Review

MicroRNAs: A Puzzling Tool in Cancer Diagnostics and Therapy

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Abstract. *MicroRNAs (miRNAs) constitute a dominating class of small RNAs that regulate diverse cellular functions. Due the pivotal role of miRNAs in biological processes, a deregulated miRNA expression is likely involved in human cancers. MicroRNAs possess tumor suppressor capability, as well as display oncogenic characteristics. Interestingly, miRNAs exist in various biological fluids as circulating entities. Changes in the profile of circulating miRNAs are indicative of pathophysiological conditions in human cancer. This concept has led to consider circulating miRNAs valid biomarkers in cancer diagnostics. Furthermore, current research promotes the use of miRNAs as a target in cancer therapy. However, miRNAs are an evolving research field. Although miRNAs have been demonstrated to be potentially valuable tools both in cancer diagnosis and treatment, a greater effort should be made to improve our understanding of miRNAs biology. This review describes the biology of microRNAs, emphasizing on the use of miRNAs in cancer diagnostics and therapy.*

Different classes of small non-coding RNA molecules, including microRNA (miRNA), small interfering RNA (siRNA) and PIWI-interacting RNA (piRNA) have been identified in eukaryotic organisms. Initially, non-coding RNAs

were believed to lack a particular function, hence they were classified as RNA junk (1). Although the definition of RNA junk is still elusive (1), several categories of small RNAs are now recognized for their capability to control gene expression and participate in cell signaling regulation. Distinctive features of small RNAs are their length (20-30 nucleotides), as well as their association with Argonaute-2 proteins (2). MicroRNAs constitute a dominating class of small RNAs that regulate diverse cellular functions, including apoptosis (3), metabolism (4), as well as cell growth and differentiation (5, 6). Due the pivotal role of miRNAs in biological processes, deregulation in miRNAs expression alters normal cell functions, hence participating in the development of human disease, including cancer. In this review we sought to describe the biology of microRNAs, outlining the potential use of miRNAs in cancer diagnostics and therapy.

MicroRNA Biogenesis and Function

MiRNAs are endogenous, non-coding RNA molecules, of 18-22 nucleotides length, capable of modulating negatively gene expression at the post-transcriptional level. miRNAs are made by a multistep process that initiates in the nucleus and ends in the cytoplasm.

The majority of miRNAs are generated from "independent" genes (also known as intergenic miRNA genes), so called as they retain their set of promoters and regulatory elements (7). However, about 25% of miRNA genes is a portion of introns (intronic miRNAs) placed within the sequence of canonical genes. Intronic miRNAs share mutual promoters with genes wherein they are integrated. After being produced as a single transcript, an intronic miRNA is processed from introns sequences (8). Another category of RNA molecules is represented by miRNAs generated by entire intron sequences. These miRNAs are known as mirtrons (8-10).

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The first step in the biogenesis of miRNA genes involves the activation of the RNA polymerase II enzyme that produces primary miRNA transcripts (pri-miRNAs). Pri-miRNAs are hairpin precursors of about 70 nucleotides and are further processed into shorter (10 nucleotides smaller), transcripts (pre-miRNAs) by the Drosha endonuclease complex (7). An exception to this mechanism is represented by the mirtrons pathway in which the generation of miRNAs does not require the activity of the Drosha complex; instead these pre-miRNAs are originated directly from the mirtron transcript, by splicing mechanisms (9). However, several other characteristics distinguish the processing mirtrons from canonical miRNAs. For a comprehensive study on mirtrons biogenesis, refer to Wen *et al.* (10).

Once produced, pre-miRNAs are transported to the cytoplasm where they are cleaved by the endonuclease enzyme Dicer into double-stranded 20-21 nucleotides miRNAs, each containing a guide strand, which is complementary to the target RNA messenger (mRNA), and a pairing strand referred as passenger strand. When a duplex miRNA unwinds, the passenger strand is degraded, while the guide strand (now considered as mature miRNA) is associated with Argonaute-2 proteins that initiate the loading of the mature miRNA into the miRNA-induced silencing complex (miRISC) (11). Once assembled, miRISC targets and silences specific mRNAs. The selection of mRNA by the miRISC is determined by the degree of complementarity between the region at 5' end of the miRNA-loaded in the complex and 3'UTR site on the mRNA. miRISC can perform two alternative silencing mechanisms: it can inhibit the translation of mRNA or, induce the mRNA degradation. The nature of control adopted by miRISC depends on whether the sequence of the miRNA-loaded in the complex shows partial or full complementarity with the sequence of the target mRNA: sequences that match only partially lead to repression of the translation, while a sequence perfect matching triggers mRNA degradation (8, 12).

