Using Circulating MicroRNAs as Noninvasive Cancer Biomarkers in Breast Cancer is a Cutting-Edge Application of MicroRNA Profiling Technology

Steven Talbot[1]

[1]Department of Genetics, University of Michigan

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Review

Using Circulating MicroRNAs as Noninvasive Cancer Biomarkers in Breast Cancer is a Cutting-Edge Application of MicroRNA Profiling Technology

Steven Talbot[1]*

1. Department of Genetics, University of Michigan, Ann Arbor, USA

* Corresponding Author: Email: bjqfffg@telegmail.com

Abstract

This study presents technology to detect and quantify microRNAs (miRNAs) as possible cancer biomarkers, using breast cancer as an example. The majority of breast cancer cases are identified at more advanced stages, reducing the likelihood of survival for the patient. Several microRNAs (miRNAs) have been demonstrated to have high preliminary clinical sensitivity and specificity for early cancer diagnosis or staging, and they are up- or down-regulated in breast cancer patients at different stages. Methods based on the analysis of nucleic acids, such as reverse transcription-quantitative polymerase chain reaction (RT-qPCR), microarrays, and next-generation sequencing (NGS), may be used to estimate the relative abundance of specific miRNAs of interest. For miRNA biomarker identification, NGS is the most effective technology, whereas RT-qPCR has the greatest potential for future clinical diagnostic applications.

Keywords: microRNA, reverse transcription quantitative PCR, microarray, RNA sequencing, breast cancer

Introduction

Patients with cancer have a far greater chance of survival if they are diagnosed at an early stage, before the original tumor has grown and spread to other parts of the body1,2. MicroRNA (miRNA) profiling shows potential in enabling early cancer detection, for example in breast cancer, which represents approximately one in three cancer diagnoses in the United States and is a leading cause of cancer-related deaths worldwide.1 Early breast cancer detection is essential to improve treatment outcomes and decrease mortality rates (Table 1).1, 2, 3 The American College of Radiology developed an algorithm for breast cancer screening and diagnosis (Fig. 1),4 which relies on breast examination, various imaging methods, and eventual confirmation of abnormal imaging results via histological examination of a biopsy tissue sample.3 However, mammograms and ultrasound imaging have limited sensitivity and specificity, which leads to many missed cases and also many unnecessary referrals for biopsy evaluation.3,4 Once malignancy is confirmed based on biopsy results, breast cancer treatment consists of breast-conserving or radical mastectomy, radiation therapy, or chemotherapy.5 Less invasive treatment is possible if breast cancer is detected before the tumor has grown in size and spread beyond the breast.

Mammographically, breast cancer progresses through five stages, from 0 to IV.5 Stage 0 involves the development of tiny malignancies in the ductal, lobular, or nipple cells. The cancer expands and spreads via the lymphatic system throughout Stages I through III. In Stage IV, distant metastases have spread throughout the body. On a microscopic level, breast cancer may be divided into three stages: incipient, metastatic, and recurrent. Many different mechanisms inside a somatic cell may lead to the development of breast cancer. Breast tumor initiation cells (BT-ICs) acquire self-renewal capacities and function as "cancer stem cells" at the outset of tumor development.6,7 BT-ICs then give rise to various types of differentiated cancer cells that proliferate quickly and evade apoptosis as the disease progresses. For

cancer to metastasize, cells must break out from the main tumor, enter the circulation, and travel to distant organs and tissues. The epithelial-mesenchymal transition (EMT) is a process linked to cancer metastasis in which epithelial cells undergo a de-differentiation into cells with stem cell-like properties.8

Review of microRNAs

MicroRNAs (miRNAs) are a kind of short noncoding RNA molecules that control gene expression and are encoded in the introns of protein-coding genes in the host genome.9 This discovery is quite recent. The transcription of a primary miRNA (pri-miRNA) results in the formation of a precursor miRNA (pre-miRNA) with a folded hairpin loop structure and a length of 70 nucleotides. Once in the cytoplasm, the pre-miRNA undergoes further processing to become a mature, single-stranded miRNA of around 20-22 nucleotides in length. Tumor-free miRNAs have been found in the bloodstream18, and these miRNAs seem to be coupled to proteins or encapsulated in microvesicles termed exosomes. This shields the miRNAs from the RNases found in blood, giving them an unexpectedly long half-life of about 5 days in plasma.19 Circulating miRNAs are a promising new class of cancer biomarkers that may facilitate early minimally invasive breast cancer diagnosis. Tumor staging and prognosis are two further applications for miRNAs.18,20

