Chapter 2

Circulating MicroRNA Profiling in Cancer Biomarker Discovery

Francesca Scionti, Pierosandro Tagliaferri, Pierfrancesco Tassone and Maria Teresa Di Martino

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Abstract

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules of approximately 22 nucleotides that regulate gene expression at the post-transcriptional level. Alterations in miRNA expression patterns correlate with a wide spectrum of pathological conditions, including cancer. miRNA profiling was mostly performed, in solid tissues, obtained by invasive diagnostic procedures. However, miRNAs in biofluids, such as serum and plasma, show high stability resulting from the formation of complexes with specific protein or incorporation within circulating exosomes or other microvesicles. Circulating miRNAs could be reliable biomarkers for early-stage cancer diagnosis, prognosis and response to therapy. In this chapter, we analyze the major pre-analytical and analytical challenges in experimental design for circulating miRNA detection, focusing on exosome fraction and microarray-based approach.

Keywords: miRNAs, exosomes, miRNA profiling, biomarkers discovery

1. Introduction

MicroRNAs (miRNAs) are small evolutionary conserved non-coding RNAs of 19–25 nucleotides that bind to the 3′-untranslated region (3′-UTR) of target mRNAs, resulting in a negative regulation of gene expression by suppressing translation or causing mRNA degradation [1]. The complex miRNA network plays an important role in the regulation of cellular processes such as development, proliferation, differentiation and apoptosis. Significant changes of tissue miRNA "signatures" occur in various diseases, including cancer [2–4]. More than 50% of human miRNAs are mapped to chromosomal region of genomic instability due to extensive

repetitive sequences resulting in structural mutations (deletion, duplication and translocation) during tumor development [5]. Regulation of miRNA expression is an important mechanism by which tumor-suppressor proteins and oncogenic proteins exert some of their effects. A decrease or mutation in tumor-suppressor miRNAs can lead to overexpression of oncogenic proteins, in contrast to an overexpression of oncogenic miRNAs which can reduce expression of tumor suppressors. For example, tumor suppressor of the let-7 family targets RAS oncogene, which is involved in cell growth, differentiation and survival; reduced expression of let-7 miRNAs correlates with poor survival in many cancers [6]. In contrast, oncogenic miRNAs in the cluster miR-17-92, comprising six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1), inhibits PTEN to activate AKT signaling and promote cancer cell survival [7].

miRNAs have been considered promising candidates as diagnostic and prognostic biomarkers for the strong correlation between expression patterns of miRNAs and disease status and for the differences between normal and cancer tissues. This reflects the current evidence that some miRNAs are overexpressed or downregulated exclusively or preferentially in certain cancer types.

Although miRNA profiling of tumors has been reported in solid tissues, obtained by invasive procedures, as an excellent prognostic test [8], routine biopsies from any organ for miRNA profiling are not practical options. Different studies suggest that circulating miRNAs are reliable indicators of pathological change because of their stability and protection against RNase digestion resulting from the formation of complexes with specific protein or incorporation within circulating exosomes or other extracellular vesicles (EV) [9]. In this context, accumulating evidence suggests that tumor cells are able to alter the function of both local and distant normal cells, thereby promoting tumor growth and metastasis, through the transfer of EV cargo [10]. miR-21, which targets the tumor-suppressor gene PTEN and programmed cell death 4 (PDCD4), is one of the first discovered and most investigated circulating miRNAs. Its upregulation seems to be of diagnostic and prognostic value in a variety of solid and hematological malignancies. High serum levels of miR-21 were strongly associated with lymph node metastasis, advanced-stage clinical disease and poor survival [11]. In patients with ovarian cancer, high serum levels of miR-34a were associated with lymph node disease and distant metastases [12].

Also, in patients with prostate cancer, plasma levels of miR-21, miR-141 and miR-221 were significantly higher in patients with metastases as compared to patients with localized or locally advanced-stage disease [13].

Currently, a variety of miRNA detection methods, including northern blotting, in situ hybridization, quantitative reverse transcription PCR (qRT-PCR), microarray and deep sequencing, are commonly used [14]. However, miRNA profiling in biofluid samples is affected by a range of pre-analytical and analytical challenges in experimental design, from sample collection to profiling and data analysis (**Figure 1**). In this chapter, we will propose a workflow for exosomal miRNA detection from sera samples using the Affymetrix GeneChip microarray platform as a powerful molecular approach for biomarker discovery to translate into clinical practice.

