

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. These have evolved from a simple clinical index [1, 2] using nonspecific screening tests such as the white blood cell with leukocyte differential, the erythrocyte sedimentation rate, and the C-reactive protein to the use of pro-inflammatory cytokines/chemokines [3, 4] to most recent use of microRNA (miRNA) molecules [5–7], the last of which have the greatest potential for predicting infection. A set of clinical and laboratory criteria necessary for an ideal diagnostic marker of infection have previously been proposed by Ng and his colleagues [8]. 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).

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Numerous biomarkers have been found and tested in clinical practice [1–4]. 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 [9]. However, recognition of the miRNA *let-7* and its ability to regulate *lin-14* by Ruvkun and colleagues [10] in 2000 resulted in the 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 [11]. Primary miRNA transcripts, i.e., pri-miRNAs, are transcribed by RNA polymerase II or polymerase 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 [12–14]. Mature miRNAs are single-stranded RNA molecules of about 19–25 nucleotides in length. Through partial homology to the 3'-untranslated 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 [15]. According to miRBase 21.0 (<http://microrna.sanger.org/>), which is a collective registry of currently known miRNA sequences and targets hosted by the Sanger Institute, there are currently 2588 recognized miRNA sequences in the *Homo sapiens* genome; this number is constantly growing as new miRNA sequences are discovered. Importantly, miRNAs have been detected in human body fluids, including peripheral blood plasma; these circulating miRNAs are found as extracellular nuclease-resistant entities that are strikingly stable in blood plasma [16]. Such miRNAs have been found circulating not only in serum and plasma but also in other body fluids such as saliva, tears, and urine [17]. Some of these miRNAs appear to be enriched in specific fluids [17, 18]. 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 investigated [16–19]. A number of important observations have been noted. Circulating miRNAs are present in a stable form that is protected from endogenous RNase activity [16, 19, 20]. Most extracellular circulating miRNAs in plasma completely pass through 0.22 micron filters but remain in the supernatant after ultracentrifugation at 110,000 g, which indicates the non-vesicular origin of these miRNAs [16]. Finally, circulating miRNAs have been shown to co-immunoprecipitate with the 96 kDa Ago2 protein; this Ago2 protein is part of an RNA-induced silencing complex [16] and might account for the high stability of this complex [16]. Some groups also reported a higher stability of miRNAs compared to mRNA in samples obtained

from formalin-fixed paraffin-embedded tissues [21–23]. The expression level of miRNAs has been noted as consistent among individuals of the same species [19]. Expression alteration of circulating miRNAs has been reported to be associated with pathophysiological states including various cancers, heart disease, pregnancy, and diabetes [24, 25]. Needless to say, serum, plasma, and other body fluid specimens are generally available for clinical testing. Profiling hundreds of miRNA requires only 200 ul of sera [20]. Thus, these unique and stable characteristics of circulating miRNAs potentially make them extremely useful biomarkers for disease diagnosis and prognosis. The potential use of tissue and/or circulating miRNAs for diagnosing cancer was quickly recognized [12, 13, 18–20, 22]. The discovery of virus-encoded miRNAs and the recognition that such miRNAs played multiple roles in virus infections has resulted in their use in diagnosing viral infections [5, 26–28].

Virus-Encoded miRNAs

Virus-encoded miRNAs were recognized as having many functions in viral infections, including controls for viral replication and thus potentially limiting antiviral responses, inhibition of apoptosis, and stimulation of cellular growth [26]. Moreover, unique host cell miRNAs expression profiles have been revealed in response to various microbial infections [27, 28]. Host miRNA thus appears to play an important role in viral replication and may be used by host cells to control viral infection. The first virus-encoded miRNA was described by Pfeffer and his colleagues in 2004 when they identified five EBV-encoded pre-miRNAs [29]. 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 1. Bewilderingly, more than 95% of the virus-encoded miRNAs known today are of herpesvirus origin [30]. Herpesvirus miRNAs were initially determined to not be required for lytic replication, but were thought to strongly enhance viral pathogenesis, including oncogenesis, and also to promote latently infected cells [30]. Subsequently, the role of herpesvirus miRNAs in virus latency and persistence has been confirmed, with specific cellular miRNAs being identified as inhibiting reactivation of herpesviruses, thereby promoting latent infections [31, 32]. Interestingly, almost all virus-encoded miRNAs are encoded by DNA viruses except those encoded by retroviruses, 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 [33, 34], HBV [35], and HCV [36].

The interactions between viral and cellular miRNAs in viral diseases and virus-associated cancers are complex [37]. Viral miRNAs target perfectly complementary viral mRNAs as well as imperfectly complementary viral and/or cellular mRNAs.

Table 1 Virus-encoded miRNAs related to human infection

Name of virus	Number of precursors	Number of mature
Bovine foamy virus	2	4
Bovine herpesvirus 1	10	12
Bovine herpesvirus 5	5	5
BK polyomavirus	1	2
Bovine leukemia virus	5	10
Bandicoot papillomatosis carcinomatosis virus type 1	1	1
Bandicoot papillomatosis carcinomatosis virus type 2	1	1
Duck enteritis virus	24	33
Epstein-Barr virus	25	44
Herpes B virus	12	15
Human cytomegalovirus	15	26
Human herpesvirus 6B	4	8
Human immunodeficiency virus 1	3	4
Herpes simplex virus 1	18	27
Herpes simplex virus 2	18	24
Herpesvirus saimiri strain A11	3	6
Herpesvirus of turkeys	17	28
Infectious laryngotracheitis virus	7	10
JC polyomavirus	1	2
Kaposi sarcoma-associated herpesvirus	13	25
Mouse cytomegalovirus	18	29
Merkel cell polyomavirus	1	2

Adapted with permission from miRBase 21.0 (<http://microrna.sanger.org/>)

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 [26–28, 37–39]. Another primary function of virus miRNAs is to regulate the latent-lytic switch. During latency, the host cell 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 [38]. 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, promotion of latency, etc.) [5, 26–28, 37–39]. The miRNAs themselves show poorly primary sequence conservation [39]. 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 down-modulate at least some cellular mRNAs and thereby exert physiological effects [40]. Microbial infections induce changes in the host miRNA expression profile, which may also have a profound effect on the outcome of infection [6, 41, 42]. Host miRNA may directly or indirectly affect virus replication and pathogenesis. For example, liver-specific miR-122 is required for HCV replication [43, 44]. 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 [45]. 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 [45]. However, it is unclear whether these miRNAs are actively inhibited by viral factors or whether their deregulation is due to host responses. Host miRNAs expression profiles have been noted to represent specific pathophysiological states [24, 25]. 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 such miRNA profiles in specific infection diseases will be discussed in the next sections.