Breast cancer and the function of microRNAs

MiRNAs 21, 30a, 145, 155, 195, 200b, and 200c are only some of the circulating miRNAs that have been studied as potential biomarkers for the diagnosis or staging of breast cancer (Table 2). These microRNAs control processes crucial to the development, spread, and metastasis of breast cancer. Downregulation of miR-30, miR-200b, and miR-200c during breast cancer development suggests that these miRNAs function as oncosuppressors. By downregulating ubiquitin-conjugating enzyme 9 (Ubc9), miR-30 indirectly suppresses pluripotency and cell renewal.16 Moreover, miR-30 represses integrin 3 (ITG3), which mediates apoptosis based on environmental cues.21,22 miR-200c strongly represses expression of Bmi-1,23 a downstream product of the Hedgehog pathway (HH) that induce

However, miR-21 is significantly overexpressed in breast carcinoma and is involved in all stages of tumorigenesis16. miR-21 represses the oncosuppressor genes programmed cell death 4 (PDCD4), tropomyosin 1 (TPM1), and phosphatase and tensin homolog (PTEN),26,27 which induces EMT,28 promotes breast cancer metastasis,29 and leads to cell proliferation. Although miR-21 has been linked to many different forms of cancer,16,31 it is unlikely to be breast cancer specific due to the fact that it controls other genes, leading to enhanced breast cancer invasion30 and encouraging carcinogenesis.

It seems that the differential expression levels of other miRNAs are more stage-dependent and varied in cell culture, tissue samples, and blood, with occasionally contradictory findings. For example, miR-145 is downregulated in cancer cells in vitro,32 but one clinical study reported decreased miR-145 levels in the plasma of breast cancer subjects,33 whereas another study reported upregulation of miR-145 in the serum of primary breast cancer subjects.34 As another example, upregulation of miR-155 in vitro has been shown to suppress FOXO3a and its downstream targets, which results in increased cancer cell survival and drug resistance.35 Whereas several clinical studies have demonstrated upregulation of miR-155 in breast cancer patients,36,37 downregulation of miR-155 has been observed in metastatic breast cancer patients in at least one study.38 Likewise, miR-195 appears to be upregulated in unclotted whole blood samples from breast cancer subjects,39 but it is downregulated in breast cancer tissue and cell lines.40 Some of these discrepancies may be due to changes in DNA methylation during disease progression,37,38 which may play a role in tumorigenesis by miRNA dysregulation.40. Area under the curve (AUC) of the receiver-operator characteristic (ROC) curve (Fig. 2) is used to evaluate the therapeutic value of prospective miRNA biomarkers in many clinical validation studies (Table 2), where AUC = 1 for a perfect assay and AUC = 0.5 for a random result. The sensitivity and specificity that can be achieved in the clinic as a function of different cutoffs may also be extrapolated from the ROC curves.

The miRNAs reviewed here are only a few of the many that have been proposed as biomarkers for breast cancer26,33,41,42,43,44. Currently, it appears that assessing the differential expression of a small panel of miRNAs will provide a better predictive value for early-stage diagnosis and prognosis compared to profiling single miRNAs.45 Furthermore, the majority of clinical studies reported to date have included a relatively small number of cases and controls. To definitively confirm the clinical sensitivities and specificities of potential miRNAs as reliable breast cancer biomarkers, much bigger, prospective investigations are required. The difficulty of validating biomarkers is compounded by the fact that studies frequently disagree on how much a given miRNA's expression should fluctuate. Differences in research design, subject numbers, inclusion of various breast cancer subtypes, and disproportionate representation of particular breast cancer stages may all contribute to these divergent findings. Variability in relative expression level measurements may also result from discrepancies in sample preparation methods and analytical technology platforms. This means that the methods used to find and verify miRNA biomarkers must be thoroughly examined.

Some microRNAs were shown to have higher expression in BC tumor relative to surrounding tissues, as found in a research by Mojdeh Mahmoudian and colleagues. Fourteen microRNAs were upregulated: hsa-miR-25-3p, -29a-5p, -105-3p, and -181b1-5p, while two were downregulated: hsa-miR-335-5p and -339-5p. Except for hsa-miR-339-5p, these putative microRNAs were shown to have an up or downregulation related with TNM stages. Furthermore, all potential microRNAs linked with HER-2 status except for hsa-miR-105-3p. The ROC curve analysis also showed that the combination of these six miRNAs has the potential to act as a biomarker for distinguishing tumorous from nontumoral breast tissue samples.

