

Review Article

Circulating microRNAs: Challenges with their use as liquid biopsy biomarkers

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Abstract. Circulating microRNA (miRNA) is a major focus in liquid biopsy studies. The circulating levels of certain miRNAs have been suggested to reflect specific physiological conditions, and several studies have reported their potential use as biomarkers for the detection and prognosis of cancer, as well as for predicting responses to chemotherapy or radiotherapy. Alongside these biomarker studies, research into the effects of specific background factors on circulating miRNA levels is progressing. Indeed, several studies have shown that a number of factors, including blood sample collection and processing methods, as well as subject-specific factors such as age, sex, and other physiological conditions, can affect the normal levels of circulating miRNAs. Unfortunately, the evidence supporting these effects is not yet strong enough to support a definite conclusion and further research is warranted. Here, we summarize the findings of several studies that have addressed these concerns and identify important topics that should be considered when analyzing circulating miRNA levels in liquid biopsy studies.

Keywords: miRNA, biomarker, cancer, serum, plasma

1. Introduction

Liquid biopsy is a hot topic in the field of exploring new diagnostic methods. The most popular liquid biopsy sample is circulating DNA, which can be used to detect specific mutations in genomic DNA released from cancer cells. Practical use of this type of liquid biopsy has already brought in a new era in cancer diagnosis. In addition, new protein, RNA, and metabolite biomarkers have also been identified in liquid biopsies, and some commercial products for the detection of these biomarkers have now been developed. Nonethe-

less, the detection of these new biomarkers in body fluids is often hampered by a lack of suitable laboratory techniques or clinical assays. In particular, optimal methods for sample handling, biomarker detection, and data preprocessing and normalization have not been established or standardized.

Circulating miRNAs are extracellular forms found in body fluids such as serum, plasma, urine, cerebrospinal fluid, and tears. Circulating mRNA occurs at very low levels and microRNA (miRNA) is more stable and abundant in serum/plasma than mRNA. Alongside circulating tumor cells and DNAs, circulating miRNAs have been attracting attention as candidate biomarkers for cancer detection and predictive prognosis. However, the specific features of circulating miRNAs are still under investigation, and factors such as sample handling methods and control selection can impact the results of circulating miRNA analyses. Here, we summarize some

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of the challenges that need to be overcome if circulating miRNAs are to be used for clinical applications. Although the studies discussed below are sometimes conflicting, we think this article will promote further discussion of this important research field.

2. Physiological and background variables affecting circulating miRNA levels

Most studies of circulating miRNAs aim to establish disease biomarkers by comparing the miRNA profiles of disease cases and healthy controls. In this setting, the concept of “healthy control” is often controversial. Researchers typically use age- and sex-matched controls who do not suffer from the disease of interest; however, there is no clear evidence of which miRNAs are associated with age or sex. In addition, it is unclear whether some other clinical factors should also be matched between controls and disease cases. A limited number of reports have tackled this issue. For example, Ameling et al. [35] examined the expression levels of 179 miRNAs in plasma samples from 372 healthy volunteers that were randomly selected from a previous population-based cohort study. After adjusting for blood cell parameters, 12 and 19 miRNAs were found to be significantly associated with age and BMI, respectively. Notably, although 35 sex-associated miRNAs were identified, this number was reduced to 7 following adjustment for age, BMI, and blood cell parameters (hematocrit and platelet counts). Tonge et al. [9] also reported sex-related changes in the expression levels of circulating miRNAs and reported that erythroid-specific miR-486-5p was the only miRNA to be expressed at higher levels in male plasma than in female plasma. Mooney et al. [7] examined time-dependent changes in the levels of 754 miRNAs in plasma samples collected from 20 healthy males and females. Unlike in the study by Ameling et al., Mooney’s group did not find significant differences in circulating miRNA levels between sexes or collection time-points. In addition, Foye et al. [4] reported no sex-related differences between circulating miRNA levels, and Max et al. [24] reported that the female menstrual cycle does not affect the extracellular miRNA profile. Moreover, several other studies have reported contradictory findings regarding the relationship between sex and circulating miRNA levels (Table 1). The cause of this contradiction is unclear; however, the correlation of miRNA profiles with blood cell status and sex reported by Ameling et al. and Tonge et al. sounds a warning for the selection of

“healthy controls” and interpretation of the relationship between disease marker miRNAs and sex.

