Expression Levels of Candidate Circulating microRNAs in Early-Onset Neonatal Sepsis Compared With Healthy Newborns

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ABSTRACT: The high mortality rate of neonatal sepsis is directly connected with time-consuming diagnostic methods that have low sensitivity and specificity. The need of the hour is to develop novel diagnostic techniques that are rapid and more specific. In this study, we estimated the expression levels of circulating microRNAs (miRNAs) that are involved in regulating immune response genes and underlying inflammatory responses, which may be used for sepsis diagnosis. The total circulating miRNA was isolated and the candidate miRNAs (miR-132, miR-146a, miR-155, and miR-223) were quantified by real-time polymerase chain reaction technique. Statistical analysis revealed that miR-132 ($P < .01$) and miR-223 ($P < .05$) were downregulated in septic newborns compared with healthy babies. The decrease in expression of miR-132 and miR-223 may be associated with increased expression of immune-related genes involved in TLR (Toll-like receptor) signaling pathway. Further case-control studies with large sample size are required to identify the potential of miRNAs in neonatal sepsis diagnosis.

KEYWORDS: Neonatal sepsis, diagnosis, miRNA, inflammation, septic shock

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Introduction

Sepsis is a complex syndrome that is initiated by an infection and is characterized by a systemic inflammatory response. The global mortality rate of neonatal sepsis is around 1 million per year, which may be due to lack of potential diagnostic measures.[1] Bacterial culture remains the gold standard in sepsis diagnosis; moreover, it takes at least 24 to 48 hours to provide the results, having only 60% sensitivity.[2] Biological markers such as C-reactive protein (CRP), interleukins (ILs), procalcitonin, and erythrocyte sedimentation rate (mESR) have high sensitivity but poor specificity. As the search for potential diagnostics expands, the possibility of linking genetic variations with severity and susceptibility to sepsis and using them as prognostic/diagnostic markers is being explored.

MicroRNAs (miRNAs) are short noncoding RNAs that are involved in posttranscriptional gene regulation. The role of miRNAs is vital in biological processes such as cell growth, development, and activities. The biological significance of miRNAs is being analyzed by experimental methods such as induced deletion of specific miRNAs or using artificial miRNA. They also function as key regulators at different stages of host immune response, for example, miR-155 regulates proliferation of CD4+ CD25+ Treg cells; miR-146a downregulates interleukin-1 receptor–associated kinase (IRAK1), IRAK2, tumor necrosis factor receptor–associated factor 6 (TRAF6); miR-150 was found to be involved immune pathways such as MAP kinase pathway, Wnt signaling pathway, and insulin resistance; and miR-181b regulates vascular inflammation mediated by nuclear factor κB (NF-κB).[3–6] The differential expression of some miRNAs circulating in serum and plasma during sepsis is listed in Table 1. However, the role of miRNAs in neonatal sepsis is not much explored. In this study, we attempted to estimate the expression levels of 4 candidate circulating miRNAs in small cohorts of septic and healthy newborns.

Materials and Methods

Study subjects and sample size

The study was approved by Institutional Human Ethics Committee (IHEC), JIPMER, Puducherry. To estimate the level of circulating candidate miRNAs in plasma, newborns with early-onset sepsis (n = 25) and without sepsis (n = 25) were recruited. Early-onset sepsis usually occurs within 72 hours, after birth. The inclusion criteria for cases are as follows: age ⩽3 days, term birth, clinically diagnosed sepsis with at least 2 screening tests positive such as mESR >age in days + 3 mm/h, CRP >4 mg/dL, band cell count (>20%), and no antibiotic treatment before blood sample collection. The exclusion criteria are as follows: surgical procedures, maternal history of infections, inflammations or antibiotic therapy before delivery, APGAR score <6 at 5 minutes, and presence of any congenital malformations.

In silico selection of candidate miRNAs

The candidate miRNAs were selected based on their significance in sepsis-associated inflammatory pathways through literature, bioinformatics tools such as mirPath v2.0, microT-CDS v5.0, miRTarBase v6.0, and TargetScanHuman v6.2. The retrieved data were validated with miRecords (Bioleads,

Table 1. Differential expression of miRNAs in serum and plasma during sepsis.

