Chapter 46
Diagnosis and Assessment of Microbial Infections with Host and Microbial microRNA Profiles

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Introduction

Biomarkers are continuously being sought in the field of diagnostic microbiology for the laboratory diagnosis and assessment of microbial infections. A set of clinical and laboratory criteria necessary for an ideal diagnostic marker of infection have previously been proposed by Ng and his colleagues [1]. According these criteria, an ideal biomarker should possess at a minimum the following characteristics: (a) biochemically, a biomarker should be stable and remain significantly deregulated in the body fluid compartment for at least 12–24 h even after commencement of appropriate treatment that may allow an adequate time window for specimen collection or storage without significant decomposition of the active compound until laboratory processing; (b) its concentration should be determined quantitatively and the method of measurement should be automatic, rapid, easy, and inexpensive; (c) the collection of a specimen should be minimally invasive and require a small volume (e.g., <0.5 mL blood). Numerous biomarkers have been found and tested in clinical practice. Currently, microRNA (miRNA) molecules are without a doubt the biomarkers with the greatest potential capacities in the diagnostic microbiology field.

The first miRNA, lin-4, was inadvertently discovered in *Caenorhabditis elegans* in 1993 by Lee and colleagues [2]. However, recognition of the miRNA let-7 and its ability to regulate lin-14 by Ruvkun and colleagues [3] in 2000 resulted in the

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establishment of this new class of regulatory nucleic acids and their potential value in diagnostic microbiology.

The majority of the characterized miRNA is intergenic and is located in introns. Primary miRNA transcripts, i.e., pri-miRNAs, are transcribed by RNA polymeraseII or III. In the nucleus, ribonuclease Drosha cleaves pri-miRNAs and releases 60–80-nt stem-loop intermediate structures named pre-miRNAs. The pre-miRNAs are transported to cytoplasm by exportin-5 protein, and then are cleaved by Dicer RNase III to form a mature double-stranded miRNA. One strand of the miRNA duplex is subsequently unwound and then incorporated into an effector protein complex termed RNA-induced silencing complex (RISC), which is responsible for the gene silencing in a posttranscriptional manner [4–6]. Mature miRNAs are single-stranded RNA molecules of about 19–25 nucleotides in length. Through partial homology to the 3′-untranscribed region (UTR) in target mRNAs, miRNAs control of gene expression via repression of translation as well as reducing mRNA levels directly.

A large number of miRNAs have been found in various animal and plant tissues. According to miRBase 18.0 (http://microrna.sanger.org/), which is a collective registry of currently known miRNA sequences and targets hosted by the Sanger Institute, there are currently 1,527 recognized miRNA sequences in the *Homo sapiens* genome; this number is constantly growing as new miRNA sequences are discovered. Recently, virus-encoded miRNAs have been discovered; these miRNAs may function as controls for viral replication and thus limit antiviral responses, inhibit apoptosis, and stimulate cellular growth [7]. Moreover, unique host cell miRNAs expression profiles have been revealed in response to various microbial infections [8, 9]. Host miRNA appears to play a role in viral replication and may be used by host cells to control viral infection.

Beginning in 2008, miRNAs have been found circulating in serum and plasma as well as other body fluids such as saliva, tears, and urine. Some of these miRNAs appear to be enriched in specific fluids [10, 11]. These circulating miRNAs subsequently have become the focus of ongoing research. The properties, origin, function, and relationship with disease of circulating miRNAs have been intensively explored. A number of important observations have been noted. Circulating miRNAs are present in a stable form that is protected from endogenous RNase activity [12, 13]. Some groups also reported a higher stability of miRNAs compared to mRNA in samples obtained from formalin-fixed paraffin-embedded tissues [14–16]. The expression level of miRNAs has been noted as consistent among individuals of the same species [12]. Expression alteration of circulating miRNAs has been reported to be associated with pathophysiological states including various cancers, heart disease, pregnancy, and diabetes [17, 18]. Needless to say, serum, plasma, and other body fluid specimens are generally available for clinical testing. Profiling hundreds of miRNA requires only 200 μL sera [13]. Thus, these unique and stable characteristics of circulating miRNAs potentially make them extremely useful biomarkers for disease diagnosis and prognosis.

Virus-Encoded miRNAs

The first virus-encoded miRNA was described by Pfeffer and his colleagues in 2004 when they identified five Epstein–Barr virus (EBV)-encoded pre-miRNAs [19]. Since then, hundreds of virus-encoded miRNAs have been described in humans, animals, and plants. Examples of human virus-encoded miRNA are shown in Table 46.1. Bewilderingly, more than 95 % of the virus-encoded miRNAs known today are of herpesvirus origin. Moreover, almost all virus-encoded miRNAs are encoded by DNA viruses except those encoded by retroviruses (HIV-1), which reverse-transcribe and integrate their genetic material into host DNA. Aberrantly expressed circulating miRNAs have been explored for the diagnosis and prognosis of several infectious diseases, including sepsis [20, 21], HBV [22], and HCV [23].

