Circulating microRNAs in Medicine

Subjects: Biotechnology & Applied Microbiology

Contributor: Tetiana Pozniak , Dzmitry Shcharbin , Maria Bryszewska

Circulating microRNAs (c-microRNAs, c-miRNAs), which are in almost all biological fluids, are promising sensitive biomarkers for various diseases (oncological and cardiovascular diseases, neurodegenerative pathologies, etc.), and their signatures accurately reflect the state of the body. The discovery of microRNAs was a scientific breakthrough, and the study of their functional potential opened up the possibility of influencing protein synthesis at the gene level. Moreover, microRNAs of this class are highly sensitive biomarkers of various diseases, allowing not only to detect a disease at early asymptomatic stages, but also to predict therapeutic efficacy.

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1. Introduction

More than 20 years ago, a mechanism of negative regulation of gene expression at the level of translation was discovered: mRNA is blocked by small non-coding RNAs, repressing translation or promoting mRNA degradation. This process has been called RNA interference and it leads to “gene silencing”. Several subclasses of small non-coding RNAs (snRNAs) are involved in this powerful post-transcriptional gene silencing process. RNA interference was first described by Andrew Fire and Craig Mello in *Nature* in 1998. They received the Nobel Prize in Physiology or Medicine (2006) for this discovery [1]. They showed that short double-stranded RNAs can silence homologous genes.

There are many groups of snRNAs and the list is growing rapidly. This details the components of RNA interference, such as microRNAs (miRNAs) and small interfering RNAs (siRNAs). These two species have similar structures and are short (21–23 and 20–24 nucleotides, respectively) single- and double-stranded RNAs that inhibit gene expression. The main differences between them lie in their mechanisms of formation and their degrees of homology with respect to targeting of mRNAs.

In 1993, it was determined that the *lin-4* gene in *Caenorhabditis elegans* produced a snRNA [2][3]. In 1998, *lin-4* was shown to encode a 61-nucleotide precursor that matured into a 22-nucleotide RNA, later called miRNA. This short RNA contains sequences that are partially complementary to the 3′-untranslated region (3′-UTR) of mRNA transcribed from the *lin-14* nematode gene and represses translation of this mRNA, inhibiting LIN-14 protein synthesis. In 2000, a second miRNA was discovered, a product of *let-7* [2], which suppressed the expression of several genes simultaneously and was later identified in a number of organisms, including humans. Although *lin-4*

and *let-7* were identified by standard positional cloning of genetic loci, most miRNA genes are detected by cloning cDNA sequences complementary to the desired RNA fragments. This method involves the isolation of a miRNA that blocks the translation of a specific messenger, followed by cDNA synthesis using reverse transcriptase. One difficulty in finding miRNA genes for further cDNA cloning is that not only fragments of the target but also fragments of other noncoding RNAs (such as rRNA, tRNA, and snRNA), together with mRNAs, are cloned from RNA samples of a selected size. However, this difficulty is easily solved by comparing the candidate miRNA sequence with known miRNA sequences in annotated databases [4]. To date, more than 2000 miRNAs have been registered in this database.

2. Circulating miRNAs (c-miRNAs) and Their Differences

All organisms transmit genetic information from parent to offspring through vertical gene transfer. Bacteria also have horizontal (lateral) gene transfer for exchanging genetic information, which allows them to diversify their populations and facilitate adaptation to new conditions. Horizontal information transfer in eukaryotes was discovered relatively recently through the intercellular transport of miRNAs [5]. In 2008, circulating miRNA (c-microRNA) was first detected in maternal plasma [6]. Later, c-microRNAs were also found in other biological fluids, such as blood (serum, plasma), urine, cerebrospinal fluid, saliva, milk, lacrimal and seminal fluids, and bronchial lavage [7][8][9]. The appearance of c-microRNAs in the blood can result from secretion by cells or from cell death during apoptosis, necrosis, tumors, or trauma.

