Detection and Assessment of MicroRNA Expression in Human Disease

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Contents

Abstract MicroRNAs (miRNAs) are noncoding RNAs that posttranscriptionally suppress gene expression through sequence-specific interaction with the 3′ untranslated region (3′UTR) of target mRNAs. By modulating gene networks, miRNAs have been shown to regulate many aspects of cellular homeostasis and physiology, including differentiation, growth, proliferation, and apoptosis. The discovery of extracellular miRNA in blood and other body fluids has prompted investigation into their ability to serve as biomarkers for human disease. Further, many miRNAs have been implicated in the underlying pathophysiology of human disease, and there is substantial opportunity to develop novel molecular

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therapeutics through manipulation of these miRNAs. Here, we review the evidence for the use of miRNAs as diagnostic and therapeutic targets in human disease. We also describe the advantages and limitations for current miRNA profiling strategies.

Keywords MicroRNA • IsomiR • Biomarker • qRT-PCR • Northern • Microarray • Next-generation sequencing • Molecular beacons

1 Introduction

MicroRNAs (miRNAs) are short (~19–23 nucleotides), highly conserved, noncoding RNAs that repress translation or induce degradation of specific messenger RNA (mRNA) molecules (Bartel 2004; Dennis 2002; Guo et al. 2010). To date, there are 1,881 annotated human miRNAs in miRBase (Kozomara and Griffiths-Jones 2014), which is the repository for miRNA sequences from all plant and animal species. The discovery of miRNAs and their function has substantially increased our appreciation for the complexity of gene regulation in health and disease, and miRNAs are increasingly being recognized as novel diagnostic and therapeutic targets for human disease.

2 miRNAs: Regulators of Gene Networks

2.1 miRNA Function

Targeting of mRNA occurs through base pairing of the miRNA seed sequence, defined as nucleotides 2–8 of the 5′ end, to a complementary sequence in the mRNA (Bartel 2009). Since miRNA-mediated gene suppression typically requires pairing of only 7–8 nucleotides, a single miRNA can regulate the expression of hundreds of mRNAs (Lim et al. 2005), and a given target mRNA can be regulated by multiple miRNAs (Friedman et al. 2009). These features are key to the ability of miRNAs to influence entire gene networks, thereby impacting many aspects of development, physiology, and pathology (Kloosterman and Plasterk 2006; Sayed and Abdellatif 2011). In fact, over 60 % of coding genes in humans are computationally predicted to be targeted by miRNAs (Friedman et al. 2009). Adding to the breadth and complexity of miRNA-mediated gene regulation is the finding that, through targeting transcription factors, miRNAs can indirectly regulate the expression of other miRNAs (Matkovich et al. 2013).

2.2 miRNA Biogenesis

miRNAs may be transcribed from various genomic loci, including introns or exons of noncoding genes, introns of coding genes, or intergenic regions (van Rooij 2011). Also, through variations in miRNA processing, a single miRNA genomic locus can

generate multiple mature miRNAs, which are otherwise known as isomiRs. The canonical miRNA biosynthetic pathway begins in the nucleus where primary miRNA transcripts (pri-miRNAs) are generated, most often by RNA polymerase II but occasionally by RNA polymerase III (Borchert et al. 2006). The hairpin structure of a pri-miRNA includes a double-stranded stem (roughly 33 base pairs) that contains the mature miRNA sequence, a single-stranded loop at one end of the stem, and two single-stranded 5′ and 3′ flanking regions at the other end of the stem (Ha and Kim 2014; Winter et al. 2009). In the nucleus, the 5′ and 3′ flanking regions of the pri-miRNA stem are cleaved by the Microprocessor complex, consisting of an RNase II enzyme, Drosha, and its cofactor, DGCR8 (also known as Pasha). The resulting product is termed the pre-miRNA, which varies in length between 60 and 70 nucleotides (Finnegan and Pasquinelli 2013). The pre-miRNA is exported from the nucleus to the cytoplasm by Exportin-5-Ran-GTP (Lund et al. 2004). In the cytoplasm, a second RNase, Dicer, and a double-stranded RNA-binding protein, TRBP, cleave the pre-miRNA terminal loop to produce a transient double-stranded miRNA with a length equal to that of the mature miRNA. This double-stranded molecule separates into two complimentary mature miRNAs, labeled 5p or 3p, depending on whether they originated from the 5′ or 3′ end of the primary transcript. One of the miRNAs becomes a functional "guide" strand, which complexes with the Argonaute-2 (Ago2) protein and is incorporated into the RNA-induced silencing complex (RISC). The other mature miRNA is referred to as the passenger strand (often denoted with a *) (Ha and Kim 2014), which is degraded. Mature miRNAs incorporated in the RISC guide the complex to specific mRNA sequences, thereby leading to degradation, translational repression, or deadenylation of the target mRNA (Filipowicz et al. 2008; Krol et al. 2010).