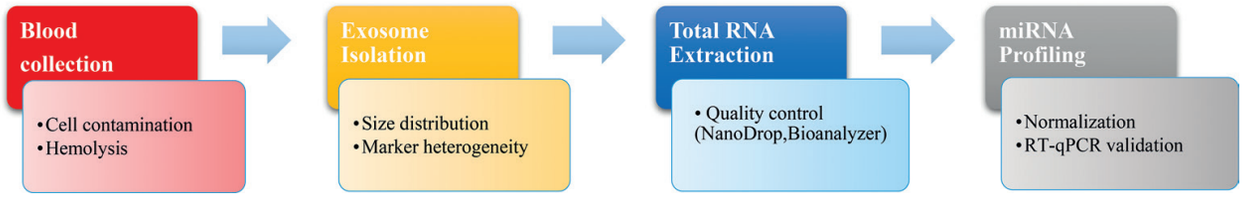

Figure 1. Summary of workflow in designing miRNA profiling from blood serum.

2. Methodological challenges in miRNA profiling design

2.1. Sample collection

Blood sample processing has a substantial impact on the results of miRNA profiling. During blood collection, it is important to avoid cellular contamination and hemolysis that can occur during phlebotomy as miRNAs derived from red and white blood cells risk to mask the intensities of truly circulating miRNA species. Residual platelets and microparticles can also affect the miRNA profile so an additional centrifugation is recommended prior to freezing samples. Moreover, biofluidics contain inhibitors of the reverse transcriptase and polymerase enzymes that can inhibit the enzymatic reactions in RT-qPCR so it is important to minimize the carryover of inhibitors into the RNA.

In our protocol, blood samples were collected and processed according to the national cancer institute (NCI's) Early Detection Research Network (EDRN) standard operating procedures for the collection and preparation of serum [15].

Whole blood samples were collected in red-top vacutainer tubes. Blood samples were incubated at room temperature for 30 min to allow complete coagulation. Coagulated samples were then centrifuged at 1500 × *g* for 20 min at room temperature to separate serum. The serum was transferred to new cryotubes with care so as to not to disturb the red blood layer and then centrifuged for 5 min at 3000 × *g* to remove cells. Aliquots of 1.5 ml of supernatant containing the cell-free serum were stored in cryotubes at −80°C until RNA extraction. Hemolyzed samples were excluded from further analysis.

2.2. Exosome isolation and characterization

The established standard for exosome isolation is ultracentrifugation [16]. However, this method cannot discriminate between exosomes and other microvesicles because different vesicles of similar size as well as protein aggregates can co-sediment at 100,000 × *g*.

Recently, methods claiming fast and simple exosome-purification procedures without ultracentrifugation are commercially available by various firms that use polymer-based precipitation or immune capture by antibody-coated beads.

However, according to the International Society for Extracellular Vesicles (ISEV) the separation of non-vesicular entities, such as protein complexes, from EV is not fully achievable by

common EV isolation protocols, including centrifugation protocols or commercial kits. On the other hand, a list of EV-specific markers that distinguish subsets of EVs from each other is not proposed [17]. The ISEV provides minimal requirements to claim the presence of EV in pellets isolated from different methods. They suggested assessing the protein composition in at least a semi-quantitative manner in any EV preparation (Western blots, flow cytometry or mass spectrometry techniques). Size distribution of EVs, such as nanoparticle-tracking analysis (NTA), dynamic light scattering or resistive pulse sensing, needs to be analyzed. However, the values acquired with these techniques should be compared with TEM, AFM or other microscopy techniques, since they do not distinguish membrane vesicles from co-isolated non-membranous particles of similar size.

In our laboratory, serum exosomes underwent isolation by miRCURY™ Exosome Isolation Kit (Exiqon, Vedbaek, Denmark). The process is based on capturing water molecules which otherwise form the hydrate envelope of particles in suspension. The samples (1.5 ml) thawed on ice or at 4°C, were mixed with a precipitation solution in order to reduce the hydration of the particles. This allows precipitation of the subcellular particles below 100 nm with a low-speed centrifugation step after incubation at 4°C for 1 h. The last pellet, containing exosomes, was resuspended using 240 μL of the provided resuspension buffer and used for further RNA extraction or stored at −20°C.

