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Extracellular microRNA: a new source of biomarkers

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Abstract

MicroRNAs (miRNAs) are a recently discovered class of small, non-coding RNAs that regulate protein levels post-transcriptionally. miRNAs play important regulatory roles in many cellular processes, including differentiation, neoplastic transformation, and cell replication and regeneration. Because of these regulatory roles, it is not surprising that aberrant miRNA expression has been implicated in several diseases. Recent studies have reported significant levels of miRNAs in serum and other body fluids, raising the possibility that circulating miRNAs could serve as useful clinical biomarkers. Here, we provide a brief overview of miRNA biogenesis and function, the identification and potential roles of circulating extracellular miRNAs, and the prospective uses of miRNAs as clinical biomarkers. Finally, we address several issues associated with the accurate measurement of miRNAs from biological samples.

miRNA biogenesis and function

miRNAs are short (19-24 nucleotides in length) non-coding RNAs that regulate messenger RNA (mRNA) or protein levels either by promoting mRNA degradation or by attenuating protein translation. Based on computational prediction, it has been estimated that more than 60% of mammalian mRNAs are targeted by at least one miRNA [1]. miRNAs were first identified in *Caenorhabditis elegans* in the early 1990s [2], but have since been reported in a wide variety of organisms ranging from single-cell algae to humans, suggesting that miRNA-mediated biological function is an ancient and critical cellular regulatory element [3,4]. The importance of miRNA function is further suggested by the extreme evolutionary conservation of both individual miRNA sequences and the miRNA processing machinery. In addition, the number of miRNAs in the genome appears to be correlated with the complexity of the developmental program, with mammals having the largest number of miRNAs.

miRNAs are transcribed in much the same way as protein-coding genes. The majority of miRNAs are transcribed by RNA polymerase II [5], though a minor fraction of miRNAs that lie within repetitive elements in the genome are transcribed by RNA polymerase III [6]. These primary miRNA transcripts (pri-miRNAs) are often several hundred nucleotides long and are modified similarly to protein-coding transcripts by the addition of a 5’ cap and a 3’ poly-A tail. Pri-miRNAs are then processed first in the nucleus and later in the cytosol by the RNase III enzymes Drosha and Dicer, respectively. This sequential processing of pri-miRNAs first yields a miRNA precursor (pre-miRNA) of ~70 nucleotides in length, and eventually a mature double-stranded miRNA of 19-24 nucleotides (Figure 1).

Typically, one strand of this mature miRNA duplex, termed the guide strand, associates with the RNA-induced silencing complex (RISC). While it is generally believed that upon incorporation into the RISC complex, the other strand (the passenger strand) is unwound from the guide strand and degraded, there is evidence that in some cases, both strands of the

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miRNA duplex are functional [7]. miRNA-RISC complexes interact with mRNA targets through partial sequence complementation, typically within the 3' untranslated region of target mRNAs. It is thought that the extent of base pairing between the miRNA and its mRNA target determines whether the mRNA is degraded or translationally repressed [8].

As with mRNAs, some miRNAs are differentially expressed among tissues or developmental stages. Examples include i) miR-122, which is preferentially expressed in the liver [9], ii) miR-133a and miR-133b, which are highly enriched in muscle [10], and iii) the miR-302 family members which are specific to stem cells [11]. Unlike some widely expressed miRNAs, these tissue- or developmental stage-specific miRNAs likely play key roles in regulating specific processes involved in the development or function of individual tissues. The liver-specific miR-122, for instance, has been implicated in lipid and cholesterol metabolism, which are both known to be important functions of the liver [12]. Because of their restricted expression profiles, these miRNAs hold promise as diagnostic markers or therapeutic targets for tissue- or biological stage-specific diseases. For example, miR-122 is required in hepatitis C virus (HCV) replication [13] and reagents that can modulate the level of miR-122 have moved into clinical development for HCV treatment [13-19].

