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MICRORNA, A CLINICAL DIAGNOSTIC AND PROGNOSTIC BIOMARKER

Anitha TS, *Scientist*
Central Inter-Disciplinary Research Facility

Sri Balaji Vidyapeeth Mahatma Gandhi Medical College and Research Institute Campus
Pillaiyarkuppam, Puducherry - 607403, India
Email:tsanitha6@gmail.com

Abstract ▶ microRNAs, small non-coding RNAs, have recently emerged as powerful regulators in a variety of cellular processes especially important roles in disease and tissue remodeling. Apart from involvement in a variety of biological processes, microRNAs were early recognized for their potential use as biomarkers in disease diagnostics and prognosis. Currently, there are number of microRNAs helping clinicians to determine the origins of cancer in disseminated tumors. The development of microRNA therapeutics has proved more challenging mainly due to delivery issues. This review focuses on the potential role of as clinical diagnostic and prognostic biomarkers. In addition, it highlights the microRNA profiling techniques, thereby leading to the advance opportunities to safely pursue microRNA as therapeutic modalities.

Key Words: miRNAs, biomarkers, prognostic markers, profiling, diabetes mellitus.

Introduction

microRNAs (miRNAs) are evolutionarily conserved small non-coding RNA molecules that regulate gene expression, and their recent discovery is revolutionizing both basic biomedical research and drug discovery. The human genome is believed to encode ~1,000 miRNAs. A repository of miRNAs from many organisms has been listed in the miRBase Sequence Database[1], that contains sequences and annotation[2]. More than 25,000 miRNAs have been described in man, worms, Drosophila, and also in the small plant *Arabidopsis thaliana*[3]. This review aims to describe the basics of miRNA and their role as biomarkers in clinical diagnostics and prognostic values. Moreover, this review also discusses the promising methods for measuring miRNA expression profiles in various biological samples such as cells, tissues and body fluids.

miRNA basics

Gary Ruvkun and Victor Ambros in 1990, first discovered miRNAs in *Caenorhabditis elegans* and their target gene[4,5]. Together, these two seminal discoveries identified a novel mechanism for post-transcriptional gene regulation. A hairpin fold-back structure from the precursor transcript separates the miRNAs from other small RNAs with expression confirmation of about 22 nucleotide-long mature sequence. Presently, based on deep-sequencing data, 1600 human miRNA precursors have been deposited into miRBase v19[6]. The nomenclature of these miRNAs is based on a "mir" or "miR" prefix with identifying numbers assigned sequentially at the time of discovery. "mir" represents a precursor miRNA whereas "miR" denotes a mature miRNA sequence. Similar or identical sequences can be given the same number.

miRNA biogenesis

miRNA genes reside either in intergenic regions, within introns of coding or non-coding genes or within exons of non-coding genes[7]. Approximately one third of miRNAs are intergenic and most of all miRNA loci contain clustered miRNAs (miRBase v19). The majority of miRNAs are transcribed as long primary transcripts by RNA polymerase II and many are capped and polyadenylated[8,9]. Analysis of miRNAs residing in intergenic primary transcripts indicates that such transcripts are shorter than protein-coding transcripts with transcriptional start sites about 2 kb upstream of the pre-miRNA and polyadenylation signals 2 kb downstream[10]. A subset of miRNAs is transcribed by RNA polymerase III. This cluster of miRNAs is located among Alu rich regions on chromosome 19[11]. Pri-miRNAs fold into hairpin structures containing imperfectly base-paired stems and are processed into 60- to 100-nt hairpins known as pre-miRNAs. The pre-miRNAs are exported from the nucleus to the cytoplasm by exportin 5, where they, in general, are cleaved by the endonuclease Dicer to yield imperfect miRNA-miRNA* duplexes[12]. The miRNA strand is selected to become a mature miRNA, whereas, most often, the miRNA* strand is degraded. The mature miRNA that is added to the RNA-induced silencing complex (RISC) identifies the potential precise targets and activates post- transcriptional gene silencing[13]. On the other hand, an alternative biogenesis pathway was revealed in which miR-451 enters RISC by direct loading of the pre-miR into RISC after Drosha processing, by skipping further processing by Dicer[14]. Transcription of miRNAs occurs through RNA polymerase II and subsequent processing is mediated by the nuclear ribonuclease III (RNase III) enzyme Drosha to form precursor miRNAs (70–100 nucleotides). Following transportation to the cytoplasm by exportin 5, a further cleavage occurs via another RNase III enzyme, Dicer, to form the mature miRNA. miRNAs modulate both physiological and pathological pathways by post-transcriptionally inhibiting the expression of a multitude of target genes. Much work has been done on the role of miRNAs in human disease, especially in cancers and infections.