NTA was performed using a NanoSight LM10-HS microscope equipped with NTA software v3.1 (NanoSight Ltd., UK). Background was extracted and the automatic setting for minimum expected particle size, minimum track length and blur settings was employed. Each sample was diluted at 1:10,000 in sterile-filtered PBS (Sigma, USA). A video of typically 60 s duration was made, with a frame rate of 30 frames per second, and particle movement was analyzed by NTA software. Only measurements with >1000 completed tracks were analyzed (**Figure 2**).

We also have used 20 μL of human CD63 coated beads (Thermo Fisher Scientific, Inc) per 50 μL sample in order to isolate CD63 positive sub-populations of exosomes from total exosome isolations derived from serum samples. The isolated exosomes were stained for typical exosome markers, such as CD63, and CD9 was analyzed with the Attune NxT Flow Cytometer (Thermo Fisher Scientific, Inc.) (**Figure 3**).

2.3. RNA purification

RNA extraction method is another important issue in miRNA profiling as the exosomal RNA pattern is highly correlated with the sample source. The principles for isolating miRNA for profiling are, in general, the same for isolation of total RNA, except that miRNA isolation protocols are adapted to retain the small RNA fraction. In different studies describing exosome RNA, a number of alternative RNA extraction methods have been used, including phenol-based techniques, combined phenol and column-based approaches and pure column-based techniques [18–22].

For RNA purification from exosomes, we used the miRCURY™ RNA Isolation Kit (Exiqon, Vedbaek, Denmark) that is based on spin column chromatography using a proprietary resin as the separation matrix. First, membrane particles and cells were lysed using the lysis solution and then proteins were precipitated using the provided protein precipitation solution.