HIV-1 and Other Human Retroviruses

The roles of microRNAs in HIV-1 replication and latency are being intensely investigated in order to provide new approaches to clear the viral reservoir [46, 47]. It has become apparent that cellular miRNAs may play crucial roles in controlling HIV-1 infection and replication [47]. Houzet and colleagues have profiled miRNAs in peripheral blood mononuclear cells (PBMCs) from HIV-1-infected patients [48]. They found the T cell-abundant miRNAs (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 [48]. 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 miR-17/miR-92 (comprises miR-17-(5p/3p), miR-18, miR-19a, miR-20a, miR-19b-1, and miR-92-1) was strongly decreased [49]. Two cellular miRNAs, miR-196b and miR-1290, have been found to contribute to HIV-1 latency [50]. Like HIV-1, human T cell leukemia virus type 1 (HTLV-1) also infects CD4⁺ T cells. As seen with HIV-1, miRNAs also play an important role in the pathogenesis of HTLV-1 infection and transformation [51, 52]. Two miRNA profiling studies have been performed in infected cell lines and ATL (adult T cell leukemia) cells [53, 54]. The studies find two common miRNAs that are consistently downregulated in the context of HTLV-1 infection. For both HIV-1 and HTLV-1, it is clear the miRNAs play a role in latency; inhibitors of these miRNA could be used to activate latent retroviruses in order to assist in clearing the reservoirs of virus.

Respiratory Viruses

The emergence of Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 following the earlier severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002–2003 marked two instances in which a highly pathogenic coronavirus was introduced into the human population in the twenty-first century [55]. Along with highly pathogenic avian influenza viruses [56], these respiratory viruses are notable due to their potential to cause pandemics [57]. The potential role of miRNAs to diagnose these respiratory tract infections is under investigation. For example, the miRNA expression profile in bronchoalveolar stem cells (BASCs) infected with SARS coronavirus (CoV) has been determined using miRNA microarray [58]. A total of 116 miRNAs were found differentially expressed. Upregulated BASC miRNAs-17*, miRNAs-574-5p, and miRNAs-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 [58]. The miRNA expression of avian influenza strains has also been investigated. 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 [59]. A total of 73 and 36 miRNAs are differentially expressed in lungs and trachea upon virus infection, respectively [59]. 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 [60]. A group of microRNAs, including miR-200a and miR-223, were differentially expressed in response to influenza virus infection, and infection by these two influenza viruses induced distinct microRNA expression profiles [60]. Finally, a novel avian-origin influenza A (H7N9) caused 137 human infection cases with a 32.8% mortality rate; characterization of the miRNA profile in response to infection by this strain revealed significant alterations in serum miRNA expression following virus infection in comparison with controls [61]. This study confirmed the potential for using serum miRNA expression for the diagnosis of viral respiratory diseases.

Adenovirus

Human adenoviruses are DNA viruses that cause infections in both immunocompetent and immunosuppressed patients [62]. Adenovirus infections are associated with viral persistence and reactivation and continue to provide clinical challenges in terms of diagnosis and treatment. Adenoviruses express large amounts of noncoding virus-associated RNAs able to saturate key factors of the RNA interference processing pathway, including Exportin 5 and Dicer [63]. Moreover, a proportion of the noncoding virus-associated RNA is cleaved by Dicer in viral miRNAs. This cleaved RNA results in miRNAs that can saturate Argonaute, which is an essential protein for miRNA function that engages in transcriptional silencing processes in the nucleus [63, 64]. Therefore, processing and function of cellular miRNAs are blocked in cells infected by adenovirus [63]. Of note is that the cellular silencing machinery is active early after infection and can be used to control the adenovirus cell cycle [63]. It is also important to realize that miRNA expression has been found to

fluctuate during the course of an adenovirus type 2 infection in human lung fibroblasts [65]. The miRNA expression profiles from adenovirus type 3 (AD3)-infected human laryngeal epithelial (Hep2) cells have been analyzed using a SOLiD deep sequencing [66]. A total of 44 miRNAs demonstrated high expression, and 36 miRNAs showed lower expression in the AD3-infected cells than in control cells [66]. The role of miRNAs in adenovirus-infected cells is relevant because of the past and future use of recombinant adenoviruses as vectors for gene therapy [67]. Manipulation of the viral genome allows the use of these vectors to express therapeutic miRNAs or to be silenced by the RNAi machinery leading to safer vectors with specific tropisms. Adenovirus is known to interact with a number of different extracellular, intracellular, and membrane-bound innate immune sensing systems [68] such as Toll-like receptor 4 [68, 69]. The investigation of miRNAs expression during adenovirus infection is likely to provide important new insights into the scope and mechanisms of these cellular defensive responses [70].

Human Herpesviruses

Among the human herpesviruses, cytomegalovirus (CMV) is an important human pathogen that has the potential to disseminate via the bloodstream to all organs, but only produces overt clinical disease if the viral load achieves high levels [71]. Normally there is a strong immune response such that the infected individual typically remains asymptomatic [71]. Over time, this immune response wanes and infected individuals can become symptomatic due to a higher viral load. For this reason, miRNA expression in latent and symptomatic infections is being studied [72]. Wang et al. monitored the time course of cellular miRNA expression in human cytomegalovirus (CMV)-infected cells using miRNA microarrays and found that 49 miRNAs significantly changed on at least 1 time point [73]. There were no global unidirectional changes, with changes for these miRNAs sometimes being transient. Fu and colleagues noted similar results in a human cytomegalovirus latent infection cell model using THP-1 cells [74]. The miR-199a/miR-214 cluster (miR-199a-5p, miR-199a-3p, and miR-214) was recently found to be downregulated in CMV-infected cells [75]. Human cytomegalovirus miRNA miR-US25-1-5p has been shown to inhibit viral replication by targeting multiple cellular genes during infection [75]. Clearly additional studies are needed, but the use of miRNAs will undoubtedly increase our understanding of the pathogenesis of cytomegalovirus.