Technical Analysis of the Product

Microarrays, next-generation DNA sequencing (NGS), and RT-qPCR are only a few of the technologies that have evolved in the last decade to assess miRNA expression. These methodologies have been designed to solve intrinsic problems linked to miRNA measurement owing to its short length and high GC content. The initial step in any of these three methods is isolating RNA from plasma, serum, or other biological materials.

Methods for Isolating RNA

MiRNA profiling results may be affected by the total RNA extraction technique that was used. There are a number of commercial kits available for miRNA isolation; some examples are the mirVana miRNA Isolation Kit (Ambion, Austin, TX),46, 47, 48 the PureLinkTM miRNA Isolation Kit (Ambion),49 a Microcon YM-100,49 and the miRNeasy Mini Kit (Qiagen, Venlo, the Netherlands).50, 51 However, after reviewing multiple protocols for RNA extraction, researchers have concluded that no single method is definitive. By separating the material into its molecular weight components, microRNAs may be purified and concentrated without contaminating it with other, bigger RNA species. The miRNeasy kit (Qiagen) employs two glass-fiber filter spin columns; the first is to immobilize total RNA, and a second is for miRNA enrichment. Ion-pair reverse-phase chromatography and conventional denaturing polyacrylamide gel electrophoresis (PAGE)52 may be used for miRNA enrichment, and RNase-free DNase can be used to eliminate contaminating DNA.

Measurement of Excellence

Given that miRNA expression analysis is very sensitive to total RNA quality, quality control is essential prior to miRNA profiling using microarrays or sequencing technologies. Total RNA integrity is quantified by the RNA integrity number (RIN) using microfluidic capillary electrophoresis on an Agilent Technologies 2100 Bioanalyzer (Agilent, Santa Clara, CA).53 The RIN is a surrogate for the degree of RNA degradation (e.g., due to RNase digestion) and is used for quality control of total RNA. To determine the quality of plasma-derived miRNA, exogenous synthetic miRNAs can be spiked into the sample before extraction and then measured in the purified RNA.58 Generally speaking, RNA samples with

RINs greater than 8 out of 10 are suitable for library construction.54 Although fresh biopsy samples and cell lines typically yield high-quality miRNA, this is not the case with miRNA derived from plasma,55 or from formalin-fixed paraffin-embedded tissue.

PCR Quantitation by Reverse Transcription (RT-qPCR)

Several commercial kits, including the Taqman miRNA Assay from Life Technologies (Carlsbad, CA), the miRCURY LNA Universal RT microRNA PCR from Exiqon (Vedbaek, Denmark), and the NCode miRNA qRT-PCR System from Invitrogen (Waltham, MA), are available for RT-qPCR analysis of miRNAs. Both the poly(A) tail and stem-loop RT primer techniques may be used to analyze microRNA expression by means of RT-qPCR (Figs. 3 and 4). Both strategies include lengthening the target miRNA during reverse transcription to provide a cDNA template of enough size for subsequent PCR amplification and detection.62**.** After enzymatic polyadenylation to lengthen all RNAs, reverse transcription is performed using a universal RT primer, resulting in cDNA synthesis from all RNA strands present in the sample (Fig. 3).48,63 This is followed by PCR amplification using a miRNA-specific forward primer and a universal reverse primer complementary to the 5′ end of the RT primer,62 with real-time detection based on intercalating dyes. High quantification yield and improved sensitivity are achieved with this method64,65; however, specificity may be compromised due to the generation of nonspecific RNA strand complements during RT.