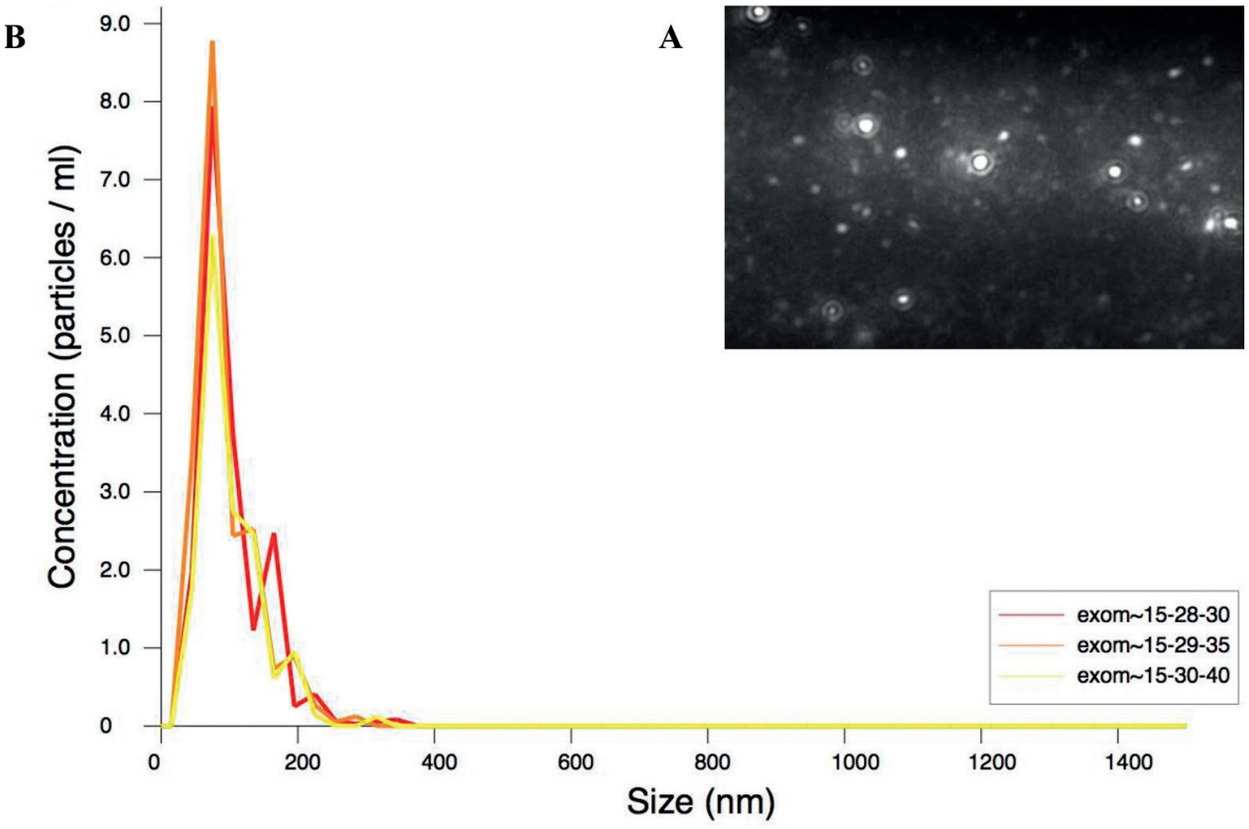

Figure 2. Extracellular vesicle NanoSight data. Example NanoSight NTA video frame (A), NanoSight NTA particle size/concentration for 3 different miRCURY™ Exosome Isolation Kit recovered exosome pellets (B).

Isopropanol was added to the supernatant and the solution was loaded onto a spin column. Thus, only molecules of RNA will bind to the resin, while proteins will be removed in the flow through. The RNA bound to the column was then washed with provided wash solution in order to remove any remaining impurities and then eluted with 100 μL of RNase free water.

We also evaluated miRNA recovery from serum samples by the use of the exogenous synthetic miRNA cel-mir-39 (Applied Biosystems, Inc.) as standard control. For this procedure during RNA isolation, a known amount of cel-mir-39 (25 fmol) was spiked in each serum sample after the denaturation step. Consistent and homogeneous miRNA recovery leads to cel-miR-39 levels that are similar in all samples. A standard curve was generated using five ten-fold dilution of cel-miR-39 (1.4×10^{-4}–1.4×10^{-8} ng) processed in parallel with qRT-PCR of experimental samples. The copy number of spiked cel-miR-39 was estimated, plotting Ct values, versus the copy number of the synthetic miRNA. The recovery of the synthetic miRNA is considered consistent if cel-miR-39 Ct values are within the range of acceptability (≤2 × standard deviation) in replicate isolations.

2.4. RNA quantification and quality control

Exosomal RNA purity was assessed by Nanodrop ND-1000 UV spectrometer (Thermo Scientific, Wilmington, DE) at the absorbance of 230, 260 and 280 nm. RNA samples extracted

using the miRCURY™ method produced RNA concentrations above the lower detection limit of 2 ng/μL of the NanoDrop instrument and less than 10 ng/μL. Moreover, the miRCURY™ RNA isolation method showed a high average A260/280 ratio (1.9 ± 0.1) but low average