Regarding age-related changes in circulating miRNA levels, Noren Hooten et al. [33] performed a comprehensive analysis of serum samples using next-generation sequencing (NGS) and quantitative RT-PCR (qRT-PCR), and found that the levels of miR-151a-5p, miR-181a-5p, and miR-1248 were lower in serum samples from older individuals (mean age 64 years) than in those from younger people (mean age 30 years). Five years later, Dluzen et al. [8] analyzed age-related changes in extracellular RNAs using NGS and qRT-PCR, and found that the levels of 5 miRNAs and 12 miRNAs (including miR-181a-5p and miR-1248) were higher and lower, respectively, in the old age group (80–85 years) than in the young group (30–32 years).

Some circulating miRNAs reported as potential cancer biomarkers have been shown to be affected by background factors. For example, circulating miR-181a-5p has been described as a biomarker of several cancers [38,46,47] and, whereas some reports have shown that its extracellular expression level is age-dependent [8,33], Wang et al. [47] found no correlation between exosomal miR-181a-5p expression and the age of prostate cancer patients. Such post evaluation within control and test groups is essential for the accurate evaluation of cancer biomarkers.

In addition to age and sex, other factors that can affect circulating miRNA levels include physiological conditions such as obesity and diabetes [25], exercise level [43], and diet and nutrient intake [13,26]. Furthermore, the profile of circulating miRNAs can differ depending on the time of day [32] and can be affected by ethnogeographical factors [48]. Clearly, for biomarker discovery studies, the use of control and test groups that are fully matched for all background factors is not easy, even for prospective studies. Consequently, the identification of candidate circulating miRNA disease biomarkers should be supported by examination of other potential confounding factors. In addition, studies examining the mechanisms of action or origin of candidate circulating miRNA biomarkers are also recommended.

3. Sample-specific effects on circulating miRNA levels

3.1. Serum versus plasma

Serum and plasma are often used to examine circulating miRNAs and two previous studies [16,24]

Table 1
Studies examining the effects of sample characteristics on circulating miRNA levels

| PMID | Year | Author | Analysis method | Sample | Variables examined | Variable-associated difference | No variable-associated difference | Reference |
|----------|------|---------------------|------------------------------|------------------------|---|--------------------------------|--|-----------|
| 26462558 | 2015 | Ameling et al. | qRT-PCR | Plasma N = 372 | Age BMI Sex | Age BMI Sex | | 35 |
| 26860190 | 2016 | Tonge et al. | NGS | Plasma N = 18 | Sex Smoking BMI | Sex Smoking BMI | | 9 |
| 26699132 | 2015 | Mooney et al. | qRT-PCR | Plasma N = 10 | Sample collection period Sex | | Sample collection period Sex | 7 |
| 29211799 | 2017 | Foye et al. | Nanostring | Serum/plasma N = 18 | Serum/plasma | Serum/plasma | | 4 |
| 29777089 | 2018 | Max et al. | NGS | Serum/plasma N = 13 | Serum/plasma Sample collection period Sex Menstrual cycle Food intake | Serum/plasma | Sample collection period (one year) Sex Menstrual cycle Food intake Sex Race | 24 |
| 24088671 | 2013 | Noren Hooten et al. | NGS qRT-PCR Microarray | Serum N = 31 | Sex Age Race Age | Age | | 33 |
| 29797538 | 2018 | Dluzen et al. | NGS qRT-PCR | Serum N = 23 | Age | Age | | 9 |
| 29984665 | 2018 | Shiotsu et al. | qRT-PCR | Serum/plasma N = 25 | Serum/plasma | Serum/plasma | | 16 |

Abbreviations: NGS, next-generation sequencing; PMID, PubMed reference number; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

Table 2

List of miRNAs that are abundant in serum or plasma and their putative sources

| miRNA | Putative source |
|-------------|---|
| miR-16 | Multiple blood cells [18] |
| miR-21 | Platelets [6] |
| miR-126 | Platelets [18] |
| miR-144 | Red blood cells [6] |
| miR-199a-3p | Mononuclear cells [6] |
| miR-199a-5p | Mononuclear cells [6] |
| miR-223 | Platelets and peripheral blood mononuclear cells [6] Granulocytes [18] |
| miR-451 | Red blood cells [6,18] |
| miR-486 | Red blood cells [6] |

have identified a number of miRNAs that are abundant in these body fluids (Table 2). However, the miRNA profiles of serum and plasma are not always consistent [2,16,24,40]. During blood coagulation, several miRNAs can leak into the serum from platelets. In addition, procedural variations can cause slight partial coagulation during plasma preparation. Differences in centrifugal conditions can also affect the concentration of platelets in plasma samples, resulting in the preparation of platelet-poor or platelet-rich plasma (PPP/PRP). All of these factors can affect the levels of miRNAs in plasma and serum samples.