As a result of genomic duplications, many miRNAs share similar seed sequences, and miRNAs with identical seed sequences are referred to as a "miRNA family" (Ha and Kim 2014). In general, seed sequences are highly conserved; slight variations in seed sequence can lead to the suppression of completely different groups of targets (Kim et al. 2013). While miRNA biogenesis is often portrayed as a linear, uniform process, further investigation has revealed multiple layers of regulation and alternative pathways that appear to be miRNA specific (Winter et al. 2009). A detailed review of the alternative synthetic pathways and regulatory mechanisms involved is beyond the scope of this chapter and has been recently reviewed elsewhere (Finnegan and Pasquinelli 2013; Ha and Kim 2014).

2.3 Intracellular and Extracellular miRNAs

miRNAs are found within all cells and tissues, where they function as important modulators of cell differentiation (Chen et al. 2004, 2006), organ development (Carrington and Ambros 2003), homeostasis, and physiology (Kloosterman and Plasterk 2006). Efforts to comprehensively describe miRNA profiles in human and animal tissues (Landgraf et al. 2007; Liang et al. 2007) have revealed that some miRNAs are tissue specific, while others are widely and more uniformly expressed

(Liang et al. 2007). Intracellular abundance of miRNAs varies broadly, from only a few molecules per cell to greater than 10,000 molecules per cell (Liang et al. 2007). Repositories of miRNA expression profiles in healthy and diseased human tissues can be found at mirna.org or mirnabodymap.org.

The identification of stable, extracellular miRNAs was surprising given the known presence of highly active RNases in serum, plasma, and other body fluids (Kamm and Smith 1972). Extracellular miRNAs are remarkably stable, even if the sample has been left at room temperature for a prolonged period of time or has been exposed to multiple freeze/thaw cycles (Chen et al. 2008; Mitchell et al. 2008). Circulating miRNAs are protected from RNases through encapsulation within cell-derived membranous vesicles or by binding to circulating proteins or lipoproteins. Cell-derived membranous vesicles include exosomes, microparticles, and apoptotic bodies (Huang et al. 2013; Kosaka et al. 2010; Valadi et al. 2007). Interestingly, the miRNA profiles of circulating microvesicles are different from the contents of their maternal cells, implying an organized and regulated system for packaging and exporting particular miRNAs (Hunter et al. 2008). While little is known about the underlying mechanisms that govern this process, there is evidence that miRNA content within microvesicles changes in response to cell activation or systemic disease and that these conditions may affect the ability of cells to load miRNAs into the microvesicles (Alexy et al. 2014; Diehl et al. 2012; Finn et al. 2013). Importantly, neighboring cells can take up circulating microvesicles, resulting in the intercellular transfer of miRNAs and other bioactive molecules (Turchinovich et al. 2013). In a manner similar to paracrine signaling, transferred miRNAs may then influence gene expression and phenotype of the recipient cells (Hergenreider et al. 2012; Jansen et al. 2013; Zernecke et al. 2009).