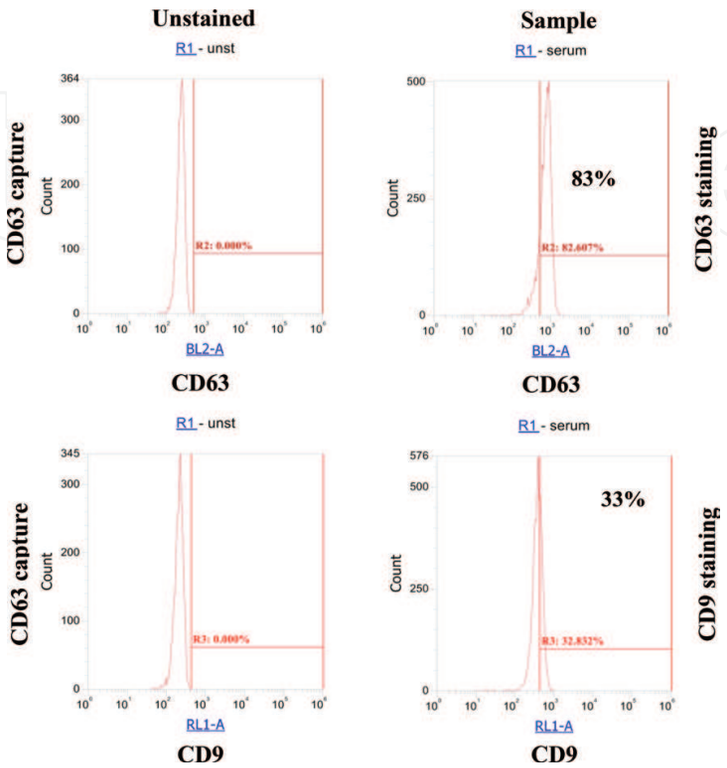

Figure 3. Flow analysis on exosome sub-populations (A). Exosomes isolated with CD63 showed that serum-derived exosome in our samples express high level of CD63 marker (82.6%) and a moderate level of CD9 markers (32.8%) (B).

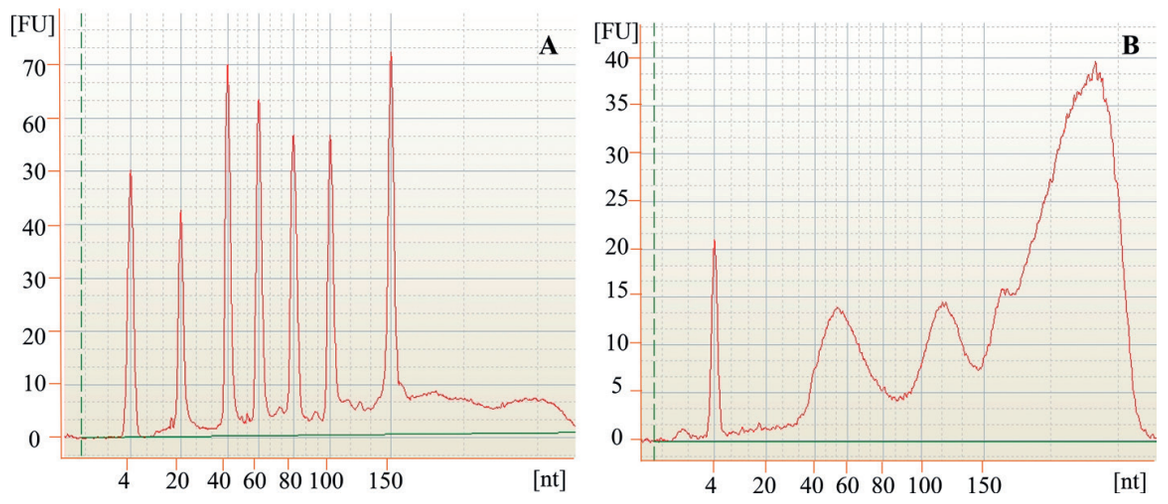

Figure 4. Bioanalyzer analysis of serum exosomal small RNA. Exosome total RNA derived from serum was analyzed using Small RNA Kit in an Agilent 2100 Bioanalyzer. The electropherograms show the size distribution in nucleotides (nt) and fluorescence intensity (FU) of ladder (A) and exosome total RNA (B). The peak at 4 nt is an internal standard. The Small RNA region is visible in the interval of 0–150 nucleotides, including the miRNAs in the sizes between 10 and 40 nucleotides.

A260/230 ratio (<2.0). These values are consistent and acceptable in samples containing very low amounts of RNA, as biofluid samples.

Because of the absence of 18S and 28S rRNA species in serum, the quality of isolated RNA samples might not be assessable by means of RNA integrity number (RIN), as a rule for total RNA samples to use for microarray procedures. On this basis, we used the Agilent Small RNA assays that are able to resolve fragments in the range 0–150 nucleotides (**Figure 4**).

3. MiRNA profiling and data analysis

miRNA expression profiling has allowed the identification of miRNAs that are involved in many biological processes, including organism development and establishment and maintenance of tissue differentiation [23, 24]. Thus, miRNAs are being explored as elements for cell-fate reprogramming in stem-cell applications or as biomarkers for identifying the origin of cancers of unknown primary sites. The expression pattern of miRNAs is widely different being tissue specific and related to developmental stages. Measuring miRNA expression can also be useful for system-level studies of gene regulation, especially when miRNA profiles are integrated with mRNA profiling. Circulating extracellular miRNAs, including exosome miRNA, are quantifiable in a range of specimen types including serum, plasma, urine and formalin-fixed tissue block. Hence, they are important as non-invasive biomarkers for many molecular diagnostic applications, including cancer [25, 26], cardiovascular and autoimmune diseases [27] and forensics [28]. **Table 1** shows new and promising miRNAs as potential biomarkers for diagnosis and prognosis of different cancers.