Herpes Simplex Viruses (HSV)

Herpes simplex viruses (HSV) are evolutionarily ancient viruses that are ubiquitous, having a worldwide prevalence [76]. There are two serotypes, HSV-1 and HSV-2, both of which primarily infect humans through epithelial cells. HSV infections are extremely common; seropositivity occurs in 50–90% of adult populations [76]. The success of HSV-1 and HSV-2 as human pathogens is due to the virus first infecting epithelial cells and then entering sensory neurons via nerve termini [76, 77]. Latency within long-lived neuronal cell bodies and subsequent mucocutaneous shedding is central to the survival of this neurotrophic virus [77]. The generally mild sequelae of HSV infection reflects a balance between the host and the virus in most immunocompetent persons [76, 77]. However, HSV infections of the central

nervous system are recognized, but relatively rare complications of this infection [78]. The miRNAs of HSV are of particular interest due to the latency of this virus as well as its role in CNS infections [79]. Infection of human primary neural cells with a high phenotypic reactivator HSV-1 (17syn+) can induce upregulation of a brain-enriched microRNA (miRNA)-146a [80]. Both miR-101 and miR-132 are also found to be highly upregulated after HSV-1 [81, 82].

Another factor in terms of the involvement of HSV in CNS infections is the fact that miRNAs are key regulators of neuroinflammation [83]. Several miRNAs have been found to play an important role in the microglia-mediated inflammatory response including miR-155 and miR-146a [83]. Another miRNA, miR-125, plays a critical role in the adaptation of microglia and macrophages to the CNS microenvironment [84]. Traumatic brain injury has been shown to produce profound and lasting neuroinflammation; microRNAs have been implicated in the regulation of inflammation after traumatic brain injury [85]. In particular, miR-155 is induced after traumatic brain injury and is thought to play an important role in the regulation of the IFN response and neurodegeneration following brain injuries [85]. The net sum of this regulation is thought to be neuroprotective, which in turn may predispose the brain to viral infections such as herpes simplex encephalitis [86].

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) is the etiological agent of KS; this virus is also known as human herpesvirus 8 (HHV-8) [87]. Kaposi's sarcoma is a mesenchymal tumor with poorly understood molecular and cytogenetic changes. The predicted target genes for differentially expressed miRNAs include genes that are involved in cellular processes such as angiogenesis and apoptosis, which suggests a role for these miRNAs in the pathogenesis of Kaposi's sarcoma [88]. KS tumor cells are latently infected with KSHV, which express only a subset of viral genes, among them 12 miRNAs [89]. The metabolic properties of KSHV-infected cells are similar to those of cancer cell and display features of lymphatic endothelial differentiation [89]. The M type K15 protein of KSHV induces the expression of microRNAs miR-21 and miR-31 via this conserved motif [90], while K13 strongly stimulated upregulation of miR-146a [91]. KSHV miRNAs expression decrease mitochondrial biogenesis and induce aerobic glycolysis; this metabolic shift favors latency and offers a growth advantage [89].

Epstein–Barr Virus (EBV)

Epstein-Barr virus (EBV) is a gammaherpesvirus that infects most humans during their lifetime [92, 93]; these infections are usually asymptomatic but result in a lifelong latent infection [94] that is controlled by the host's immune system [95]. EBV also is an oncogenic herpesvirus [96] that is endemic in humans and is found in about 15% of patients with diffuse large B-cell lymphoma (DLBCL) [97]. Multiple cellular functions are mediated by the miRNAs of EBV [98]. For example, EBV de novo infection of primary cultured human B-cells results in a dramatic downregulation of cellular miRNA expression, with 99.5% of the miRNAs detected being downregulated, with an average downregulation of 19.92-fold [99]. Imig et al. found that expression of hsa-miR-424, hsa-miR-223, hsa-miR-199a-3p, hsa-miR-199a-5p, hsa-miR-27b, hsa-miR-378, hsa-miR-26b, hsa-miR-23a, and

hsa-miR-23b were upregulated and those of hsa-miR-155, hsa-miR-20b, hsa-miR-221, hsa-miR-151-3p, hsa-miR-222, hsa-miR-29b/c, and hsa-miR-106a were downregulated more than twofold due to EBV-infection of DLBCL [100]. 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 [101].

Bacterial Infections

The role of miRNAs in mammalian host signaling and defense against bacterial pathogens has been recognized [102] and will provide both insights and diagnostic opportunities. The use of miRNA patterns for diagnosing bacterial infections is evolving [6]. *Helicobacter pylori* is a bacterium that utilizes multiple colonization factors and virulence factors to persist in the human stomach for life [103]. This persistent colonization of the gastric mucosa results in an inflammatory process that may remain asymptomatic for decades or progress to a more serious disease such as gastric carcinoma [104]. The host immune response along with the *H. pylori* gene expression and miRNAs is involved in this process; specific miRNA patterns may prove to be useful for detecting a shift from asymptomatic carriage to gastric carcinoma [104]. In vitro infection assays have revealed that *H. 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 [105]. More recently, expression patterns of miRNA in gastric mucosa infected with *H. pylori* using endoscopic biopsy specimens were determined by microarray. There were 31 differentially expressed miRNAs between the *H. pylori*-infected and *H. pylori*-uninfected mucosa (more than twofold), and miRNA expression profiling could distinguish *H. pylori* status, with the eight miRNAs yielding acceptable sensitivity and specificity [106]. Overexpression of miR-223 has been described in *H. pylori*-associated gastric cancer and appears to contribute to cancer cell proliferation and migration [107]. Muscle-specific miRNAs miR-1 and miR-133 were significantly downregulated in the stomachs after long-term infection with *H. pylori* in mouse model [108].

Salmonellae cause a wide range of human infections, including gastroenteritis, bacteremia, enteric fever, and focal infections such as osteomyelitis [109]. *Salmonellae* have been shown to render human host cells more susceptible to infection by controlling host cell cycle progression through the active modulation of host cell miRNAs [110]. 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 [111]. 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/let-7c/let-7d/let-7f/let-7g/let-7i 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 [111].