Origin and function of circulating miRNAs

While the majority of miRNAs are found intracellularly, a significant number of miRNAs have been observed outside of cells, including various body fluids [20-24]. These miRNAs are stable and show distinct expression profiles among different fluid types. Given the instability of most RNA molecules in the extracellular environment, the presence and apparent stability of miRNAs here is surprising. Serum and other body fluids are known to contain ribonucleases [25], which suggests that secreted miRNAs are likely packaged in some manner to protect them against RNase digestion. miRNAs could be shielded from degradation by packaging in lipid vesicles, in complexes with RNA-binding proteins, or both [26,27]. Despite accumulating evidence for the presence of miRNAs in body fluids, the origin and especially the function of these circulating extracellular miRNAs remains poorly understood. One of the more intriguing ideas is that extracellular miRNAs are used as mediators of cell-cell communication [22,28-30]. If this is the case, then certain miRNAs are presumably targeted for export in one cell, and can be recognized, taken up, and utilized by another.

Using cell lines in culture, we have studied the miRNA export phenomenon in detail [22]. Our results revealed distinct intra- and extracellular miRNA spectra, which suggests the existence of a specific miRNA export system. Similar findings have been demonstrated by Pigati et al. in mammary epithelial cells, where the bulk of miR-451 and miR-1246 produced by malignant mammary epithelial cells, but not by non-malignant mammary epithelial cells, was released into the culture medium [31]. In addition to extracellular miRNAs, we have identified several RNA-binding proteins in the medium of cells cultured in the absence of serum [22]. In *in vitro* assays, one of these RNA-binding proteins, nucleophosmin 1 (NPM1), was able to protect synthetic miR-122 miRNA from RNase A digestion. However, further work is needed to determine whether other miRNA-protective proteins exist, as well as whether the apparent NPM1-miR-122 complex is sequence-specific or whether NPM1 acts as a general escort for miRNAs. Recently, Kosaka et al. showed that the release of miRNAs is controlled by neutral sphingomyelinase 2 (nSMase2) and through ceramide-dependent secretory machinery [32]. These results suggest the existence of miRNA export system and protein chaperones that could selectively export miRNAs and protect them in the extracellular environment.

Recent studies have also identified both mRNAs and miRNAs in two types of cell-derived lipid vesicles; microvesicles and exosomes. Microvesicles are relatively large (~100nm-1um) vesicles released from the cell through blebbing. Exosomes, on the other hand, are smaller vesicles (~30-100nm) released when endosomally-derived multivesicular bodies fuse with the plasma membrane. miRNAs have been identified in both exosomes and microvesicles derived from a variety of sources, including human and mouse mast cells [27], glioblastoma tumors [33], plasma [34], saliva [35] and urine [36]. It has recently been reported that miR-150 secreted in microvesicles from human blood cells or cultured THP-1 cells can be taken up by HMEC-1 microvascular endothelial cells and can regulate the expression of c-Myb, a known miR-150 target [37]. Furthermore, Pegtel et al. have shown that miRNAs released in exosomes by Epstein–Barr virus (EBV)-infected cells can be taken up by peripheral blood mononuclear cells and can suppress confirmed EBV target genes [38]. These findings strongly support that at least some exported miRNAs are used for cell-to-cell communication, although much more study is needed to determine how miRNAs are specifically targeted for secretion, recognized for uptake, and what information can be transmitted via this process.

miRNA potential as biomarkers

The ideal biomarker should fit a number of criteria depending on how the biomarker is to be used (Table 1). It should be accessible through non-invasive methods, specific to the disease or pathology of interest, a reliable indication of disease before clinical symptoms appear (early detection), sensitive to changes in the pathology (disease progression or therapeutic response), and easily translatable from model systems to humans. Most of the current blood biomarkers are based on the levels of specific proteins in the blood, such as troponin for cardiovascular conditions, carcinoembryonic antigen (CAE) for various cancers, prostate specific antigen (PSA) for prostate cancer, and aminotransferases (alanine aminotranferase, ALT and aspartate aminotransferase, AST) for liver function. Challenges for developing new protein-based biomarkers include the complexity of protein composition in blood, the diversity of post-translational modifications, the low relative abundance of many proteins of interest, the sequence variations among different clinically relevant species, and the difficulties in developing suitable high-affinity detection agents. While proteins are more diverse and therefore potentially more informative, the challenges listed above have made the discovery and development of new protein-based biomarkers with proper characteristics an expensive and time-consuming task.