FLUID	REGULATION	miRNAs
Serum	Downregulated	miR-223[7], miR-146a[4], miR-15a[8], miR-122[9]
	Upregulated	miR-133a[10],miR-150[11], miR-16[12]
Plasma	Downregulated	miR-297[13]
	Upregulated	miR-574-5p[13]

Abbreviation: miRNAs, microRNAs.

Minnesota, MN, USA), which contains experimentally verified miRNA-target interactions.

Isolation of circulating miRNAs and synthesis of complementary DNA

After getting informed consent from the parent/guardian, 0.5 mL of peripheral venous blood was collected from the neonates in a vacutainer containing EDTA (ethylenediaminetetraacetic acid). The plasma was separated by centrifugation at 3000 rpm for 10 minutes at 4°C, and the total RNA (messenger RNA, miRNA, and other RNAs) was extracted using miRNeasy Serum/Plasma extraction kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The complementary DNA (cDNA) was synthesized through reverse transcription using miScript II RT Kit (Qiagen).

Expression levels of candidate miRNAs

The quantification of candidate miRNAs was done through real-time polymerase chain reaction (PCR) approach in a 48-well, StepOne Real-Time PCR platform (Applied Biosystems, Foster City, CA, USA). The PCR reaction mixture was prepared using miScript SYBR green PCR assay kit (Qiagen) as follows: 2× miScript SYBR green master mix—10 µL, 10× miScript universal primer—2 µL, 10× miScript primer assay—2 µL, RNA sample—2 µL, and nuclease-free water—4 µL. The steps of thermal cycling conditions were as follows: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 70°C for 30 seconds. Real-time PCR amplification was followed by melt curve analysis wherein the PCR products were melted from 60°C to 85°C with a gradual increment of 0.2°C/min. The melting temperature (T_m) of the amplified products was measured with fluorescence intensity (−dF/dT) using the software StepOne v2.1 (Applied Biosystems).

Normalization controls

The spike-in control (*Caenorhabditis elegans* miR-39 mimic) was used as exogenous control for normalization, to monitor the bias factors such as variation in input RNA concentration, the presence of RNA inhibitors, possible RNA degradation, and errors in sample handling. The spike-in control was reconstituted in 300 µL of nuclease-free water, resulting in 2×1010 copies/µL of miR-39, from which 4 µL was taken and added to 16 µL nuclease-free water, reducing the concentration to 4×109 copies/µL. The working concentration (1.6×108 copies/µL) was prepared by adding 2 µL of diluted control with 48 µL of nuclease-free water. The primer assay for SNORD61 was used as endogenous control, whose expression is constant in all cell types and tissues with an amplification efficiency of 100%. The expression levels of candidate miRNAs were normalized with that of endogenous control.

Standard curve preparation and miRNA estimation

For the preparation of standard curve, 2 µL of the diluted spike-in control (4×109 copies/µL) was added to 78 µL of nuclease-free water, resulting in 1×108 copies/µL. This spike-in control was reverse-transcribed to cDNA, following the protocol mentioned earlier. The components of revere transcription mixture were as follows: 2.2 µL spike-in control (1×108 copies/µL), 2 µL template RNA sample, 4 µL 5× miScript Hi Spec buffer, 2 µL 10× miScript nuclease buffer, 2 µL miScript reverse transcriptase mix, and 7.8 µL nuclease-free water. The standards were prepared by mixing different aliquots of spike-in control cDNA with nuclease-free water. The cycle threshold (C_t) values obtained for standard cDNAs were used to create a standard graph, in which the C_t values for cDNAs from patient samples were extrapolated and the corresponding miRNA expression is found. The T_m of the amplicons was used to verify nonspecific amplification.

Statistical analysis

Statistical analysis was performed with SPSS v19 (IBM, Armonk, NY, USA) and Microsoft Excel at 95% confidence interval. Categorical data were represented as numbers and percentages. Independent Student *t* test was used to compare the expression levels of candidate circulating miRNAs between the groups.

Results

The cases and controls were matched for baseline demographic parameters such as age, sex, and birth weight. There is no significant difference between the cases and controls.