Other Microbial Agents

Mycobacterium avium subspecies *hominissuis* is an opportunistic pathogen of immunocompromised individuals [112]. 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 RT-qPCR [113]. They that found expressions of *let-7e*, miR-29a, and miR-886-5p were increased in response to mycobacterial infection at 48 h [113].

The human papillomavirus (HPV) is associated with a number of oral, genital, and cutaneous conditions that may be benign or malignant [114, 115]. The association of HPV and cervical cancer is well-known because most cervical cancers contain HPV DNA, notably HPV types 16 and 18 [116]. The contribution of HPV to anogenital, oral, and oropharyngeal cancers is less clear [115]. The altered expression of miRNAs in these HPV-associated cancers has been investigated as a marker for possible diagnosis and therapy [117]. Expressions of miR-23b, miR-34a, and miR-218 are significantly reduced by HPV E6 infection, while HPV E7 infection downregulates expression of miR-15a/miR-16-1 and miR-203 [118].

Methods of miRNA Detection

Accurate determination of miRNA 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. Firstly, mature miRNA is 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. Secondly, miRNAs are heterogeneous in their GC content, which results in melting temperatures (T_m) 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 non-active pri-miRNA and pre-miRNA precursor species do not contribute to the detection signal [119]. 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 included among the current standard methods [120].

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; and (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 increase the affinity between LNA probes and target miRNA which results to at least tenfold increase of sensitivity [121]. Using soluble carbodiimide cross-link 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 [122].

RT-qPCR

The most widely used method for detection and qualification of miRNA appears to be real-time quantitative RT-PCR (RT-qPCR). 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 RT-qPCR used for the quantitative analysis of miRNAs. The first step in RT-qPCR 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. 1a) and a linear primer (Exiqon, Fig. 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.

Another approach is done as follows: miRNAs are first tailed with adenosine nucleotides at 3'-end of miRNA with poly(A) polymerase (Fig. 1c) or linker adaptor with T4 RNA Ligase 1 (Fig. 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 [119]. 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 RT-qPCR assay. LNA modification is a widely used

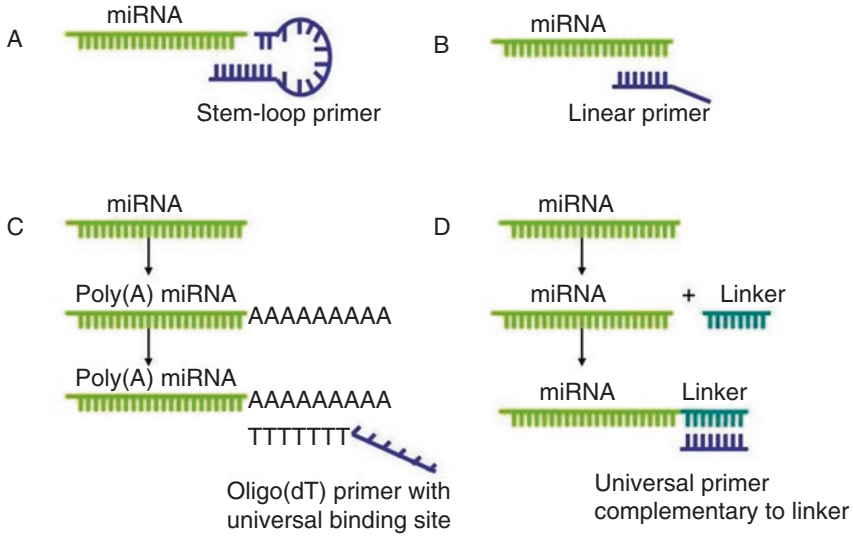


Fig. 1 Schematic representation of reverse transcription methods used to generate cDNA. Reverse transcription of individual mature miRNAs is done using stem-loop (a) or linear (b) primer. miRNAs are first tailed with adenosine nucleotides at 3'-end of miRNA with poly (a) polymerase (c) or linker adaptor 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

method for increasing the T_m and the specificity of primer. Each incorporated LNA monomer increases the T_m up to 2–8 °C, depending upon the position of the LNA moiety in the oligonucleotide primer [123].

There are two approaches available for detection of RT-qPCR 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 is 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 [119]. 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 only 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 RT-qPCR method has demonstrated a high sensitivity and specificity with ability to accurately detect miRNAs in a single stem cell [124, 125]. 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 RT-qPCR

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 [126]. 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 include (a) miRNA probe design and array preparation, (b) isolation of miRNA and labeling, and (c) hybridization and signal detection. A schematic flow chart of the miRNA profiling microarray is shown in Fig. 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 [127, 128]. 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

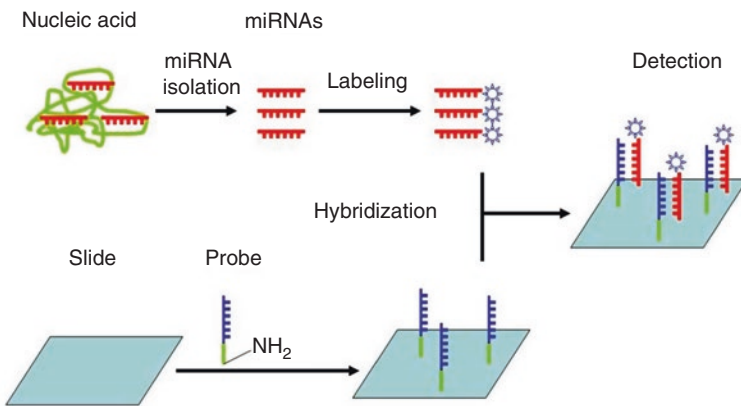


Fig. 2 Schematic flow chart of the microRNA profiling microarray. A miRNA microarray prepared by amine-modified microRNA probes that consist of “linker” sequences (yellow) and capture sequences (blue) spotting to amine-reactive glass slides. After miRNAs are isolated from samples and labeled with fluorescence dye, they are hybridized with microarray and then the expression signal can be detected by fluorescence detector

combined with a commercialized kit (e.g., the mirVana™ microRNA Isolation Kit of Ambion, Inc. and the PureLink™ microRNA Isolation Kit of Invitrogen Co) has 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 include labeling through poly(A) polymerase, labeling through 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 [129, 130]. Indirect labeling methods include 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 is 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 [131]. Microarray technology has proven to be standard technique for profiling miRNA expression. However, due to relatively low specificity and reduced dynamic range compared to other methods, the results obtained from microarray often require the validation via RT-qPCR.