It has been suggested that the majority of extracellular miRNAs in plasma, serum, or cell culture media are mostly non-encapsulated (Arroyo et al. 2011; Creemers et al. 2012; Turchinovich et al. 2011) and bound to extracellular proteins or lipoproteins (Arroyo et al. 2011; Vickers et al. 2011). Non-encapsulated miRNAs appear to be primarily associated with Argonaute proteins, the same proteins to which they are bound intracellularly (Arroyo et al. 2011). Lipoproteins, including high-density lipoprotein (HDL) and low-density lipoprotein (LDL), have also been shown to bind and transport miRNAs in the circulation (Vickers et al. 2011), although lipoprotein-bound miRNAs may represent a relatively low proportion of the total circulating miRNA pool. Whereas the ability of protein-bound extracellular miRNAs to function as intercellular communicators has not been well studied, lipoprotein-bound miRNAs do not appear to be efficiently transferred to recipient cells, and their role in intercellular communication appears be limited (Wagner et al. 2013). Despite uncertainty whether the majority of circulating miRNAs are encapsulated or non-encapsulated, it has become clear that the extracellular localization of miRNAs is miRNA dependent. Some miRNAs are predominantly found in microvesicles, while others are primarily bound to circulating Ago-2 (Arroyo et al. 2011). The distinct extracellular transport modalities of miRNA likely represent different cellular export mechanisms (Wang et al. 2010b) that are influenced by the cell type and the activating stimulus or disease state (Finn et al. 2013; Jansen et al. 2013).

2.4 Importance of miRNAs in Disease

High conservation of miRNA sequences across species is indicative of their biological significance. miRNAs are clearly important for development, as demonstrated by studies in which deletion of specific miRNAs or the miRNA synthetic machinery results in severe developmental defects and/or nonviability (Harfe et al. 2005; Thai et al. 2007; Yang et al. 2005; Zhao et al. 2007). The discovery of disease-specific miRNA signatures in tissues (Volinia et al. 2006), along with the identification of stable extracellular miRNAs, has prompted the search for circulating miRNA profiles that might be utilized as noninvasive biomarkers of disease (Mitchell et al. 2008; Weber et al. 2010). Indeed, unique circulating miRNA profiles have been shown to be associated with a variety of human disorders, including, but not limited to, cancer (Calin and Croce 2006; Lu et al. 2005; Mitchell et al. 2008; Schultz et al. 2014; Schwarzenbach et al. 2014), cardiovascular disease (Creemers et al. 2012; Fichtlscherer et al. 2011; Gupta et al. 2010), rheumatologic disease (Kloosterman and Plasterk 2006; Wang et al. 2010a), liver disease (Pirola et al. 2014; Wang et al. 2009), and neurologic disease (Leidinger et al. 2013). Furthermore, accumulating evidence suggests that circulating extracellular miRNAs are not only passive markers of disease, but that they are also biologically active mediators of cell-to-cell communication implicated in the underlying pathophysiology (Valadi et al. 2007; Zhu and Fan 2011).

miRNA expression can be manipulated in vivo, which has led to the development of miRNA-based pharmacotherapies (van Rooij et al. 2012). Antagomirs (antimiRs, blockmirs) are synthetic oligonucleotides with complementary sequence to mature miRNAs and are capable of binding to and inhibiting the function of a given miRNA (Krutzfeldt et al. 2005). Another strategy for modulating miRNA levels is miRNA mimicry, in which synthetic oligonucleotides are modified to enhance stability and cellular uptake and delivered to mimic the function of endogenous miRNAs (Bader et al. 2010). Off-target effects of miRNA mimics in tissues in which they are not normally expressed remain a challenge, and adeno-associated viral vectors have been suggested as tissue-specific miRNA delivery vehicles (Kota et al. 2009). Animal models suggest that manipulation of miRNA levels can mitigate disease, demonstrating the potential for novel, miRNA-based therapeutics (Hinkel et al. 2013; Hu et al. 2010; Jansen et al. 2013; Lanford et al. 2010; Son et al. 2013). The development of human miRNA-based drugs is underway, and there has been early success using this approach to treat hepatitis C in clinical trials (Janssen et al. 2013).

Overall, miRNAs are important modulators of gene expression that become dysregulated in many human diseases. The development of improved strategies to detect and study these molecules is becoming increasingly important, which will aid in identifying novel biomarkers, uncovering new disease mechanisms, and guiding novel therapeutic approaches.

3 miRNA Profiling

3.1 Sample Preparation

Sample preparation and proper RNA extraction techniques are crucial, but often overlooked, aspects of high-quality miRNA profiling. Variations in sample collection methods can influence subsequent assessment of miRNA levels. For example, patient fasting status, phlebotomy technique, hemolysis, cell contamination, and anticoagulants such as heparin have all been identified as factors that influence miRNA detection in serum and plasma (Moldovan et al. 2014). Furthermore, serum and plasma have similar miRNA profiles for some miRNAs, but not for all (Wang et al. 2012). There is concern that, when preparing a serum sample, the clotting process activates platelets, thereby inducing miRNA release in platelet microvesicles and altering miRNA levels in serum (Wang et al. 2012).

