Journal of Cardiovascular Disease Research
ISSN:0975-3583,0976-2833 VOL12, ISSUE03,2021

The Value of Serum miRNA-223 in Early Diagnosis of Neonatal Sepsis

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ABSTRACT

Background: Sepsis is leading cause of newborn death and morbidity. As a result, biomarkers that can either detect early sepsis or predict its fate are essential.

Objectives: to investigate the clinical value of miR-223 on neonatal sepsis diagnosis and prediction of prognosis.

Methods: Ninety neonates were included in this case-control study divided into three equal groups of matched age and sex. The sepsis group consisted of 30 neonates with neonatal sepsis confirmed with positive blood cultures. The systemic inflammatory response syndrome group (SIRS group) consisted of 30 neonates with clinical manifestations of sepsis but with negative blood culture and the third group of 30 apparently healthy neonates. The relative serum expression level of miRNA-223 was detected by qRT-PCR.

Results: The relative serum expression of miRNA-223 was significantly lower in sepsis and SIRS groups than the control group ($p< 0.001$), in late onset sepsis rather than early onset sepsis ($P <0.027$). and in non-survived sepsis patients than survived ones ($P < 0.001$).

Serum miR-223 expression could discriminate between neonatal sepsis and SIRS with 100%

sensitivity, 85.3% specificity and 87% accuracy. It also could distinguish survived from non-survived neonates in sepsis group, with 90% sensitivity, 78.3 % specificity.

Conclusion: Serum expression of miR-223 may be a promising predictor for sepsis diagnosis and outcome prediction among the high-risk neonates.

Keywords: Neonatal Sepsis, miRNA-223, SIRS.

INTRODUCTION

As one of the leading causes of death and impairment in infants, neonatal sepsis can be difficult to diagnose, evaluate and monitor as well as predict the outcome. [1]. The term neonatal sepsis has no broadly accepted definition. Neonatal sepsis is commonly referred to as the "systemic inflammatory response syndrome," or "SIRS." [2]. Clinical diagnosis of sepsis is difficult due to the ambiguity of its signs and symptoms. [3]. It is common practise to classify newborns with "clinical sepsis" when their blood culture is negative, despite the fact that blood culture confirmation is the gold standard for diagnosing sepsis. [4]. There are two types of sepsis: early-onset (EOS) and late-onset (LOS), the latter of which occurs more than 72 hours after delivery. [5]. Early detection of sepsis is crucial for improving survival rates because traditional screening approaches and biomarkers lack specificity. [6].

Molecular diagnostics and therapeutics are now possible because to the discovery of the microRNA (miRNA) family of small single-stranded non-coding regulatory RNAs of 19 to 22 nucleotides. As a result of miRNA's ability to bind to and regulate specific mRNA molecules, it has the ability to inhibit the production of target genes or degrade the levels of mRNA. [7]. Mice that lack miR-223 are less likely to develop disorders like diet-induced inflammation and insulin resistance, both of which are associated with increased insulin resistance. A blood cell-specific microarray known as miR-223 is involved in the development of myeloid lineages, granulocyte differentiation and red blood cell inhibition. [8].

This study's goal was to see if miR-223 had any clinical relevance in the early detection and

prognosis of newborn sepsis.

SUBJECTS and METHODS

In this case-control study, 90 neonates were divided into three groups: the sepsis group (blood culture positive group), the SIRS group (blood culture negative group), and the control group (blood culture negative group). Septicemia testing was not performed, and the control group included 30 healthy neonates with no clinical or laboratory indications of sepsis. Between February and October 2020, a random sample of babies in the paediatrics department's neonatal intensive care unit (NICU) was drawn at random from the Benha University Hospital. Published in 2003, at the Kunming Neonatal Sepsis Definitions Conference, sepsis criteria were used to make the diagnosis [9]. Preterm infants, those with genetic abnormalities, those with intrauterine growth retardation, and those who had suffered perinatal asphyxia were not included in the study.