Next-Generation Sequencing (NGS)

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 technique 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 [132, 133]. 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 reduces cloning biases observed with traditional capillary sequencing since sequence reads are generated from fragment libraries that don't 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 [132]. 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 (1000–1200 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. RT-qPCR 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 intra-reproducibility, the inter-platform reproducibility among different platforms is low [134]. An “industry standard” for analysis of miRNA expression awaits further advances in both technology and computation [135].

Quantification of miRNAs in Biological Specimens: Normalization Approaches

The growing interest in developing circulating miRNAs as blood-based biomarkers in the diagnostic microbiology field necessitates very careful consideration of the effects of various pre-analytical and analytical parameters on their quantification. To ensure that miRNA quantification is not affected by the technical variability that may be introduced at the multiple different analysis steps and to minimize any other potential effect of non-biological variation in the quantification results, it is important to select and identify stable miRNAs as normalizers and to choose the right normalization approaches.

The correct quantification of miRNA transcripts in clinical samples should include data normalization using both endogenous and exogenous control miRNAs [136–138]. The selection of endogenous control miRNAs is necessary to avoid false negative results due to a bad sample quality, but it is difficult. In this case a miRNA gene that is expected to be stably expressed in all analyzed samples should be selected as an endogenous control. Preferably target mRNA levels should be normalized using as miRNA normalizers control genes belonging to the same RNA class [139]. Based on the same concept, normalization of miRNA levels should be

based on endogenous control genes that belong to the small noncoding RNA family (ncRNA) of RNAs, such as small nuclear RNAs (snRNA). It is important to note that the endogenous miRNAs should meet the following characteristics in order to be used as miRNA normalizer: (a) the miRNA normalizer should be highly expressed in most samples, (b) the miRNA normalizer should show invariable expression across the test sample, and (c) the miRNA normalizer should have equivalent extraction and quantification efficiency with the target miRNAs. In order to avoid misinterpreted data and to identify true changes in miRNA expression levels, it is important to select the correct endogenous miRNA normalizer. Different algorithms can be used to select the best endogenous miRNA normalizer including geNorm [140], NormFinder [141], and BestKeeper [142]. In most cases reported so far, researchers select their endogenous reference genes for miRNA quantification according to reports in the literature or based on distinguishable low standard deviations (SD) in miRNA microarrays data. In the majority of studies, a relative quantification (RQ) step is included to compare the expression levels of target miRNA gene with the expression of an endogenous reference gene, based on the $\Delta\Delta C_q$ approach as described by Livak and Schmittgen [143]. Therefore, in each case different miRNA normalizers should be first evaluated and then established for different sample types. The combination of several normalizers might be more appropriate than a single universal normalizer [144]. It is crucial to mention that the selection of a gene as a miRNA normalizer should always follow validation screening tests on a subset of samples under analysis.

The inclusion of synthetic miRNAs as exogenous controls added to samples prior to any analysis step is also very important for miRNA quantification. This is the only way to correct the different recovery rates for each sample during the various steps of miRNA isolation and PCR amplification between individual clinical samples. Exogenous synthetic miRNAs have been used as external controls for data normalization of sample-to-sample variations in RNA isolation [136, 137, 145]. The synthetic miRNA is added to all the plasma aliquots as an exogenous miRNA spiked-in control after the addition of the denaturing solution to avoid differences in template quality and warrant efficiency of the reverse transcription reaction. Several synthetic miRNAs have been used so far, including *C. elegans* miRNA *cel-miR-39* which is the almost widely used [136, 146], miRNAs *Quanto EC1* and *Quanto EC2* [147], and the *simian virus gene SV40* [148]. We have to point out that using only spike-in miRNA controls for the quantification of miRNA expression in clinical samples is not correct since in this case only the handling of experiments is considered, but not the sample quality. Several studies have shown that normalization should be based on a combination of an endogenous and an exogenous control miRNA, since in this case differences in miRNA recovery and differences in cDNA synthesis between samples are compensated [136, 137, 145].

Concerning miRNA profiling by using microarrays technology, the normalization methods have not been investigated in detail so far. One of the most widely used normalization methods in this case is based on the mean expression value of all miRNAs [149]; however, quantile normalization is also a popular method for large-scale mRNA array expression [150–152], while rank-invariant set normalization [153]

has been also used. The first normalization methods that were used with miRNA array data employed centering to median values [154, 155] or scaling based on total array intensities [156]. Variance stabilizing normalization (VSN) methods have also been applied to miRNA array data [157, 158]. Normalization procedures based on the set of invariants and quantile were the most robust over all experimental conditions tested. Suo et al. and Pradervand et al. evaluated the effectiveness of these methods by comparing the normalized microarray data to qPCR data. The correlation between the microarray and qPCR data tended to be low [157, 158].

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 will be discussed in the next section.

Virus-Encoded miRNAs

Epstein-Barr Virus

Chronic active Epstein-Barr virus (EBV) infection has high mortality and morbidity [92–94]. To explore the biomarkers for disease severity and prognosis, Kawano et al. assessed the 12 plasma miRNA expression levels encoded by EBV [159]. They found that virus-encoded miR-BART1-5p, miR-BART2-5p, miR-BART5, and miR-BART22 levels in patients with chronic active EBV infection were significantly greater than those in patients with infectious mononucleosis and in controls. Plasma miR-BART2-5p, miR-BART4, miR-BART7, miR-BART13, miR-BART15, and miR-BART22 levels were significantly increased in the patients with systemic symptoms, compared with levels in patients without systemic symptoms. The levels of miR-BART2-5p, 13, and 15 showed clinical cutoff values associated with specific clinical conditions, in contrast to plasma EBV loads which can serve as the potentially biomarkers of disease severity or progress [159].