3.2. Sample preparation and storage

Analyses of circulating miRNA levels in serum or plasma typically involves the use of whole blood samples in standard collection tubes. In addition, several providers offer specific products to stabilize miRNAs in samples. There is some evidence that the type of collection tube and the methods used for the preparation, handling, and storage of samples can have confounding effects on circulating miRNA measurements. Conventional tubes used to collect blood samples for plasma preparation contain heparin, EDTA, or citrate as anticoagulants. Glinge et al. [5] examined the effects of these anticoagulants on the stabilities of three different miRNAs using qRT-PCR analyses. The levels of miR-1, miR-21, and miR-29 were comparable between serum, EDTA-plasma, and citrate-plasma samples. Samples collected in heparin tubes were unsuitable for analysis because heparin is a well-known inhibitor of PCR, an effect that was confirmed for the circulating miRNA analysis. The levels of miR-1 and miR-21 in whole blood were stable for at least 24 h at room temperature, whereas those in the separated fractions did show alterations within 24 h. Gahlawat et al. [3] evaluated the stabilities of circulating miRNAs in blood samples stored in conventional EDTA tubes for up to 18 h, or in long-

term storage blood collection tubes from four different manufacturers (Streck, Roche, PAXgene, Norgen) for up to 7 days. In the conventional EDTA tubes, the levels of miR-16 and miR-451a, both of which are expressed at high levels in blood cells, were stable for up to 12 h at room temperature or 4°C. The tubes designed for long-term storage maintained stable levels of miRNAs for up to 7 days, depending on the manufacturer. Notably, the level of miR-451a increased gradually in the Streck and Roche tubes. Murray et al. [30] also compared the levels of several miRNAs in blood samples collected in serum tubes and conventional EDTA, Streck-DNA, and Streck-RNA plasma tubes. In qRT-PCR analyses, the Ct values of multiple housekeeping miRNAs in plasma samples collected in the EDTA, Streck-DNA, and Streck-RNA tubes were significantly higher than those of the miRNAs in serum samples. For multiple miRNAs, including miR-451a, miR-23a-3p (hemolysis markers), miR-30b-5p, miR-30c-5p, miR-191-5p (suggested internal controls), and the spike control cel-miR-39-3p, the standard deviations of the Ct values were highest for samples collected in the Streck-RNA tubes. These findings suggest that Streck-RNA tubes may cause hemolysis of blood samples, leading to increases in the levels of hemolysis-related miRNAs such as miR-451a or miR-23a-3p, as well as those of other miRNAs. Murray et al. [30] also found that the EDTA and Streck-DNA tubes could only prevent hemolysis of blood samples for up to 4 days, but suggested that normalization of miRNA data using an internal spike control miRNA and a hemolysis marker would generate more accurate results. We recommend avoiding hemolysis as much as possible when analyzing circulating miRNA levels for biomarker identification. Long-term storage (up to 1 week) of serum/plasma is suggested to be acceptable for samples collected in these special tubes.

Binderup et al. [14] studied the effect of pre-storage centrifugation conditions on miRNA levels in plasma samples. In this study, plasma was prepared from EDTA-treated anticoagulated blood using three different methods: PPP was made by two sequential centrifugations at $3000 \times g$ for 15 minutes, standard plasma samples were prepared by a single centrifugation at $2000 \times g$ for 10 minutes, and “biobank” plasma samples were prepared by a single centrifugation at $2000 \times g$ for 10 minutes, followed by storage at -80°C , and then thawing and centrifugation at $3000 \times g$ for 15 minutes. The platelet count was high in the standard sample centrifuged at $2000 \times g$ for 10 minutes, whereas there were few platelets in the PPP and freeze-

thawed biobank samples. However, the levels of several miRNAs were higher in the freeze-thawed biobank samples than in the freshly made PPP samples. This phenomenon suggests that freeze-thawing of moderately centrifuged plasma allows residual platelets to release miRNAs, thereby confounding circulating miRNA profile analyses. Binderup et al. also reported that the miRNA expression level was dependent on the specific method of centrifugation after thawing. These findings remind us that the characteristics of the samples used for miRNA analyses require careful consideration.