3.1. General consideration for high-throughput miRNA profiling

The yield of RNA extracted from biofluids is usually very low, in the order of 1–10 ng. If we consider that miRNAs represent only 0.01% of total RNA, the strategies used for their detection and quantification are crucial. Some general consideration must be made in high-throughput miRNA profiling. First, the length of mature miRNA (19–25 nucleotides) is too low to allow annealing to the traditional primers during the reverse transcription step. Second, unlike mRNAs, miRNAs lack poly(A) tail, a region frequently used to anneal complementary and universal primers for RNA enrichment or reverse transcription. Third, miRNAs exist in different isoforms, so-called isomiRs, that are functional and evolutionarily important and, inside the same family (e.g., the let-7 family), can differ by a single base to the reference miRNA sequence. Depending on the goals of an miRNA profiling experiment, measurement of different forms may be required, even if the large majority of miRNAs typically show only modest-length heterogeneity. Another challenge for miRNA high-throughput profiling is variance in miRNA GC content that is reflected in different melting temperatures (Tm) of annealing reactions. To date, three major approaches are used for miRNA profiling: quantitative reverse transcription PCR (qRT-PCR), hybridization-based methods (microarrays) and next-generation sequencing (NGS) (RNA-seq). Microarrays were among the first hybridization-based methods to be used for parallel analysis of large numbers of miRNAs.

Cancer	Samples	miRNAs		Types of biomarkers	Reference
Head and neck	Plasma	miR-21	qRT-PCR	D	[29]
Lung cancer	Serum	miR-182, miR183, miR-210, miR-126	qRT-PCR	D	[30]
	Serum	miR-125b	qRT-PCR	R	[31]
Breast cancer	Serum	miR-1, miR-92a, miR-133a, miR-133b	Microarray	D	[32]
	Serum	miR-125b	qRT-PCR	R	[33]
Ovarian cancer	Serum	miR-34a		P	[12]
Prostate cancer	Plasma	miR-21, miR-141, miR-221	qRT-PCR	D	[13]
Gastric cancer	Serum	miR-1, miR-20a, miR-27a, miR-34, miR-423-5p	qRT-PCR	D	[34]
Renal cancer	Serum	miR-378, miR-451	qRT-PCR	D	[35]
Pancreatic cancer	Serum	miR-16 and miR-196a	qRT-PCR	D	[36]
		miR-21	qRT-PCR	R	[37]
Colorectal cancer	Plasma	miR-409-3p, miR-7, miR-93	qRT-PCR	D	[38]
		miR-126	qRT-PCR	R	[39]
Hepatocellular cancer	Exosome	miR-101, miR-221, miR-221, miR-224	qRT-PCR	D	[40]
Melanoma	Serum	miR-221	qRT-PCR	D, P	[41]
Lymphoma	Serum	miR-221	qRT-PCR	P	[42]
Leukemia	Exosome	miR-29a	qRT-PCR	P	[8]

D = diagnostic; P = prognostic; R = response predictor.

Table 1. Circulating miRNAs as potential biomarkers in different cancers.

3.2. Microarray miRNA profiling

Microarrays provide a high-throughput approach to profile all annotated miRNAs, in different types of samples including biological fluids. This method is relatively less expensive than others, such as qRT-PCR and next-generation sequencing (NGS). Moreover, the integrated analysis of the expression profile of miRNAs and their target genes, together with the analysis of each miRNA gene target pathway, is able to provide information on the function of each miRNA for a given sample. The technique generally begins with the enzymatic or chemical marking of targets followed by their hybridization to oligonucleotides fixed on a solid support. The signal generated by each probe is detected by a scanner and analyzed by specific software able to process the signal intensity. The variables related to the method include the

different hybridization efficiencies of each probe, due to their different content in GC, the different melting temperature, due to the reduced size of miRNAs, the bias due to the enzymatic labeling and the relative low dynamic range. Microarray-based methods generally require a larger amount of starting material than qRT-PCR, and it can be challenging to develop probes and hybridization conditions that work well to detect many different miRNAs at once [43, 44]. miRNA profiling by NGS platforms may be the most promising approach, as it largely avoids many miRNA measurement pitfalls [45]. However, NGS remains expensive and labor intensive, both in the sample preparation and data analysis.