MicroRNAs and Cancer

Due the pivotal role of miRNAs in biological processes, deregulation in miRNAs expression participates in cancer initiation and progression (13). Our collaborative study by Cattaneo *et al.* has determined a specific miRNA signature with a potential biomarker feature in acute myeloid leukemia (14). Similarly, numerous investigations have approached the identification of specific miRNAs as biomarkers and biotargets in human cancer (15).

MicroRNAs either have tumor suppressor capability (*e.g.*, miR-29b and miRNA-30-5p) (16, 17) or display oncogenic characteristics (*e.g.*, mir-17-92 cluster) (18), both of which influence cell growth (19).

Similarly to proteins, miRNA dysfunction depends on several reasons: alteration in miRNA expression can be a result of miRNA gene mutations (20), epigenetic modifications (21) or deficiency in the miRNA processing pathway (22). Also, genetic mutation, as well as epigenetic modification of miRNAs genes, have been correlated with cancer (8).

Circulating miRNAs in Cancer Diagnosis

Successive to their initial identification in tissues, microRNAs have been found in various biological fluids, including plasma, serum, saliva, milk and cerebrospinal fluid. Non-cellular microRNAs, also known as circulating miRNAs, display a high grade of stability despite their RNA nature (23). Studies have demonstrated that circulating miRNAs are packed into particular carriers and, therefore, protected from the degrading activity of RNase enzymes, highly abundant in body fluids (23). Exosomes, microvesicles and apoptotic bodies, as well as lipoprotein complex (high-density lipoprotein (HDL)), all have been identified as non-cellular miRNAs carriers. However, the majority of circulating miRNAs seems associated in a stable complex with Argonaute-2 proteins (23). Despite the initial belief that non-cellular miRNAs were products of cell degradation with no specific functions, it has been suggested that these non-cellular miRNAs may represent signaling molecules with a definite role in cell-cell communication (24). Likely to cellular miRNAs, studies suggested that changes in the profile of circulating miRNAs are correlated with a pathophysiological condition of human cancer. In line with this concept, data achieved from our studies indicated that blood levels of miR-22, miR-24 and miR-34a are correlated with advanced stages of non-small cell lung cancer (NSCLC) (25). Also, several other cell-free miRNAs (*e.g.* miR-429, miR-205, miR-200b, miR-203, miR-125b and miR-34b) have been validated as diagnostic tools in NSCLC (26), as well as gastric cancer (27). Furthermore, studies demonstrated that high plasma levels of miR-19a are a favorable prognostic factor in patients with metastatic HER2+ inflammatory breast cancer (28).

At present, several clinical trials are investigating circulating miRNAs as biomarkers. For example, in patients with glioma associated with neurofibromatosis type 1 (NF-1) (ClinicalTrials.gov Identifier: NCT01595139), the microRNAs blood profile is being evaluated to identify circulating microRNAs that would be suitable as predictive/diagnostic markers for glioma associated with NF-1. Also, many clinical studies are focusing on detecting circulating miRNAs biomarkers in breast cancer (ClinicalTrials.gov, NCT02065908 and NCT0172285), hepatocellular carcinoma (ClinicalTrials.gov, NCT02448056), as well as pediatric cancer (ClinicalTrials.gov, NCT01541800).

Challenges of Circulating miRNAs in Cancer Diagnosis

The discovery of circulating miRNAs has suggested the use of these microRNA molecules as biomarkers. In fact, non-cellular miRNAs characteristics, namely their extraordinary resistance to degrading processes, and miRNAs presence in body fluids, which makes them detectable with non-invasive techniques, together with their correlation with pathological conditions, have made these cell-free molecules suitable candidates as predictive factors, as well as diagnostic markers.

