Clinical Implication of MicroRNAs in Molecular Pathology: An Update for 2018

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KEYWORDS

• miRNAs • Fine-needle aspirates • Serum • Pancreatic cancer • Prostate cancer

KEY POINTS

- The microRNAs (miRNAs) have immense potential in the clinical arena because they can be detected in the blood, serum, tissues (fresh and formalin-fixed paraffin-embedded), and also, fine-needle aspirate specimens. Recently novel in situ hybridization techniques have been described to detect miRNAs in tissues, which enables direct miRNA and histomorphologic correlation.
- Identification of novel molecular miRNAs and their target oncogenomic signatures have the potential to significantly impact clinical management.
- Incorporating miRNA expression profiling on tissue samples in the future may not only confirm diagnosis categorizing diseases and their subtypes but also may predict drug response in helping clinicians define the precise therapy to each individual.
- Increasing the knowledge of disease progression and tumor recurrence might also improve the development of personalized therapies.
- The most attractive feature of miRNA-based therapy is that a single miRNA could be useful for targeting multiple genes that are deregulated in cancers, which can be further investigated through systems biology and network analysis that will allow designing cancer-specific personalized therapy.

INTRODUCTION

In this era of personalized medicine, a plethora of molecular markers are emerging to be used as novel tools in molecular pathology. The role of the pathologist is no longer

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confined to being behind the glass slide. Rapid advances in the fields of molecular biology and medicine have led to the development of the newly maturing field of molecular pathology, which has ramifications for the therapeutic management of patients. This field incorporates the use of cellular molecules in the clinical arena for early and accurate diagnosis, determining prognosis and risk stratification of disease, as therapeutic targets for designing molecular therapies, disease surveillance, and more recently prevention of disease progression and metastasis.

The field of molecular pathology has revolutionized clinical medicine. It further directs to the development of a novel branch of molecular pharmacotherapeutics. This is a newly developed branch of pharmacology that evaluates the impact of human genetic variations that affect individual drug responses and further analyzes mechanisms to overcome drug resistance and tailor pharmacologic drug response based on molecular alterations within cells. It includes the potential and challenges of drug optimization, the implications for drug development and regulation, ethical and social aspects of pharmacogenomics, signal transduction, the use of knockout mice, and informed consent process in pharmacogenetic research. Based on gene expression patterns seen in different tumors in individualized patients, the tumor is classified into different genotypes and treated based on an independent set of molecular and genetic characteristics.[1] This molecular tumor profile is then used to select specific targeted treatment approaches for patients with specific types of cancer.[2] Furthermore, the response to molecular-based therapy is then evaluated, taking into consideration the patients' individual drug response and drug resistance of the tumor cells if any, and methods to overcome the same are explored.[3]

Recent literature reveals a deluge of several small molecule inhibitors with possible clinical use in future clinical trials.[4,5] However, before they can be brought into the clinical arena, there is an ongoing process of drug evaluation in vitro and in animal models. This has resulted in significant expansion of the responsibilities of the pathologist in identifying druggable targets, prognostic biomarkers, histopathologic risk predictors, and further assisting in developing molecular targeted therapies. It is in the realm of the pathologist to identify these small molecules, which can be targeted through these small molecule inhibitors. After the small molecular alterations are identified in a subset of tumor types, these are stratified by the pathologist into those that develop early in the course of carcinogenesis, making them relevant biomarkers of disease identification for early and accurate diagnosis. Additionally, the pathologist evaluates whether these small molecules can be used in risk stratification and to determine prognosis in specific tumors. Furthermore, the pathologist can assist in the drug trials in determining the efficacy of small molecule inhibitors in reducing the size of the tumor, reducing the number of tumor cells, and the overall tumor burden leading to pathologic and microscopic identification of druggable target molecules.

One recently described class of molecule that is showing far-reaching clinical effects in molecular pathology is that encompassing microRNA (miRNA) biology and technology.[6,7] These are small, noncoding endogenous single-stranded RNAs comprising only 19 to 25 nucleotides in length but on average of approximately 22 nucleotides in length.[6,7] First described in 1993, these molecules are actively comprised in the regulation of multiple physiologic and pathologic procedures in humans, animals, and plants. They regulate the physiologic embryonic stem cell differentiation,[7,8] and recent studies have also demonstrated their key roles in the pathogenetic evolution, progression, and metastasis of carcinomas.[9–11]

The proposed mechanism of action of miRNAs is through the posttranscriptional gene expression regulation through the 3′ untranslated region binding of target mRNAs.[7,8] This causes mRNA degradation or suppression of their translation to

functional proteins.[4] Transcripts complementary to the 3′ untranslated region govern the translation by the RNA-RNA interaction. This results in the translational suppression or cleavage of the targeted mRNA because of the damaged complementarity between miRNA and mRNA. The miRNA genes transcript occurs by RNA polymerase II or III, which then yield primary miRNA. The location of maximum miRNA genes is in intergenic regions approximately 1 kilobase away from annotated genes.[7,8]

In addition to general transcriptional regulation of mRNA expression and translation, miRNAs also influence the development, progression, and metastasis of cancers.[6–8,12–14] Their functional effect may differ depending on their expression levels. They have either an oncogenic potential or tumor-suppressor effect depending on their downstream impact on target genes and thereby controlling the biologic manifestations of cancers.

Emerging evidence suggests that cancer stem cells (CSCs) and epithelial-to-mesenchymal transition phenotypic cells are regulated by the expression of miRNAs, implicating their role in chemo resistance and cancer metastasis.[15–17] There is evidence that these molecules are critical in the formation of CSCs, making them potential targets for overcoming drug resistance. Additionally, they have been proposed to have a part in the epithelial-to-mesenchymal transition phenomenon with implications in cancer drug resistance and metastasis.[17]

CLINICAL PERSPECTIVE

Cancers are a common clinical problem worldwide, leading to immense mortality, morbidity, and escalating health care costs. Despite rapid technologic and clinical advances, the cancer-related mortality and morbidity remain high, which also impacts patients' quality of life. Recent advances in the imaging and diagnostic modalities have resulted in early diagnosis of many cancers wherein select treatments have led to miraculous results with significant reduction in patient anguish. However, many malignancies remain occult until they have reached the late stages of the disease or have metastasized. The best of treatment options, including multimodal therapeutic approaches, have yielded minimal success in such instances. This underlines the need to use novel technologic advances in molecular biology from bench to patient bedside for clinical patient management. Moreover, the molecular mechanism of carcinogenesis, progression, and metastasis in some cancers is still largely unknown despite the "omics" revolution, which clearly suggests that further development in the areas of molecular signatures of disease aggressiveness is urgently needed.

