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The diagnostic and prognostic role of MiRNA 15b and MiRNA 378a in neonatal sepsis

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ABSTRACT

Background and objectives: Sepsis is one of the major factors for both term and preterm babies with morbidity and mortality. On the basis of recent clinical trials, altered circulating micro-RNAs (miRNAs) may serve as possible biomarkers in sepsis for diagnosis and prognosis. The aim of this research is to assess the diagnostic and prognostic biomarkers of miRNA 15b and miRNA 378a for neonatal sepsis.

Subjects & methods: This study was carried out 25 neonates with sepsis admitted to neonatal ICU of Menoufia University Hospital and 25 healthy controls from February 2019 to May 2020. The relative quantification (RQ) of miRNA-15b and miRNA-378a expression was assessed using real time PCR technique. Results: Our results demonstrated that patients with sepsis had significantly higher level of MiRNA-15b than the healthy volunteers. On the other hand, patients with sepsis had significantly lower level of MiRNA-378a than the healthy volunteers. The ROC curve showed that the serum MiRNA-15b was a significant discriminator of sepsis with a combined sensitivity and specificity of 76% and 88% with cutoff point of 3.24. In addition, serum MiRNA-378a was a significant discriminator of sepsis with a combined sensitivity and specificity of 60% and 88% with cutoff point of 0.361. The miRNA-15b expression significantly correlated positive with respiratory rate (r =0.415,p =0.039), WBCs (r = 0.408, p =0.043), and CRP (r =0.407, p=0.043). Likewise, miRNA-378a expression significantly correlated negative with respiratory rate (r =-0.415p =0.024), WBCs (r =- 0.442, p =0.027), and CRP (r =-0.459, p=0.021).

Conclusion: Both MiRNA 15b and 378a are promising biomarker for neonatal sepsis.

1. Introduction

Sepsis is a widespread, medical condition characterized by inappropriate infection responses and associated abrupt organ failure. Overall, sepsis is one of the most prevalent causes of mortality of hospital-based patients in the ICU, a big public health concern with significant financial implications [1]. Neonatal sepsis appears to be one of the major causes in both term and preterm births morbidity and mortality [2]. There are wide range of cases of neonatal sepsis, with bacterial infection by Group B streptococcus (GBS) as the commonest etiology [3]. The progression of neonatal sepsis has a lethal consequence on a effected patients, previous reports demonstrated that the development of septic shock reduced the survival rate for every hours of delayed treatment [4].

Therefore, it is imperative to diagnose sepsis as early as possible before the onset of septic shock. Over the past few decades, a number of diagnostic and prognostic factors were developed for early identification of sepsis [5]. Several indicators for early detection of sepsis have also been proposed. These proteins are C-reactive protein (CRP), procalcitonin (PCT), pancreatic glyco-protein (PSP), fibronectin, haptoglobin, lactoferrine, neopterin, and oromucoside [6].

With our knowledge of the genetic origin of human pathology, recent biomarkers have been employed for the diagnosis of sepsis, which involves molecular markers such as genetic modifications in DNA, adjustments in transcriptome profiling (mRNA, miRNAs, lncRNAs and circRNAs), epigenetic markers have been employed in diagnosis of sepsis [7]. MicroRNAs (miRNA) are non-coding single-stranded RNAs with post-transcriptive gene silencing features. They usually control.

Numerous intracellular incidents like the Toll-like Receptor (TLR) downstream signaling cascade to inhibit systemic inflammation during

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infections. In comparison, unusual miRNA expression allows sepsis progress in addition to its clinical symptoms and signs [8].

MicroRNAs (miRNAs) are not protein coded but influence the expression of the genes by inhibiting translation or transcription of their target mRNAs. MiRNAs have lately been found to spread and the distribution of circulating miRNAs may be altered in different clinical circumstances, such as inflammation, injury and sepsis. Any miRNAs associate with the stage of the illness, suggesting that miRNAs play a crucial role in the detection and prediction of sepsis in critically ill patients. [9].

miR-15b related to the miR-15/16 family known as the miR-15b/16 group, which performs a variety of roles in numerous cell and tissue forms. This miRNA was observed to be highly expressed in hepatocellular carcinoma and other tumours that suggest that miR-15b could be essential for cancer prediction [10]. Previous reports demonstrated that miRNA 15b is down regulated in many forms of malignancy, particularly chronic lymphocytic leukemia [11]. Also, a previous study was applied on animals models demonstrated that 15b miRNA played a pivotal role in the development and progression of sepsis [12].

On the other hand, miRNA 378a is a key player in various metabolic pathways through its transcriptional regulation of oxidative energy metabolism; under physiological conditions, miRNA 378a was found to contribute significantly to the development and regulation of myocytes, as well as mitochondrial metabolism and energy production [13]. A previous study was applied on (SIRS) systemic inflammatory response syndrome patients in which also the term sepsis is used. The report demonstrated that miRNA 378a affected by intensity of this disease and produced by immune cells upon activation which indicate it to be promising biomarker for sepsis [14].