Viral miRNAs target perfectly complementary viral mRNAs as well as imperfectly complementary viral and/or cellular mRNAs. Viral miRNAs modulate expression of host gene involved in cell proliferation and survival, stress responses, and antiviral defense pathways, which are pivotal for viral replication. Another primary function of virus miRNAs is to regulate the latent-lytic switch. During latency, the host cell

Table 46.1 Virus-encoded miRNAs related to human infection (Adapted with permission from miRBase 18.0 (http://microrna.sanger.org/))

Virus family	Virus	No. of pre-miRNA	Names
α-Herpesviruses	HSV-1	16	hsv1-mir-H1 to hsv1-mir-H8, hsv1-mir-H11 to hsv1-mir-H18
	HSV-2	18	hsv2-mir-H2 to hsv2-mir-H7, hsv2-mir-H9 to hsv2-mir-H13, hsv2-mir-H19 to hsv2-mir-H25
β-Herpesviruses	hCMV	11	hcmv-mir-UL22A, hcmv-mir-UL36, hcmv-mir-UL70, hcmv-mir-UL112, hcmv-mir-UL148D, hcmv-mir-US4, hcmv-mir-US5-1, hcmv-mir-US5-2, hcmv-mir-US25-1, hcmv-mir-US25-2, hcmv-mir-US33
γ-Herpesviruses	EBV	25	ebv-mir-BART1 to ebv-mir-BART22, ebv-mir-BHRF1-1 to ebv-mir-BHRF1-3
	KSHV	13	kshv-mir-K12-1 to kshv-mir-K12-9, kshv-mir-K12-10a, kshv-mir-K12-10b, kshv-mir-K12-11, kshv-mir-K12-12
Polyomaviruses	Simian virus 40	1	sv40-mir-S1
	JC polyomavirus	1	jcv-mir-J1
	BK polyomavirus	1	bkv-mir-B1
	Merkel cell polyomavirus	1	mcv-mir-M1
Retroviridae	HIV-1	3	hiv1-mir-H1, hiv1-mir-N367, hiv1-mir-TAR

maintains the viral genome and only a limited portion of virus genome is expressed. Viral gene expression is restricted, but virus miRNAs and their precursors are regularly detected [24]. There is an accumulating amount of evidence that has demonstrated that virus-encoded miRNAs mediate evolutionarily conserved functions (e.g., immune evasion, cell cycle control, and promotion of latency, etc.). The miRNAs themselves show poorly primary sequence conservation [25]. These phenomena raise an important question: can viral miRNAs be used as the detection maker for virus infection during the latent infection phase despite the fact that little or no viral protein is being produced? Further studies are needed to evaluate this hypothesis.

Host miRNA Response in Relation to Microbial Infection

Microbial infections are known to downmodulate at least some cellular mRNAs and thereby exert physiological effects. Microbial infections induce changes in the host miRNA expression profile, which may also have a profound effect on the outcome of infection. Host miRNA may directly or indirectly affect virus replication and pathogenesis. For example, liver-specific miR-122 is required for HCV replication [26, 27]. Moreover, miR-28, miR-125b, miR-150, miR-223, and miR-382 are over-expressed in resting CD4+ T lymphocytes compared to their activated counterparts. These miRNAs are able to target sequences near the 3′ portion of HIV-1 mRNA. This finding suggests that miRNAs may contribute to viral latency [28]. However, it is unclear whether these miRNAs are actively inhibited by viral factors or whether their deregulation is due to host responses. Host miRNA expression profiles have been noted to represent specific pathophysiological states [17, 18]. Theoretically, a characteristic profile should be potential biomarkers for disease diagnosis and prognosis. A number of studies have been conducted to demonstrate this theory and promising results have been seen in a number of altered physiological states including various cancers, heart disease, pregnancy, diabetes, injury, and infection. The use of these miRNA profiles in infection diseases is discussed in next section.

HIV-1 and Other Human Retroviruses

Houzet and colleagues have profiled miRNAs in peripheral blood mononuclear cells (PBMCs) from HIV-1 infected patients. They found the T cell abundant miR-NAs (miR-223, miR-150, miR-146, miR-16, and miR-191) were downregulated three- to ninefold compared to cells from uninfected controls, depending on the disease stage of the patient [29]. Triloubet et al. reported increased expression of 11 miRNAs including miR-122, miR-370, miR-373*, and miR-297 in HIV-1 infected Jurkat cells, whereas expression of the polycistronic miRNA cluster miR17/92 (comprises miR-17-(5p/3p), miR-18, miR-19a, miR-20a, miR-19b-1, and miR-92-1) was strongly decreased [30]. Like HIV-1, human T cell leukemia virus type 1

(HTLV-1) also infects CD4+ T cells. Two miRNA-profiling studies have been performed in infected cell lines and ATL (adult T-cell leukemia) cells [31, 32]. The studies find two common miRNAs that are consistently downregulated in the context of HTLV-1 infection.