The severity of sepsis was gauged by a method known as the haematological scoring system. Sepsis is given a score of 1 by the HSS for each of the following seven findings: Increased levels of immature polymorphonuclear neutrophils (PMNs) as well as an abnormally low total leukocyte count (TLCcomplete immaturity), (I:T) The immature PMN ratio is: mature (I:M) Blood platelet count 150,000/mm3, PMN ratio of 0.3, and obvious degenerative or toxic changes in PMNs are all symptoms of this condition. Sepsis was considered rare with a score of 2, but it is possible with a score of 34, and with a score of 5, sepsis or infection is quite likely. [10]. A group of sepsis-infected neonates were categorised into survivors and non-survivors after 28 days of observation in the hospital. .

In accordance with "The World Medical Association's code of ethics," the study was authorised by the Ethical Committee of the Faculty of Medicine, Benha University. (Helsinki's Declaration). The children's legal guardians granted their agreement after being fully apprised of the study's protocols prior to registration.

Laboratory investigations

Six ml peripheral blood was withdrawn from each neonate; two ml were added to pediatric Bactec blood culture bottle, one ml EDTA anticoagulated blood was used to assess complete blood count and two ml were used to separate serum by centrifugation at room temperature for 10 minutes at 2,000 xg to detect CRP titer by latex agglutination method (CRP- Latex Cromatest). The last 1 ml was put into RNA-protect Animal Blood Tubes and stored at -80°C for further molecular testing of miR-223.

Relative quantitation of miR-223:

Total RNA including miRNA was purified using miRNeasy Mini Kit (Cat. # 52304, Vilnius, Lithuania), followed by cDNA production by reverse transcription using miScript II RT Kit (Cat. # K0251, California, USA). The quantitative detection of miR-223 was done on Step-One™ Real-Time PCR (Life Technologies, USA) using miRNA-specific miScript Primer Assay (forward primer) and miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix. For accurate and reproducible miRNA quantification, target miRNA was normalized by an endogenous reference RNA; RNU6 gene. Quantification of miR-223 was expressed as relative expression level (fold) compared to reference RNA (RNU6) according to the $2^{-\triangle\triangle ct}$ method.

Statistical analysis:

SPSS version 2.0 was used to compile and analyse the data (SPSS Inc, Chicago, USA). Numbers and percentages were used to represent categorical data, whereas means, SDs, and ranges were used to represent quantitative data. Tests for statistical significance included the chi-square (X2), Fisher's exact (FET), Man-Whitney (U), and Spearman's coefficient of correlation (r) tests. For newborn sepsis diagnosis and prognosis prediction, ROC curves were utilised to identify the cutoff value of miR-223 with the best sensitivity and specificity. Sepsis was predicted with the help of a logistic regression model. This study's recognised

level of significance was 0.05 (a significance threshold of P 0.05 was used).

RESULTS

A total of 90 neonates, ranging in age from 4 to 14 days, were included in the study. They were divided equally among three groups: those with sepsis and positive blood cultures, which included 19 men (63.3 percent) and 11 women (36.7 percent). SIRS group, 30 infants with clinical signs of sepsis and negative blood cultures, there were 16 (53.3 percent) males and 14 (46.7 percent) girls in this group. While the control group included 21 (70 %) males and 9 (30%) females. The studied groups were matched in age, sex, gestational age, and weight (P> 0.05). The baseline laboratory data revealed significant lower levels of hemoglobin, platelets count and random blood sugar, and higher total leukocytes count in sepsis group. Serum CRP was positive in sepsis and SIRS groups, and negative in controls. **(Table 1)**

Regarding the possible risk factors of sepsis and its outcome; 25 cases (83.3%) of the sepsis group have premature rupture of membrane (PROM), 23 cases (76.7%) have maternal urinary tract infection, 7 cases (23.3%) have history of prolonged use of antibiotics and 14 cases (46.7%) have history of fever during pregnancy. The outcome of the neonates was significantly different among the studied groups as 6 neonates (15%) died in sepsis group while all neonates in SIRS group were survived.

In the sepsis group, early onset sepsis was detected in 17 cases (56.7%) and late onset sepsis in 13 cases (43.3%). Blood culture revealed gram-positive cocci in 20 cases (66.6%); including 15 cases (50%) group B streptococci and 5 cases (16.7%) staphylococcus aureus; and gram-negative bacilli in 10 cases (33.3%); including 5 cases (16.7%) E. coli, 3 cases (10%) Listeria Monocytogens and 2 cases (6.6%) Klebsiella.