On the other hand, secreted miRNAs have many requisite features of good biomarkers. miRNAs are stable in various bodily fluids, the sequences of most miRNAs are conserved among different species, the expression of some miRNAs is specific to tissues or biological stages, and the level of miRNAs can be easily assessed by various methods, including methods such as polymerase chain reaction (PCR), which allows for signal amplification. The changes of several miRNA levels in plasma, serum, urine, and saliva have already been associated with different diseases [39-59] (Table 2). For example, serum levels of miR-141, have been used to discriminate patients with advanced prostate cancer from healthy individuals [41], the ratio of miR-126 and miR-182 in urine samples can be used to detect bladder cancer [39], and decreased levels of miR-125a and miR-200a in saliva is associated with oral squamous cell carcinoma [50]. In addition to these potential uses in detection of various cancers, another intriguing possibility is the use of levels of organ-specific miRNAs in body fluids to monitor the physiopathological conditions of specific organs.

Using acetaminophen overdose as a model, we have previously demonstrated the possibility of using specific miRNA levels in blood to detect drug-induced liver injury [60]. The severity of liver injury in this well-established model system can be precisely detected and

monitored by measuring the levels of miR-122, a liver-specific miRNA, in plasma using quantitative PCR (qPCR). While elevated plasma ALT levels usually indicate impairment of liver function, plasma ALT originating from extra-hepatic sources have also been reported from patients with burns, muscle inflammation, hypothyrodism, or myopathies [61]. This makes the use of ALT levels alone in diagnosis of liver diseases unreliable, whereas the expression of miR-122 is fairly restricted to liver. In our hands, this miRNA-based method is more sensitive and probably more reliable than the current serological method, plasma ALT level, to detect and monitor drug-induced liver injuries. Besides miR-122 for liver injury, we and others [62] have demonstrated that the plasma level of miR-499, a heart specific miRNA, shows a perfect correlation with blood troponin levels in patients with myocardial infarction (Figure 2). This clearly demonstrates the possibility of using the level of a tissue-specific miRNA in circulation to reflect the health status of targeted tissues.

Issues associated with miRNA measurement

As mentioned above, extracellular circulating miRNAs offer several potential advantages as informative biomarkers when compared to protein-based blood biomarkers. For instance, while low abundance can significantly hinder the detection of some protein-based biomarkers, most circulating miRNAs can be readily detected by PCR. Additionally, protein-based biomarkers may have different post-translational modifications which can affect the accuracy of measurement, but miRNA species are relatively homogenous. Furthermore, the highly specific expression profile of several miRNAs such as miR-122 in the liver and miR-499 in the heart add the possibility of using the levels of these miRNAs in blood to precisely monitor the health status of specific organs. However, to further develop miRNA-based biomarkers, there are several fundamental issues associated with miRNA measurements that still need to be addressed (Table 3).

As with mRNA measurement, there are two major global miRNA profiling platforms in use today; microarrays and qPCR-based methods. Each of these methods has benefits and drawbacks. Although qPCR methods for miRNA quantitation are relatively inexpensive, widely available, and allow measurements of very small quantities of miRNAs, the primer design can heavily influence the results. Microarray-based measurement methods generally require more starting material than qPCR, and it can be challenging to develop probes and hybridization conditions that work well to detect many different miRNAs at once. In addition to microarrays and qPCR, there are other less frequently used methods, such as traditional northern blotting, ligation based measurement [63], and direct sequencing using next generation sequencing (NGS) platforms [64]. Of these methods, miRNA profiling by NGS may be the most promising, as it largely avoids many miRNA measurement pitfalls. The use of NGS technology offers the possibility to obtain comprehensive and accurate measurement for transcripts as well as miRNAs. Since the NGS approach is largely sequence independent, it does not rely on the design of primers or probes specific to each miRNA. Instead, NGS sequencing provides the number of counts for each miRNA or transcript present in the sample. Despite these advantages, NGS remains expensive and labor intensive, both in the sample preparation and in data analysis.