Use of miRNA as biomarkers in clinical diagnosis

miRNAs exhibit strict developmental and tissue-specific expression patterns in organ and immune system development. For example, miR-1 is involved in mammalian heart development[15], miR-375 regulates pancreatic insulin secretion[16], miR-181 influences the differentiation of hematopoietic cells toward the B-cell lineage[17], and miR-430 is required for zebrafish brain development[18]. These studies highlight the participation of miRNAs in diverse cellular processes. Hence, it is not surprising that dysregulation of miRNA function is associated with many human diseases such as diabetes, neurological disorders, and cancer.

miRNA as biomarker for cancer

miRNAs play a critical role in the development of cancer and can influence cancer-promoting and cancer-suppressing genes. The first documentation of a miRNA abnormality in cancer stemmed from studies of human chromosome 13q14[19]. In cancer, miRNA expression variations are evident across different stages of cancer progression[20]. In tumorigenesis, overexpression of certain miRNA down-regulates tumor suppressor genes. These miRNAs can be exploited as potential biomarkers due to their tissue specificity and to tumor type and its origin[21]. An increasing number of miRNAs are now identified and utilized as prognostic miRNAs to predict drug response.

Glioblastoma

The primary brain tumor, glioma arises from glial cells of the central nervous system (CNS). Even after aggressive treatment like surgical resection and chemotherapy, glioblastoma multiforme patients show the least promising prognosis, where the reappearance is frequent and mean survival is only 12–15 months[22]. The intricacy in determining an explicit biomarker for glioma lies in part with the complex heterogeneous nature of the cancer itself. miRNA signatures have been recognized in both glioblastoma tissue and in blood circulation of glioblastoma patients. Recently, deep sequencing method has produced one of the largest sets of miRNA profiles for glioblastoma and control brain tissue. This study identified 33 up-regulated miRNA in the glioblastoma tissue and 40 down-regulated. In addition, 18 novel miRNAs and 16 novel miRNA-3ps were identified (miRNA-3p, miR-3676, miR-204, miR-539, miR-758, miR-382, miR-1271miR-98, miR-1307, miR-181b1-miR-873, miR-212, miR-135a-2, miR-511-1, miR-301a, miR-381, miR-487a)[23].

Breast cancer

Profiling studies of miRNA have led to the categorization of miRNAs that are abnormally expressed in human breast

cancer, with down regulation of miR-125b, miR-10b and miR-145 and up-regulation of miR-21, miR-9 and miR-155. When comparing breast cancer tissue with the normal tissues, 29 differentially-expressed miRNAs were acknowledged, and a subset of 15 miRNAs could be used to discriminate tumor from normal. In recent years, the discovery of upregulation of miR-10b in promoting invasion and metastasis in most cancers, exhibited a downregulation in metastatic breast cancers, which is validated by migration and invasion assay[24]. miR-9 up-regulation in breast cancer cells, directly targets *CDH1,* the E-cadherin-encoding mRNA, leading to increased cell motility and invasiveness[25]. Interestingly, miR-378(β) was recognized as a molecular switch in cancer cell bioenergetics pathway, also known as the Warburg effect, by the regulation of ERBB2 expression[26]. On the other hand, increasing the expression of few tumor suppressor miRNAs can alleviate development of breast tumors[27]. For example, the expression of tumor suppressor miR-127 down-regulates the expression profile of proto-oncogene BCL6, a potential target of miR-127[28].