Alongside the effects of platelet contamination, hemolysis also has an impact on the circulating miRNA profile. Expression of the locus encoding miR-144/miR-451 is required for erythroid homeostasis. As mentioned above, miR-451a is enriched in erythrocytes and is often used as an indicator of hemolysis in circulating miRNA analyses [29]. In a recent study, Wakabayashi et al. [17] reported significant correlations between the serum expression levels of 11 erythrocyte-derived miRNAs. In particular, four major erythrocyte-derived miRNAs (miR-16-5p, -451a, -486-5p, and -92a-3p) were found to have a strong correlation and have been suggested as markers of hemolysis or erythrocyte contamination. At present, clinical analyses of plasma potassium, aspartate aminotransferase, and lactate dehydrogenase levels require the use of samples that meet minimum hemolysis limits; samples that are confirmed to be hemolyzed are discarded and new samples are obtained. We suggest that a similar protocol should be implemented for samples used in analyses of circulating miRNAs. In addition to hemolysis-related effects on circulating miRNA levels, the effects of chyle require investigation because miRNAs could potentially bind to lipid bodies in serum.

Appropriate sample storage is also critical to maintain the quality of miRNAs in serum/plasma. Circulating miRNAs are encapsulated in lipid vesicles such as exosomes or are bound to proteins [18], high-density lipoproteins [15], or other components of serum/plasma. Although miRNAs are reported to be stable in serum/plasma, prolonged storage of these samples at high temperatures can affect the miRNA profile. For example, the levels of miR-16, miR-21, miR-122, and miR-142-3p reportedly decrease in a time-dependent manner when serum samples are stored at room temperature [42]. Aiso et al. [39] reported that storage of serum samples at 4°C for periods as short as 24 h causes degradation of miRNAs. This degradation was inhibited by a RNase inhibitor, suggesting that RNases in serum (or plasma) may degrade miRNAs that are bound to pro-

teins or lipids but are at least partially exposed. Other studies have shown that miRNAs in plasma samples are stable during storage at -80°C for multiple years [10], whereas storage of serum samples at -20°C for 4 years or longer can result in miRNA degradation [37].

4. RNA purification methods

Analyses of circulating miRNA levels require their extraction from biological samples; however, the choice of RNA purification method can impact the results. Most researchers examining circulating miRNA levels use commercial kits for RNA extraction and purification. The classical acid guanidinium thiocyanate-phenol-chloroform extraction method is used widely, but newly designed tools incorporating protein lysis and precipitation reagents have been welcomed because of their ease of use. Commercial kits use columns to extract RNA from serum, plasma, or other body fluids. The efficiencies of several commercial kits for extracellular miRNA isolation were examined by Klotten et al. [41], who found that methods relying on phenol extraction (mirVana and miRNeasy S/P) resulted in lower qualities and smaller quantities of miRNAs than those without phenol (miRNeasy Advanced S/P, P/S RNA Purification Mini, miRCURY Biofluids). Klotten et al. [41] also found a method-dependent bias between kits for NGS results. Clearly, the efficiencies of these commercial kits depend on their unique designs; however, the specific components of their constituent reagents and columns are generally proprietary. Nonetheless, the choice of which kit to use for miRNA extraction should be considered when analyzing circulating miRNA levels. We suppose that the underlying causes of the differences between these extraction methods will be analyzed by both biological and chemical researchers.

5. Selection of a suitable intrinsic control

The existence of a suitable control for circulating miRNA analyses is still a matter of debate. Studies examining miRNA levels in tissues or cells typically use 5S, U6, or other snoRNAs as intrinsic controls. However, RNAs derived from nuclear or cytosolic compartments are thought to be unstable in serum/plasma [11,31]. The lack of a validated intrinsic miRNA control in serum/plasma is one of the causes of the paucity of research into the use of circulating miRNAs as biomarkers. Nonetheless, several endogenous

miRNAs have been suggested as suitable intrinsic controls for the analysis of circulating miRNAs. MiR-15b, miR-16, and miR-24 [34] are used widely as intrinsic controls in such studies. In addition, a number of other miRNAs, including miR-4644 [22], miR-22-3p, miR-93-5p [44], miR-1228 [19], and the combination of miR-149-3p, miR-2861, and miR-4463 [1], have been used as endogenous controls. The validity of this combination of three miRNAs has been confirmed in other cancer biomarker studies [23,45].

Several methods for the identification of intrinsic nucleic acid controls have been proposed, particularly for qRT-PCR analyses. These methods include geNorm [21], NormFinder [6], and delta Ct calculations. The geNorm algorithm evaluates the difference in expression levels between two genes across a panel of samples to identify the most stably expressed genes. The use of a combination of two or more control genes enables more robust normalization than using a single gene. The NormFinder software uses the variation in expression levels within/between sample groups to identify stably expressed genes among a set of candidate normalization genes. Both methods (and other approaches) have unique characteristics, so it is better to try a number of methods to identify the best intrinsic controls.