JC and BK Polyomavirus

Polyomaviruses are ubiquitous, species-specific viruses that belong to the Papovaviridae family [160]. JC and BK polyomaviruses were first described in the 1970s and are the two most commonly recognized human polyomaviruses [160]. JC polyomavirus causes a fatal central nervous system demyelinating disease known as

progressive multifocal leukoencephalopathy (PML) in immunocompromised individuals or individuals being treated with potent immunosuppressive therapies [161, 162]. JC polyomavirus is a DNA tumor virus that has a double-stranded DNA genome encoding a well-studied oncogene, large T antigen [163]. The expression of the JC polyomavirus miRNAs has been investigated after infection in vitro [164]. The JC polyomavirus expressed several miRNAs, JC-miRNA-3p and JC-miRNA-5p [164]. JC polyomavirus also encodes another microRNA, jcv-miR-J1. The expression of jcv-miR-J1-5p and its variant jcv-miR-J1a-5p in 50 healthy subjects was investigated [165]. The overall detection rate of JCPyV miRNA was 74% (37/50) in plasma and 62% (31/50) in urine. The detection rate was 86% (12/14) and 57% (8/14) of plasma and urine samples in seronegative subjects, while the detection rate was 69% (25/36) and 64% (23/36) in seropositive subjects. Furthermore, in seropositive subjects shedding virus in urine, higher levels of urinary viral miRNAs were observed, compared to non-shedding seropositive subjects [165].

BK polyomavirus is the cause of nephritis in renal transplant patients and often results in graft loss [160, 166]. BK polyomavirus is latent in the urogenital tract and is able to reactivate and replicate in the nucleus of renal epithelial tubular cells of the transplanted kidney [160, 167]. BK polyomavirus-specific bkv-miR-B1-5p, JC polyomavirus-specific jcv-miR-J1-5p, and bkv-miR-B1-3p/jcv-miR-J1-3p, sharing identical sequences between the two viruses, were analyzed from body fluids diagnosed with, or suspected of, a severe polyomavirus-associated disease [167]. The miRNAs frequently amplified from human plasma, urine, and cerebrospinal fluid samples. Bkv-miR-B1-5p was amplified from one-third of the samples which often contained high viral DNA loads. Their diagnosis and management significances in severe polyomavirus-associated diseases need further clinical evaluation [167].

Host-Encoded miRNAs

Sepsis

Diagnosis and monitoring of sepsis can be difficult because many of its signs and symptoms can be caused by other noninfectious disorders [1, 2, 4, 8]. 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, IL-6, IL-10 and TNF- α), chemokines (IL-8, MCP-1 and G-CSF), procalcitonin, and metabonomic [1, 2, 4, 8]. 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 [33, 34, 168, 169]; some of these studies use *Escherichia coli* lipopolysaccharide (LPS) stimulation as a trigger [170]. 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 [171]. Upregulation of miR-155, miR-223, and 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. [172]. Schmidt et al. screened for differentially expressed miRNAs in circulating leukocytes using an in vivo model of acute inflammation also triggered by LPS [170]. They found that four miRNAs were downregulated (miR-146b, miR-150, miR-342, and let-7 g) 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 [33]. 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 RT-qPCR assay [34]. 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.

Pulmonary Tuberculosis

With approximately nine million new cases of tuberculosis (TB) each year, tuberculosis remains a global scourge [173, 174]. Moreover, the emergence and increase in highly resistant strains as well as the emergence of functionally untreatable TB have made the diagnosis and control of TB particularly important [175]. The gold standard for the diagnosis of TB continues to be the growth of *Mycobacterium tuberculosis* in selective media, but this culture in clinical specimens requires long incubation time (3–12 weeks) due to the slow growth of *M. tuberculosis* [173]. The diagnostic usefulness of interferon-gamma-releasing assays has been reviewed, the T-SPOT. TB assay has proven to be a helpful adjunct test for diagnosing TB [176]. Additional accurate, tuberculosis-specific biomarkers are needed [177]. Human and mycobacterial miRNAs are being evaluated for their usefulness as tuberculosis-specific biomarkers [7, 178, 179]. Studies have shown that miR-155 and miR-155* in peripheral blood mononuclear cells (PBMCs) isolated from active TB (ATB) patients exhibited characteristic expression under purified protein derivative (PPD) challenge [180]. MiRNA expression profiles have been shown to be different in PBMCs from patients with active TB, latent TB infection (LTB), and healthy controls [181]. Differences in miRNA expression of whole blood between TB and sarcoidosis (SARC) were also detected [182]. The expression levels of miRNAs in serum samples from 30 patients with active tuberculosis have been profiled [183]. Ninety-seven miRNAs were differentially expressed in pulmonary TB patient sera compared with healthy controls (90 upregulated and 7 downregulated). Following RT-qPCR confirmation and receiver operating characteristic (ROC) curve analysis, three miRNAs (miR-361-5p, miR-889, and miR-576-3p) were shown to distinguish TB-infected patients from healthy controls and other microbial infections with moderate sensitivity and specificity (area under curve (AUC) value range,

0.711–0.848). Multiple logistic regression analysis of a combination of these three miRNAs showed an enhanced ability to discriminate between these two groups with an AUC value of 0.863 [184]. Fu et al. also explore the potential roles of circulating miRNAs in active pulmonary tuberculosis infection. They found that 59 miRNAs were downregulated and 33 miRNAs were upregulated in the TB serum compared to their levels in the control serum. Interestingly, only two differentially expressed miRNAs were increased not only in the serum but also in the sputum of patients with active pulmonary tuberculosis compared to the levels for the healthy controls. Their results indicated that upregulated miR-29a could discriminate TB patients from healthy controls with reasonable sensitivity and specificity [185]. Zhang et al. employed qPCR assay to detect the expression level of miR-183 in blood from TB patients and healthy individuals. Expression level of miR-183 was found to be increased in serum samples from TB patients, compared with healthy controls. Further analysis revealed that miR-183 level is positively associated with the activity of macrophages from TB patients [186]. References 158–163 don't make sense in that the topics in these references are not about TB.