Table 2. List of microRNAs selected along with their target genes and pathways.

GENE NAME	NO. OF TARGET GENES	EXAMPLES	NO. OF PATHWAYS	SOME PATHWAYS
miR-132	294	PIK3CA, MAPK1, VDAC2, ADCY3, BIRC4, GHR, EP300	62	TLR signaling pathway, cell cycle, ubiquitin-mediated proteolysis, cell communication, NK cell–mediated cytotoxicity, cytokine-cytokine receptor interaction, T-cell receptor signaling pathway, B-cell receptor signaling pathway, calcium signaling pathway, apoptosis
miR-146a	143	TRAF6, CD80, IRAK1, HDAC1, NFAT5, ERBB4	31	
miR-155	340	FOS, MAP3K14, NFAT5, IFNAR1, SOS1, SMAD2	73	
miR-223	228	UBE2W, STAT1, MAPK10, PPP3CB, IL6ST, E2F1	46	

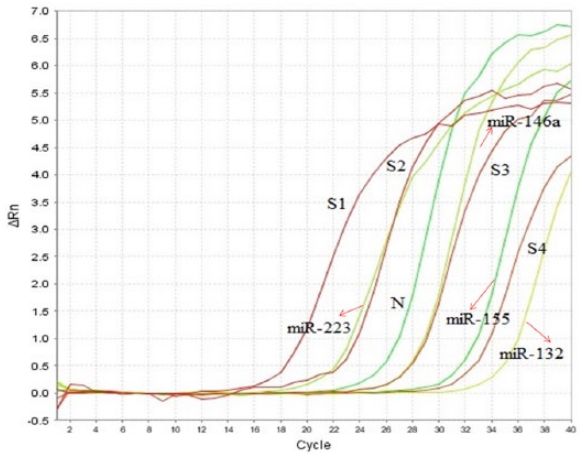

Figure 1. Amplification plots of the microRNAs and standards showing the C_t values.

Four candidate miRNAs, miR-132, miR-146a, miR-155, and miR-223, were selected for the study via bioinformatics screening. The targeted genes and pathways of these miRNAs are given in Table 2.

The amplification plots of miRNAs and standards are shown in Figure 1. The standards were denoted as S1, S2, S3, and S4 that showed gradual increase in the C_t value due to decrease in miRNA concentration. The endogenous control is denoted as "N". The normalized C_t values of candidate miRNAs were extrapolated in the standard graph to estimate their expression levels. The melt curves for the candidate miRNAs are shown in Figure 2. The melt curve analysis was performed from 65°C to 95°C to find out the melting temperature of the amplicon. As the amplicon size was around 250 bp (base pairs), the melting temperature falls in the range of 75°C ± 1°C.

The expression levels of circulating miRNAs determined from the standard graph were compared between the cases and controls. The levels of all candidate miRNAs were found to decrease in cases compared with controls, although only miR-223 and miR-132 were statistically significant (Figure 3). The negative expression shown in the figure was due to normalization with endogenous reference control. Positive expression of miR-223 showed that its expression level was higher than the reference control, whereas other miRNA expression levels were lower. The miR-146a expression was found to be very low in both cases and controls, compared with other miRNAs.

Discussion

Microarray analysis of peripheral blood leukocytes revealed differential expression levels of miRNA and was found to be associated with plasma cytokine levels.[14] Candidate miRNA study found that lipopolysaccharide (LPS)-induced inflammation is downregulated by miR-15a/16.[15] In this study, the expression levels of 4 candidate miRNAs, miR-146a, miR-223, miR-155, and miR-132, were analyzed. The circulating miRNAs were selected, as miRNAs originated from cells were like monocytes, and endothelial cells were differentially expressed during microbial infections.[16] Although the expression of all miRNAs was downregulated in cases, only miR-132 and miR-223 were found to be statistically significant.