Rapid progress occurring in technology and knowledge of molecular pathology has prompted a paradigm shift in the therapeutic patient management in the clinical arena. There is evidence of increasing integration of molecular markers with clinical and morphologic disease criteria to yield clinically relevant diagnostic, prognostic, and therapeutic algorithms for patient management. Moreover, selection of molecular targeted therapies and determination of prognosis and risk stratification of patients are being based on genomic and proteomic molecular diagnostic and prognostic signatures for patient care.

There is a unique opportunity to think outside the box and look at clinical problems in an analytical manner to solve cancer research dilemmas for the ultimate benefit of patients. Newly developed high-throughput, quantitative image-based methodologies for analysis and subcellular identification of alteration in cancers hold extreme promise in this regard. However, before these can be clinically applicable, they need to be correlated with the morphologic and clinical findings.

Cellular molecules, such as miRNAs, have an immense potential in the clinical realm of molecular pathology. Furthermore, one particular miRNA may target multiple genes

in a context-dependent manner, suggesting that targeted deregulation of 1 miRNA will have effects on multiple targets, which seems to be an attractive attribute for cancer therapy. Therefore, modulating activity of miRNAs may provide openings for novel cancer interventions. They have widespread clinical application in various aspects of patient care because their expression levels vary in different tumors and also alter with the disease progression.

Several miRNAs with oncogenic potential have been demonstrated to be upregulated in cancers and miRNAs with tumor-suppressive effect are downregulated in malignancies. Translating the application of miRNAs in clinical context has been enhanced by the applicability of several novel high-throughput multiplex technologies on a wide variety of patient samples, including blood, serum, tissues (fresh and formalin-fixed paraffin-embedded [FFPE]), and cerebrospinal fluid.[12,13,18–20] Apart from being able to decipher small molecules, these technologies lower laboratory costs, increase operational productivity, and enhance yield.[21–24] These are an integral part of the clinical armamentarium of the new-age pathologist, which has strengthened diagnostic capabilities in the move forward into the era of precision medicine.

Therapeutic decision-making and patient clinical management in the current clinical practice is being dictated more by alterations occurring in the tumor microenvironment at the molecular level, namely genetic, epigenetic, miRNA, and multispectral protein levels than by the histomorphology spectrum alone. This has led to a multisystem approach to a patient that includes a team composed of several clinicians with expertise in medical, surgical, pathology, molecular biology, and pharmacotherapeutics working together in synergy for the maximum benefit of the patient, minimizing the side effects and using drugs with targeted approach to achieve the goal of personalized medicine.

USE OF MOLECULAR PATHOLOGY PRACTICE

Molecular diagnostic applications are now an integral part of the management algorithms of several solid tumors. With the use of molecular diagnostics in oncology, pathologists hope to assist early and accurate diagnosis of malignant disease processes during initial workup. Molecular diagnostics also can help in risk stratification based on molecular parameters. Additionally, one can use the molecular biomarkers for disease surveillance during treatment and follow-up. Emerging evidence directed to the complex molecular changes involved in the development and progression of different malignancies produced innovative diagnostic molecular tools leading to the introduction of targeted therapies. In lung cancer, miR-27a regulation of MET, EGFR, and Sprouty2 is being explored for targeted therapies.[25] The promising therapeutic targets for patients with osteosarcoma include integrin, ezrin, statin, NOTCH/HES1, matrix metalloproteinases, and miR-215.[26] The miR-205BP/S3 is a possible promising therapeutic modality for melanoma.[27] The miR-34a may act as a tumor-suppressor miRNA of hepatocellular carcinoma, and current efforts are under way to evaluate strategies to increase miR-34a level as a critical targeted therapy for hepatocellular carcinoma.[28]

Promising candidate biomarkers are being discovered that may soon switch to the realm of clinical management of malignancies. There is a need for new and improved molecular-based treatment options to improve on the modest outcome in patients with cancer. Prognostic molecular biomarkers require validation, which may be challenging at times, to help clinicians classify patients in need of early diagnosis. Recognizing predictive biomarkers that will stratify response to developing targeted therapeutics is additionally required in arenas of cancer research and patient management. Furthermore, there is a need to identify clinically strong molecular tests to

classify patients who are further responsive to certain drugs, in the early treatment design based on well-certified molecular prognosticators.

Scientific discoveries of molecular markers are often prematurely highlighted before the completion of clinical trials to establish appropriate application to disease. Before clinicians can use the molecular findings for clinical patient use, there is a need for evidence-based guidelines established by knowledgeable clinicians to communicate emerging molecular clinical standards.

ROLE OF microRNAs IN CLINICAL SPECIMENS

miRNA research has advanced within a decade from one publication to thousands of publications describing their role in gene regulation. miRNA expression profiling has been recently evaluated as a reliable diagnostic biomarker for differentiating between normal and tumor specimens.[6–8,12,29,30] It has shown to be deregulated in multiple cancers in human and mouse models, and has proved to play a critical role in the development and progression of the tumor.[6,7,12,29,30] Most of the miRNAs are differentially expressed, whereas some of them discriminate totally between normal and tumorigenic samples. The miRNA expression enhances (oncogenic miRNA) or reduces (tumor suppressor) as the tumor progresses and was found to be associated with drug resistance.[31]