Respiratory Viruses

The miRNA expression profile in bronchoalveolar stem cells (BASCs) infected with SARS coronavirus (CoV) has been determined using miRNA microarray [33]. A total of 116 miRNAs were found differentially expressed. Upregulated BASC miRNAs-17*, -574-5p, and -214 are co-opted by SARS-CoV to suppress its own replication and evade immune elimination until successful transmission takes place. In contrast, viral nucleocapsid and spike protein targets seem to co-opt downregulated miR-223 and miR-98 respectively. Differentially expressed miRNAs in chicken lung and trachea infected with a low pathogenic strain of H5N3 avian influenza virus were analyzed by a deep sequencing approach [34]. A total of 73 and 36 miRNAs are differentially expressed in lungs and trachea upon virus infection, respectively. Lung cellular "microRNAome" of mice infected by reconstructed 1918 influenza virus was compared with that of mice infected by a nonlethal seasonal influenza virus, A/Texas/36/91. A group of microRNAs, including miR-200a and miR-223, was differentially expressed in response to influenza virus infection and infection by these two influenza viruses induced distinct microRNA expression profiles [35].

Adenovirus

We have previously analyzed the miRNA expression profiles from adenovirus type 3 (AD3) infected human laryngeal epithelial (Hep2) cells using a SOLiD deep sequencing. A total of 44 miRNAs demonstrated high expression and 36 miRNAs showed lower expression in the AD3 infected cells than in control cells [36].

Human Herpesviruses

Wang et al. monitored the time course of cellular miRNA expression in human cytomegalovirus (CMV) infected cells using miRNA microarrays. Forty-nine miRNAs significantly changed on at least one time point [37]. There were no global unidirectional changes, with changes for these miRNAs sometimes being transient. The miR-199a/214 cluster (miR-199a-5p, miR-199a-3p, and miR-214) has recently been found to be downregulated in CMV-infected cells [38].

Herpes Simplex Viruses

Infection of human primary neural cells with a high phenotypic reactivator herpes simplex viruses-1 (HSV-1) (17syn+) can induce upregulation of a brain-enriched microRNA (miRNA)-146a [39]. Both miR-101 and miR-132 are also found to be highly upregulated after HSV-1 [40, 41]. Kaposi's sarcoma (KS) associated herpes-virus (KSHV) is the etiological agent of KS. The M type K15 protein of KSHV induces the expression of microRNAs miR-21 and miR-31 via this conserved motif [42], while K13 strongly stimulated upregulation of miR-146a [43].

Epstein–Barr Virus

EBV is an oncogenic herpes virus that is endemic in humans and also can be found in about 15 % of patients with diffuse large B-cell lymphoma (DLBCL). EBV de novo infection of primary cultured human B-cells results in a dramatic down-regulation of cellular miRNA expression, with 99.5 % of the miRNAs detected being downregulated, with an average downregulation of 19.92-fold [44]. Imig et al. found that expression of hsa-miR-424, -223, -199a-3p, -199a-5p, -27b, -378, -26b, -23a, -23b were upregulated and those of hsa-miR-155, -20b, -221, -151-3p, -222, -29b/c, -106a were downregulated more than twofold due to EBV-infection of DLBCL [45]. Cameron et al. demonstrated differential expression of cellular miRNAs in type III versus type I EBV latency including elevated expression of miR-21, miR-23a, miR-24, miR-27a, miR-34a, miR-146a and b, and miR-155. In contrast, miR-28 expression was found to be lower in type III latency [46].

Bacterial Infections

In vitro infection assays have revealed that *Helicobacter pylori* infection can affect miRNA expression profiles: specifically, miRNAs such as miRNA-155, miRNA-16, and miRNA-146a are significantly upregulated in human gastric epithelial cells during infection [47]. More recently, expression patterns of miRNA in gastric mucosa infected with *H. pylori* using endoscopic biopsy specimens have been determined by microarray. There were 31 differentially expressed miRNAs between the *H. pylori*-infected and -uninfected mucosa (more than twofold) and miRNA expression profiling could distinguish *H. pylori* status, with the eight miRNAs yielding acceptable sensitivity and specificity [48]. Muscle-specific miRNAs miR-1 and miR-133 were significantly downregulated in the stomachs after long-term infection with *H. pylori* in mouse model [49].

Schulte et al. identified differentially regulated miRNAs by comparative deep sequencing of a total of 14 cDNA libraries prepared from the small RNA population

of host cells before or after *Salmonella* infection, or in mock-treated cells. In murine RAW 264.7 cells, upregulation of miR-21, miR-146a/b, and miR-155 was observed after infection, they also observed significant downregulation of several let-7 family members, namely, let-7a/c/d/f/g/i, and miR-98. In HeLa cells, a significant upregulation of miRNAs by *Salmonella* was limited to miR-1308. In contrast, miR-21, miR-146a/b, or miR-155 remained unaffected. Intriguingly, downregulation of let-7 miRNAs also occurred in HeLa cells [50].