Kits for RNA isolation are commercially available, including some that isolate total RNA and others that preferentially enrich for small RNAs. The method by which RNA is extracted is not trivial—one paper was recently retracted because results were confounded by the selective loss of low-GC-content miRNAs during standard TRIzol RNA extraction (2012). Qualitative and quantitative assessment of miRNA isolation may also be challenging. For many profiling studies, RNA is extracted from tissues that have low-abundance miRNA, such as serum and plasma. Because miRNA levels tend to be below the threshold of accurate detection by spectrophotometry, some groups have used the Agilent Bioanalyzer to assess quantity and quality of isolated miRNAs.

3.2 Techniques to Detect and Quantify miRNA

The short length, high degree of sequence homology between closely related miRNAs, varying GC content, and relatively low abundance of miRNAs in some cells/tissues are all factors that impede accurate miRNA detection. Here we will review several of the most common techniques available for miRNA quantification.

3.2.1 Northern Blot Analysis

Northern blot analysis has been a widely used method for miRNA quantification. miRNA is isolated from cells or tissues, fractionated by gel electrophoresis, and then transferred to a membrane. miRNA molecules are fixed to the membrane by either UV cross-linking or baking. Labeled, miRNA-specific probes are hybridized to the membrane, and miRNA abundance is assessed by the signal intensity of bound probe.

Radiolabeled miRNA-specific probes can be generated using the StarFire system from Integrated DNA Technologies (IDT), in which a DNA polymerase is used to add 10 α-^{32}P-dA residues to the 3′ end of a synthetic oligonucleotide probe. Alternatively, predesigned fluorescently labeled, locked nucleic acid (LNA)-enhanced probes can be purchased from Exiqon (miRCURY). As probe specificity is determined by sequence homology and position of the base mismatches, the short length and high sequence similarity of miRNAs make it challenging to generate probes that are highly specific. Through incorporation of a class of nucleic acid analogs, LNA technology improves miRNA probe specificity by increasing the melting temperature (T_m) and binding affinity of the probe. LNA probes can also be 3′ end-labeled with γ-^{32}P-ATP or tagged at the 5′ and/or 3′ end with digoxigenin or biotin. After hybridization to miRNA, the tagged probes are detected by the reaction of a chemiluminescent enzyme that has been attached to anti-digoxigenin monoclonal antibody or streptavidin. In general, radiolabeled poly(A) probes produce stronger signals compared to non-radiolabeled LNA probes (van Rooij 2011).

The main advantage of Northern blot analysis is its ability to assess miRNA size, in particular the ability to distinguish mature and precursor forms of miRNAs in a sample. However, the Northern blot protocol has low throughput, is time consuming, and requires a relatively large amount of RNA (8–10 μg). Despite improvements in detection specificity provided by LNA technology, probe cross-reactivity with miRNA family members with high sequence homology remains a concern.

3.2.2 qRT-PCR

Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) or real-time PCR is the most commonly used method for assessing miRNA abundance and has the advantage of being a familiar, readily available technique in many laboratories. In qRT-PCR, a cDNA template is first generated by reverse transcription of the isolated RNA/miRNA, followed by PCR. However, since mature miRNAs are short transcripts of only 19–23 nucleotides, quantification using traditional qRT-PCR methods is challenging. In particular, cDNA produced by reverse transcription of a mature miRNA is not long enough for subsequent specific binding of two PCR primers. PCR primers, which are typically 22–25 nt in length, require a cDNA template that is at least 50 base pairs (bp) in length. Therefore, mature miRNAs must first be extended prior to the RT reaction. There are two main methods to increase the length of miRNA for reverse transcription: (1) miRNA extension with a hairpin-loop primer and (2) miRNA extension by polyadenylation.