Selection of a suitable intrinsic control for circulating miRNA analyses is particularly difficult; the short and sometimes GC-rich sequences of miRNAs, as well as their limited amounts in serum/plasma, can complicate the analysis. Selection of an appropriate intrinsic control miRNA requires a robust analysis of a large number of samples. As an alternative to the use of intrinsic control nucleic acids, global normalization or quantile normalization can be useful for comprehensive analyses. The use of synthetic nucleotide spike-in controls has also been examined by several groups, although this method is not able to correct for sample variation/degradation that occurs before addition of the spiked control. To date, a gold standard method for the normalization of circulating miRNAs has not been developed; therefore, careful consideration of normalization methods is required for such analyses.

6. Experimental miRNA analysis methods

Generally speaking, there are three major methods to measure miRNA levels: qRT-PCR, microarray, and NGS. The nCounter platform is another alternative for multiplex analysis. High sensitivity is required for cir-

culating miRNA analyses because the amounts of these species in serum/plasma are much lower than those in tissues or cell lines. High sensitivity can be achieved by the amplification of template miRNA extracted from serum/plasma and/or by improvement of the detection technique. In general, qRT-PCR and NGS analyses adopt the former strategy, whereas some microarray and nCounter analyses adopt the latter.

To date, comprehensive comparisons or validations of techniques for the detection of circulating miRNAs have not been performed, although some well-known platform quality control research could be helpful. For mRNA analysis validation, the MAQC (I–IV) project started in 2006 and has evolved from microarray platform comparison (MAQC) [27] and sequencing platform comparison (MAQC-III, SEQC) [28] to data analysis method validation (MAQC-II) [36] and establishing standard analysis protocols and quality control metrics for NGS (MAQC-IV, SEQC-II). Two groups have attempted to compare methods for analyzing miRNA levels: Sato et al. compared the performances of five different miRNA microarray platforms [12], and Klotten et al. performed a multicenter evaluation of five circulating RNA extraction protocols and two exosome miRNA extraction protocols, including detection via qRT-PCR and NGS [41]. Both reports suggested that each technique and platform differs in terms of sensitivity and reproducibility. In addition to the selection of analysis tools, control of the analysis site is very important. Inter-site differences in generated data can be problematic, so each analysis site should control their techniques by periodic testing with predefined universal control samples. Unfortunately, universal control samples have not yet been developed for circulating miRNA analyses. The identification and sharing of such universal controls is required to facilitate future development of miRNA biomarkers in clinical liquid biopsies.

7. Conclusion

Circulating miRNA is likely to be a useful tool in the analysis of liquid biopsy samples, and the number of papers reporting circulating miRNAs as potential disease markers is increasing constantly [20]. The identification of suitable miRNA biomarkers must be based on stable and objective analyses, and the validation of suitable analysis methods is a critical first step. However, method development and validation have been lagging behind biomarker research. This phenomenon is not specific to miRNA/liquid biopsy analyses, but seems to

be universal for every new technology. Nonetheless, the situation appears to be changing for circulating miRNA analysis. As described in this manuscript, several comprehensive studies describing circulating miRNA analyses have been published in authoritative journals and discussion is heated.

For future studies of circulating miRNA biomarkers, the existing research supports the following recommendations: (1) selection of an appropriate control group (matching of background factors and use of a sufficient number of samples); (2) independent validation; (3) post-analysis of candidate biomarkers, examining the potential confounding effects of background factors; and (4) studies of the mechanisms of action or potential causes of differential miRNA expression in body fluids. More comprehensive research focusing on the potential confounding effects of background factors will also be necessary to validate the effects of test factors or conditions on circulating miRNA levels.

Studies of circulating miRNAs as cancer biomarkers also require consideration of their source. In this review, we have discussed the analysis of whole miRNA pools in serum/plasma; however, circulating miRNA is suggested to exist within exosomes or other microvesicles, or in a protein-bound form [18]. In analyses of biomarkers for the early diagnosis of cancer, good performance has been obtained without separating exosomes, suggesting that the development of diagnostic tools can proceed without this complicated process. In the future, when expanding miRNA biomarkers for purposes other than the early diagnosis of cancer, it is possible that whole serum/plasma will not exhibit sufficient performance, and this limitation could possibly be overcome by exosome isolation. In this case, it will be necessary to pay close attention to the exosome separation technology.

To date, several reports of circulating miRNAs as cancer biomarkers have been published and some clinical studies are currently being performed in Japan. We anticipate that a number of circulating miRNAs will soon be validated in an independent analysis and may enter the market as approved clinical tests for cancer.

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