Colorectal cancer

The major reason for the failure of treating advanced colorectal cancer is chemoresistance[29]. Several miRNAs have been found to be associated (miR-192, miR-215, miR-140, miR-129, let-7, miR-181b, miR-200 s) with chemoresistance by regulating key cell death pathways such as apoptosis and autophagy. Several important miRNA were described that regulate targets such as Bcl2, thymidylate synthase, dihydrofolate reductase, histone deacetylase, and E2F. For example, miR-215 was identified to suppress the expression of both thymidylate synthase and dihydrofolate reductase[30]. In addition, the expression of miR-215 was directly regulated by p53. The expression of miR-215 was significantly associated with colorectal cancer patient survival. Another miRNA, miR-140, was found to modulate chemosensitivity by suppressing HDAC4 expression, and the levels of miR-140 and miR-215 were elevated in colon cancer stem cells[31]. Furthermore, miR-194 was identified to regulate BMI-1 protein expression (BMI-1 is involved in epithelial to- mesenchymal transition)[32]. Moreover, miR-502 regulates autophagy in colon cancer by targeting Rab1B[33]. Taken together, these miRNAs can be utilized in predicting patient's prognosis and survival.

miRNA as biomarker for diabetes mellitus (DM)

The circulating miRNA (serum and plasma) characterizes a unique form of disease initiation and development. Due to a variety of pathogenesis of diverse types of D, the differential regulatory roles of miRNA leading to disease outcomes, including β-cell deficiency and insulin resistance, have recently been defined. In type 1 DM, the role of miRs in controlling β-cell genesis, β-cell death (miR-21), insulin production (miR-30d, miR204, miR-124a) and β/ β-cell mass balance (miR-375) or its susceptibility to immune-mediated β-cell destruction has been described[34-39]. In regard to type 2 DM, insulin resistance miRNAs, the key regulators for homeostasis, has been characterized based on the differential expression of the candidate miRNA in insulin targeted DM patients. Some of the insulin sensitivity related miRNA in adipocytes (miR-21, miR-29, miR-93, miR-103, miR-143, miR-320), muscle (miR-1, miR-106b, miR-133a, miR-223), and liver (let-7, miR-130a-3p, miR-143, miR-181a, miR-802) were essential in maintaining physiological homeostasis and energy balance[40-42]. Studies by Zampetaki et al.[43] showed decreased levels of 10 miRNAs in plasma of diabetic patients (miR-15a, miR-20b, miR-21, miR-24, miR-126, miR-191, miR-197, miR-223, miR-320 and miR-486).

miRNA as biomarker for neurodegenerative disease

Neurodegenerative diseases include several central nervous system disorders characterized by the progressive loss of neural tissues and CNS damage. Hence, early diagnosis is essential to maximize the effectiveness of disease-modifying therapies. In recent years, much effort has been taken to recognize the neuropathological, biochemical, and genetic biomarkers of the diseases so that the diagnosis could be established in the earlier stages. The biomarkers for Alzheimer's, Parkinson disease and other neurodegenerative diseases must be reliable and specific, and they should be useful in guiding us to make more accurate diagnosis and better treatment of the diseases.

Alzheimer's disease (AD)

miRNAs has been demonstrated as potential non-invasive biomarkers from blood and serum for a wide variety of human pathologies[44]. A deregulation of miRNA expression might be involved in neurological dysfunction or neurodegenerative processes. Interestingly, Liang et al.[45] showed a specific expression signature pattern of brain and blood mononuclear cells as a useful biomarker for AD and other neurological diseases. Though altered miRNA expression patterns have been extensively investigated in AD patients' tissue samples or cell cultures[46], yet less information on circulating miRNAs in AD is known. A recent serum profiling of AD

patients provided first evidence that expression changes of circulating miRNAs may be valuable biomarkers for AD[47]. Recently, Leidinger et al.[48] has reported 140 unique differentially expressed miRNAs between AD patients and healthy controls. Further, Lugli et al.[49] has reported that the expression of microRNAs in plasma fraction enriched in exosomes showed twenty miRNAs with significant differences in the AD group (miR-23b-3p, miR-24-3p, miR-29b-3p, miR-125b-5p, miR-138-5p, miR-139-5p, miR-141-3p, miR-150-5p, miR-152-3p, miR-185-5p, miR-338-3p, miR-342-3p, miR-342-5p, miR-548at-5p, miR-659-5p, miR-3065-5p, miR-3613-3p, miR-3916, miR-4772-3p, miR-5001-3p). Recently, in CIDRF, one of the Scientist, Dr.Doulathunnisa had been working on to determine the effect of artificial sweeteners on miRNA expression that leads to the regulation of AD.