3.3. Affymetrix GeneChip microarray platform

To date, oligonucleotide-miRNA microarray analysis is the most common high-throughput technique for assessment of disease-specific expression of hundreds of miRNAs. The Affymetrix GeneChip® miRNA microarrays platform provides the most sensitive, accurate and complete measurement of small non-coding RNA transcripts involved in gene regulation. It represents miRNA sequences from all organisms present in miRBase (http://www.mirbase.org), as well as small nucleolar RNAs (snoRNA) and small Cajal body-specific RNAs (scaRNA) included in snoRNABase (http://www.snorna.biotoul.fr/) and Ensembl (http://www.ensembl.org). This platform has been used in our laboratory with success to profile exosome non-coding RNA from serum samples (unpublished data, manuscript in preparation). The protocols for non-coding RNA profiling include the use of the Affymetrix FlashTag Biotin HSR RNA Labeling Kit (Affymetrix, Santa Clara, CA, USA) and the detection by fluorescent emission. Total RNA samples or total RNA samples enriched for low molecular weight (LMW) RNA were starting materials. The process begins with a poly(A) tailing reaction followed by the ligation of a biotinylated signal molecule to the target RNA samples. The labeled RNA samples are included in the hybridization mix and hybridized overnight to Affymetrix GeneChip miRNA arrays (Affymetrix, Santa Clara, CA, USA), followed by a procedure to wash, stain and scan the array to acquire probe cell intensity data (CEL file). To date, the GeneChip miRNA 4.0 array is the updated array designed to interrogate all mature miRNA sequences contained in miRBase release 20, including 30,424 probe sets, covering 203 organisms and requiring low sample input (130–1000 ng total RNA). CEL files analysis by the Expression Console Software coupled with Transcriptome Analysis Console (TAC) Software (Affymetrix, Santa Clara, CA, USA) leads to a simple, fast and free analysis for Affymetrix GeneChip expression arrays.

3.4. Analysis of raw data

Raw data processing begins with quality control analysis by assessing the performance of internal controls and analyzing replicates to detect biases. For example, microarrays have well-known geographic biases, because some areas of the array perform differently from others. Data normalization, which is the next step after quality assessment, is crucially for obtaining accurate results [46]. The goal of normalization is to adjust the data to remove variation across samples not related to the biological condition and therefore allowing the identification of relevant biological differences. The normalization step is particularly important because some of the discrepancies between miRNA profiling studies are in part due to the application

of different normalization approaches. GeneChip array data are normalized by the use of a tool that uses Robust Multichip Analysis (RAM) plus detection above background (DABG) algorithms, as default analysis. RAM is a robust linear normalization model, to minimize the effect of probe-specific affinity differences, and consists of three steps: background adjustment, quantile normalization and summarization. DABG is a detection metric generated by comparing perfect match probes to a distribution of background probes.

miRNA profiling experiments typically involve comparisons between two or more groups, and therefore the next stage of analysis is usually the calculation of differential miRNA expression between groups. The degree of fold difference that constitutes a meaningful difference depends on the experimental context, although it is always useful to assess the statistical significance and false discovery rate that is associated with the differential miRNA expression. This comparison yields a p-value, which is, then, combined into a probe set level p-value using the Fischer equation. Statistical analysis is performed using TAC and a fold change of two is commonly adopted to describe the signal changes between groups.