Other Microbial Agents

Sharbati et al. performed miRNA as well as mRNA expression analysis of human monocyte-derived macrophages infected with several *Mycobacterium avium hominissuis* strains using microarrays as well as qRT-PCR. They found that the expression of let-7e, miR-29a, and miR-886-5p were increased in response to mycobacterial infection at 48 h [51]. Expression of miR-23b, miR-34a, and miR-218 are significantly reduced by human papillomavirus (HPV) E6 infection, while HPV E7 infection downregulates expression of miR-15a/miR-16-1 and miR-203 [52].

Methods of miRNA Detection

Accurate determination of miRNAs expression levels in a specific cell, tissue, or fluids is prerequisite to assess their biological, pathological, and clinical roles in health and disease. Theoretically, all mRNA detection methods should be useful for miRNA analysis. However, the following characteristics of miRNA sequences make quantification of miRNAs expression a technical challenge. First, mature miRNA are short (only 19–25 nucleotides; nts) and miRNAs within the same family may differ by a single nucleotide, which makes it difficult to design specific primers and probes and to reliably amplify or label each miRNA without introducing signal bias. Second, miRNAs are heterogeneous in their GC content, which results in melting temperatures (Tm) of these nucleic acid duplexes that vary widely. Finally, the target sequence is present in the primary miRNA transcript (pri-miRNA), the precursor miRNA (pre-miRNA), and the mature miRNA. It is therefore important to ensure that the nonactive pri-miRNA and pre-miRNA precursor species do not contribute to the detection signal [53]. Several standard methods for quantification of mRNA levels have been successfully adapted to miRNA including northern blotting, cloning, in situ hybridization, RT-PCR, and microarrays. In addition, emerging techniques based on colorimetric, fluorescence, bioluminescence, enzyme, and electrochemical, hold immense promise for the future of miRNA detection. However, technical issues must be addressed before they are accepted among the current standard methods [54].

Northern Blotting

Northern blotting was the first technique used to detect miRNAs and is considered the "gold standard" for characterizing miRNA expression. The basic procedures of miRNA northern blotting are similar to traditional blotting and are done as follows: (a) the small RNA molecules are separated by using high percentage denaturing urea–acrylamide gels rather than the usual agarose electrophoresis gels; (b) the small RNA molecules are transferred from the gel onto a membrane; (c) the miRNA molecules are fixed on the membrane through various cross-linking procedures; (d) the membrane is hybridized with radiolabeled oligonucleotide probes. Northern blotting methods are able to determine the absolute amount of miRNA in a sample by blotting a dilution series of synthetic oligo miRNA molecules of known concentrations in parallel with the sample. The concentration of miRNA in the sample can be calculated by a standard curve obtained from the dilution series. However, short length as well as low prevalence of mature miRNA molecules can lead to poor sensitivity of such routine northern analysis. It requires a large amount of total RNA for each sample (generally, more than 5 μg). Other disadvantages of this method include low throughput and potential environment hazards of radiolabeling. Several technical modifications have been used to improve detection sensitivity. LNA (locked nucleic acid)-modified oligonucleotide probes increases the affinity between LNA probes and target miRNA which results at least tenfold increase of sensitivity [55]. Using soluble carbodiimide cross-linking method increases the efficiency of miRNA that are fixed on the membrane, which can increase by 25–50-fold miRNA detection sensitivity compared to the traditional UV cross-linking method [56].

qRT-PCR

The most widely used method for detection and qualification of miRNA appears to be real-time quantitative RT-PCR (qRT-PCR). The small size of the mature miRNA sequences as well as sequence homology between the mature and precursor miRNA forms limits the direct application of conventional RT-PCR protocols to miRNA detection. To solve these problems, innovative solutions have been applied for each step of qRT-PCR used for the quantitative analysis of miRNAs. The first step in qRT-PCR of miRNAs is the accurate and complete conversion of miRNA into cDNA. Two different approaches for reverse transcription of miRNAs have been reported. In the first approach, miRNAs are reverse transcribed individually by using miRNAs-specific reverse transcription primers. Both stem-loop (Applied Biosystems Co, Fig. 46.1a) and a linear primer (Exiqon, Fig. 46.1b) containing partial complementary sequence of 3′-end of miRNA can be annealed to miRNA in order to prime the reverse transcription. The double stranded structure of the stem-loop primer prevents its nonspecific binding to pre- and pri-miRNAs, thereby increasing the specificity of the assay. Unlike stem-loop primer, the design of the linear primer is simpler. But the linear primer cannot discriminate mature miRNA from their precursors.
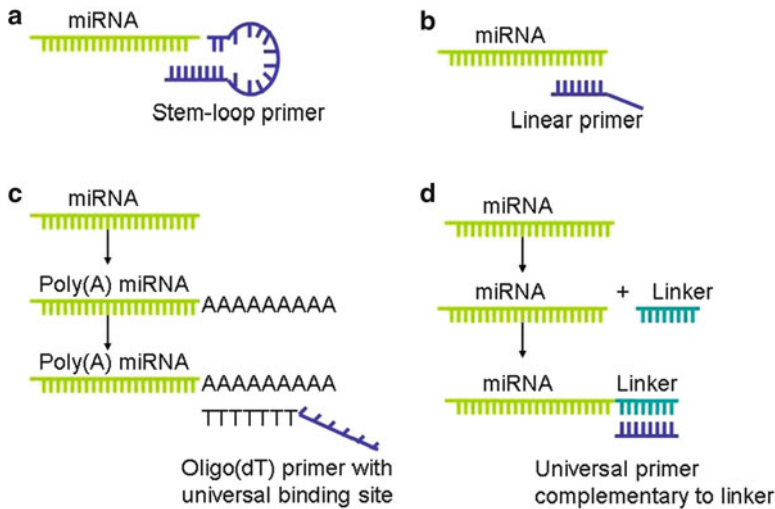
Fig. 46.1 Schematic representation of reverse transcription methods used to generate cDNA. Reverse transcription of individual mature miRNAs is done using stem-loop (**a**) or linear (**b**) primer. miRNAs are first tailed with adenosine nucleotides at 3′-end of miRNA with Poly(A) Polymerase (**c**) or linker adaptor with T4 RNA Ligase 1 (**d**). Then, a primer consisting of an oligo(dT) sequence with a universal primer-binding sequence at its 5′-end (**c**) or a universal primer complementary to the 3′-end of the linker (**d**) was used to prime reverse transcription