This discovery of miRNA a decade ago, as a diagnostic and prognostic marker, has now led to miRNA-based targeted therapy in vitro, and may selectively predict better treatment outcome for patients with cancer. In addition, classification of an unknown tumor may be possible by the alteration of tumor-specific miRNA.[32] The nomenclature for assigning names to novel miRNAs for publication in peer-reviewed journals is done by miRBase, which is the central repository for miRNA sequence information.[33] It has an online database with all published miRNA sequences with links to the primary literature and to other secondary databases. Although some miRNAs are tumor specific,[32] miR-21 has proved to be the global oncogenic miRNA in many solid tumors.[34–39] The miRNAs with oncogenic potential include miR-155, miR-17 to 92, and miR-21,[7] but it is not limited to these alone. The level of expression of miR-155 is upregulated in various carcinomas.[40–42] However, they are specifically significant in pancreatic cancers where they have prognostic relevance.[12,40,42,43]

Similarly, miRNA let-7 family and miR-200 family is frequently downregulated in many types of cancer, suggesting their role as a general tumor suppressor.[29,44–48] Low levels of let-7 miRNA[49] have been shown to have poorer prognosis with shorter postoperative survival in human lung cancer.[50] The tumor-suppressor miR-34 is directly transactivated and induced by p53 signaling in the inhibition of human pancreatic cancer tumor-initiating cells.[51] Based on a recent literature review, it has been suggested that modulation of miRNAs is a novel molecular targeted therapeutic approach for cancer in vivo.[52] This has led to the development of an emerging field of miRNA pharmacotherapeutics, which involves altering the expression of miRNAs, which can inhibit cancer growth.[44] The therapeutic strategies suggested using miRNAs include the inhibition of upregulated miRNAs and reexpression of tumor-suppressor miRNAs. They have also been shown to affect CSCs in overcoming drug resistance.[14] Regulation of miRNA levels in patients with ongoing cancer therapies would likely enhance the efficacy of their ongoing therapies.

Several synthetic small molecule inhibitors of miRNAs, such as chemically modified antisense oligonucleotides called “antagomiRs,” are currently in use in vitro, targeting against specific oncogenic miRNAs. These antagomiRs silence the overexpressed oncogenic miRNAs in cancers by blocking their function.[7,30] In animal experiments,

antagomiRs against miR-16, miR-122, miR-192, and miR-194 were found to be efficacious in reducing the levels of miRNA in the liver, lung, kidney, and ovaries.[52] Reexpression of tumor-suppressor miRNA, such as let-7, is another proposed miRNA therapeutic strategy to upregulate tumor-suppressor miRNA by exogenously transfecting with pre-let-7 that led to the inhibition of growth in vitro and in vivo.[15,44] These characteristics of miRNA suggest their potential role as novel biomarkers for diagnostic, prognostic, and therapeutic targets (Fig. 1).

METHODOLOGY AND CLINICAL IMPLICATIONS

Purification of microRNA from Human Plasma

The discovery of circulating miRNAs and their stability in plasma and serum is an interesting characteristic that could be used as noninvasive biomarkers for a variety of cancers, providing valuable tools to observe the changes during tumor progression.[29] Isolating miRNA of appropriate quality and quantity from blood is critical. Exosome RNA seems to be the richest source of miRNA in the serum or plasma. We have successfully isolated RNA from plasma samples,[29] and the detailed methodology is described next. Initially, the steps are carried out on ice, but later at room

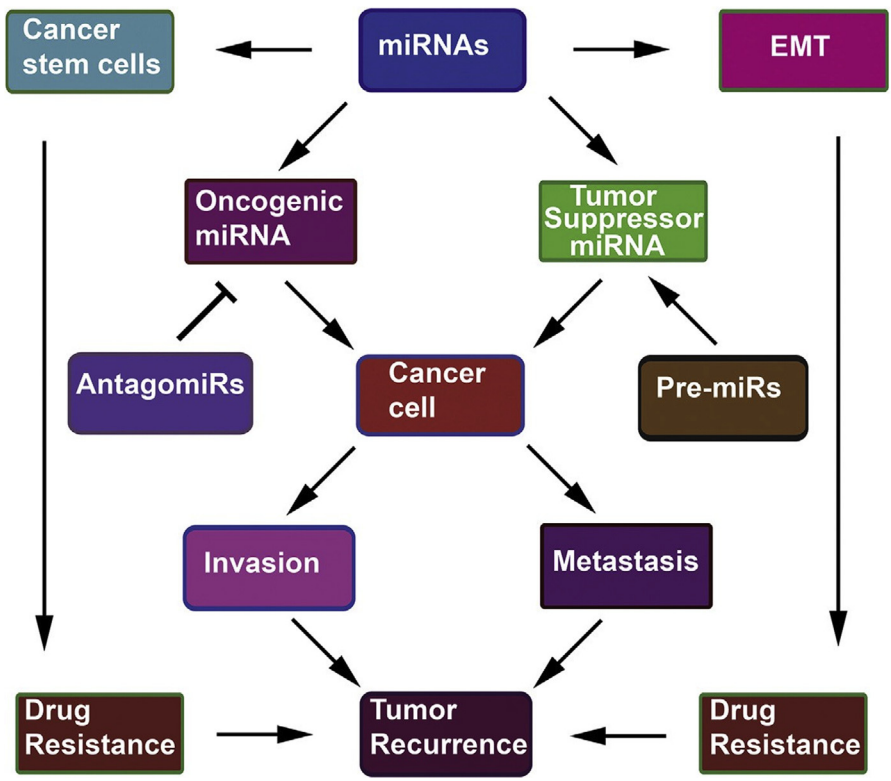

Fig. 1. Schematic representation on the relationship between CSCs and miRNAs with tumor aggressiveness, and the role of antagomiRs and pre-miRNAs on oncogenic and tumor-suppressive miRNAs. EMT, epithelial-to-mesenchymal transition. (*From* Sethi S, Ali S, Kong D, et al. Clinical implication of microRNAs in molecular pathology. Clin Lab Med 2013;33(4):778; with permission.)