In a similar study by Wang et al,[7] miR-146a and miR-223 were found to be significantly low in septic cases when compared with patients with systemic inflammatory response syndrome and controls. MiR-146a was also found to regulate IL-6 expression, controlling the proliferation of monocytes.[17] In contrast to the observations from this study, Vasques-NÓvoa et al[18] found increased plasma miR-155 expression in both human and experimental septic shock. Similar observation of increased miR-155 expression in plasma was observed by Wang et al,[3] among sepsis patients when compared with healthy controls.

MiR-223 is a blood cell–specific miRNA with significant roles in the development of myeloid lineages, differentiation of granulocytes, and suppression of red blood cell differentiation.[19–22] MiR-223 was also found to play a critical role in regulating polarization of macrophages in a specific pattern, thus preventing mice from conditions such as diet-induced inflammation and insulin resistance. It was also found that

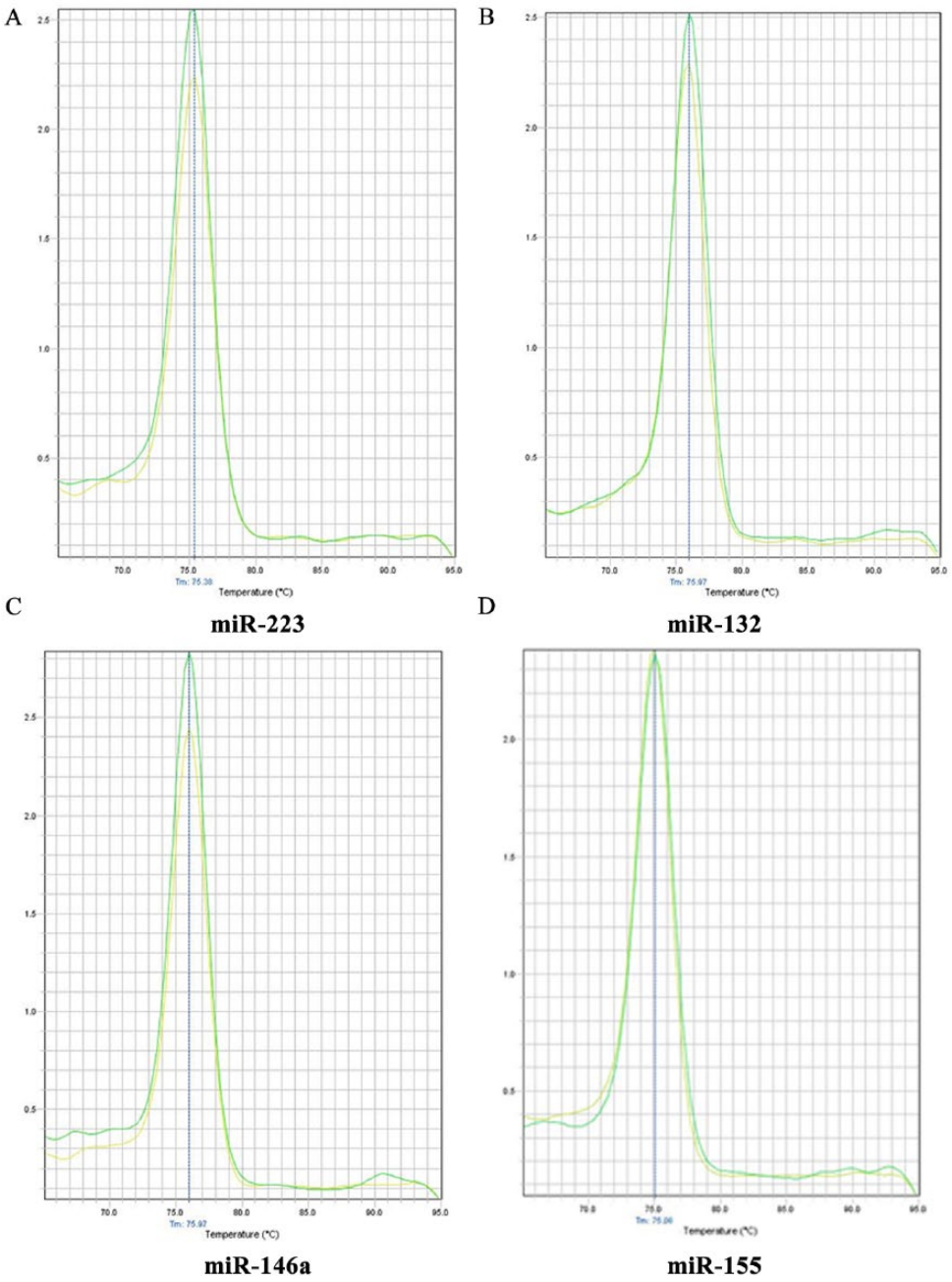

Figure 2. Melt curves showing the melting temperature of amplicon for each microRNA: (A) miR-223, (B) miR-132, (C) miR-146a, and (D) miR-155.