We aimed by that research to assess the role of miRNA 15b and miRNA 378a as diagnostic and prognostic biomarkers for neonatal sepsis.

2. Subject and methods

2.1. Subjects

This research has been done by collaboration between Medical Biochemistry & Molecular Biology, Faculty of Medicine, Menoufia University and faculty of Science, Menoufia University, Egypt in the period from (February 2019 to May 2020).

This study was conducted on 25 neonates (Males = 12, females = 13) with faster sepsis onset who were admitted to neonatal intensive care units (NICU) of Menoufia University hospital through fifteen month from (February 2019 to May 2020). In addition, 25 neonates were included as age and sex-matched control group. The study's protocol was approved by local ethics committee of Menoufia University.

2.2. Inclusion and exclusion criteria

Full term neonates of both sex with symptoms and signs suggestive of neonatal sepsis, according to clinical sepsis score, were included. We excluded neonates with congenital infection, suspected inborn errors of metabolism, perinatal asphyxia, congenital anomalies, chromosomal abnormalities and infant of diabetic mother. Written informed consents were obtained from parents before the initiation of enrollment.

We recorded the following data from eligible patients: demographic characteristics, gestational age, mode of delivery, maternal history, perinatal and antenatal history, clinical examinations findings, complete blood count (CBC) with differential, CRP, blood culture (BACTIC) and assessment of serum miR-15b and miR-378a levels using real time PCR technique.

3. Methods

3.1. Blood sampling

5 ml of venous blood from septic neonates and the control are collected. 2 ml of blood was transferred into EDTA tube to perform CBC and blood culture. While, 1 ml was transferred into a plain tube to perform (CRP). Likewise, 2 ml was transferred into a plain tube and centrifuge at 15000 rpm for 10 min at room temperature to precipitate cell debris, then the supernatants were stored at -80 °C until the RNA extraction. The CBC was done by Sysnex XN-1000 (Japan (19723), B.M EGYPT company).

3.2. Real-time PCR

Total RNA including miRNA were isolated by using RNeasy mini kit (QIAGEN, USA). Once purification of microRNA then quantified by NanoDrop® N50 nanophotoemter Implen GmbH and Implen,Inc. Schatzbogen 5281829 Munich, Germany to estimate both RNA concentration and purity. The isolated miRNA was kept at -80 until transcription step.

microRNA was reverse transcribed (RT) synthesizing single-stranded cDNA by the Qiagen® miScript II RT Kit. RT master mix incorporated in a nuclease-free micro centrifuge tube which prepared on ice as the following: 4 μl 5 × miScript HiSpec Buffer, 2 μl 10 × miScript Nuclease Mix, 2 μl RNase-free water, 2 μl miScript Reverse Transcriptase Mix, then a 10 μl Template RNA. The incubation was done at 37 °C for 60 min and 95 °C for 5 min by using Biosystems 2720 thermal cycler (Bioline, Singapore, USA). cDNA product has stored at -20 °C till real-time PCR step. Until amplification, cDNA samples were diluted to 5 ng/ul for real-time quantitative PCR estimation. Diluted cDNA developed by the miScript II RT Kit was used as a prototype for real-time PCR with Qiagen's miScript SYBR Green PCR Kit. Preparation of reaction mixed by: 12.5 μl 2x QuantiTect SYBR Green PCR Master Mix, 2.5 μl 10x miScript Universal Primer, 2.5 μl 10x miScript Primer focused on mRNA sequences (miRNA-15b and miRNA-378a), 2.5 μl Template cDNA and 3.5 μl RNase-free water. Program Real-time cycler Applied Biosystems ® 7500 thermal cycler (Applied Biosystems, Foster City, CA, USA) Under the following conditions: 95 °C for 15 min (initial denaturation step), denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, extension for 30 s at 70 C° for 40 cycles. U6-small RNA amplification was conducted with each experimental sample as endogenous control.

Comparative 2−Ct equation with Applied Biosystems 7500 software version 2.0.1 demonstrated relative quantification (RQ) amounts. Where the aim level miRNA-15b and miRNA-378a were normalized to a limited and control-related endogenous model U6. Using melting curve analysis to validate the accuracy of amplification and unavailability of primer dimers, each run was completed.

3.3. Statistical analysis

Data interpretation were performed by SPSS v25 (SPSS Inc., Chicago, IL, USA). Shapiro-wilks test used to verify the normality of distribution. Quantitative parametric variables were presented as mean and standard deviation (SD). They were compared between the two groups by unpaired student's t-test and within the same group by paired T test. Quantitative non-parametric variables were presented as median and range and compared between the two groups by Mann Whitney (U) test and within the same group by Wilcoxon test. P value < 0.05 was considered significant.