temperature. Approximately 250 mL of plasma from each sample is centrifuged at 1000 *g* for 5 minutes to remove debris. The plasma is then transferred (200 mL) into a new tube along with 750 mL of QIAzol master mix containing 800 mL of QIAzol and 1 mg of MS2 RNA, and incubated for 5 minutes at room temperature. To this, 200 mL of chloroform is added, mixed well, and incubated for 5 minutes at room temperature, followed by centrifugation at 12,000 *g* for 15 minutes. All the subsequent steps are carried out at room temperature (20–25°C). The upper aqueous phase is moved into a new microtube containing a 1.5 volume of ethanol. After mixing well, the solution is then transferred onto the RNeasy Mini Spin Column with approximately 750 mL each time, and centrifuged at 13,000 *g* for 30 seconds. The flow-through is discarded, and the steps are repeated until the entire sample is used. The RNeasy Mini Column is then washed once with 700 mL of RWT, and 3 times with 500 mL RPE provided with the kit, and centrifuged for 13,000 *g* for 1 minute. The flow-through is discarded each time. The RNA (containing miRNA) is eluted with approximately 25 mL of water in a new collection tube by centrifugation and stored at 80°C.

The amount of RNA obtained from blood is usually too low a yield compared with RNA obtained from tissue samples to be quantified using single-drop spectrophotometry technology (ie, NanoDrop). However, there are several miRNAs that are detectable in plasma or serum samples thus displaying the presence of miRNAs. One such miRNA is miR-21, which can be detected by conventional real-time reverse-transcriptase polymerase chain reaction (RT-PCR) methodology, and is described next with significant modification using Exiqon-Universal cDNA synthesis kit (Exiqon, Woburn, MA).[12]

Reverse-Transcriptase Reaction

The RT reaction is performed by using Exiqon-Universal cDNA synthesis kit. The cDNA for standard curve is synthesized by reverse transcription using the template mature miRNAs, which can be prepared by diluting it with water. The RT reaction is set up with 20 mL of sample containing 4 mL of 5x RT buffer, 2 mL of enzyme mixture, 10 mL of water, and 4 mL of either the plasma miRNA or 250 nM of standard miRNA for 1 hour at 42°C, and 5 minutes at 95°C. The cDNA samples can be stored at −20°C.

Polymerase Chain Reaction

Multiple housekeeping genes are used in triplicate for data normalization and for analysis by the standard C_t method of quantification using StepOnePlus Real-Time PCR (Applied Biosystems, Foster City, CA). They also serve as controls for variability in sample loading. The sample testing is performed in equivalent with standards to evade batch effects as described next.

The miRNA standard cDNA is diluted in water. The standard curve is set up in triplicate with 5 points starting at 10,000, 5000, 2500, 1250, and 625 copy number. The plasma cDNA is diluted to 20-fold. The real-time PCR reaction is set up with a total volume of 10 mL containing 5 mL of SYBR Green (Applied Biosystems), 1 mL of PCR primer mix, and 4 mL of either the diluted cDNA from miRNA standard or the plasma cDNA, using standard curve model. The plasma miRNA concentration is calculated in 10^{-2} pM units using the Quantity value *3.125/6.02/1000. The reproducibility of the quantitative RT-PCR (qRT-PCR) assay data confirms the efficient extraction of miRNAs from plasma samples.

The method described previously has no limitations or interferences with respect to miRNA extraction and RT-PCR.

METHODOLOGY USING THE MAIN RESOURCES OF FORMALIN-FIXED TISSUES

Because the availability of fresh or frozen tissue is typically limited, archival collections of FFPE tissues are highly desirable and provide a rich source for the study of human disease. Tissue blocks are available worldwide from nearly every patient with well-documented clinical data including histopathologic reports, which makes it ideal to carry out research studies. The summarized methodology of plasma and fine-needle aspirate (FNA) samples is shown in Fig. 2. Of interest is that miRNA, because of its strong structure and smaller size, remains protected from degradation during the fixation process, and requires only a small amount to perform miRNA expression profiles or RT-PCR. A recent study using clinical specimens from prostate cancer has demonstrated the use of this in the clinical context.[6] The following section highlights the collection of FNA from tumor mass and the fixation method.

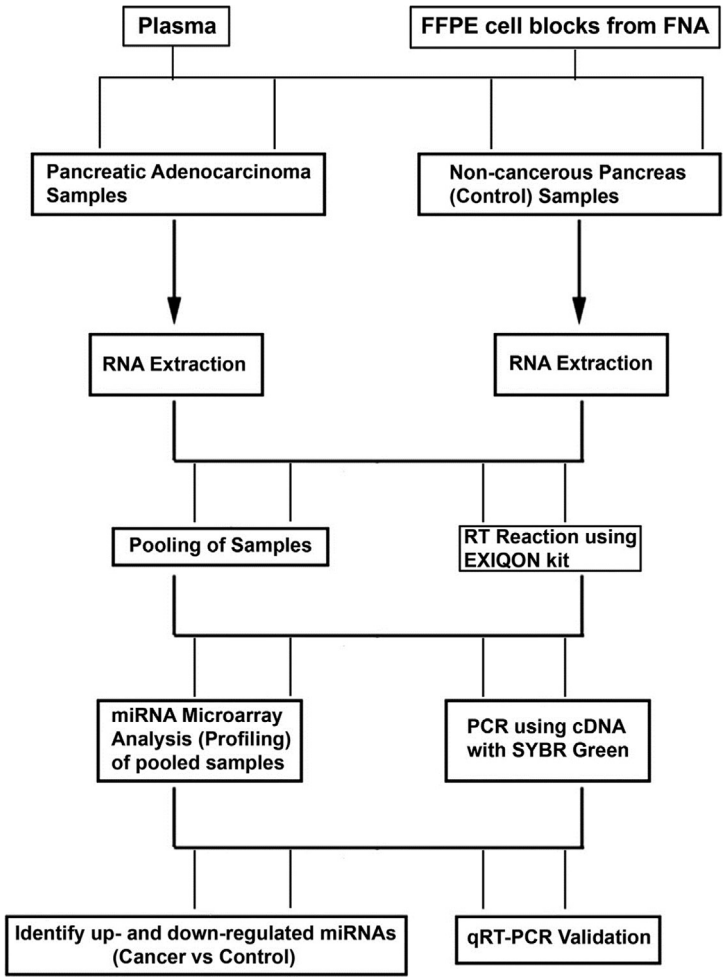

Fig. 2. Flow chart summarizing the methodology for miRNA research using plasma and FFPE cell blocks of FNA samples. (*From* Sethi S, Ali S, Kong D, et al. Clinical implication of microRNAs in molecular pathology. Clin Lab Med 2013;33(4):780; with permission.)