miR-223 targeted the gene *Pknox1*, resulting in the suppression of macrophage infiltration.[23] MiRNA-223 was found to be associated with sepsis, rheumatoid arthritis (RA), and hepatic ischemia and type 2 diabetes.[24–26] In this study, the expression of miR-223 was found to be decreased in cases when compared with controls. In contrast to this finding, a recent study stated that miR-223 was upregulated among pediatric patients with sepsis and was correlated with inflammatory cytokines such as IL-10 and TNF-α.[27]

Expression of miR-132 was found to modulate infection-associated inflammation at the early stages, which was stimulated by LPS.[28] The plasma levels of miR-132 were found to be decreased in patients with RA, when compared with healthy controls.[29] MiR-132 was shown to target the gene, *SirT1*, resulting in the synthesis of the inactive SirT1 enzyme. Due to the absence of SirT1, its substrate NF-κB gets activated which promotes inflammation, followed by increased production of IL-8 in adipocytes.[30] In this study, a highly significant decrease in expression levels of miR-132 in septic cases was observed, compared with nonseptic controls. Increased expression of miR-132 was found to reduce inflammation by targeting acetylcholinesterase, whereas decreased miR-132 levels resulted in exaggerated inflammation through production of a pro-inflammatorycytokines such as IL-6 and TNF-α.[31]

In conclusion, this study shows that miR-132 and miR-223 were downregulated in newborns with early-onset sepsis. Downregulation of these miRNAs may cause aberrant changes in inflammation associated with infection. The major

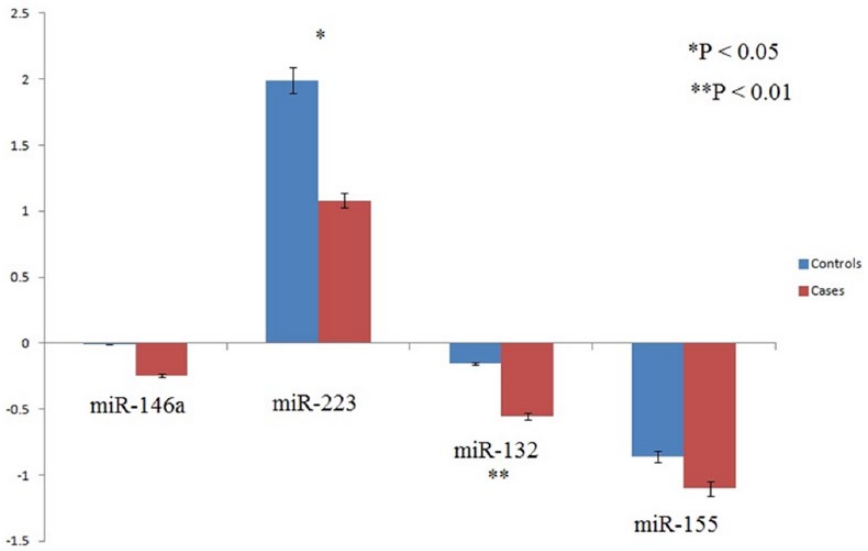

Figure 3. Plasma levels of microRNAs compared between cases and controls.

limitations of the study are small sample size and lack of microorganism-specific analysis. Further research with large sample size and differential analysis is required to evaluate the potential of miRNAs as diagnostic markers.

Author Contributions

BVB conceived the idea and postulated the hypothesis. BBD carried out the laboratory works, analyzed the results and prepared manuscript. VRD was involved in analyzing the data and preparation of manuscript.

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