Mann Whitney test for abnormally distributed quantitative variables, to compare between two studied groups. Spearman coefficient To correlate between two distributed abnormally quantitative variables. Receiver operating characteristic curve (ROC) generated by plotting sensitivity (TP) on Y axis versus 1-specificity (FP) on X axis at different estimated values. The area under the ROC curve indicate test

performance. for the test area more than 50% reveals significant performance and area about 100% reports the best performance. The ROC curve permits also a comparison of performance between two tests. Sensitivity, the capacity of the test to surely identify diseased cases in a population "TRUE POSITIVES". If the sensitivity is high, the unidentified cases "false negatives" is low. Specificity, the power of the test to surely reject cases who are normal "TRUE NEGATIVES". If specificity is high, "false positives" cases is low. Positive Predictive value (PPV) The probability of the disease being present, among those with positive diagnostic test results. Negative Predictive value (NPV) The probability that the disease was absent, among those whose diagnostic test results were negative.

4. Results

Analysis of data obtained in this study revealed no significant difference between all septic neonate and control regarding to age, sex, maternal age, maternal diseases, maternal medications, family history, mode of delivery and gestational age. On the other hand, patients with sepsis had statistically significant higher heart rate, respiratory rate and temperature (Table 1).

Regarding laboratory characteristics, It was found that patients with sepsis had significantly lower platelet count and higher CRP levels. As regard to the leading causes of sepsis, our results showed that the most commonly encountered diagnosis in the present study was respiratory tract pathogens followed by gastrointestinal pathogens (Table 2).

4.1. Expression of miRNA-15b in serum

There was statistically significant difference observed between both groups in terms of miRNA15b ($p < 0.001$). Patients with sepsis had significantly higher level of miRNA15b than the healthy volunteers (7.49 ± 4.71 versus 1.38 ± 0.75, respectively) (Table 3) (Fig. 1).

4.2. Expression of miRNA-378a in serum

Likewise, patients with sepsis had significantly lower level of MiRNA-378a than the healthy volunteers (0.32 ± 0.24 versus 0.82 ± 0.19, respectively) (Table 4) (Fig. 2).

The ROC curve showed that the MiRNA-15b was a significant discriminator of sepsis with a combined sensitivity and specificity of 76% and 88% with cutoff point of 3.24. In addition, MiRNA-378a was a significant discriminator of sepsis with a combined sensitivity and specificity of 60% and 88% with cutoff point of 0.361 (Table 5)(Fig. 3).

In our result, The miRNA 15b expression significantly correlated positive with respiratory rate ($r = 0.415$, $p = 0.039$), WBCs ($r = 0.408$, $p = 0.043$), and CRP ($r = 0.407$, $p = 0.043$). Likewise, miRNA 378a expression significantly negative correlated with respiratory rate ($r =$

Table 1
The baseline characteristics of the included patients.

Variable	Cases (n =25)	Control (n =25)	P-value
Male, No. (%)	12 (48%)	11 (44%)	0.777
Age (days), mean ± SD	1.72 ± 2.11	2.40 ± 3.06	0.207
Gestational Age (days), mean ± SD	37.80 ± 0.50	37.80 ± 0.41	0.94
Maternal Age (Years), mean ± SD	22.12 ± 3.55	24.04 ± 3.87	0.074
Maternal diseases	2(8%)	0.0	0.49
Maternal Medications	2(8%)	0.0	0.49
Family History, No. (%)			
Consanguinity	3 (12%)	5 (20%)	0.702
Mode of delivery, No. (%)			
CS	22 (88%)	22 (88%)	———
VD	0	0	
Heart rate (Bpm), mean ± SD	102.0 ± 15.0	112.80 ± 13.16	0.009
Respiratory rate/min, mean ± SD	49.68 ± 12.10	35.56 ± 6.02	0.001
Temperature, mean ± SD	37.39 ± 0.61	36.88 ± 0.60	0.001

Table 2
Comparison between the two studied groups according to laboratory data.

	Cases (n = 25)		Control (n = 25)		p
WBC, mean ± SD	13.90 ± 7.30		11.84 ± 3.32		0.587
HB, mean ± SD	12.31 ± 2.01		13.71 ± 3.27		0.047
PLT, mean ± SD	202.0 – 214.46		288.79 ± 179.03		0.023
CRP, mean ± SD	92.39 ± 71.97				—
Blood culture, No. (%)					
No growth	2	8.0	25	100.0	<0.001*
Gram -ve	22	88.0	0	0.0	
Gram + ve	1	4.0	0	0.0	
Bacilli most probably klebsiella	2	8.7	0	0.0	–
Bacilli mostly klebsilla	12	52.2	0	0.0	–
Bacilli most probably enterobacter	4	17.4	0	0.0	–
Most probably enterobacter	1	4.3	0	0.0	–
Bacilli mostly E coli	2	8.7	0	0.0	–
Multidry resistance terobacter	1	4.3	0	0.0	–
Most probably aphylococcus	1	4.3	0	0.0	–

Table 3
Comparasion between two studied groups according to RQ miR (15b).