Fine-Needle Aspiration Tissue Collection

Diagnostic FNAs from tumor mass can be collected using a 20-gauge to 23-gauge needle from patients with pancreatic cancer (or other cancers) who underwent computerized tomography or endoscopic ultrasound–guided biopsy. Diagnostic smears are stained using Diff-Quick staining (Mercedes Medical, Sarasota, FL). The aspirates are put in fixative fluid, centrifuged, and embedded in paraffin using standard protocol, and are stained by hematoxylin-eosin for the presence of tumor cells. Approximately 50 cells are required to obtain excellent quality and quantity of total RNA (consisting of miRNA) to perform qRT-PCR. For the comparative purpose, normal pancreatic tissues that are farther away from the pancreatic tumor (typically obtained from surgical specimens) also can be similarly fixed to serve as controls.

RNA Isolation

The miRNA is isolated from FFPE tissue using RNeasy Kit (QIAGEN, Valencia, CA) following the manufacturer's protocol with a few modifications, which are described next. Four 10-mm thick and approximately 1 cm in diameter tissue curls are placed in 1 mL of xylene, mixed, and centrifuged for 2 minutes at room temperature. One milliliter of ethanol is added to the pellet and centrifuged for 2 minutes, and mixed with 240 mL of Buffer PKD along with 10 mL of proteinase K and incubated at 55°C, and then at 80°C for 15 minutes each. The mixture is then centrifuged, and the collected flow-through is mixed with 500 mL of buffer RBC to adjust binding conditions. The solution is mixed with ethanol and applied to the RNeasy column, and washed with buffer solution. The total RNA containing miRNA is eluted with RNase-free water. The purity of RNA is measured by the absorption ratio at 260/280 nm and quantified using NanoDrop 2000 (Thermo Scientific, Pittsburgh, PA). The ratio of 260/280 typically ranges between 1.8 and 2.1.

Real-Time Reverse-Transcriptase Polymerase Chain Reaction

The RT reaction is performed with SYBR Green miRNA-based assay using Exiqon-Universal cDNA synthesis kit (Exiqon). The RT reaction is set up with 10 mL of sample containing 2 mL of 5 RT buffer, 1 mL of enzyme mixture, 2 mL of water, and 10 ng of total RNA in 5 mL for 1 hour at 42°C and 5 minutes at 95°C. All reactions for PCR are carried out in triplicate using StepOnePlus Real-Time PCR (Applied Biosystems). The expression level of miRNAs is analyzed using C_t method.

microRNA Profiling

The prognosis of patients with cancer remains poor. Hence, new biomarkers are essential for early detection of cancer progression. The miRNA profiling can be useful as biomarkers for differentiating tumor from normal samples from plasma, FNA, or surgical tissue samples as discussed in many recent reports.[6,12,29,38,53] For example, equal amount of RNA containing miRNA and combined separately from control and patient samples can be analyzed for miRNA microarray profiling (LC Sciences, Houston, TX). The data are normalized using multiple housekeeping genes. Differentially regulated miRNAs between normal and cancer samples are analyzed using various statistical methodologies (eg, hierarchical clustering). Network analyses are accomplished using World Wide Web–based bioinformatics software programs, such as Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA) functional network analysis. Pathway analysis is a new innovative tool that reveals the expression of deregulated[12] miRNAs and their putative targets in a signaling pathway.[6,54] Because each miRNA may have more than 1 target, miRNA-based

therapy can be beneficial to target multiple signaling pathways, which include inhibition of oncogenic miRNAs by antisense oligonucleotides or by overexpression of tumor-suppressor miRNA by precursor miRNAs.[6,30,44]

ADVANCES MADE IN CHARACTERIZING microRNA PROFILING

Because there is an urgent need to investigate potential biomarkers for early detection of malignancies in a fast and straightforward way, miRNA research has made a significant impact on many types of cancer research, revealing basic gene expression changes toward identifying major signaling pathways involved in biomedical research. It empowers efficient profiling of deregulated miRNAs in plasma, serum, FFPE, and many other sample types because of their stability, which enables their detection and analysis, thus leading to a potentially reliable biomarker. Profiling of miRNAs not only provides access to hundreds of expressed miRNAs in the sample, but also differentiates between healthy and diseased state. Some of the miRNAs are substantially upregulated or downregulated in cancer cells or tumor tissues relative to normal cells or normal tissues, permitting the identification of miRNA signature. Differential expression of lead candidate miRNAs can be individually further validated by RT-PCR; however, it is unlikely that any one of the conventional housekeeping genes will be sufficient to normalize the data. Hence, the use of multiple housekeeping genes for data normalization using the standard method of quantification may be the ideal way of performing miRNA profiling. Overall, miRNA profiling is one of the most current methods to differentiate abnormally expressed miRNAs in a set of samples.