For miRNA measurement, it is well acknowledged that there is low correlation of results obtained from different platforms or even from the same platform using products from different vendors (such as microarrays from Agilent and Affymetrix) [65,66]. The short length and high sequence similarity among some miRNAs likely contributes to the inconsistency of measurement results due to the problems in designing specific primers for qPCR or probes for microarrays. Additionally, sequencing of miRNAs has revealed significant sequence heterogeneity at the 3’ and 5’ ends (termed isomirs) that may further complicate measurements [67]. This miRNA sequence variation probably results from

imprecise processing of miRNAs. However, our preliminary results indicate that there may be functional association with differences in the abundance of individual isomirs [67]. Further supporting the possibility of functionally significant isomirs, dynamic changes in the isomir population have been observed during *Drosophila* development [68]. Using synthetic isomir sequences, we have shown that sequence heterogeneity at the ends of isomirs may further complicate the miRNA measurement results, especially for some qPCR-based methods [67]. We have created an isomir database (http://galas.systemsbiology.net/cgi-bin/isomir/find.pl), to collect observed miRNA sequence variations which we hope may facilitate better miRNA probe or primer design in the future.

Another critical issue associated with miRNA measurement is assessing the quantity and quality of miRNA isolated from biological samples. In contrast to isolated mRNA, where the sizes and relative abundance of ribosomal RNAs can be used to assess the integrity of the RNA preparation, there is currently no good way to gauge the quality of isolated miRNA. In addition, degraded mRNA can interfere with the quantitation of miRNA preparations. Measuring the amount of miRNA isolated from body fluid samples is especially difficult due to the low concentration of RNA typically obtained from these samples. Therefore, it is difficult to establish the amount of input RNA in any measurement platform, which may make sample-to-sample and cross platform comparisons challenging.

Most protein-based blood biomarkers are measured using enzyme linked immunosorbent assays (ELISA) on serum. EDTA-plasma, on the other hand, is the preferred sample type for blood proteome studies, since it offers more reproducible protein measurement [46,69]. However, for blood miRNA measurements, both plasma and serum have been used in various studies (examples see Table 2). Our preliminary results suggest that there are notable differences in miRNA levels between serum and plasma. However, a comprehensive and systematic comparison is needed to establish the difference, if any, between serum and plasma miRNA content, as well as the effects of various anticoagulants on miRNA measurement.

Another issue that remains to be addressed is how to normalize or compare miRNA measurement results between samples. There are several well-established protocols for normalization of gene expression measurements across different samples. These include using global normalization approaches such as the level of housekeeping genes. Similar approaches can be used to normalize cellular miRNA measurements. However, for extracellular miRNAs, there are currently no known extracellular housekeeping RNAs that can be used for normalization. In many cases, normalization by input volume remains the best option when measuring circulating miRNAs. One interesting possibility is to use the level of other biomolecules in body fluids such as creatinine levels in blood and urine to normalize miRNAs. The levels of creatinine have been used to “normalize” other biomolecules in urine, and it provides an interesting possibility as a normalization basis for miRNAs in plasma and urine [70,71].