Expression levels of miRNA -223

The relative expression of serum miRNA-223 was significantly lower in sepsis and SIRS groups than the control group (p< 0.001). **(Table 2, Fig. 1)**

Sepsis patients with late onset had a significant drop in the relative expression of miRNA-223. (P <0.027). The expression of miRNA-223 was considerably lower in sepsis patients who did not survive than in those who did. (P < 0.001). **(Table 2)**

Furthermore, the correlation analysis between miR-223 expression and studied clinical laboratory parameters revealed that only CRP showed significant negative correlation with miR-223 in sepsis groups (r= -0.682, P <0.001) and SIRS group (r= -0.542, P <0.001).

Diagnostic value of miRNA-223

ROC curve analysis of data was performed to detect the utility of miR-223 expression in discriminating between sepsis and SIRS groups. The best obtained cut-off value was ≤0.541 relative units, with area under the curve (AUC) (95% CI) 0.921 (0.87-0.97) with 100% sensitivity, 85.3% specificity and 87% accuracy. **(Fig 2A)**

In the sepsis group, the expression of miRNA-223 could discriminate non-survivors from survivors with an AUC of separating neonates who survived from those who did not. (95% CI) 0.872 (0.81–0.94) with 90% sensitivity, 78.3 % specificity at a cut-off value ≤ 0.31 relative units. **(Fig 2B)**

Multivariable logistic regression analysis

MiR-223 expression levels and sepsis outcomes were examined using binary logistic regression. Premature rupture of membranes, an infection of the urinary tract, elevated CRP levels, and decreased expression of miRNA-223 were found to be independent risk factors for mortality in sepsis. **(Table 3)**

Table (1). Comparison of the study groups' pre-interventional clinical and laboratory characteristics .

Variables	Sepsis group (n=30)	SIRS group (n=30)	Control group (n=30)	P
Age (days)	4.9±2.9	5.15±2.4	4.3±3.5	0.054
Gestational age (weeks)	37.8±0.3	37.4±0.49	37.7±0.47	0.071
Weight (Kg)	3.28±0.3	3.21±0.22	3.09±0.12	0.074
Total leucocytic count ($x10^9$/L)	18.9±16.15	11.2±4.66	10.3±2.78	<0.001
Hemoglobin (g/dL)	12.5±1.21	14.2±3.78	15.3±4.22	0.04
Platelet count ($x10^9$/L)	110±19.9	195.1±33	267±60.5	0.038
Random blood sugar (mg/dl)	45±12.6	50±14.2	78±10.8	0.041
CRP (mg/L)	36.6±14.19	15.6±3.18	3.6±0.5	<0.001

Data presented as mean ±SD.

Table (2). Comparison between the studied groups regarding miRNA-223.

Studied groups	miRNA-223	P
	Median (Range) / mean ±SD	
Sepsis group (n=30)	0.21 (0.001-0.500)	<0.001
SIRS group (n=30)	0.46 (0.22-0.80)	
Control group (n=30)	0.94 (0.83-1.76)	
Early onset sepsis (n=17)	0.245±0.17	<0.027
Late onset sepsis (n=13)	0.11±0.11	
Survived cases (n=24)	0.352 (0.215-0.500)	<0.001
Non-survived cases (n=6)	0.142 (0.115-0.254)	

Table (3). Regression analysis for prediction of sepsis within high-risk group.

Variable	n= 30	P	OR (95% CI)
Rupture of membrane >18 h		0.002	2.503 (0.025-3.024)
Yes	25		
No	5		

Urinary tract infection		0.0124	3.124 (1.254-5.235)
Yes	23		
No	7		
Prolonged use of antibiotics		0.79	0.91 (0.31-2.65)
Yes	7		
No	23		
Fever during pregnancy		0.54	1.09 (0.39-3.07)
Yes	14		
No	16		
CRP >6 mg/L		0.0235	3.46 (1.11-10.78)
Yes	30		
No	0		
miR-223 <0.541		0.0024	0.92 (0.87-0.97)
Yes	28		
No	2		

OR: odds ratio, CI: confidence interval.