The hairpin-loop RT primer (Life Technologies, TaqMan PCR assays) has a 3′ overhang that specifically binds to a few bases in the miRNA template, as well as a double-stranded stem and a loop. This primer serves two purposes: it extends the miRNA of interest to a longer template for RT and its loop structure provides a binding sequence for a universal primer. The RT reaction using the hairpin-loop primer is specific for the miRNA of interest; it will not reverse transcribe other miRNAs or mRNAs. The resulting cDNA is then subjected to PCR using one

miRNA-specific primer and a second, universal primer. While this assay is generally more difficult to design, the structure of the RT and PCR primers reduces nonspecific amplification of the pre- and pri-miRNAs. Also, the specificity of the RT primer is helpful in the detection of miRNAs with low abundance. A major disadvantage of this method for miRNA reverse transcription is the requirement for a separate RT reaction for each miRNA of interest, so screening a sample for expression levels of multiple miRNAs can be labor intensive.

Reverse transcription after miRNA polyadenylation is very similar to a regular RT reaction with oligo-dT primers. In the case of miRNA reverse transcription, an additional polyadenylation step has to be performed that adds a tail of adenines (As) to all miRNAs in the sample. Reverse transcription of the polyadenylated miRNA involves a universal oligo-dT RT primer with a special 3′ anchor and a universal tag sequence in the 5′ end. Using the QIAGEN miScript PCR system or Exiqon's miRCURY system, polyadenylation and reverse transcription can be performed simultaneously in the same tube, producing cDNAs from all miRNAs in the sample. The subsequent PCR uses a forward primer specific to the miRNA-specific sequence and a universal reverse primer that binds to the oligo-dT end of the cDNA. SYBR Green is typically used to quantify the PCR product of this cDNA. Since the RT reaction is not miRNA specific, this method has the advantage of being relatively cost-efficient; the cDNA generated from a single RT reaction can be used as a template to assess expression levels of any miRNA, pre-miRNA, and mRNA of interest. Also, the use of SYBR Green with this qRT-PCR method allows a melting curve analysis, indicating purity of the product. The disadvantage of this method is its reduced specificity compared to the method that uses the hairpin-loop RT primer. Also, since all RNA/miRNA transcripts in the sample are polyadenylated and reverse transcribed, accurate detection of low-abundance miRNAs may be challenging.

Two methods for the assessment of PCR product are available: one uses a DNA intercalating dye, such as SYBR Green described above; the other uses a nonspecific short fluorescent DNA probe that has a fluorophore and a quencher attached. SYBR Green has a very high affinity for double-stranded DNA. During the amplification and synthesis of DNA during PCR, SYBR Green intercalates into the double-stranded DNA product and fluoresces—the intensity of fluorescent signal is directly proportional to the amount of cDNA. In the other method of PCR product detection, the short DNA probe with fluorophore–quencher combination binds to the template DNA, and the fluorophore is quenched when the probe is bound. During the PCR reaction, exonuclease activity of DNA polymerase degrades the probe while the polymerase also extends the primers and synthesizes the nascent strand. Degradation of the probe separates fluorophore from quencher and fluorescence is emitted. The registered fluorescence intensity is directly proportional to the amount of DNA in the sample.

LNA technology, as discussed above, has been utilized to improve primer sensitivity and specificity (e.g., Exiqon's miRCURY LNA Universal RT microRNA PCR kit). Incorporation of LNAs increases primer T_m and binding affinity. This approach requires as little as 1 pg of total RNA to perform

qRT-PCR (Moldovan et al. 2014). Preamplification kits are also available for samples in which low amounts of cDNA are produced after reverse transcription. For example, Megaplex from Applied Biosystems can be used to increase template availability for PCR. Finally, several companies also offer array-based, high-throughput qRT-PCR options. TaqMan Low Density Arrays (Applied Biosystems), miRNome panels (Exiqon), or miScript miRNA PCR Arrays (QIAGEN) allow users to select from pre-plated miRNA panels. These assay panels can also be custom designed to suit the users' preferences.

Whereas the use of various small nucleolar RNAs (e.g., RNU48, RNU6b, RNU444, RNU43) to normalize for technical and biological variation in qRT-PCR data obtained from cells or tissues is well established, normalization of data obtained for extracellular miRNAs has proven to be a challenging issue. For extracellular miRNAs, several normalization strategies have been developed and used, although each has drawbacks. Technical variation can occur between samples as a result of differences in RNA extraction efficiency, reverse transcription, or the presence of PCR inhibitors. A common method employed to normalize for technical variation between samples is to "spike in" an exogenous miRNA that does not have homology with human miRNAs, such as cel-miR-39 from *C. elegans*. During RNA isolation, after endogenous RNases have been denatured, a known concentration of exogenous miRNA is added to the isolate and subsequently assessed by qRT-PCR. After PCR, technical variation is controlled for by normalizing the value for the miRNA of interest to that of the spike-in miRNA.