Another approach is done as follows: miRNAs are first tailed with adenosine nucleotides at 3′-end of miRNA with Poly(A) Polymerase (Fig. 46.1c) or linker adaptor with T4 RNA Ligase 1 (Fig. 46.1d). Then, a primer consisting of an oligo(dT) sequence with a universal primer-binding sequence at its 5′-end or a universal primer complementary to the 3′-end of the linker is used to prime reverse transcription [53]. The universal reverse primer sequence is introduced into the cDNA during reverse transcription. The design of the miRNA specific forward primer is critical for the specificity and sensitivity of the qRT-PCR assay. LNA modification is a widely used method for increasing the Tm and the specificity of primer. Each incorporated LNA monomer increases the Tm up to 2–8 °C, depending upon the position of the LNA moiety in the oligonucleotide primer [57].

There are two approaches available for detection of qRT-PCR products. One approach uses SYBR Green dye whose fluorescence increases approximately 100-times upon intercalating into dsDNA chain; this property is used to monitor real time amplification products as they accumulate during the PCR reaction. One limitation of the SYBR Green-based method is that target PCR products and nonspecific products cannot be discriminated. Therefore, a melting point analysis usually required following the PCR amplification, which can be reached by using a dual-labeled hydrolysis TaqMan probe. The basis for this type of TaqMan probe detection has been reviewed by Benes and Castoldi [53]. The characteristics of TaqMan miRNA assays make them ideally suited for detection of mature miRNAs. Moreover, these TaqMan miRNA assays can discriminate related miRNAs that differ in only
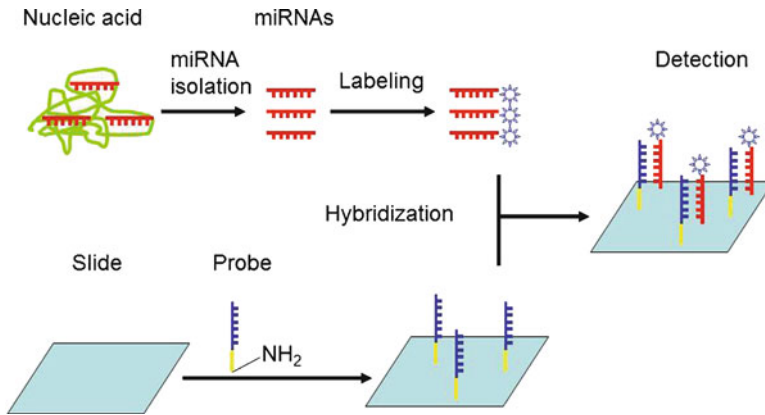
Fig. 46.2 Schematic flowchart of the microRNA profiling microarray. A miRNA microArray prepared by amine-modified microRNA probes that consist of "linker" sequences (*yellow*) and capture sequences (*blue*) spotting to amine-reactive glass slides. After miRNAs are isolated from samples and labeled with fluorescence dye, they are hybridized with microArray and then the expression signal can be detected by fluorescence detector

one nucleotide. However, the cost of the TaqMan probes is higher than the SYBR Green method, which limits its routine use in most laboratories.

The qRT-PCR method has demonstrated a high sensitivity and specificity with ability to accurately detect miRNAs in a single stem cell [58, 59]. Only low amounts of starting material (in the range of nanograms of total RNA) are needed and quantitative results can be acquired within 3 h. This method also has a considerably larger dynamic range compared to microarray analysis. Recently, the qRT-PCR technique has been adapted for increased throughput by developing a miRNA PCR array that can detect hundreds of miRNAs at one reaction tube. Examples include the TaqMan Low density microRNA Array (TLDA, Applied Biosystems) and the RT^2 miRNA PCR Arrays (SABioscience, a Qiagen Company).