Specific primers and probes prevent nontarget amplification when using the stem-loop RT primer approach.48 This method is direct since it does not need polyadenylation before reverse transcription. To prevent nonspecific amplification and increase specificity, RT is performed using miRNA-specific stem-loop RT primers that stabilize and lengthen the miRNA (Fig. 4).63 A miRNA-specific forward primer then initiates qPCR amplification, with real-time fluorescence detection mediated by Taqman probes.60,63. Synthetic miRNA spikes and/or endogenous miRNA of high abundance can be used as a positive control and quantification standard in RT-qPCR, with the latter having the advantage of exhibiting natural physiochemical properties during quadruplex formation, as measured by the cycle number at which the fluorescence measurement in the real-time amplification curve passes a fixed fluorescent threshold amount.60,66 MiRNAs in a sample may be measured using a standard curve, generated by plotting the log of known standard quantities against their Ct value.67 Synthetic miRNA spikes, derived from Arabidopsis, for example, may be preferable since they do not demonstrate similarity with human miRNAs. The problem with using external standard curves is that they don't take into account the fact that extraction and amplification efficiencies might vary greatly from sample to sample. Thus, the 2-Ct approach may be used to calculate relative changes in miRNA expression by comparing the Ct value of the target miRNA to the Ct value of the positive control and quantification standard.68.

The poly-A tail technique of RT-qPCR, which relies on dye intercalation for detection, can only be run as a singleplex test. Low-level multiplexing within the same process is possible, however, when using the stem-loop approach with detection using fluorescently tagged probe oligonucleotides. Either way, it takes hundreds of separate RT-qPCR reactions per sample to determine expression-level changes for large panels of miRNAs. The miRCURY human miRNome panel, for instance, includes two 384-well plates for analysis of 742 distinct miRNAs, one duplicate for each, plus reference genes, calibrators, and controls.69 In general, RT-qPCR provides great analytical sensitivity, needing just 500 ng extracted RNA. However, its sensitivity is diminished when the material is distributed over several RT-qPCR procedures. Microarrays and NGS allow for more multiplexing than conventional methods. In order to profile the differential expression of a large number of miRNAs, this may help solve the problem of insufficient RNA.70

Expression Analysis of MicroRNAs Using Microarrays

Affymetrix, Agilent, Exiqon, and Life Technologies are only few of the companies that provide commercialized microarray technologies tailored for miRNA quantification. While some microarray technologies focus on only a few hundred miRNAs, others provide a high-throughput approach for scanning the whole genome using only mature miRNAs that have been verified.71

Fluorescently labeled miRNAs are isolated from a sample and hybridized to an array of thousands of miRNA-specific oligonucleotide probes immobilized on individual spots of a solid surface. Imaging and data processing follow after the array has been exposed to successive, more stringent wash procedures designed to eliminate unwanted, nonspecifically bound sequences. Both two-color and single-color detection methods may be used for microarrays. One-color methods include using a single fluorophore to mark miRNAs from breast cancer and healthy controls before hybridizing the cDNA to two arrays. The miRCURY LNA miRNA array platform (Exiqon), the Eppendorf DualChip and Silverquant (Eppendorf, Hamburg, Germany), and the Affymetrix GeneChip (Affymetrix, Santa Clara, CA), the Illumina BeadChip (San Diego, CA), the Agilent single-channel arrays, the Applied Microarrays CodeLink arrays (Applied Microarrays, Tempe, AZ), and the Eppendorf DualChip. Alter-natively, miRNAs isolated from breast cancer and healthy controls may be tagged with two distinct fluorophores (e.g., Cy3 and Cy5), mixed, and then hybridized to the same microarray. Two-color arrays include Agilent's Dual-Mode platform and Eppendorf's DualChip platform for colorimetric Silverquant labeling. Figure 5 depicts the standard process flow for miRNA quantification using two-color microarrays.

Many different fluorescent labeling methods have been devised, all with the same overarching goal: maximize labeling yield to improve signal to noise while minimizing the effects of labeling bias. Direct labeling of the miRNA or indirect labeling of a cDNA derived from the miRNA via reverse transcription both provide fluorescent labels (Fig. 6). Direct labeling may be accomplished by using T4 RNA ligase to attach a fluorescent-modified nucleotide to the 3′-OH of the miRNA's terminal. Due to the potential for substrate sequence bias introduced by direct enzymatic-labeling methods,74 direct chemical-labeling approaches have been developed. These include chemical alkylation (Mirus Bio Label IT, Mirus Bio, Madison, WI) and platinum coordination chemistries [Kreider et al.