Finding informative biomarkers is not only key to understanding physiopathological processes of diseases, but is also critical for therapeutic development. It has been one of the major focuses of biomedical research in the past two decades. Despite the identification of several new protein-based biomarkers, very few of them actually pass rigorous validation processes. Circulating extracellular miRNAs clearly possess many desirable properties when compared to protein biomarkers. However, they require the same stringent validation processes as protein-based biomarkers to demonstrate their specificity and selectivity. The most fundamental challenges in miRNA-based biomarker development are the issues associated with accurate measurement. Building better reagent sets is one of the key steps to

obtaining more comprehensive and accurate miRNA measurements. Setting up a standardization process for sample preparation and developing a more accurate method to assess the quality and quantity of miRNA are also urgently needed.

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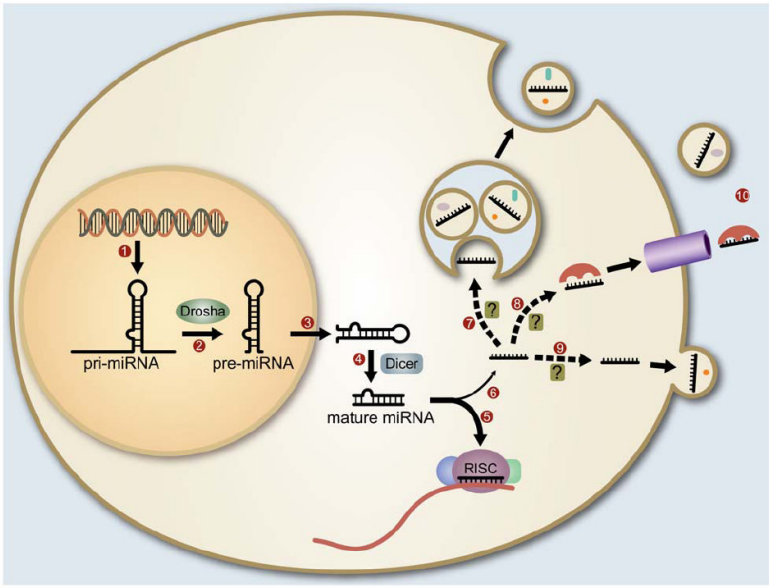

Figure 1. The biogenesis of miRNAs

miRNA biogenesis begins with transcription of pri-miRNA transcripts by RNA polymerase II or III (1). In the nucleus, pri-miRNAs are processed by Drosha to produce pre-miRNA hairpins (2), which are then exported into the cytosol (3). Here, pre-miRNA hairpins are processed into 19-24 nucleotide mature miRNA duplexes by Dicer (4). One strand of the mature miRNA duplex is incorporated into the RISC complex where it can regulate expression of target mRNAs (5). The other strand may either be degraded, or possibly prepared for export from the cell (6). Some miRNAs have been found packaged in exosomes derived from multivesicular bodies (7). Others may be exported in the presence of RNA-binding proteins (8). Still others might be exported microvesicles shed during membrane blebbing (9). Once in the extracellular space, these miRNAs could be taken up by other cells, degraded by RNases, or excreted (10).

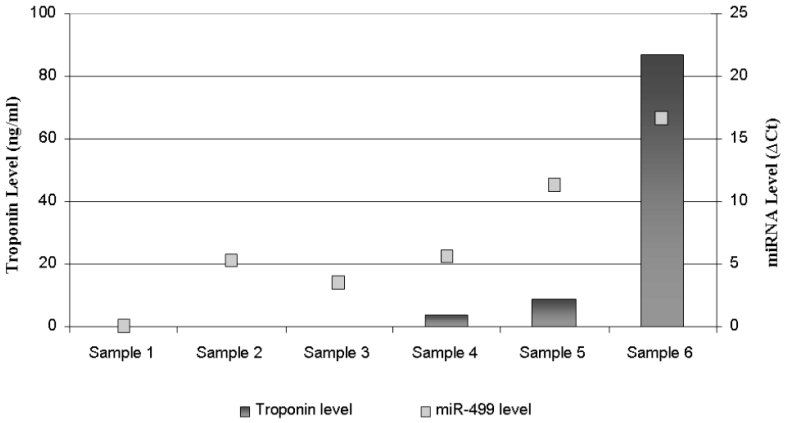

Figure 2. Plasma miR-499 levels in patients with myocardial infarction

The concentration of plasma troponin (bars) and miR-499 (gray squares) was determined from 6 individuals (X-axis) whom may suffer myocardial infarction. The concentration of troponin was displayed as ng/ml and the level of miR-499 was presented in ΔCt value (40-Ct).