However, studies in both cancer and non-neoplastic field, devoted in placing circulating miRNAs among biological markers, remain still much elusive. The effort in finding specific miRNAs for each disease has failed (29). In fact, several miRNAs has been found associated with a broad range of conditions. For example, the non-cellular miR-141 has been reported in the blood of pregnant women (30), as well as in patients with prostate and several other cancers (31). That has raised an important question: What does a change in the circulating miRNA profile mean? Is it a biomarker of a particular condition or a simple sign of a general state of the disease? (29). As suggested by Witwer the answer to this question depends on what we contemplate as cell-free miRNAs (29). We can assume that the pool of circulating miRNAs contains two types of miRNAs: miRNAs complexed with the Argonaute-2 proteins and vesicle-packed miRNAs (23). Currently, the common idea is that microRNAs encapsulated in the exosomes participate in intracellular signaling. Conversely, miRNAs-Argonaute-2 proteins complexes represent only cell by-products that accumulate in the extracellular fluids (23). This suggests that one way to identify miRNAs suitable as biomarkers should be in focalizing the attention on vesicular miRNAs, instead of continuing the study of the entire circulating pool of molecules (29).

MicroRNA as Therapeutic Target

MicroRNAs have been demonstrated to regulate fundamental cellular processes and, thus, their alteration has been correlated with a variety of human cancers. Due to their central role in human biology, a great enthusiasm has been posed in the use of miRNAs as a target in cancer therapy. Currently, several technologies are available to manipulate specific miRNAs, resulting in either their activation or inhibition (32, 33). The most common approaches used to abolish the miRNAs functionality consists of synthetic antisense oligonucleotides (ASOs) (34, 35). Despite the poor stability of these molecules, ASOs can be chemically manipulated to enhance their pharmacokinetics (33). Regardless the chemical modification, ASOs are commonly

referred as anti-miRs (33). However, cholesterol-conjugated ASOs are termed antagomirs (34, 35). Anti-miRs bind native miRNAs, through base pair matching, and prevent miRNA-mRNA interaction (33). The same concept of antisense complementarity has been used to realize miRNA sponges. A miRNA sponge consists of multiple miRNAs antisense molecules linked together to form a unique structure that can sequester and knockdown the activity of an entire pool of sequence-correlated miRNAs (36). The miRNA inhibition transcript can also be carried out by using specific drugs that alter the biogenesis of the mature miRNA transcript (37, 38).

The last decade has witnessed a considerable increase in cancer researchers devoted to exploring the therapeutic capability of miRNAs (39). The manipulation of miRNAs has been showed to undermine cancer progression. The reactivation of tumor suppressor miRNAs, such as miR-29b or miRNA-30-5p, in myeloma cells, has therapeutic effects (40, 41). On the other hand, miR-21 has been demonstrated to have an oncogenic feature (42). We proved that inhibition of the miR-21 halts the progression of multiple myeloma (MM) (43). In addition to blood cancer, the therapeutic potential of miRNAs has been addressed in different types of cancer including, but not limited to, glioblastoma (44), retinoblastoma (45) and leukemia (46).

Challenges of miRNA-based Therapies

MiRNA-based therapies pose challenges correlated to miRNA tissue-specific delivery and toxicity. The poor relative stability in the biological fluid, as well as the negative charge of mRNA molecules, make difficult the cellular uptake of miRNAs and their specific distribution into tissue (47, 48). To overcome these obstacles many strategies, including viral vector transportation, nanoparticle and cationic lipids inclusion, as well as chemical modification, have been investigated (49-53). However, approaches, such as anti-miRs and cationic lipids-linked miRNAs delivery, have elicited toxicity *in vivo* (39). Given miRNA-based therapies, an additional aspect to consider is that miRNAs regulate several diverse genes. Therefore, manipulation of these molecules could create side-effects and increase the risk of toxic phenotypes (47, 49).

Conclusion

As miRNAs are still an evolving research field, our awareness of miRNA biogenesis function is still at its infancy stage. Overall, miRNAs have demonstrated to be potentially valuable tools both in cancer diagnostic and therapy. However, a greater effort should be made to improve our approach, both technically and rationally, toward miRNAs biology, to overcome current challenges linked to the use of miRNAs. The consideration of Witwer

about microRNAs in cancer diagnostics is remarkable (29). The author suggested placing our attention on investigating only the fraction of vesicular miRNAs, as this is the unique pool of circulating miRNAs that likely retain a biological function. In our point of view, an advanced understanding of the mechanism governing the biology of these molecules represents the only valid approach to design a useful diagnostic miRNA-based tool.

Nevertheless, from the therapeutic standpoint, Garzon *et al.* consider critical to establish new methods that enable the efficient delivery of specific miRNAs in cancer tissue (39). In agreement with these authors, we believe that this approach would help find a suitable therapeutic solution against the progression and recurrence of cancer, as well as to minimize the risk of toxic effects elicited by the administration of miRNA-based drug.

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