Reverse transcription of miRNAs is necessary for indirect labeling and may be performed using random primers. Priming using a random primer, on the other hand, is not very specific and might lead to errors.75 Instead, try priming with a poly(dT) primer after attaching a poly(A) tail to the 3' end of the miRNAs. Fluorophore-conjugated deoxynucleotide triphosphates (dNTPs) can be added to the RT reaction to fluorescently label the cDNA.76 Alternatively, primary amine-containing dNTPs can be added to the cDNA synthesis reaction, and the cDNA products can be conjugated to fluorophores via amide bond formation, using an N-hydroxysuccinimide ester. This two-step method is advantageous because it allows for the incorporation of additional amino-modified nucleotides into each miRNA, hence increasing the detection sensitivity.75 The cDNA products are then purified and hybridized to the microarray, where they act as surrogate markers for the RNA. The vast majority of miRNAs on the market are already prescreened using commercially accessible microarrays. Agilent offers customizable arrays that can be tailored to interrogate a user-defined set of miRNAs, whereas for other platforms, the fabrication process makes customization less feasible and more expensive.70 Custom arrays that encompass a lower number of potential miRNA biomarkers may be more suitable for biomarker validation using a larger set of clinical samples. Likewise, numerous in vitro diagnostic systems have been authorized by the US Food and Drug Administration (FDA) to employ targeted microarrays as a detection method.77, 78, 79, 80

Furthermore, bespoke arrays provide probe tuning, which may help overcome the sensitivity and specificity hurdles presented by hybridization microarrays. High-affinity cDNA fragments or highly specific synthesized oligonucleotides are ideal capture probes for miRNA microarrays. Capture probes with lower than average guanine-cytosine (GC)

content and melting temperature (TM) values will yield lower fluorescent signals, whereas capture probes with higher GC content and TM values will display higher signals but impaired nucleotide discrimination and thus lower specificity.81 To resolve these issues, locked nucleic acid (LNA)-modified capture probes can be used to improve overall duplex stability (Exiqon). When complexed with complementary DNA or RNA, LNAs enhance thermal stability compared to DNA.82 They are called "locked" because the 2′ oxygen and 4′ carbon sites in the ribose ring are joined. Due to the enhanced heat stability, shorter PCR primers can be designed, and two miRNA-specific PCR primers may be created for each miRNA. When profiling miRNAs within the same family, for example, LNA probes can tell the difference between sequences that are quite similar.83

To further distinguish the target miRNA from nontarget RNA carrying the same sequence (e.g., pre- and pri-miRNA), hairpin structures are appended to the 5′ end of the probe during the probe design process. The probe's 3'-end hybridizing sequence is joined directly to the 5'-end hairpin structure. The hairpin destabilizes hybridization to larger nontarget RNAs, and it can provide additional stabilization if the target-probe duplex stacks with the probe hairpin.81 Overall, the physicochemical characteristics of the target sequences help determine the specificity and sensitivity of the microarray.

The miRNAs and their relative abundances are determined by scanning the array and reading the fluorescence intensity values at the various array sites (Fig. 6). Background correction and normalization utilizing several control probes are required for microarray data. This eliminates errors caused by inconsistencies in dye labeling, hybridization, and scanning.84 Controls must be included at various stages of processing to separate random variations from changes of biological significance. The use of control probes allows for reliable meta-analyses of miRNA expression studies and allows for easier comparisons of miRNA expression across different platforms. Microarray-based miRNA expression analysis can be validated, the sensitivity and specificity improved, and contamination bias (from things like scratches and dust) reduced by retesting and averaging the intensity values for data analysis 85. This is done after normalization, when the log2 ratios between sample and reference miRNAs are calculated. The miRNA expression profile is then analyzed to find genes with significantly differing expression levels. If there is a shortage of biological material, the fact that most microarrays need for 100-1000 ng of RNA extract as starting material81,86 may provide a problem. Affymetrix's GeneChip miRNA 2.0 Array technology, for instance, has been shown to be unreliable at low input levels, which equate to the quantity of miRNAs that can be retrieved from a 250 l aliquot of plasma.87