Microarrays

Another widely used high-throughput technique for analysis of the expression level of miRNA molecules is microarray technology. The microarray technology was firstly applied to miRNA studies in 2003 [60]. Since then, numerous approaches based on different microarray platforms have been developed for miRNA quantification. The basic procedures of miRNA quantification using a microArray platform including (a) miRNA probe design and array preparation, (b) isolation of miRNA and labeling, (c) hybridization and signal detection. A schematic flow chart of the miRNA profiling microarray is shown in Fig. 46.2. The design of the microRNA probes, isolation of microRNA from samples and the labeling of miRNAs are the most critical procedures in the miRNA microarray assay.

The short length of miRNAs makes design of the probes more difficult because the design of the probe is almost exclusively determined by sequence of the miRNA itself. Sequence of the miRNA determines the wider Tm distribution of the probes. Therefore, Tm normalization of the full set of probes is absolutely required since the hybridization is usually carried out at one temperature. To solve this problem, two techniques, LNA utilization or adjusting the lengths of the probes, have been successfully used to normalize Tm value [61, 62]. Quantity of miRNA is also very important for miRNA microarray assay, since the abundance of miRNAs in total RNA is very low and a relatively large amount of miRNA is needed in comparison to a RT-PCR assay. Conventional denaturing urea–acrylamide gel electrophoresis combined with a commercialized kit (e.g., the mirVana™ microRNA isolation kit of Ambion, Inc. and the PureLink™ microRNA isolation kit of Invitrogen Co) have demonstrated excellent efficiency in isolating miRNA.

Labeling of miRNA is an absolutely key step for the overall sensitivity of the microarray. Many methods have been developed to label miRNA, and these can be classified into two main categories: direct labeling and indirect labeling. Direct labeling with fluorescent dye can be accomplished enzymatically. Currently used methods including labeling through poly(A) polymerase, labeling though T4 RNA ligase, guanine labeling, and labeling microRNA through a RNA-primed array-based Klenow enzyme assay (RAKE). Fluorescent dye also can conjugate with adjacent 3′-OH of mature miRNAs using a chemical reagent [63, 64]. Indirect labeling methods including labeling through miRNA reverse transcript, the RT-PCR product of miRNA or the in vitro transcript of miRNA. The advantages of indirect labeling are obvious, since the reverse transcription product of miRNAs are more stable and easy to preserve. In addition, miRNA can be amplified and labeled synchronously through PCR or in vitro transcription, which is very useful for low-abundance miRNAs. Although direct labeling is simple, this method has inherent problems including the fact that guanine labeling is not suitable for miRNAs lacking G residues, that T4 ligase labeling can introduce base bias, and that the procedure of chemical labeling is somewhat complicated. Though indirect labeling is more sensitive, this method may introduce artificial errors during the ligation and PCR amplification procedures.

Another microarray technique, which uses biotin labeled miRNAs to hybridize with LAN probes, has been coupled to xMAP suspension microspheres (Luminex Co) to offer more rapid and reproducible results than does solid planar array due to its favorable reaction kinetics in liquid phase. Using this technique, a single nt difference can be discriminated [65]. Microarray technology has proven to be standard technique for profiling miRNA expression.

Next-Generation Sequencing

Next-generation sequencing (NGS) is also called massively parallel or deep sequencing and is becoming the most effective method for miRNA analysis. Sequence

throughput of NGS is unapproachable by other miRNA analysis techniques, since they lay DNA fragments on a single chip and simultaneous sequencing up to millions of these fragments in parallel. The principle of NGS has been described in other chapter of this book and elsewhere [66, 67]. Currently, three main platforms are in widespread use for miRNA profiling and discovery: the Roche (454) GS FLX sequencer, the Illumina Genome Analyzer and the Applied Biosystems SOLiD sequencer. The methodologies of the NGS for miRNA analysis are similar and include sRNA isolation, library preparation, sequencing, and data analysis. In addition to increased throughput, NGS technique significantly reduce cloning biases observed with traditional capillary sequencing since sequence reads are generated from fragment libraries that do not need to be cloned and amplified.

Another key advantage of NGS over microArrays in miRNA study is that it can profile unknown genes since no sequence specific probes are needed for detection. Currently, most novel miRNAs have been discovered and characterized through NGS. NGS can also gauge miRNA expression level by counting clone frequencies, which has demonstrated more sensitivity than microArrays [66]. One disadvantage of NGS to comprehensively profile mRNA expression is that this technique is rather expensive compared to microArray though its cost has significantly decreased with the development of commercial platforms. Another limitation of NGS is that the read length is relatively short (35–500 bp) compared to traditional capillary sequencing (1,000–1,200 bp). This limits their use for de novo assembly of complete genomes but makes them become the ideal instruments for miRNA profiling since the length of miRNAs (21–35 nucleotides) is shorter than the read length of NGS.