Schizoprenia

A hemizygous deletion of a 1.5–3-Mb region of chromosome 22 can lead to the 22q11 deletion syndrome (22q11DS), which is characterized by multiple physical and psychiatric abnormalities. A previous study determined that ~30% of 22q11DS patients may develop schizophrenia[50]. miR-25 and miR-185 are regulators of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA2), which is responsible for loading Ca^{2+} into the endoplasmic reticulum. Earls *et al.,* found that miR-25 and miR-185 were depleted in mouse models of 22q11DS and restoration of these miRNAs to presynaptic neurons rescued the long-term potentiation of $DGCR8^{+/-}$ mice[51]. The authors concluded that miRNA-dependent SERCA2 dysregulation is a pathogenic event in 22q11DS and schizophrenia. Gardiner *et al.*[52] investigated the expression profile of miRNA in PBMCs of 112 patients and identified 83 miRNAs that were significantly downregulated in the schizoaffective group on chromosome 14q32. Similarly, Lai *et al*.[53] identified a signature of seven miRNAs in an initial cohort of 30 patients with schizophrenia which included the upregulated miR-34a, miR-449a, miR-564, miR-548d, miR-572 and miR-652, and downregulated miR-432.

miRNA as biomarkers for pulmonary disease

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis.* Some studies have identified a group of miRNAs that are expressed specific and play regulatory roles in the interaction between *M.tuberculosis* and host cells; these include miR-223, miR-144, and miR-421. In a collaborative project between CIDRF and Pulmonary medicine, sputum and serum miR-144 levels among newly diagnosed pulmonary tuberculosis patients before and after treatment will be correlated.

Use of miRNA as Prognostic markers

microRNAs (miRNAs), endogenous small noncoding RNAs, are found to be detected in plasma, serum, saliva and urine. The diagnosis and prognosis of different pathological conditions are linked with individual miRNAs and their signature patterns[54]. Tumor-specific miRNAs have been identified in cancer patients[55]. Plasma miRNAs derived from tissues have been used as biomarkers for injury[56]. Also, alterations in circulating miRNAs have been found in cardiovascular diseases, diabetes melletius, neurodegenerative disease, as well as autoimmune diseases[57-59]. A list of miR as prognostic markers has been listed in Table 1.

Table 1: List of miRNA as prognostic marker in cancerous tissues.

miRNA	Cancer type	References
miR-205; Let-7F miR-145 miR-124 miR-23b	Ovarian cancer	Zheng et al., 2013[67] Zhang et al., 2013[68] Wang et al., 2013[69]
miR-200 and miR-203, miR-30a and miR-155	Metastatic breast cancer	Madavan et al., 2016[70]
let-7c, let-7e, miR-30c, miR-622, and miR-1285, miR-141 and miR-375; exosomal miR-1290 and miR-375	castration-resistant prostate cancer	Huang et al., 2015[71]

miR-148b, miR-376c, miR-409-3p, and miR-801	Primary breast cancer	Cuk et al., 2013[72]
miR-25 and miR-223, miR-574-5p and miR-1254, miR-21, miR-126, miR-486-5p, and miR-210	non-small-cell lung cancer	Shen et al., 2011[73]
miR-17-3p and miR-92, miR-601 and miR-760, miR-221, miR-29b	Colorectal cancer	Wang et al., 2012[74], Inoue et al., 2015[75]
miR-328	Glioblastoma	Yuan et al., 2016[76]

Cardiovascular disease

Circulating miRNAs have been investigated as possible novel prognostic markers for prediction of CHD progression and adverse outcomes. Several studies have explored associations between cardiac function and miRNAs as a predictor of future adverse outcomes in CHD. miR-134, -198, and -370, a miR signature pattern was detected by Hoekstra et al.[60] in view to differentiate unstable from stable angina pectoris suggesting a potential prognostic tool in cardiovascular disease. miR-370 has been identified as a possible prognostic biomarker in an earlier study in mice. Also, increased miR-370 expression levels had been found in response to induced ischemia[61]. Zampetaki et al.[62] has reported three signature miRNAs ((miR-126, miR-197 and miR-223) for the prediction of myocardial infarction (MI), where miR-126 levels are found to be positive, while the other miRs are inversely associated with future MI.