Table 1

Characteristics of an ideal biomarker

Specific
Specific to diseased organ or tissue
Able to differentiate pathologies
Sensitive
Rapid and significant release upon the development of pathology
Predictive
Long half-life in sample
Proportional to degree of severity of pathology
Robust
Rapid, simple, accurate and inexpensive detection
Unconfounded by environment and unrelated conditions
Translatable
Data can be used to bridge pre-clinical and clinical results
Non-invasive
Present in accessible fluid sample

Table 2

Circulating microRNAs proposed as diagnostic biomarkers in human diseases

Type of cancer	Biomarker candidate	Reference
Prostate cancer		
	Fifteen **serum** miRNAs were over-expressed from stage 3 & 4 prostate cancer patients (*miR-16, -92a, -103, -107, -197, -34b, -328, -485-3p,-486-5**p**, -92b, -574-3p, -636, -640, -766, -885-5p*) when compared to normal individuals.	[40]
	Expression levels of *miR-141* in **serum** can distinguish prostate cancer patients from healthy controls	[41]
Breast cancer		
	Forty eight **serum** miRNAs were differentially expressed in breast cancer patients (*22 up-regulated, 26 down-regulated*) when compared to controls	[42]
	Increased expression levels of *miR-10b and -34a* in **serum** was observed in breast cancer patients	[43]
	Decreased expression levels of *miR-195 and let-7a* in **serum** was observed in breast cancer patients	[44]
Ovarian cancer		
	miR-21, 92, 93, 126 and 29a were over-expressed in **serum** samples from cancer patients compared to controls and *miR-155, 127* and *99b* were under-expressed	[52]
	Eight **serum** exosomal miRNAs were elevated in ovarian cancer patients: *miR-21, -141, -200a, -200b, -200c, -203, -205, -214*.	[45]
Lung cancer		
	Eleven **serum** miRNAs (including *miR-7i, -146b, -206, and and -21)* were changed more than five-fold by NGS between longer-survival lung cancer patient groups and shorter-survival groups. Levels of four miRNAs (*miR-486, -30d, -1, -499*) were associated with overall survival	[46]
Colorectal cancer		
	Both *miR-29a and miR-92a* showed elevated levels in **plasma** from 37 advanced colorectal cancer patients.	[47]
	The levels of two **plasma** miRNAs (*miR-17-3p and -92a)* were significantly elevated.	[51]
Bladder cancer		
	The ratio of two **urinary** miRNAs (*miR-126 and -182)* enabled detection urinary bladder cancer.	[39]
Oral cancer		
	The level of **plasma** *miR-31* was significantly elevated in oral squamous cell carcinoma patients compared to the control groups.	[72]
	Two miRNAs (*miR-125a and -200a)* showed decreased levels in **saliva** with oral cancer	[50]
Heart conditions		
	From acute myocardial infarction patients, the level of **plasma** miR-208b and -499 was highly elevated and correlated with plasma troponin level.	[53]
	Level of six **plasma** miRNAs including miR-423-5p was elevated in patients with heart failure.	[54]
	Plasma miR-1 level was significantly elevated from patients with acute myocardial infarction.	[55,56]

Table 3

Issues associated with miRNA measurement

Measurement
Low correlation between different measurement platforms
Short and conserved sequences in paralogs make it difficult to measure specific miRNA levels
Difficult to distinguish between precursor and mature forms
Sample
Lack of standard protocols for sample preparation
Concentration measurement for miRNA in sample is difficult
Data processing
Normalization among different samples, especially for extracellular miRNA
Difficult to validate results especially when using different protocols and platforms