeper review, please check reference 34.

Regulation of the Epigenome by Non-Coding Rnas

Epigenome regulation by the miRNome: The turn of epi-miRs

The idea that miRNAs could act as regulators of the epigenome first came with the work published by Fabbri et al.,[35] in which members of the miR-29 family (29a, 29b, and 29c) were shown to negatively modulate DNMT3A and DNMT3B enzymes in lung cancer, causing lower survival rates in patients with higher levels of DNMT3A. These miRNAs were then named epi-miRs, referring to a group of miRNAs capable of targeting effectors of the epigenetic system—directly or indirectly—via a regulatory loop, by affecting its regulating enzymes.[12] Also, DNMT3B was described as a target for miR-29b in AML[36]; however, no clinical significance was assessed for this tumor.

In hepatocellular carcinomas, downregulation of miR-22, known to target HDAC4, was correlated with decreased disease-free survival and worse prognosis, suggesting that it may be involved in the carcinogenesis and progression of this tumor.[37]

Other miRNAs have also been implicated as regulators of the epigenetic machinery, through the target of crucial enzymes. Some of the examples are miR-449a targeting HDAC in prostatic cancer,[38] miR-144, targeting EZH2 in bladder cancer,[39] and miR-101, also targeting EZH2 in bladder,[40] prostate,[41,42] and breast cancer.[42] DNMTs have been demonstrated as targets of miR-152 in hepatobiliary cancer[43] and in ALL,[44] and as target of miR-148 in various cell lines.[45] Although the field of epi-miRs is very promising, the literature is still scarce in regard to clinical aspects in cancer detection, prognosis and predictive values of miRNAs.

lncRNAs as regulators of the epigenome

XIST (X-inactive-specific transcript), one of the first discovered lncRNAs in mammals,[46] is 17 kb long and possesses a very important and well known function: it is responsible for the epigenetic regulation in the inactivation of X chromosome. Through coating one of the X chromosomes, XIST is capable of inactivating it,[47] driven by its binding to PRC2.[48] Unmethylated

XIST is a frequent finding in testicular germ cell tumors, and the methylation status of this gene is being considered for evaluation in male-derived plasma as a biomarker for early tumor detection and prognosis of these types of tumors.[49]

HOTAIR (homeobox transcript antisense RNA), a lincRNA, is overexpressed in laryngeal squamous cell carcinomas when compared with adjacent tumors, and associated with poorly differentiated tumors, lymph node metastasis and advanced clinical stages, suggesting this lincRNA as a marker of poor prognosis.[50] HOTAIR seems to bring this oncogenic phenotype through promotion of PTEN methylation[50] and was also described as capable of altering histone H3 methylation in a PRC2 regulation-dependent fashion. HOTAIR expression was increased in both primary breast and metastasis tumors, posing as a predictor of metastasis and shorter overall survival.[51]

piRNAs regulation of the epigenome

piRNAs have been shown to be capable of maintaining the integrity of the genome by epigenetically silencing transposons through DNA methylation. piRNAs interact only with members from the Piwi family, but its human ortholog, Hiwi, has been described as aberrantly expressed in many cancer types.[47] In human esophageal squamous cell carcinoma, cytoplasmic expression of Hiwi was associated with evidences of poor prognosis.[52]

Concluding Remarks: Challenges and Opportunities

The accurate comprehension of the molecular machineries involved in cancer development and progression is critical for diagnosis, prognosis, and treatment. Much data have been generated in the last years regarding ncRNAs, their regulation by the epigenetic machinery and, in turn, their role in the regulation of epigenetic processes. The knowledge of how these two mechanisms of regulation are integrated in the cellular context is opening up new avenues for the identification of precise predictive and prognostic factors for new therapeutic strategies in oncology. Still, much has to be done. Cancer is part of an intricate network; clarifying the complex relationship between epigenetics and other mechanisms, such as ncRNAs, and translating these into the clinical practice, remains a big challenge.

The wide range of interactions between the non-coding RNome and the epigenome present new challenges and opportunities for the development of new therapeutic strategies in oncology. Even though ncRNAs are promising candidates for their role in the regulation of the epigenetic machinery, few studies have explored their function in a clinicopathological standpoint. The study of their capacity to regulate one of the most important machineries in cancer remains not only a big challenge, but also a big opportunity in oncology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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