Diabetes mellitus

miRNAs are being available as biomarkers for monitoring of disease onset and progression. They have a great tendency to serve as accurate diagnostic and prognostic markers, as well as being viable therapeutic targets for treating diabetes complications. A large number of specific miRNAs have appeared as major regulators of particular aspects of disease pathologies including diabetes complications.

Cancer

As an important factor in tumorigenesis, microRNAs (miRNAs) are anticipated as potential biomarkers for early cancer detection and accurate prognosis as well as targets for more efficient treatment. Alterations in miRNAs lead to resistance for anticancer drugs and are well-known to be dysregulated in cancer; current literature revealed that miRNA levels in biological samples may be interrelated with chemotherapy response. miRNA expression profiles vary between normal tissues and cancerous cells derived from the same organ, and also between cancer types miRNAs can either function as oncogenes nor tumor suppressors, thereby leading to various pathways in tumorigenesis[63]. They may be used for prognostic purposes and they also constitute novel targets for cancer treatment. Recently, the evidence for the roles of miRNAs in determining drug sensitivity/resistance has been emerging. miRNA that can be used as prognosis in different types of cancer have been listed in table.

Also, human mesenchymal stromal/stem cells generally have the potency to differentiate into various mesenchymal cell lineages that makes them a challenging cell source for the use in tissue repair strategies. Georgi et al.[64] has recently investigated that profiling of hMSC donors for specific panel of miRNAs could serve as a prognostic marker for selecting donors with high differentiation potential to improve hMSC-based tissue repair approach. Peripheral blood miRNA has shown expression patterns that serve as a challenging prognostic tool in primary CNS lymphoma patients[65].

miRNA profiling

miRNA expression profiling is mainly been done to identify miRNAs that play a critical role in organismal development, establishment and maintenance of tissue differentiation as biomarkers and serve as reagents for the reprogramming of cell fate in stem cell applications. miRNA profiling has attracted many researchers to work in various research areas of biology and medicine. miRNA profiling is defined as the measurement of the relative abundance of a cohort of miRNAs, ranging from a group of several miRNAs of specific biological interest to comprehensive profiling of all miRNAs in a given species (typically numbering in the several

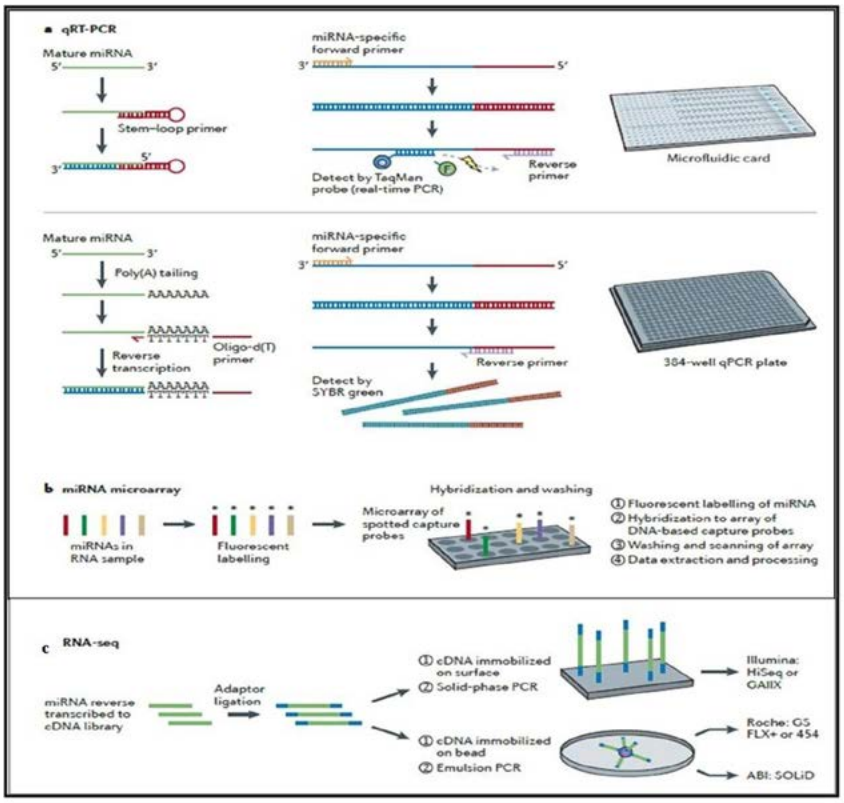

Figure 1. miRNA profiling techniques (Pritchard et al., 2012)

hundred)[66]. Assaying such small RNA molecules poses some inherent challenges, but technological advances in recent years have overcome many of these barriers, and a wide range of approaches and platforms is now available for miRNA profiling (Figure 1).