Sequencing for the Next Generation

In comparison to conventional Sanger sequencing, the computational accuracy, throughput, and cost of next-generation sequencing (NGS) have all greatly improved.88 The NGS platforms developed by Roche, Illumina, and Life Technologies55 use in vitro clonal fragment amplification via PCR, which greatly simplifies the preparation of sequencing libraries. These systems can support millions to billions of individual reads per run, which is a substantial advance over the 96 reads that can be sequenced on a high-end Sanger system. The average number of times a nucleotide is read throughout the sequencing process is known as the sequencing coverage. When the human genome is sequenced, for instance, a coverage of 30x means that there are, on average, 30 sequence reads for each nucleotide. The abundance of a miRNA in a sample is proportional to the number of reads collected for that miRNA during miRNA sequencing (miRNA-seq), which in turn is related to the miRNA's relative expression level. Therefore, in miRNA-seq, coverage is reported in terms of the number of sequence reads, in the millions. The number of reads needed to identify low-abundance miRNAs is proportional to the sensitivity of the experiment. An estimated minimum depth of 200 million paired-end reads is needed to discover novel transcribed elements and to obtain strong quantification of known transcript isoforms in human samples.89 Achieving ultra-deep sequencing allows for the identification of mutations in miRNAs, such as inversions, deletions, and translocations,82 even if these occur in only a small fraction of the sample. However, such high coverage incurs significant costs per sample, leading many investigators to consider profiling miRNAs at a lower number of reads.91, 92, 93 Specifically, five million reads per sample have been considered

sufficient to provide the statistical power necessary for differential expression analysis and to achieve a high discovery rate of novel mRNAs.

Extraction of total RNA is the first step in miRNA-seq sample preparation (Fig. 7), which is then followed by size fractionation (e.g., by PAGE) to concentrate RNAs between 17 and 25 nucleotides in length. Therefore, RNA must be transformed into cDNA in order to be utilized with the vast majority of currently available sequencing technology. RNA adaptors are ligated to the 5′ and 3′ ends of the isolated short RNAs, which act as primer-binding sites during reverse transcription and PCR amplification,95, 96, 97 and are also critical during the following sequencing run. The sample preparation method takes at least 4 days to complete.99 Size selection may be conducted either before or after adaptor ligation and reverse transcription.

These cDNA amplicons are sequenced using NGS platform-specific protocols after the library has been constructed. Denatured cDNA strands from an Illumina NGS analyzer's library anneal to complementary oligonucleotides bonded to a glass surface in a flow cell (Fig. 8). After polymerase extension and denaturation, single-stranded cDNA is linearized and covalently bonded to the surface. Then, bridge amplification occurs inside the fluidic channels, producing clusters of identical cDNA molecules. One adapter sequence is then cleaved, leaving clusters of single-stranded templates of the sense strand that are ready for sequencing. Polymerase-mediated incorporation of fluorescence-labeled nucleotides, known as reversible dye terminators, is the basis of this sequencing approach. To ensure that only one nucleotide incorporation event occurs per fragment population per sequencing cycle,46 these nucleotides are chemically blocked at the 3′ hydroxyl position. This fluorescence signal is then used to determine which base was incorporated. To probe the subsequent nucleotide base, the fluorescent dye and 3′ blocking group are chemically cleaved, and the cycle is restarted.

Pre-processing the short reads to remove adapters and low-quality sequences is the first step in miRNA-Seq data analysis and interpretation.100 The reads are then mapped onto a genome reference sequence using a miRNA sequencing software tool like miRDeep, mirTools, or MIReNA.101 In miRNA-Seq, miRNA quantification is expressed as the ratio of the number of sequence reads for a given miRNA to the total number of reads mapped to Inconsistencies in sample preparation, for example, might lead to discrepancies in miRNA abundance measurements between samples taken from the same patient, since relative miRNA quantification is reliant on the size of the library.

Evaluate Current Technologies

Table 3 compares the cost, complexity, throughput, accuracy, sensitivity, and dynamic range of a number of different miRNA profiling techniques. Both the number of miRNAs per sample and the number of samples that can be examined in a single run contribute to throughput. Assaying the expression levels of hundreds to thousands of miRNAs or miRNA variants in a single experiment55 makes microRNA profiling by microarrays or NGS a powerful tool for early biomarker identification. The accurate identification of miRNA sequences is made possible by NGS, and the discovery of new miRNAs is made possible as well. To quantify hundreds of miRNAs, RT-qPCR panels like the miRCURY platform are used, although this requires a lengthy and expensive series of individual reactions. For the planned clinical applications, however, it is expected that just a handful of miRNAs will need to be tested each sample,45 a task that is more easily achieved using RT-qPCR or bespoke microarrays. The high-throughput afforded by NGS is unnecessary if breast cancer diagnosis simply needs relative quantification of a small number of miRNAs per sample. Multiplex barcodes, like those found in Illumina's TruSeq small RNA kits, have increased throughput by allowing the user to sequence up to 48 samples in a single flow cell.99 This means that the cost of NGS can be reduced by sequencing multiple patient samples in a single run.