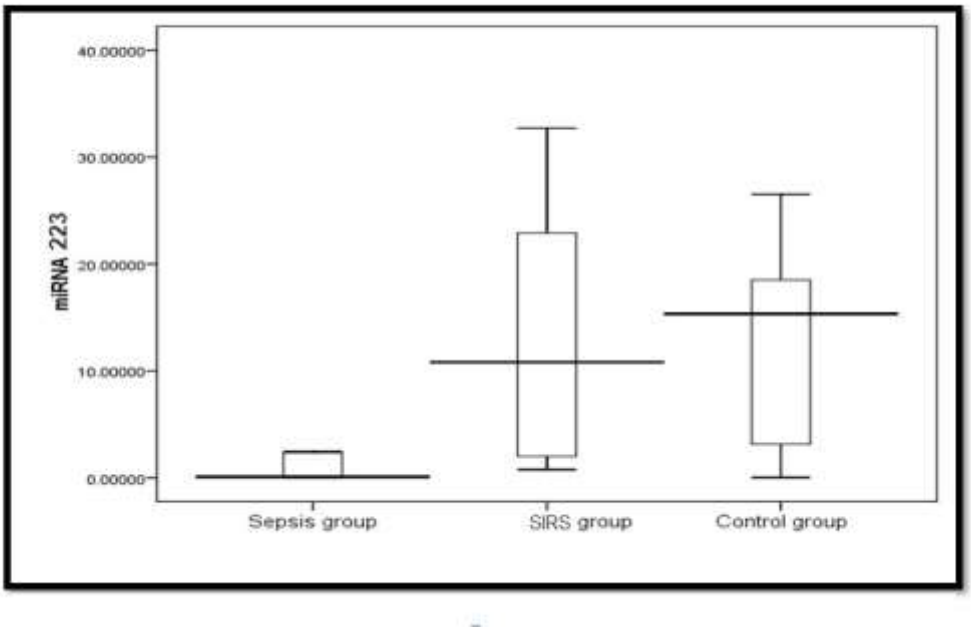
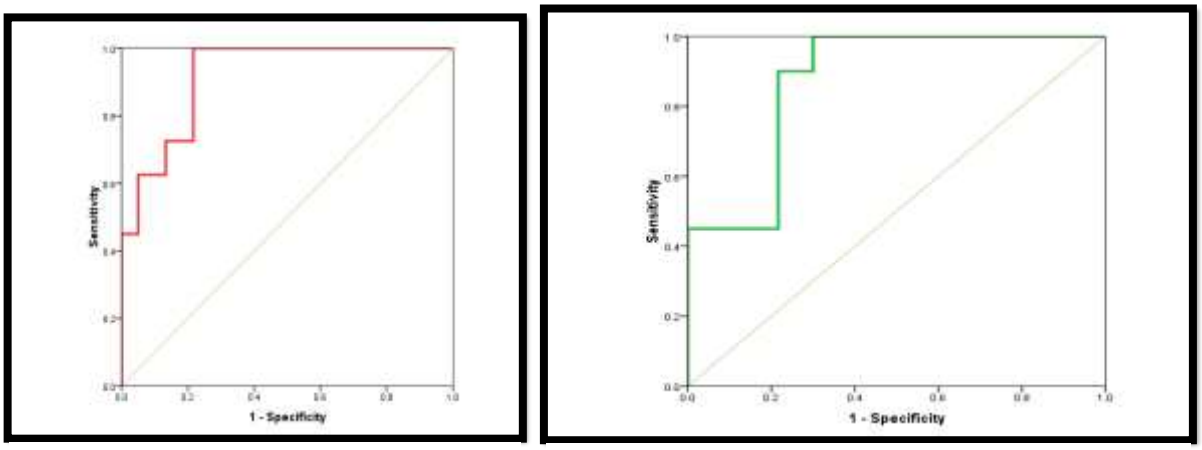


Figure (1). Median and range of miRNA 223 among the studied groups



A: **B:**

Figure 2: A) ROC curve for the performance of miR-223 in diagnosis of sepsis (in comparison with SIRS and controls). B) Performance of miRNA-223 in distinguishing survived from non-survived neonates in s