3.5. Circulating miRNA profiling challenges

Circulating exosome miRNAs are surprisingly stable and show distinct expression profiles among different fluids. Given the instability of most RNA molecules in the extracellular environment, the presence and apparent stability of miRNAs in body fluids such as serum and other body fluids, that are known to contain ribonucleases, suggest that secreted miRNAs are packaged in some manner to protect them against RNase digestion. miRNAs could be shielded from degradation by packaging in lipid vesicles, like exosomes, in complexes with RNA-binding proteins or both [47]. This view supports the idea that extracellular miRNAs are prepared for export in one cell. They can be recognized, taken up and utilized by another cell, working as mediators of cell-cell communication [48–50]. The growing interest in developing circulating miRNAs as blood-based biomarkers requires very careful consideration of the effects of various pre-analytical procedures, such as handling and storage conditions of the sample before processing, which can affect the reliability and reproducibility of circulating miRNA quantification. The main technical difficulty to study miRNA expression profiles is the efficient extraction of miRNAs from biological samples, because of their small size and their attachment to lipids and proteins. The use of commercial extraction kits has become available to optimize the extraction of small RNAs and normalize sample-to-sample variations in isolation procedures. Therefore, it is important to establish standardized protocols for blood collection, sample storage conditions, inclusion of exogenous and endogenous miRNA controls for each clinical sample and standardized calculations for normalization of the results to ensure the reproducible and accurate quantification of circulating miRNA levels so that miRNA analysis can be implemented in the clinical laboratory setting.

3.6. Validation of microarray results by qRT-PCR

miRNA microarrays are less expensive but inclined to have a lower sensitivity and dynamic range and are therefore best used as discovery tools rather than as quantitative assay platforms. Current publication guidelines require that all microarray results are confirmed by

an independent gene expression profiling method. Most researchers choose qRT-PCR as the preferred method for the validation of microarray data using both TaqMan and SYBR-green assay.

The TaqMan qRT-PCR method uses a stem-loop RT primer, specifically designed to detect the 3′ end of individual mature miRNAs generating a unique template for RT. In the qPCR step, cDNA is amplified with specific primers and product accumulation is monitored using a fluorogenic probe (TaqMan probe), complementary to the target gene. In SYBR-green-based qRT-PCR, miRNA is typically poly-adenylated at the 3′ end, and oligo-d(T) is used as an RT primer while a double-strand DNA binding dye (SYBR-green) allows for the detection of PCR products during qPCR.

We routinely use TaqMan® microRNA assays (Applied Biosystems, Inc.) to validate microarray data on the same clinical samples as described [51] and perform qPCR in triplicate reactions and the $2^{-\Delta\Delta Ct}$ method to estimate the relative quantity of each miRNA [52]. An important issue is the choice of normalizer. Typically, "housekeeping" genes selected as endogenous controls allow normalization of qPCR data as they are affected by the same experimental variability as the target genes. In a cellular context, stable small RNA controls, such as RNU44, RNU48 and RNU6, are usually used. However, for circulating miRNAs, there is growing evidence that the abovementioned small RNAs are highly variable or not stably detectable [53]. This lack of consensus has resulted in the generation of various normalization strategies. An approach widely used and employed in our group is the selection, from each microarray study, of several genes as a normalizer based on their stable expression.

4. Conclusion

Circulating miRNAs are attractive as clinical biomarkers for diagnostic purposes, as well as for monitoring disease progression and response to treatment. However, the nature of circulating miRNAs places several challenges. The success of the circulating miRNA profiling requires rigorous control of pre-analytic and analytic variables, specifically when investigating potential circulating miRNA markers. Here, we provided a consistent and reproducible method for circulating miRNA detection, profiling and analysis. We discussed the main issues associated with miRNA measurement that is crucial for miRNA profiling, especially for exosomal circulating miRNA. In addition, it needs to take into account that it is difficult to measure specific miRNA levels because they are short and conserved sequences, paralogs or distinguish between precursor and mature forms. In our work experience, the use of standard protocols for sample preparation, and of exogenous synthetic miRNA as the standard control, helps to solve a part of these problems. Microarray data processing such as normalization procedures among different samples is challenging especially for extracellular miRNA. In our work experience, it can be concluded that the use of robust algorithms and software may avoid errors and false positive discovery. However, the validation of array results by the use of an alternative methodology, especially when using different protocols and platforms for profiling purposes, is mandatory.

Objective assessment of these technical metrics is an important step toward understanding the appropriate use of microarray technology application in general and more specifically for circulating miRNA to be used as clinical markers as well as in regulatory settings.

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Conflict of interest

The authors declare no conflicts of interest.

Author details

Francesca Scionti, Pierosandro Tagliaferri, Pierfrancesco Tassone and
Maria Teresa Di Martino*

*Address all correspondence to: teresadm@unicz.it

Department of Experimental and Clinical Medicine, Medical Oncology, Magna Graecia University of Catanzaro, Italy

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