Quantitative reverse transcription PCR-based methods.

One major approach relies on reverse transcription of miRNA to cDNA, followed by qPCR with real-time monitoring of reaction product accumulation (known as 'real- time PCR'). An appealing aspect of this approach is the ease of incorporation into the workflow for laboratories that are familiar with real-time PCR. In order to scale this approach for miRNA profiling, reactions are carried out in a highly parallel, high-throughput form (that is, hundreds of qRT-PCR reactions measuring different miRNAs using the same reaction conditions). Two common strategies used for priming the reverse transcription reaction to generate cDNA are enzymatic addition of a poly (A) tail and generation of a reverse transcription primer binding site using a stem–loop primer. A hurdle in performing highly parallel qRT-PCR is that optimal reaction conditions may vary substantially between miRNAs owing to sequence-specific differences in primer annealing. Although different vendors have sought to solve this problem using various approaches, one effective strategy has been the incorporation of locked nucleic acids (LNAs) into primers to standardize optimal miRNA primer hybridization conditions for the hundreds of PCR assays that are to be run simultaneously.

Hybridization-based methods

The first method to perform parallel analysis of large number of miRNAs is done by microarrays, where different approaches for fluorescent labelling of the miRNA in a biological sample for subsequent hybridization to DNA- based probes on the array will be performed. One commonly used labelling approach is the enzymatically catalysed ligation of a fluorophore-conjugated nucleotide or short oligonucleotide to the terminal 3β-OH of the miRNA using T4 RNA ligase. Another enzymatic-labelling approach involves 3β tailing of the miRNA (for example, with poly (A), following which a fluorophore-conjugated oligo-nucleotide may be ligated using a splinted ligation. Alternative chemical approaches to miRNA labelling exist, that includes chemical alkylation-based labelling

along the miRNA and approaches based on platinum coordination chemistry with nucleic acids. It should also be kept in mind that other cellular RNAs, in addition to miRNAs, may be labelled by both enzymatic and chemical approaches, which can contribute to background signal as well as to cross-hybridization with specific miRNA probes.

RNA-sequencing

The advent of next-generation sequencing platforms has enabled a third major approach for miRNA expression profiling. The general approach begins with the preparation of a small RNA cDNA library from the RNA sample of interest, followed by the 'massively parallel' sequencing of mil- lions of individual cDNA molecules from the library. Bioinformatic analysis of the sequence reads identifies both known and novel miRNAs in the data sets and provides relative quantification using a digital approach (that is, the number of sequence reads for a given miRNA relative to the total reads in the sample is an estimate of relative abundance of the miRNA). The major advantages of next-generation sequencing for miRNA profiling are detection of both novel and known miRNAs and precise identification of miRNA sequences (for example, RNA-seq can readily distinguish between miRNAs that differ by a single nucleotide, as well as isomiRs of varying length). However, it should be noted that RNA-seq-based miRNA-profiling studies typically identify a plethora of small RNAs of novel sequence (that is, putative miRNAs), but not all of these may be bona fide miRNAs. Potential limitations of next-generation sequencing include the high cost, although this is dropping with the introduction of newer versions of the instruments, and the use of DNA 'barcoding', which permits multiplexing of many samples in a single run.

Conclusion

Taking into account of the use of miRNA as biomarkers for clinical diagnostics and prognostics values outlined in this review will allow for more precise understanding for their potential use in clinical applications. Further elucidation of miRNA biogenesis and functionality will enable the development of more specific and sensitive assays. Enhancing the art of performing research and implying its application in clinical set-up will lead to exciting novel gene regulators. Also, their specific functions will augment the opportunities to safely pursue them as therapeutic modalities.

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