Pertussis

Pertussis, also known as whooping cough, is caused by *Bordetella pertussis* (*B. pertussis*) [187]. Despite high levels of vaccination, *B. pertussis* continues to circulate in Asia, Europe, the United States, Australia, and other countries, making pertussis a reemerging disease [188–192]. It is clear that the diagnosis of pertussis is still relevant despite ongoing efforts to improve pertussis vaccines [192]. The serum miRNA profile in pertussis patients was investigated in order to explore its potential as a novel diagnostic biomarker for pertussis [193]. Serum miRNA profile in pertussis patients was analyzed using a miRNA array; 50 miRNAs were overexpressed, and 81 were under-expressed in the serum of pertussis patients [193]. Expression levels of seven candidate miRNAs were further evaluated by real-time RT-qPCR. A panel of five miRNAs (miR-202, miR-342-5p, miR-206, miR-487b, miR-576-5p) was confirmed as being overexpressed in pertussis patients [193]. Risk score and receiver operating characteristic (ROC) curve analysis showed that the area under the curve of the five-member miRNA profile was 0.980. At an optimal cutoff value (0.707), this panel of miRNAs yielded a sensitivity of 97.4% and a specificity of 94.3%. These data suggest that this five-member serum miRNA profile may serve as a new biomarker for pertussis diagnosis with high specificity and sensitivity [193].

Varicella

Varicella, also called chickenpox, is a highly contagious disease caused by varicella-zoster virus [194–196]. Although varicella vaccination has become routine for all children at 12–15 months of age in the United States, Germany, Australia, and Korea [196], outbreaks of varicella are still seen in the community [194]. Expression levels of miRNAs in serum samples from 29 patients with varicella were analyzed using TLDA [197]. The array results showed that 247 miRNAs were differentially expressed in sera of the varicella patients compared with healthy controls (215 upregulated and 32 downregulated). Through the following RT-qPCR confirmation

and receiver operating characteristic (ROC) curve analysis, five miRNAs (miR-197, miR-629, miR-363, miR-132, and miR-122) were shown to distinguish varicella patients from healthy controls and other microbial infections with moderate sensitivity and specificity [197]. Li et al. found that six miRNAs, including miR-190b, miR-571, miR-1276, miR-1303, miR-943, and miR-661, exhibited significant higher expression levels (more than fourfold) in herpes zoster (HZ) patients, compared with those of healthy controls and herpes simplex virus (HSV) patients [197]. The altered miRNA could be potentially used as biomarkers to test for latent HZ infection [198, 199].

Avian Influenza A (H7N9) Virus

Novel human influenza A virus strains continue to emerge and evolve from avian influenza strains and result in yearly epidemics and occasional pandemics [200]. The latest of these zoonotic avian influenza A strains to infection humans is the H7N9 avian influenza strain [201, 202]. MiRNA regulates host immune response and pathogenesis during influenza A infection and modulated viral replication [203]. Serum miRNA profile in response to H7N9 virus infection has been characterized using TLDA [61]. Upon infection, a total of 395 miRNAs were expressed in the serum pool of patients, far beyond the 221 in healthy controls. Among the 187 commonly expressed miRNAs, 146 were upregulated and only 7 were downregulated in patients. Further analysis by quantitative RT-PCR revealed that the serum levels of miR-17, miR-20a, miR-106a, and miR-376c were significantly elevated in patients compared with healthy individuals. ROC curves were constructed to show that each miRNA could discriminate H7N9 patients from controls with AUC values ranging from 0.622 to 0.898, whereas a combination of miR-17, miR-20a, miR-106a, and miR-376c obtained a higher discriminating ability with an AUC value of 0.96. These findings reveal significant alterations in serum miRNA expression following influenza virus infection [61] and confirm the great potential of circulating miRNAs for the diagnosis of influenza and other viral diseases [5, 203].

HIV

Infection with HIV-1 leads to a systemic destruction of T cells and diminished cell-mediated immunity resulting in a wide range of opportunistic infections as well as cancers [204]. Although treatment with antiretroviral therapy increases the survival of HIV-infected individuals, it does not result in eradication of infection [205]. Moreover, efforts to vaccinate against HIV-1 have not been successful [206]. HIV-1 is known to persist in resting T cells and also may persist in different cell types [205]. Understanding the role that miRNAs may play in the pathogenesis of HIV-1 may allow different approaches to both antiretroviral therapy and vaccine development [45–47, 207]. Studies have shown the different expression pattern of miRNAs in peripheral blood mononuclear cells (PBMCs), CD4⁺ T cells, CD8⁺ T cells, and monocytes from HIV-1-infected subjects [207]. For instance, Wang et al. have reported that four miRNAs (miRNA-28, miRNA-150, miRNA-223, and miRNA-382) showed different expression levels between monocytes and macrophages in HIV infection [50]. A cohort of 128 plasma samples from HIV-1-infected subjects and 37 samples from healthy donors have been analyzed in the light of

HIV-1-infected patients with low (<200 cell/ μ L), medium (200–350 cell/ μ L), and high (>350 cell/ μ L) CD4⁺ T cell count (LTC, MTC, and HTC). Of the 754 host miRNAs (excluding endogenous controls) incorporated in the array, 232, 346, 316, and 258 miRNAs were detected in plasma of healthy controls, LTC, MTC, and HTC groups, respectively. A total of 297 miRNAs differentially expressed in LTC subjects, of which 273 were upregulated and 24 were downregulated compared to healthy controls. Similarly, a total of 257 miRNAs (236 upregulated and 21 downregulated) were differentially regulated in MTC subjects compared to healthy controls. However, in the HTC group, only 127 miRNAs (85 upregulated and 42 downregulated) were differentially regulated compared to healthy controls. Fifteen miRNAs (miR-29a, miR-223, miR-27a, miR-19b, miR-151-3p, miR-28-5p, miR-766, miR-30a-3p, miR-136*, miR-125b, miR-18a, miR-769, miR-942, miR-1197, and miR-518b) were randomly selected for further analysis. Among these, seven miRNAs (miR-1197, miR-766, miR-136*, miR-151-3p, miR-518b, miR-769, and miR-942) were commonly dysregulated in all three groups. The other eight miRNAs were upregulated expression in LTC, and MTC groups compared controls. A combination of nine miRNAs (miR-29a, miR-223, miR-27a, miR-19b, miR-151-3p, miR-28-5p, miR-766, miR-30a-3p, and miR-136*) were found to distinguish the HIV-1-infected patients from healthy controls with sensitivity of 96.1% and specificity of 97.3% and AUC = 0.994 [208]. Seven of them were significantly associated with CD4⁺ T cell count and thus have a great potential to serve as biomarkers for monitoring the HIV immune status.