DIAGNOSTIC AND PROGNOSTIC IMPLICATIONS OF microRNAs

Biomarkers that are predictive, prognostic, and diagnostic can be a valuable tool to clinicians in making important decisions about patient care.[6] The discovery of circulating miRNAs in plasma and serum, because of their noninvasive nature, are excellent biomarkers for a variety of cancer detection and prognostic purposes. We have previously shown circulating miRNAs as biomarkers in pancreatic cancer. A previous report by Ali and colleagues[29] has identified a group of 7 miRNAs including 2 oncogenic and 5 tumor-suppressor miRNAs that were recognized and validated as a diagnostic marker in 50 plasma samples from patients with pancreatic cancer in comparison with healthy control subjects. Similar reports on FNA from FFPE cell blocks in patients with pancreatic cancer also identified 7 miRNAs that were differentially expressed in tumor cells compared with normal tissues.[12] These 7 miRNAs both from plasma and FNA showed substantial differences in their expression level and hence may serve as diagnostic biomarkers in the detection of cancer. In addition, the Ingenuity networking pathway analysis in FNA study provided a unique strategy to study various signaling pathways, which revealed 15 biofunctional network groups relating to cancer, genetic disorder, and gastrointestinal disease that are expected to improve prognosis and response to certain therapies.[12] Other investigators also performed miRNA profiling in various other solid tumors to discover a differentially expressed panel of miRNAs in pooled samples that reached excellent diagnostic properties to classify them as biomarkers for cancer detection.[34,38,53,55] Novel high-throughput multiplex technologies hold immense promise in this regard. However, before these can be clinically applicable, these need to be correlated with the morphologic and clinical findings to decipher those that are clinically relevant. Additional studies are needed to edify miRNA biomarkers to reduce patient mortality, morbidity, and introduce early diagnosis, thereby reducing health care costs and applying these approaches to patient care.

RECENT ADVANCES IN THE FIELD OF microRNAs

miRNAs are rapidly evolving as significant molecular markers in the fields of oncology, clinical diagnostics, and therapeutics. Rapid advances in the diagnostic technologies of miRNAs have led to identification of these molecules in several body fluids as circulating biomarkers of disease.[56] This has led to markedly increased potential of miRNAs in the clinical arena.[57] miRNA therapeutics is currently evolving with clinical applications in several disease conditions, including cardiac disorders[58] and malignancies.[59] Recent techniques describing purification and detection of miRNAs in the urine specimens has tremendously increased the noninvasive clinical potential of this molecule in early diagnosis, prognosis, and surveillance of different malignancies, especially urothelial carcinomas.[60] Not only are the miRNAs being used for oncology but also have wide-ranging potential applications in several clinical conditions, including sepsis,[61] hepatic diseases,[62] including drug-induced liver injury,[63] atherosclerosis,[64] and Parkinson disease,[65] among others. Prior knowledge of the action of miRNAs targeting CSCs is now being used in the field of oncotherapeutics as a new paradigm for cancer treatment and prevention of tumor recurrence.[59] miR-17 miRNA family is currently being evaluated as a therapeutic strategy against vulvar carcinoma.[66] miRNAs are also being used to predict response to therapy in patients with malignancies, including prediction of response to radiation therapy in non–small-cell lung carcinoma.[67] Overall, miRNAs hold great promise as molecular biomarkers with immense potential in early diagnosis, prognosis, and therapeutics in wide-ranging malignancies including ovarian cancer,[68] prostate carcinoma,[69] head and neck carcinomas,[70] and breast carcinomas[71] in this area of personalized medicine.

SUMMARY

The miRNAs have immense potential in the clinical arena because they can be detected in the blood, serum, tissues (fresh and FFPE), and FNA specimens. Identification of novel molecular miRNAs and their target oncogenomic signatures has the potential to significantly impact clinical management. The discovery of miRNAs and their expression profile in a wide variety of cancers has led investigators to understand the key role of miRNAs as biomarkers during cancer progression. Incorporating miRNA expression profiling on tissue samples in the future may not only confirm diagnosis categorizing diseases and their subtypes, but also may predict drug response in helping clinicians define the precise therapy to each patient. Moreover, increasing the knowledge of disease progression and tumor recurrence might also improve the development of personalized therapies. The most attractive feature of miRNA-based therapy is that a single miRNA could be useful for targeting multiple genes that are deregulated in cancers, which can be further investigated through systems biology and network analysis that allows designing cancer-specific personalized therapy. In summary, miRNAs are poised to provide diagnostic, prognostic, and therapeutic targets for several diseases, including malignancies for precision medicine.

REFERENCES

1. Lei Z, Tan IB, Das K, et al. Identification of molecular subtypes of gastric cancer with different responses to PI3-kinase inhibitors and 5-fluorouracil. Gastroenterology 2013;145:554–65.
2. Elghannam DM, Ibrahim L, Ebrahim MA, et al. Association of MDR1 gene polymorphism (G2677T) with imatinib response in Egyptian chronic myeloid leukemia patients. Hematology 2014;19(3):123–8.