Correct identification of fold-change differences, or relative expression level changes, in a biological sample is what we mean when we talk about accuracy in terms of a platform or technology. Several studies have characterized the reproducibility of miRNA profiling methods, both within and between platforms72,103,104,105,106. The analytical

sensitivity, or limit of detection, determines how much input RNA must be extracted from the sample and how many miRNAs can be queried. In addition, a wide dynamic range is desirable to enable accurate identification of fold-change differences for miRNAs present in both high and low abundance.107 Traditionally, RT-qPCR has been considered to offer the highest accuracy, sensitivity, and dynamic range, and it is therefore frequently used to validate microarray and NGS results.93,108 For example, the stem-loop RT primer chemistry in combination with Taqman has been found to offer a dynamic range of up to seven orders of magnitude.107 miRNA microarrays provide the lowest dynamic range of four to five orders of magnitude.81,109 Various studies have demonstrated that RT-qPCR and NGS offer higher sensitivity and accuracy than microarray analysis.72,103,110 NGS technologies enable highly sensitive miRNA identification and can provide a dynamic range of ≥10 orders of magnitude.111 However, the dynamic range depends on read counts and biological sample types.112 Highly abundant miRNAs reduce the number of available reads for other miRNAs, which cannot be accounted for with current normalization methods.55 Consequently, comparisons between samples with high variance in miRNA distribution of expression may not be reliable.82 Concerning accuracy, a performance comparison study investigated miRNA profiling on human breast cancer cell lines via miRCURY RT-qPCR and two NGS platforms (SOLiD4 and Illumina HiSeq).69 The results indicate that the relative fold-change difference in expression level (malignant versus normal) was comparable between RT-qPCR and NGS, and between the two NGS platforms, for miRNAs present at both high and low abundance (Fig. 9). However, more heterogeneity was seen across the various platforms when the relative change in expression level was less than threefold. The major benefit of miRNA-seq is the detection of both novel and known miRNAs,82 and miRNA-seq studies with ultra-deep coverage enable relative quantification of miRNAs, which is necessary for definitive determination of potential miRNA breast cancer biomarkers.69 Discrepancies between studies reported to date for less abundant miRNAs may thus be due to differences in platform sensitivity and specificity.

The complexity of a technique comprises the experimental methodology, instrumentation, and data analysis. There are two components to the total price: the price per sample and the price per miRNA. Most research and many clinical labs now use RT-qPCR, and it is the method with the lowest complexity70. When hundreds of miRNAs need to be evaluated in a single sample, the cost of RT-qPCR might skyrocket. However, only a small number of miRNAs will be tested per sample in clinical settings, making RT-qPCR the most cost-effective method. Microarray-based miRNA quantification, although more complex than RT-qPCR, is a widely adopted method with reasonably cost, simple workflow, 114 and well-defined guidelines.115 NGS-based miRNA profiling currently entails the highest cost and complexity, and it is typically performed in sequencing centers with suitable infrastructure and personnel. Computational assistance, such as advanced data storage, data transport, analysis, and training of workers, is essential for the study of datasets on NGS systems. miRNA-seq may be obtained on a fee-for-service basis for the broader scientific community. More affordable and accessible sequencing is on the horizon thanks to the development of benchtop NGS platforms like the Illumina MiSeq, the Life Technologies IonTorrent PGM, and the Roche 454 GS Junior (Roche, Basel, Switzerland).116 Moreover, using barcoded adaptors allows analysis of more samples in a single sequencing reaction, which further reduces the cost per sample.

Conclusion

The development of more advanced profiling methods for miRNA now offers the infrastructure for identifying and validating miRNA as a biomarker. Method standardization and guidelines for RNA expression analysis using RT-qPCR,117 microarrays,115 and NGS RNA-seq.118 have helped improve intra- and interlaboratory reproducibility, but there are still technical hurdles to overcome, including the need to standardize protocols for miRNA extraction from biological specimens like serum or plasma and the normalization of measured values and controls.102 The observed discrepancies in miRNA biomarker validation studies are summarized in Table 2. To far, most clinical validation studies have included less than 100 participants. More extensive prospective studies are needed to definitively confirm a subset of miRNAs and move them toward application in clinical practice. Early noninvasive breast cancer diagnosis

and prognosis is one of the primary focused applications of this rapidly developing area, as indicated by the increased incidence of circulating miRNA publications.

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