Viral Hepatitis B

Hepatitis B virus (HBV) infects the liver and causes acute and/or chronic liver diseases that may progress to cirrhosis of the liver or to hepatocellular carcinoma [209]. HBV infection is also known to modulate the expression of host cellular miRNAs, which then participate in development of HBV-related liver diseases [210, 211]. The miRNA profiles in chronic hepatitis B patient tissues or in HBV-expressing cells have been reviewed by Liu et al. [212]. 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 [34]. 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 $99.9 \pm 0.1\%$ (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, $99.6 \pm 0.4\%$; sensitivity, 97.9%; specificity, 99.4%). The control and HBV-positive HCC group could be clearly separated by five markers (miR-23b, miR-423, miR-375, miR-23a, and miR-342-3p; AUC, $99.9 \pm 0.1\%$; 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, $99.2 \pm 0.6\%$; sensitivity, 98.5%; specificity, 98.5%) [34].

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 [213]. 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

Hepatitis C virus (HCV) also infects the liver and causes hepatitis, cirrhosis of the liver, and hepatocellular carcinoma [214]. miRNAs are thought to regulate multiple aspects of HCV life cycles; certain miRNAs appear to serve as essential mediators for interferon-based antiviral therapy [215, 216]. 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 [217]. 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 [218]. They found that miR-21 expression correlated with viral load, fibrosis, and serum liver transaminase levels, while miR-122 expression inversely correlated with fibrosis, liver transaminase levels, and patient age. Morita et al. has described hepatic miR-122 expression that was weakly and positively correlated with the serum HCV load but was not correlated with HCV load in the human liver [219].

Bihrer et al. found that sera from patients with chronic HCV infection contained higher levels of miR-122 than sera from healthy controls [36]. 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

Enteroviruses are common causes of human infections with a diverse array of clinical features ranging from gastroenteritis to meningoencephalitis and myocarditis to pleuritis [220]. The role of miRNAs in the pathogenesis of enterovirus infections are becoming appreciated although not yet fully understood [221]. A comprehensive miRNA profiling in EV71-infected Hep2 cells using deep sequencing has been performed [222]. A total of 64 miRNAs were found whose expression levels

changed for more than twofold in response to EV71 infection [222]. Ho et al. found that upregulation of miR-141 upon enterovirus infection can facilitate viral propagation by expediting the translational switch [223]. 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 have been compared [224]. 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 [224].

Cytomegalovirus

Cytomegalovirus (CMV) is an important human pathogen that is often asymptomatic until the infected individual becomes immunosuppressed [71]. CMV miRNA is currently under investigation [72]. Plasma levels of 11 human- and 3 CMV-encoded miRNAs were quantitated by real-time PCR in 13 infants with congenital CMV infection. The levels of miR-183-5p and miR-210-3p were significantly higher in patients with congenital CMV infection than in control infants. The results indicated that plasma miRNAs could be associated with the pathogenesis of congenital CMV infection and could be used as disease biomarkers [225].

Ebola Virus

Ebola virus (EBOV) is a filovirus that initially infects dendritic cells and macrophages, which leads to lethal infections in humans and primates [226]. EBOV miRNAs have been identified and may serve as biomarkers for the diagnosis and therapy of Ebola viral infections [227]. EBOV-induced changes in circulating miRNA populations of nonhuman primates and humans have been investigated [228]. Eight miRNAs, including hsa-miR-146a-5p, hsa-miR-18b-5p, hsa-miR-21-3p, hsa-miR-22-3p, hsa-miR-29a-3p, hsa-miR-432-5p, hsa-miR-511-5p, and hsa-miR-596, can correctly categorize infection status in 64/74 (86%) human and nonhuman primates samples [228].

Dengue Virus Type 1

Dengue is currently regarded as the most prevalent and rapidly spreading mosquito-borne virus [229]. Efforts have been made at increasing our understanding of the pathogenesis and immunology of this viral infection [230, 231]. Among these advances in knowledge are the roles of miRNAs in dengue [232]. Expression levels of miRNAs in serum samples from three patients with dengue virus type 1 (DENV-1) and three healthy volunteers were separately analyzed using miRNA PCR arrays [210]. The expressions of the five selected miRNAs were verified by RT-qPCR. Serum miR-21-5p, miR-146a-5p, miR-590-5p, miR-188-5p, and miR-152-3p were identified as promising serum indicators for dengue infection [233].

Parasitic Infections

Parasitic infections continue to have high morbidity and mortality rates on humans. Newly recognized insight has resulted from genetic studies [210] and microRNA studies [234–236]. The expression levels of circulating miRNAs were also analyzed in filarial-, *Toxoplasma gondii*-, and *Plasmodium vivax*-infected patients [237–239]. These studies proved that altered plasma or serum miRNAs were useful as the biomarker for the detection of parasite infection. For example, serum miR-223 could serve as a potential new biomarker for the detection of schistosome infection and the assessment of the response to chemotherapy. Plasma miR-451 and miR-16 are relevant biomarkers for malaria infection. miR-71 and miR-34 discriminated *Onchocerca volvulus*-infected samples from uninfected samples.

Concluding Remarks

Circulating miRNAs have been investigated as the diagnosis or prognosis marker for microbial infections. Studies on host miRNA profiles for microbial infections are underway. Host miRNA profiles have a considerable way to go before they will be ready for use in clinical practice. Several issues remain to be clarified 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 source should be considered prior to selecting the source. (B) Though differences in circulating miRNAs between males and females have not been found with the exception of differences associated with pregnancy [240, 241], miRNA levels in plasma, and serum from a large number of normal individuals of both genders and various ages, even the same individual over time should be extensively studied. (C) No acknowledged reference genes have been found in serum/plasma. Commonly used endogenous controls, such as miR-16, are dysregulated in some diseases; RNU6B is degraded in serum. Spiking into RNA isolation processes with synthetic exogenous miRNA only acts as a normalizers for differences in recovery between samples. (D) The methods of miRNA quantification including RNA isolation should be standardized since inter-platform reproducibility among different platforms is low. (E) It seems to lack of specificity using single miRNA as biomarker since the miRNA commonly regulated in various disease. A panel of miRNAs would be a best choice.

Acknowledgments The study was supported by the Jiangsu Provincial Natural Science Foundation (BK20161583) and the “333” Projects of Jiangsu Province (BRA2017552).

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