Interestingly, despite the presence of various nucleases, circulating endogenous miRNAs remain stable while pure exogenous miRNAs added to plasma degrade rapidly [7]. Subsequent studies established that endogenous miRNAs are highly stable and resistant not only to endogenous ribonucleases but also to extreme temperatures, pH levels, and freeze–thaw cycles [10]. To ascertain what protects them from enzymatic degradation, researchers consider the mechanisms of miRNA entry into extracellular fluids and the forms in which they are found there. Initially, c-miRNAs can be secreted into the extracellular space in microvesicles (late endosomal compartments [11]), ectosomes [12], exosomes [13], or microvesicles (liposomes), or they can be associated with high-density lipoproteins (HDL) [14], or released as parts of apoptotic bodies. However, most of them, according to Reiner et al., form complexes with the proteins AGO2 or NPM1 (nucleophosmin 1) [15]. Regardless of the form in which they enter the extracellular space, miRNAs then pass into other biological fluids, for example, the general bloodstream.

Microvesicles (microparticles or ectosomes) are plasma membrane-derived particles released into the extracellular space by budding out and detachment from the plasma membrane. They range in size from 100 nm to 1 μm and are formed by outward protrusion of the plasma membrane, followed by separation [16]. Ectosomes are secreted by various cells, including tumor cells, polymorphonuclear leukocytes, aging erythrocytes, and activated platelets. One of their characteristic features is the appearance of phosphatidylserine (PS) on their membrane surfaces. Unlike exosomes, ectosomes bind well to annexin V and can also bind to prothrombin and blood coagulation factor X to form the prothrombinase complex [7].

Exosomes are small, membrane-bound vesicles of endosomal origin, 30–100 nm in diameter, that form intracellularly via endocytic invagination and are released into a structure known as the multivesicular body (MVB). The MVB then fuses with the plasma membrane, releasing the exosome contents into the extracellular space [17]. Exosomes are secreted by almost all normal cells (T-cells, B-lymphocytes, dendritic cells, reticulocytes, neurons, intestinal epithelial cells, platelets, etc.) and by pathological cells. It was previously believed that exosomes function only as protein scavengers, but in 2007, Valadi et al. found that they can carry nucleic acids (NAs), in particular, RNA [18]. It is now known that they can contain various components of their donor cells, including proteins (proteolytic proteins, chaperones (Hsp70 and Hsp90), apoptotic proteins (Alix), translation factors, metabolic enzymes), lipids, mRNA, microRNA, small interfering RNA (siRNA), and DNA. They can also carry viruses and prions from an infected cell [19]. Thus, exosomes function as horizontal carriers (between cells of the same organism, donor–recipient) of information, mRNA, viruses, and other materials, such as proteins and microRNAs. Some proteins are exosome-specific and do not depend on the donor cell; however, there are also specific ones, so it is possible to identify a subpopulation of specific exosomes and infer the cell type of origin [20]. However, how the various components making up exosomes are chosen remains an open question. In 2012, 4563 proteins had been found in exosomes [21], and the list of exosomal miRNAs numbered about 800. Cells can interact with each other by transferring exosomes loaded with miRNAs [22][23]. The researchers of [23] showed that monocyte exosomes deliver miR-150 to endothelial cells and enhance their migration by reducing c-myb 9 expression. The miRNA content of exosomes is critical in this type of intercellular communication and determines the fate of the recipient cell. Thus, exosomes derived from mesenchymal stromal cells of the bone marrows of myeloma patients promote tumor growth, and this effect depends on their miR-15a content.

Exosomes produced by stressed cells also provide information that induces resistance in surrounding cells through a paracrine effect. For example, miRNAs regulate the activity of pancreatic β-cells by transfer via exosomes [24]. Exosomes are also involved in transmitting the immune response; miRNAs transferred by exosomes from T-cells to antigen-presenting cells (APCs) can regulate gene expression in the recipient cells [25]. Exactly this process accounts for the suppression of antitumor immunity, including inhibition of T-lymphocyte and natural killer activities, and the suppression of APC differentiation. Tumor exosomes also enhance the activity of immunosuppressive cells and increase their number. There are indications that tumor cells dispose of chemotherapy drugs (in particular, doxorubicin) by secreting them in exosomes. This process underlies the acquisition of resistance to anticancer therapy by malignant cells [26]. Considering these properties, exosomes can be considered promising tools for delivering drugs and engineering exogenous miRNAs for high-precision treatment of various diseases. Details about exosomes, their functions, mechanisms of action, and clinical applications have been researched[19][27].