Ideally, to control for biological variation in qRT-PCR assessment of extracellular miRNAs, a known invariant endogenous small RNA should be used. Unfortunately, no universally invariant miRNA has been discovered to serve this function, especially in body fluids like serum and plasma (Moldovan et al. 2014). Invariant miRNAs in a given dataset can be identified using software, such as geNorm or Normfinder that identifies the best "normalizer" in the dataset. Alternatively, statistically based methods that utilize global measures of miRNA expression can be used, including mean normalization, which was recently shown to be highly effective (Mestdagh et al. 2009). Unfortunately both of these methods rely on having a large dataset and are not suitable for smaller experiments. A third option is to generate a separate standard curve for each PCR reaction by assessing serial dilutions of a known amount of template cDNA. Unknown samples can then be fit to the standard curve for absolute quantification. However, this process requires performing multiple PCRs for the assessment of one miRNA, which is time consuming and uses substantial quantities of PCR reagents.

In summary, qRT-PCR is the most sensitive method for miRNA detection, and it is an established method in many laboratories. Throughput is improving, especially with the array-based options that are commercially available. However, this method may not be able to distinguish between closely related miRNAs that might have only one or two bases different in their sequence. If the qRT-PCR is not performed with standard curve, it can only provide information on relative changes in miRNA expression, not absolute differences in miRNA abundance. Otherwise, with the

exception of normalization issues for extracellular miRNAs, data analysis is relatively straightforward.

3.2.3 miRNA Microarray

Microarrays allow for parallel analysis of a large number of predefined miRNAs through probe hybridization. Multiple platforms for microarrays are available, all varying slightly in their chemistry, labeling, and probe design. Basically, miRNA is isolated and then labeled, usually by using T4 RNA ligase, which attaches one or two fluorophore-labeled nucleotides to the 3′ end of the miRNA. Either synthetic oligonucleotides or cDNAs are used as capture probes. Perhaps the greatest challenge for microarrays is that, based on their sequence, miRNA melting temperatures can vary widely between 45 °C and 74 °C (van Rooij 2011). This variation means that the binding affinities of the miRNA probes will differ at a given temperature; thus, absolute quantification of miRNA abundance is not possible. However, probe designs can be modified in such a way as to equalize annealing temperatures thereby making array binding more homogeneous (Moldovan et al. 2014; van Rooij 2011).

The Affymetrix GeneChip miRNA array version 4.0 covers all miRNAs in miRBase for 203 organisms. After RNA isolation, a poly(A) tail is added to the 3′ end of the miRNA followed by ligation of a biotinylated 3DNA dendrimer. The biotin-labeled miRNA is hybridized to the capture probes on the chip, and binding is detected by streptavidin–phycoerythrin fluorescence. This experiment requires 100 ng of total RNA. Agilent oligonucleotide microarrays offer up-to-date coverage of human, mouse, and rat miRNAs that also require at least 100 ng of total RNA per sample. The Agilent protocol uses RNA dephosphorylation and ligation of Cyanine 3-pCp, followed by hybridization and detection. Results generally correlate well with qRT-PCR data (Ach et al. 2008). Exiqon's miRCURY LNA microRNA arrays offer good coverage of human, mouse, and rat miRNAs from miRBase. It has 3,100 capture probes, including 146 viral miRNAs. This platform requires as little as 30 ng of input RNA. As discussed, the incorporation of LNAs helps to standardize hybridization conditions. This array includes 52 "spike-in" control miRNAs for normalization. Exiqon claims their system provides higher sensitivity and specificity as a result of LNA technology.