As described above, each technique for miRNA detection has its advantages and disadvantages. The method used should best fit the research goal and experimental conditions. For example, for new miRNA discovery and identification, in addition to sequencing technique, cloning of miRNA may be the simplest method. In situ hybridization is more suitable for location of miRNA in tissue. Northern blotting is very sensitive, but it is very time-consuming and not practical in large clinical studies for routine detection of the expression of hundreds of miRNAs. qRT-PCR is able to detect low copy numbers with high sensitivity and specificity. When studying the expression levels of multi-miRNAs simultaneously, microarray and sequencing may be the best choice. Another important problem is that although each platform is relatively stable in terms of its own microRNA profiling intrareproducibility, the interplatform reproducibility among different platforms is low [68]. An "industry standard" for analysis of miRNA expression awaits further advances in both technology and computation [69].

Application of Circulating miRNAs for Diagnosis and Prognosis of Microbial Infection

Although altered miRNA expression profiles have been detected in various tissues or cells following microbial infection, these profiles have not yet been used as biomarkers in clinical practice because obtaining infected tissues and cells is difficult without

invasive procedures. As described above, circulating miRNAs in serum/plasma seem more suitable for biomarkers that can be easily used for the diagnosis or prognosis of these infections. Some potential uses are discussed in the next section.

Sepsis

Diagnosis and monitoring of sepsis can be difficult because many of its signs and symptoms can be caused by other noninfectious disorders. The current gold standard for diagnosing septicemia is the blood culture, which generally takes several days or longer. Other early biomarkers of sepsis are being investigated; these include acute phase proteins (C-reactive protein), cytokines (IL-1, -6, -10 and TNF-a), chemokines (IL-8, MCP-1, and G-CSF), procalcitonin, and metabonomic. To date, these biomarkers have not demonstrated sufficient sensitivity and/or specificity to guide clinical management. Host miRNA expression profiles have been intensively studied using both in vitro or in vivo models of inflammation that are triggered by *Escherichia coli* lipopolysaccharide (LPS) stimulation. For example, expression of miR-146, miR-155, and miR-132 increased in human acute monocytic leukemia cell line THP-1 in response to LPS stimulation [70]. Upregulation of miR-155, miR-223, miR-146a and downregulation of miR-125b, miR-144, and miR-142-5p have been observed in human monocyte-derived dendritic cells by Ceppi et al. [71]. Schmidt et al. screened for differentially expressed miRNAs in circulating leukocytes using an in vivo model of acute inflammation also triggered by LPS [72]. They found four miRNAs were downregulated (miR-146b, miR-150, miR-342, and let-7g) and one was upregulated (miR-143).

Vasilescu et al. profiled genome-wide miRNAs by microarray in peripheral blood leukocytes of sepsis patients and found that miR-150, miR-182, miR-342-5p, and miR-486 expression profiles differentiated sepsis patients from healthy controls [20]. Moreover, miR-150 levels were significantly reduced in plasma samples of sepsis patients and correlated with the level of disease severity. Finally, these investigators noted that the plasma levels ratio for miR-150/interleukin-18 can be used for assessing the severity of the sepsis. More recently, Wang et al. have analyzed seven miRNAs expression levels in patients diagnosed with sepsis, systemic inflammatory response syndrome (SIRS), and healthy controls using a qRT-PCR assay [21]. They determined serum miR-146a and miR-223 were significantly reduced in septic patients compared with SIRS patients and healthy controls. The areas under the receiver operating characteristic curve of miR-146a, miR-223, and IL-6 were 0.858, 0.804, and 0.785, respectively.

Viral Hepatitis B

HBV infection is also known to modulate the expression of host cellular miRNAs, which then participate in development of HBV-related liver diseases. The miRNA

profiles in chronic hepatitis B patient tissues or in HBV-expressing cells are reviewed by Liu et al. [73]. Li et al. profiled serum miRNAs of healthy controls, HBV-, HCV-, and HBV-positive hepatocellular carcinoma (HCC)-affected individuals by Solexa sequencing followed by validation with quantitative RT-PCR assay [21]. These investigators successfully identified 13 miRNAs that are differentially expressed in HBV serum. This 13-miRNA-based biomarker accurately discriminated not only HBV cases from controls and HCV cases, but also HBV-positive HCC cases from control and HBV cases. For example, when using four markers (miR-375, miR-10a, miR-223, and miR-423) to separate the control and HBV groups, the AUC was 99.9±0.1 % (sensitivity: 99.3 %; specificity: 98.8 %). Similarly, two markers (miR-92a and miR-423) could separate the control and HCV groups with a high specificity and sensitivity (AUC: 99.6±0.4 %; sensitivity: 97.9 %; specificity: 99.4 %). The control and HBV-positive HCC group could be clearly separated by 5 markers (miR-23b, miR-423, miR-375, miR-23a, and miR-342-3p; AUC: 99.9±0.1 %; sensitivity: 96.9 %; specificity: 99.4 %). Similarly, the HBV and the HBV-positive HCC group could be separated by two markers (miR-10a and miR-125b; AUC: 99.2±0.6 %; sensitivity: 98.5 %; specificity: 98.5 %).