In the extracellular fluid, some c-miRNAs are complexed with HDL [14][28]. Cellular export of miRNAs to HDL is regulated by neutral sphingomyelinase. The researchers have demonstrated that reduced HDLs injected into mice form complexes with various miRNAs in normal and atherogenic models. The HDL-miRNA profile differs significantly between a healthy person and one with hereditary hypercholesterolemia. It is noteworthy that HDL-miRNAs in atherosclerosis induce differential gene expression with a significant loss of conserved mRNA targets in cultured hepatocytes. Taken together, these observations indicate that HDL is involved in intercellular communication, including the transport and delivery of miRNAs.

Other vesicles that transport miRNAs in the intercellular fluid are apoptotic bodies. These are vesicles with a diameter of 50 nm to 4 µm, formed from cells undergoing apoptosis [29].

The foregoing shows that c-miRNAs function as secreted signaling molecules and affect the phenotypes of recipient cells. Numerous studies have correlated the levels of vesicular and protein-bound c-miRNAs with various pathologies. Moreover, secreted extracellular miRNAs can reflect molecular changes in the cells from which they originate, so their profiles differ depending on physiological and pathological conditions [30]. They can therefore be considered potential diagnostic markers for diabetes, systemic lupus erythematosus, asthma, arthritis, Alzheimer's disease, cardiovascular diseases, various tumors, etc. [31][32][33].

In 2011, the existence of c-miRNAs in vesicular form was questioned, since only 10% of circulating microRNAs are in this form [34]; the remaining 90% circulate in association with proteins of the Argonauts family (AGO2), HDL, or other RNA-binding proteins [35]. However, it was later found that the non-vesicular forms of c-miRNA are non-specific products of physiological activity and cell death.

The basis on which one method of miRNA transport is chosen over another for delivery into the intercellular space is not clear. However, selective "packaging" of various c-miRNAs into microvesicles and exosomes has been demonstrated [35][36][37]. For example, the let-7 miRNA family in a metastatic gastric cancer line is selectively secreted into the extracellular environment exclusively by exosomes. According to the researchers, this contributes to the maintenance of oncogenesis and metastasis [35]. Wang et al. showed that some human cell lines (HepG2, A549, T98, and BSEA2B) actively release miRNAs for an hour immediately after serum deprivation [38], suggesting that miRNAs are secreted in response to stress. In this experiment, the researchers noticed that most of the extracellular c-miRNAs were complexed with RNA-binding proteins, not inside microvesicles or exosomes. Characteristic miRNA sequences promote interaction with the A2B1 fish nucleoprotein, while Ago2 facilitates miRNA loading into the vesicle. A mechanism for miRNA sorting based on the 3′-terminal structure was proposed [39]. These researchers found that 3′-terminal-adenylated microRNAs were predominantly intracellular, while their 3′-terminal-uridylated isoforms were found in exosomes. This confirms the influence of post-transcriptional modifications on the microRNA sorting method.

miRNA is loaded into the vesicle by the interaction of characteristic miRNA motifs with the A2B1 fish nucleoprotein, which facilitates the process. Interestingly, the number of microRNAs selectively released from cells into a particular body fluid can correlate with malignant neoplasms [40]. Pigati et al. found that most of the miR-451 and miR-1246 produced by malignant mammary epithelial cells were released into the extracellular space, while most of the same miRNAs produced by normal mammary epithelial cells were retained in the cell. These results confirm a cellular selection mechanism for miRNA release and indicate differences between their extracellular and cellular profiles. This selective release makes it possible to consider c-miRNAs as biomarkers for various diseases.

The mechanisms that regulate and control exosome release into the recipient cell remain completely unknown. However, some proteins involved in this process have been identified: TAT-5 and Rab27. TAT-5 is a cell membrane-associated protein that regulates vesicle detachment [41]. Rab27 is involved in exosomal release and

uptake by recipient cells [42]. Exosome release from mammalian cells is also facilitated by the ceramide pathway, which also inhibits the export of miRNAs in combination with HDL.

3. Advantages and Disadvantages of miRNA as a Disease Predictor

Although the class of microRNAs was discovered relatively recently [2], they have been successfully studied for more than 10 years as biomarkers for various diseases. The expression of these molecules changes in cardiovascular diseases, tuberculosis, oncology, Alzheimer's disease, epilepsy, ischemic stroke, and many other pathologies [43]. The advantage of miRNAs over other known markers is their easy accessibility, i.e., they can be detected in any body fluid, including saliva, urine, and breast milk. Their high stability is associated with encapsulation in lipid vacuoles or complexation with proteins, which protects them from denaturation.