DISCUSSION

Sepsis occurs when toll-like receptors (TLRs) are activated by microbial components such lipopolysaccharides (LPSs), which activates the inflammatory response. The hyper-immune phase, which includes enhanced production and release of pro-inflammatory mediators, resulted in a cytokine storm and the systemic inflammatory response as a result of the TLRs being activated. [11]. Initial activation of both pro- and anti-inflammatory pathways is seen in sepsis, with the pro-inflammatory response predominating for the first few days of illness [12]. A hyper-immune or immunosuppressive phase occurs when inflammatory mediators overrun and paralyse the immune system if the pro-inflammatory response is not handled early. [13] adaptive feedback mechanism to reduce tissue damage during the early stages of the inflammation [14]. Suppression of the anti-inflammatory response has been shown to increase vulnerability to subsequent infections and tissue damage. [15] It is associated with a higher rate of death [14].

Severe sepsis can only be effectively treated if it is diagnosed and treated early. [16] especially if they are being treated with high-quality methods. The blood contains circulating miRNAs, which can be evaluated immediately in a therapeutic context, unlike microbial cultures, which can take a long period. [17]. As a result of this, miRNAs can be employed as biomarkers for sepsis detection in the clinical setting. [18] clinical decision-making may benefit from this information .

MiR-223 serum expression decreased dramatically in patients with proven sepsis and SIRS compared to healthy controls, as it was significantly lower in sera from sepsis patients than from SIRS patients. Late onset sepsis and non-survivors had lower levels of miRNA-223 than early onset sepsis and survivors. Severe neonatal sepsis has previously been linked to reduced plasma levels of miR-223 relative to healthy neonates. **[**8, 16, 19].

Interestingly, the current data are consistent with the fact that miR-223 transcription is elevated in a dose dependent manner in response to the bacterial endotoxin lipopolysaccharide (LPS) [20]. miR-223 has also been demonstrated to be down-regulated in immunological competent cells in response to bacterial infections such as Mycobacteria species, Salmonella enterica and Helicobacter Pylori [21].

When cells are exposed to bacterial components, toll like receptors (TLR2, 4 and 5) on their surface are activated. The nuclear factor-κB(NF-κB)-dependent pathway is then used to induce intracellular signaling. Of interest, NF-κB is a transcription factor that binds to the promoters of numerous immune responsive genes including miR-223, and induces their induction [22]. MiR-223 is a blood cell-specific miRNA that plays an important role in the development of myeloid lineages, granulocyte differentiation, and red blood cell differentiation suppression. It was shown that miR-223 had a substantial role in modulating macrophage polarization in a specific manner, and this protected mice against diet-induced inflammation and insulin resistance. **[**23].

While some studies have shown an increase in plasma levels of miR-223 [24-27]. Such disparities could be related to changes in patients' characteristics such as age, sepsis stage, and presence of co-morbidity or therapy lines employed in those trail.

In the newborn sepsis group, the current study likewise found a strong negative relationship between miR-223 and CRP levels. This is consistent with the finding of ***Dhas et al.,*** **[**8] who found that miR-223 were much lower in sepsis patients, whereas CRP levels increased. miR-223 was discovered to be significantly lower in plasma of septic neonates compared to healthy newborns in early- onset sepsis, negatively regulate inflammatory response. The down-regulation of miRNA-223 may result in abnormal alternations in infection related inflammation. It is possible that the lower levels of miR-223 levels are linked to higher expression of immune-related genes implicated in the TLR signaling pathway [28].

In the current study, the optimum cut-off value for miR-223 was 0.541 relative units, which could predict sepsis in neonates, with an area under the curve (AUC) of 0.921 and a confidence interval of 0.87-0.97 and 100%sensitivity, and 85.3%specificity. The pooled sensitivity, specificity, AUC, and diagnostic odds ratio for miR-223 were 0.77 (95% CI 0.67–0.84), 0.91 (95% CI 0.73–0.97), 0.87 (95% CI 0.84–0.90), and 33 (95% CI 8–142), respectively in studies of gathered research [16, 24, 25, 26].

Taking in consideration the limitation of the relatively small sample size; large-scale prospective studies are required to confirm our findings, with special emphasis on kinetics of miR-223 expression level during neonatal sepsis course and its relation to treatment.

CONCLUSION

miRNA-223 linked to the severity of neonatal sepsis and the levels of inflammatory markers , suggesting that it could be used as a new diagnostic and prognostic biomarker for sepsis in the high-risk neonates.

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