Another study profiled miRNA expression on pooled sera obtained from identified groups of chronic asymptomatic carriers (ASC), patients with chronic hepatitis B (CHB) and HBV-associated acute-on-chronic liver failure (ACLF), as well as healthy controls (HC) using Applied Biosystems TaqmanArray assay [74]. A total of 37 miRNAs were amplified from HC, whereas 77, 101, and 135 were amplified from ASC, CHB, and ACLF, respectively. The expression levels of most miRNAs were also upregulated in HBV-infected patients when compared to HC. Furthermore, the level of miRNAs in the CHB serum was upregulated most in hepatitis B e antigen-positive patients. The expression of MiR-122, the most abundant miRNA in liver tissue, was significantly higher in HBV infected groups than in HC. The expression of miR-223 was similar between HC and ASC, but increased significantly in CHB and ACLF. The expression levels of miR-122 and miR-194 correlated negatively with the age of patients with CHB or ACLF.

Viral Hepatitis C

In an in vitro acute HCV infection model, 108 human miRNAs were identified whose expression levels changed for more than 2.0-fold in response to HCV infection [75]. Marquez et al. measured miR-122 and miR-21 levels in HCV-infected human liver biopsies relative to uninfected human livers and correlated these with clinical patient data [76]. They found that miR-21 expression correlated with viral load, fibrosis and serum liver transaminase levels. miR-122 expression inversely correlated with fibrosis, liver transaminase levels and patient age. While Morita et al. described hepatic miR-122 expression was weakly and positively correlated with the serum HCV load but was not correlated with HCV load in the human liver [77].

Bihrer et al. found sera from patients with chronic HCV infection contained higher levels of miR-122 than sera from healthy controls [23]. Serum miR-122 levels

correlated well with markers of liver inflammatory activity, that is, the serum levels of alanine leucine transaminase (ALT) and aspartate transaminase, and the histologic activity index (HAI) score. In patients with persistently normal ALT levels, serum miR-122 levels did not differ from healthy controls. There was no correlation of serum miR-122 levels with serum albumin, international normalized ratio, liver fibrosis, or serum HCV RNA. Thus, serum miR-122 appears to act as a biomarker of necroinflammation in patients with chronic Hepatitis C infection.

Enteroviral Infections

We previously performed a comprehensive miRNA profiling in EV71-infected Hep2 cells using deep sequencing. A total of 64 miRNAs were found whose expression levels changed for more than twofold in response to EV71 infection [78]. Ho et al. found upregulation of miR-141 upon enterovirus infection can facilitate viral propagation by expediting the translational switch [79]. We recently compared host serum miRNA levels in patients with hand-foot-and-mouth disease caused by enterovirus 71 (EV71) and coxsackievirus 16 (CVA16) as well as in other microbial infections and in healthy individuals. Among 664 different miRNAs analyzed using a miRNA array, 102 were upregulated and 26 were downregulated in sera of patients with enteroviral infections. Expression levels of ten candidate miRNAs were further evaluated by quantitative real-time PCR assays. A receiver operating characteristic (ROC) curve analysis revealed that six miRNAs (miR-148a, miR-143, miR-324-3p, miR-628-3p, miR-140-5p, and miR-362-3p) were able to discriminate patients with enterovirus infections from healthy controls with area under curve (AUC) values ranged from 0.828 to 0.934. The combined six miRNA using multiple logistic regression analysis provided not only a sensitivity of 97.1 % and a specificity of 92.7 % but also a unique profile that differentiated enteroviral infections from other microbial infections. Expression levels of five miRNAs (miR-148a, miR-143, miR-324-3p, miR-545, and miR-140-5p) were significantly increased in patients with CVA16 versus those with EV71 ($p < 0.05$). Combination of miR-545, miR-324-3p, and miR-143 possessed a moderate ability to discrimination between CVA16 and EV71 with an AUC value of 0.761. These data indicate that sera from patients with different subtypes of enteroviral infection express unique miRNA profiles. Serum miRNA expression profiles may provide supplemental biomarkers for diagnosing and subtyping enteroviral hand-foot-and-mouth disease infections [80].

Concluding Remarks

Circulating miRNAs have been investigated as the diagnosis or prognosis marker for microbial infections. Studies on host miRNA profiles upon microbial infections are underway. There have a considerable way to go before being used in clinical

practice. Several issues should be refined in this field. (a) Source material should be fixed. Plasma, serum, whole blood, and isolated exosomes or microvesicles have been used as the source for miRNA profiling. Advantages and disadvantages of each approach should be considered ahead. (b) Though differences in circulating miR-NAs between males and females have not been found with the exception of differences associated with pregnancy [81, 82], miRNA levels in plasma and serum from a large number of normal individuals of both genders and various ages, even same individual over time should be extensively studied. (c) No acknowledged reference genes have been found in serum/plasma. Frequently used endogenous controls, such as miR-16, dysregulated in some diseases and RNU6B degraded in serum. Spiking into RNA isolation process with synthetic exogenous miRNA only act as normalizers for differences in recovery between samples. (d) The methods of miRNA quantification including RNA isolation should be standardized, since interplatform reproducibility among different platforms is low. (e) There seems to be a lack of specificity when using single miRNA as biomarker, since the miRNA is commonly regulated in various diseases. A panel of miRNAs should be a best choice.

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