3. Tan J, Yu Q. Molecular mechanisms of tumor resistance to PI3K-mTOR targeted cancer therapy. Chin J Cancer 2013;32(7):376–9.
4. Lv X, Ma X, Hu Y. Furthering the design and the discovery of small molecule ATP-competitive mTOR inhibitors as an effective cancer treatment. Expert Opin Drug Discov 2013;8(8):991–1012.
5. Rai G, Vyjayanti VN, Dorjsuren D, et al. Small Molecule Inhibitors of the Human Apurinic/apyrimidinic Endonuclease 1 (APE1) 2010 [Updated 2013]. In: Probe Reports from the NIH Molecular Libraries Program [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2010. Available at: https://www.ncbi.nlm.nih.gov/books/NBK133448/. Accessed March 27, 2018.
6. Sethi S, Kong D, Land S, et al. Comprehensive molecular oncogenomic profiling and miRNA analysis of prostate cancer. Am J Transl Res 2013;5:200–11.
7. Sethi S, Sarkar FH. Evolving concept of cancer stem cells: role of micro-RNAs and their implications in tumor aggressiveness. J Carcinogene Mutagene 2011;(S1):005.
8. Hassan O, Ahmad A, Sethi S, et al. Recent updates on the role of microRNAs in prostate cancer. J Hematol Oncol 2012;5:9.
9. Bao L, Yan Y, Xu C, et al. MicroRNA-21 suppresses PTEN and hSulf-1 expression and promotes hepatocellular carcinoma progression through AKT/ERK pathways. Cancer Lett 2013;337(2):226–36.
10. Lei H, Zou D, Li Z, et al. MicroRNA-219-2-3p functions as a tumor suppressor in gastric cancer and is regulated by DNA methylation. PLoS One 2013;8:e60369.
11. Shen SN, Wang LF, Jia YF, et al. Upregulation of microRNA-224 is associated with aggressive progression and poor prognosis in human cervical cancer. Diagn Pathol 2013;8:69.
12. Ali S, Saleh H, Sethi S, et al. MicroRNA profiling of diagnostic needle aspirates from patients with pancreatic cancer. Br J Cancer 2012;107:1354–60.
13. Qazi AM, Gruzdyn O, Semaan A, et al. Restoration of E-cadherin expression in pancreatic ductal adenocarcinoma treated with microRNA-101. Surgery 2012;152:704–11.
14. Ahmad A, Sarkar SH, Bitar B, et al. Garcinol regulates EMT and Wnt signaling pathways in vitro and in vivo, leading to anticancer activity against breast cancer cells. Mol Cancer Ther 2012;11:2193–201.
15. Kong D, Heath E, Chen W, et al. Loss of let-7 up-regulates EZH2 in prostate cancer consistent with the acquisition of cancer stem cell signatures that are attenuated by BR-DIM. PLoS One 2012;7:e33729.
16. Ahmad A, Aboukameel A, Kong D, et al. Phosphoglucose isomerase/autocrine motility factor mediates epithelial-mesenchymal transition regulated by miR-200 in breast cancer cells. Cancer Res 2011;71:3400–9.
17. Kong D, Banerjee S, Ahmad A, et al. Epithelial to mesenchymal transition is mechanistically linked with stem cell signatures in prostate cancer cells. PLoS One 2010;5:e12445.
18. Baraniskin A, Kuhnhenn J, Schlegel U, et al. MicroRNAs in cerebrospinal fluid as biomarker for disease course monitoring in primary central nervous system lymphoma. J Neurooncol 2012;109:239–44.
19. Brunet VA, Pericay C, Moya I, et al. microRNA expression profile in stage III colorectal cancer: circulating miR-18a and miR-29a as promising biomarkers. Oncol Rep 2013;30:320–6.
20. Ulivi P, Foschi G, Mengozzi M, et al. Peripheral blood miR-328 expression as a potential biomarker for the early diagnosis of NSCLC. Int J Mol Sci 2013;14:10332–42.

21. Bi S, Cui Y, Li L. Dumbbell probe-mediated cascade isothermal amplification: a novel strategy for label-free detection of microRNAs and its application to real sample assay. Anal Chim Acta 2013;760:69–74.
22. Gu LQ, Wanunu M, Wang MX, et al. Detection of miRNAs with a nanopore single-molecule counter. Expert Rev Mol Diagn 2012;12:573–84.
23. Guan DG, Liao JY, Qu ZH, et al. mirExplorer: detecting microRNAs from genome and next generation sequencing data using the AdaBoost method with transition probability matrix and combined features. RNA Biol 2011;8: 922–34.
24. Luo S. MicroRNA expression analysis using the Illumina microRNA-Seq Platform. Methods Mol Biol 2012;822:183–8.
25. Acunzo M, Romano G, Palmieri D, et al. Cross-talk between MET and EGFR in non-small cell lung cancer involves miR-27a and Sprouty2. Proc Natl Acad Sci U S A 2013;110:8573–8.
26. Yang J, Zhang W. New molecular insights into osteosarcoma targeted therapy. Curr Opin Oncol 2013;25:398–406.
27. Noguchi S, Iwasaki J, Kumazaki M, et al. Chemically modified synthetic microRNA-205 inhibits the growth of melanoma cells in vitro and in vivo. Mol Ther 2013;21:1204–11.
28. Dang Y, Luo D, Rong M, et al. Underexpression of miR-34a in hepatocellular carcinoma and its contribution towards enhancement of proliferating inhibitory effects of agents targeting c-MET. PLoS One 2013;8:e61054.
29. Ali S, Almhanna K, Chen W, et al. Differentially expressed miRNAs in the plasma may provide a molecular signature for aggressive pancreatic cancer. Am J Transl Res 2010;3:28–47.
30. Ali S, Banerjee S, Logna F, et al. Inactivation of Ink4a/Arf leads to deregulated expression of miRNAs in K-Ras transgenic mouse model of pancreatic cancer. J Cell Physiol 2012;227:3373–80.
31. Ali S, Ahmad A, Banerjee S, et al. Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF. Cancer Res 2010;70:3606–17.
32. Liang Y, Ridzon D, Wong L, et al. Characterization of microRNA expression profiles in normal human tissues. BMC Genomics 2007;8:166.
33. Griffiths-Jones S. miRBase: microRNA sequences and annotation. Curr Protoc Bioinformatics 2010;29:12.9.1–12.9.10.
34. Gall TM, Frampton AE, Krell J, et al. Blood-based miRNAs as noninvasive diagnostic and surrogative biomarkers in colorectal cancer. Expert Rev Mol Diagn 2013;13:141–5.
35. Hermansen SK, Dahlrot RH, Nielsen BS, et al. MiR-21 expression in the tumor cell compartment holds unfavorable prognostic value in gliomas. J Neurooncol 2013; 111:71–81.
36. Si H, Sun X, Chen Y, et al. Circulating microRNA-92a and microRNA-21 as novel minimally invasive biomarkers for primary breast cancer. J Cancer Res Clin Oncol 2013;139:223–9.
37. Sicard F, Gayral M, Lulka H, et al. Targeting miR-21 for the therapy of pancreatic cancer. Mol Ther 2013;21(5):986–94.
38. Tang D, Shen Y, Wang M, et al. Identification of plasma microRNAs as novel noninvasive biomarkers for early detection of lung cancer. Eur J Cancer Prev 2013;22(6):540–8.
39. Yang SM, Huang C, Li XF, et al. miR-21 confers cisplatin resistance in gastric cancer cells by regulating PTEN. Toxicology 2013;306:162–8.