Another advantage of c-miRNAs is their sensitivity. They can indicate a disease before the clinical picture is manifest (during the latent period), and the profile can differ depending on the degree and severity of the disease, which is especially important for determining the stage of an oncological disease [44] and for personalized therapy. Tak Fan et al. showed the predictive power of miRNAs for the effectiveness of therapy for hepatitis virus [45].

Despite the rapid growth of knowledge about c-miRNAs, lines of these markers for individual pathologies have not yet been developed as standards; each individual miRNA has a wide and often non-specific spectrum of action. In addition, c-miRNA signatures differ depending on the biofluid in which they are detected. However, these problems can be solved if a clear algorithm for selecting markers is created, which is possible when the database of detected miRs, which is rapidly being replenished with new samples, has been sufficiently expanded. Furthermore, no standard protocol for c-miRNA detection has been approved so far. A protocol should include methods for isolation and storage and the detection technology itself. However, all these obstacles to using c-miRNAs as biomarkers for various diseases can be overcome by further studies.

4. The Use of miRNAs as Targets in the Treatment of Diseases

The therapeutic approach to using miRNAs can be divided into two categories: (1) miRNA inhibition therapy, when they are overexpressed [46]; and (2) miRNA replacement therapy, when they are repressed. The former approach uses an anti-miRNA or miRNA inhibitor consisting of a single-stranded oligonucleotide with a sequence complementary to the mature miRNA. The latter approach uses synthetic miRNA mimics with a sequence identical to that of endogenous mature miRNA. The principle is that such miRNAs are introduced into cells as an exogenous supplementary source. They are delivered in either plasmids or viruses for further expression, or by modifying the miRNA molecules themselves to stabilize them in the cell's internal environment. The approach to treating diseases at the translational level using miRNAs has advantages, since it makes it possible to "target" a specific

gene and thereby inhibit it highly specifically without affecting the genetic material of the host cell [47]. Already, drugs based on miRNAs are undergoing clinical trials.

5. Targeted miRNA Delivery Strategies In Vivo

To control the silencing of target genes, miRNA molecules need to leave the endosome and immediately enter the cytoplasm, where they bind to the RISC complex and cleave the complementary mRNA pointwise.

MicroRNAs in a complex of nanoparticles or modified molecules are captured by target cells through receptor-mediated endocytosis. Endocytic vesicles fuse and form early endosomes, which transfer their contents to late endosomes. Late endosomal vesicles have an internal pH of 5–6 owing to membrane-bound proton pump ATPases. Their contents are then transported to lysosomes, which have an even lower pH of ~4.5. Lysosomes also contain nucleases that promote miRNA degradation. To avoid this, miRNAs (free or complexed with a carrier) must exit the endosome into the cytosol, where they can bind to the RISC complex and participate in RNA interference. Endosomal yield is another obstacle to efficient miRNA delivery [48]. If this stage is overcome, the microRNA guide strand in the RISC complex interacts with complementary mRNA regions, leading to mRNA degradation and/or blocked translation.

Thus, a primary and major barrier to miRNA delivery is the plasma membrane. Being hydrophilic and negatively charged, miRNAs cannot penetrate into the cell easily. Another difficulty in using exogenous miRNAs is their short half-life in the blood owing to the nucleases therein. After the first barrier is overcome, the miRNA must be delivered to the cytoplasm bypassing the lysosome so it can bind to RISC and perform its silencing function. Therefore, the main challenge in using miRNAs as potential therapeutic agents is the development of high-precision platforms that can overcome all the difficulties of delivery and cellular uptake. The aims of these developments are: (1) to increase the residence time of miRNAs in the circulation by reducing the rate of renal clearance; (2) to protect them from serum nucleases; (3) to ensure efficient biodistribution; (4) to facilitate accurate delivery to the cytoplasm and capture by the target cell RISC system [49].

To date, there have been many approaches to maintaining the stability of miRNAs in vivo and ensuring targeted delivery to cells. The main ones include using: phosphorothioate-containing oligonucleotides [50], 2′-O-methyl-(2′-O-Me), or 2′-O-methoxyethyl oligonucleotides (2′-O-MOE) [51], locked NA (LNA), oligonucleotides [51], peptide NA (PNA) [52], fluorine derivatives (FANA and 2′-F), and others [53][54]) for chemical modifications of miRNAs.

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