Microarrays offer a high-throughput, comprehensive option for miRNA profiling at a reasonable price. Several customizable platforms are available, and microarray-based miRNA profiling is a suitable alternative to other high-throughput methods. However, this method has several drawbacks. The wide range in miRNA expression levels in a particular sample limits the ability of microarrays to detect transcripts at relatively low abundance (low sensitivity). Similar to the methods above, microarrays only probe previously annotated miRNAs, so detection of novel miRNAs or miRNA variants (isomiRs) is not possible. Absolute quantification of miRNA abundance using microarrays is difficult due to variability in probe hybridization efficiency, and the lack of specificity

limits the ability of microarrays to distinguish mature miRNAs from precursors. Most platforms require at least 100 ng of input material, which may pose a problem for miRNA profiling of some tissues. Microarray data analysis is not as straightforward as qRT-PCR, and inter-platform variability remains an issue (Callari et al. 2012). Changes in miRNA expression detected by microarray should be validated by Northern or qRT-PCR analysis. Continued improvements to microarray reproducibility, sensitivity, specificity, and inter-platform variability are being made (Pradervand et al. 2010), but as RNA sequencing technology becomes cheaper and more accessible, microarray-based miRNA profiling is likely to be utilized less.

3.2.4 Deep Sequencing

Next-generation sequencing (NGS) uses a massive number of parallel sequencing reactions to produce millions of sequence reads that are then mapped back to the genome of interest and quantified with advanced bioinformatics. Multiple miRNA sequencing platforms are now available through Illumina, Life Technologies, or Exiqon, among others. Total RNA or purified small RNA is isolated, and adapters are ligated onto the 3′ and 5′ ends of the fragmented products. A reverse transcriptase reaction generates cDNA libraries, and PCR is performed on an immobilized surface or beads (Moldovan et al. 2014). Expression levels are quantified based on the number of reads that map to a particular region. Sequence mapping, normalization, and downstream analysis require advanced bioinformatics software and expertise.

NGS is a highly sensitive and specific method for miRNA quantification. It is unique in that it is unbiased, offering the ability to detect novel miRNAs and to distinguish between very closely related miRNAs or isomiRs. NGS also characterizes other small RNAs that have not been well characterized thus far, but could just as well be biologically relevant species. NGS is able to measure absolute abundance of each transcript over a large range of expression (6–7 log fold range) (Moldovan et al. 2014). NGS requires relatively small amounts of starting material, with current technology able to generate high-quality data from as little as 10 ng of miRNA. These features highlight the advantages of NGS over other miRNA profiling methods, but NGS is the most expensive method for profiling. Furthermore, due to the very large amounts of data that are generated by NGS, it requires a significant amount of bioinformatics and biostatistical support. As with microRNA microarrays, results should be validated by Northern or qRT-PCR analysis.

3.2.5 Molecular Beacons

Molecular beacons are oligonucleotide (DNA or RNA) probes that have become an increasingly important tool for RNA detection both in vitro and in living cells. From their inception, molecular beacons have been used to determine the expression

levels of RNA transcripts, but they also have the specificity to identify splice variants and single-nucleotide polymorphisms. Our group has performed extensive studies on molecular beacon design, molecular beacon hybridization assays, and cellular imaging of mRNA molecules. Compared to other methods for assessing RNA transcript expression, such as qRT-PCR, the molecular beacon-based approach is potentially simpler, faster, more cost-effective, and more specific.

Molecular beacons are single-stranded DNA or RNA molecules labeled with a reporter fluorophore at one end and a quencher at the other end (Tyagi and Kramer 1996). In the absence of a complementary target, they are designed to form a stem–loop hairpin structure that results in quenching of the fluorophore's fluorescence (Tyagi et al. 1998; Tyagi and Kramer 1996; Vet et al. 1999). Hybridization of the molecular beacon's loop sequence with target nucleic acid opens the hairpin and physically separates the fluorophore from the quencher, allowing a fluorescence signal to be emitted upon excitation, which can be assessed in a standard fluorescence plate reader. In theory, the molecular beacon elicits a signal only upon direct hybridization to the complementary RNA sequence. This enables a molecular beacon to function as a sensitive and specific probe with a high signal-to-background-noise ratio (Bao et al. 2009; Guo et al. 2012; Nitin and Bao 2008; Nitin et al. 2004; Santangelo et al. 2004, 2006; Tsourkas et al. 2002, 2003). In addition, RNA abundance is directly correlated with molecular beacon fluorescence.