40. Liu R, Liao J, Yang M, et al. Circulating miR-155 expression in plasma: a potential biomarker for early diagnosis of esophageal cancer in humans. J Toxicol Environ Health A 2012;75:1154–62.
41. Yang M, Shen H, Qiu C, et al. High expression of miR-21 and miR-155 predicts recurrence and unfavourable survival in non-small cell lung cancer. Eur J Cancer 2013;49:604–15.
42. Zhang Y, Roccaro AM, Rombaoa C, et al. LNA-mediated anti-miR-155 silencing in low-grade B-cell lymphomas. Blood 2012;120:1678–86.
43. Yabushita S, Fukamachi K, Tanaka H, et al. Circulating microRNAs in serum of human K-ras oncogene transgenic rats with pancreatic ductal adenocarcinomas. Pancreas 2012;41:1013–8.
44. Ali S, Ahmad A, Aboukameel A, et al. Increased Ras GTPase activity is regulated by miRNAs that can be attenuated by CDF treatment in pancreatic cancer cells. Cancer Lett 2012;319:173–81.
45. Bhutia YD, Hung SW, Krentz M, et al. Differential processing of let-7a precursors influences RRM2 expression and chemosensitivity in pancreatic cancer: role of LIN-28 and SET oncoprotein. PLoS One 2013;8:e53436.
46. Hu X, Guo J, Zheng L, et al. The heterochronic microRNA let-7 inhibits cell motility by regulating the genes in the actin cytoskeleton pathway in breast cancer. Mol Cancer Res 2013;11:240–50.
47. Kang HW, Crawford M, Fabbri M, et al. A mathematical model for microRNA in lung cancer. PLoS One 2013;8:e53663.
48. Zaman MS, Maher DM, Khan S, et al. Current status and implications of microRNAs in ovarian cancer diagnosis and therapy. J Ovarian Res 2012;5:44.
49. Jusufovic E, Rijavec M, Keser D, et al. let-7b and miR-126 are down-regulated in tumor tissue and correlate with microvessel density and survival outcomes in non–small-cell lung cancer. PLoS One 2012;7:e45577.
50. Xia XM, Jin WY, Shi RZ, et al. Clinical significance and the correlation of expression between Let-7 and K-ras in non-small cell lung cancer. Oncol Lett 2010;1:1045–7.
51. Vogt M, Munding J, Gruner M, et al. Frequent concomitant inactivation of miR-34a and miR-34b/c by CpG methylation in colorectal, pancreatic, mammary, ovarian, urothelial, and renal cell carcinomas and soft tissue sarcomas. Virchows Arch 2011;458:313–22.
52. Krutzfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature 2005;438:685–9.
53. Callari M, Dugo M, Musella V, et al. Comparison of microarray platforms for measuring differential microRNA expression in paired normal/cancer colon tissues. PLoS One 2012;7:e45105.
54. Azmi AS, Ali S, Banerjee S, et al. Network modeling of CDF treated pancreatic cancer cells reveals a novel c-myc-p73 dependent apoptotic mechanism. Am J Transl Res 2011;3:374–82.
55. Lang MF, Yang S, Zhao C, et al. Genome-wide profiling identified a set of miRNAs that are differentially expressed in glioblastoma stem cells and normal neural stem cells. PLoS One 2012;7:e36248.
56. Jung HJ, Suh Y. Circulating miRNAs in ageing and ageing-related diseases. J Genet Genomics 2014;41(9):465–72.
57. Rocci A, Hofmeister CC, Pichiorri F. The potential of miRNAs as biomarkers for multiple myeloma. Expert Rev Mol Diagn 2014;14(8):947–59.
58. Bernardo BC, Ooi JY, Lin RC, et al. miRNA therapeutics: a new class of drugs with potential therapeutic applications in the heart. Future Med Chem 2015;7(13):1771–92.

59. Osaki M, Okada F, Ochiya T. miRNA therapy targeting cancer stem cells: a new paradigm for cancer treatment and prevention of tumor recurrence. Ther Deliv 2015;6(3):323–37.
60. Channavajjhala SK, Rossato M, Morandini F, et al. Optimizing the purification and analysis of miRNAs from urinary exosomes. Clin Chem Lab Med 2014;52(3): 345–54.
61. Dumache R, Rogobete AF, Bedreag OH, et al. Use of miRNAs as biomarkers in sepsis. Anal Cell Pathol (Amst) 2015;2015:186716.
62. Zhou B, Li Z, Yang H, et al. Extracellular miRNAs: origin, function and biomarkers in hepatic diseases. J Biomed Nanotechnol 2014;10(10):2865–90.
63. Wang Y, Chen T, Tong W. miRNAs and their application in drug-induced liver injury. Biomark Med 2014;8(2):161–72.
64. Toba H, Cortez D, Lindsey ML, et al. Applications of miRNA technology for atherosclerosis. Curr Atheroscler Rep 2014;16(2):386.
65. Hesse M, Arenz C. miRNAs as novel therapeutic targets and diagnostic biomarkers for Parkinson's disease: a patent evaluation of WO2014018650. Expert Opin Ther Pat 2014;24(11):1271–6.
66. de Melo Maia B, Ling H, Monroig P, et al. Design of a miRNA sponge for the miR-17 miRNA family as a therapeutic strategy against vulvar carcinoma. Mol Cell Probes 2015;29(6):420–6.
67. Sun Y, Hawkins PG, Bi N, et al. Serum MicroRNA signature predicts response to high-dose radiation therapy in locally advanced non-small cell lung cancer. Int J Radiat Oncol Biol Phys 2018;100(1):107–14.
68. Mandilaras V, Vernon M, Meryet-Figuière M, et al. Updates and current challenges in microRNA research for personalized medicine in ovarian cancer. Expert Opin Biol Ther 2017;17(8):927–43.
69. Souza MF, Kuasne H, Barros-Filho MC, et al. Circulating mRNAs and miRNAs as candidate markers for the diagnosis and prognosis of prostate cancer. PLoS One 2017;12(9):e0184094.
70. Hess AK, Müer A, Mairinger FD, et al. MiR-200b and miR-155 as predictive biomarkers for the efficacy of chemoradiation in locally advanced head and neck squamous cell carcinoma. Eur J Cancer 2017;77:3–12.
71. Xie X, Tan W, Chen B, et al. Preoperative prediction nomogram based on primary tumor miRNAs signature and clinical-related features for axillary lymph node metastasis in early-stage invasive breast cancer. Int J Cancer 2018;142(9): 1901–10.