Since sequences for mature forms of miRNAs are also present in miRNA precursor forms (i.e., pri- and pre-miRNAs), miRNA detection methods that are not able to readily distinguish between different miRNA forms (i.e., qRT-PCR, miRNA microarray) tend to report falsely high values for miRNA abundance. Recently, our group demonstrated that molecular beacons can readily distinguish mature and pre-miRNAs and reliably quantify their expression in the same sample (Baker et al. 2012). Molecular beacons can also distinguish between miRNA family members with similar sequences (Baker et al. 2012). LNA backbone chemistry may be incorporated into molecular beacon design to enhance affinity of the molecular beacon for target miRNA. Profiling of multiple miRNAs and pre-miRNAs in one sample can be accomplished through multiplex assays that utilize molecular beacons containing fluorophores with nonoverlapping spectra. Furthermore, molecular beacon-based assays can be performed in one step without the need for reverse transcription (RT) or amplification steps, and excess probe does not have to be removed prior to measurement.

Overall, molecular beacons are highly specific in their ability to detect both mature and precursor forms of miRNA in a biological sample as well as in their ability to distinguish miRNAs or isomiRs with one or two nucleotide differences in their sequences. However, the sensitivity of the current molecular beacon-based assays for miRNA quantification is modest compared to other methods that utilize signal amplification steps (e.g., qRT-PCR). It remains a challenge to use molecular beacons to profile miRNA expression in biological samples that have low miRNA abundance, such as body fluids. Furthermore, this method for miRNA profiling is currently still rather novel, and optimization of molecular beacon design for each

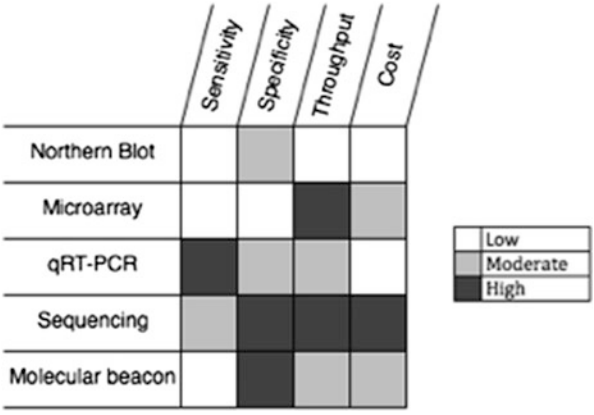
miRNA and pre-miRNA target is required, which can be costly. As use of this method becomes more widespread and molecular beacons for specific miRNAs or groups of related miRNAs are developed, this method could be a valuable tool for miRNA profiling.

4 Discussion

While enthusiasm for the clinical application of miRNA profiles as biomarkers for human disease is high, technical issues with miRNA profiling remain, particularly in regard to standardization of sample collection/processing and reproducibility of miRNA assessment assays. Causes for inconsistencies in miRNA profiles exist throughout miRNA profiling process, from pre-analysis steps such as sample preparation to variations in one of the several steps between RNA isolation and data analysis. In fact, a recent quality control study of several miRNA profiling platforms concluded that, while each method has strengths and weaknesses, the average validation rate for differentially expressed miRNAs was only 54% between any two platform combinations (Mestdagh et al. 2014). The advantages and limitations for various miRNA profiling methods are summarized in Table 1.

Several features of miRNA expression present challenges for accurate and specific quantification of miRNA abundance and could contribute to inter-assay variability (Guo and Chen 2014; Roy-Chaudhuri et al. 2014). These features include their short length, the high degree of homology between closely related miRNAs, the possibility of slight variations at the 5′ and 3′ ends due to imprecise processing (isomiRs), and the coexistence of mature and precursor forms of miRNAs in the same sample. For example, Lee et al. have demonstrated dramatic discrepancies in the assessment of miRNA abundance due to differences in

Table 1 Characteristics of miRNA profiling methods



processing at the 3′ end of specific miRNAs, an issue that influences multiple miRNA detection methodologies (Lee et al. 2010). Regardless of the method used to detect and quantify miRNA, the full repertoire of miRNA variability and its biological consequences must be taken into account if appropriate conclusions are to be drawn. As more details emerge about the biology of the intra- and extracellular expression of miRNAs, our understanding of the importance of miRNA profiling in the diagnosis and management of human disease will certainly be enhanced, and methods used to accurately assess miRNA profiles should continue to be refined.

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