RQ (15b)	Cases (n = 25)	Control (n = 25)	U	p
Min. – Max.	1.55 – 18.57	0.81 – 3.36	22.0*	<0.001*
Mean ± SD.	7.49 ± 4.71	1.38 ± 0.75		
Median (IQR)	7.30(3.25 – 10.67)	1.17(0.94 – 1.40)		

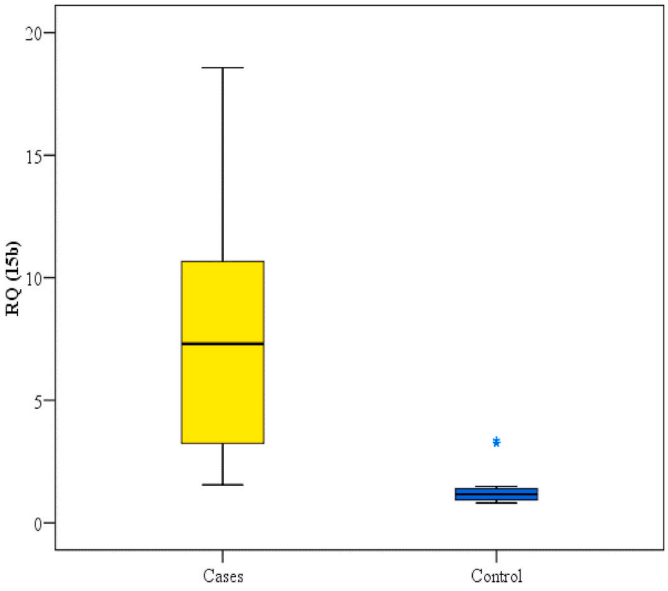

Fig. 1. Comparison between the two studied groups according to RQ of miRNA (15b).

Table 4
Comparasion between two studied groups according to RQ miR (378a).

RQ (378a)	Cases (n = 25)	Control (n = 25)	U	P
Min. – Max.	0.02 – 0.68	0.34 – 1.0	30.0*	<0.001*
Mean ± SD.	0.32 ± 0.24	0.82 ± 0.19		
Median (IQR)	0.29(0.15 – 0.58)	0.86(0.81 – 0.92)		

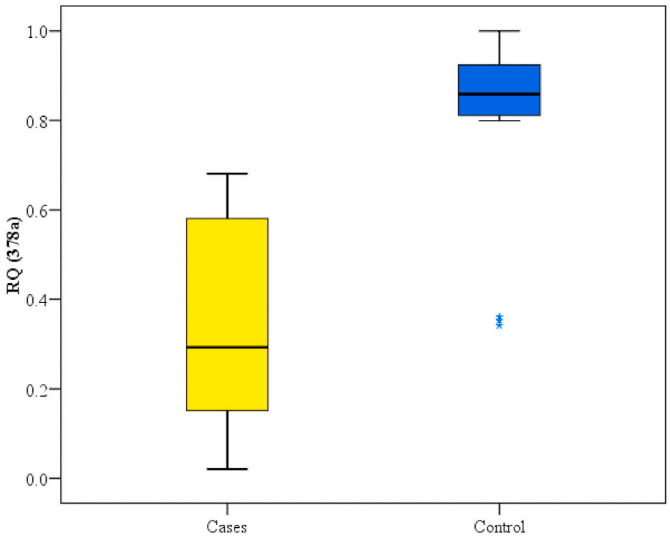

Fig. 2. Comparison between two studied groups according to RQ of miRNA (378a).

-0.415,p = 0.024), WBCs (r = -0.442, p = 0.027) and CRP (r = -0.459, p = 0.021) (Table 6).

5. Discussion

Neonatal sepsis is a clinical condition that requires prompt diagnosis and management [2]. Biomarkers play a crucial role in sepsis pathophysiology, they can demonstrate the involvement, absence or seriousness of sepsis and can distinguish from viral and fungal infection bacteria as well as from local systemic sepsis [6]. Unfortunately, the current established biomarkers are limited by low diagnostic accuracy and delayed diagnosis.

MicroRNAs (miRNAs) are non-coding single-stranded RNAs with a post-transcription gene silencing ability. They progressively exact multiple intracellular incidents including the signalling cascade of Toll-like receptors (TLRs) to prevent unnecessary inflammation following infection. In addition, unusual miRNA expression leads to septic association with its clinical common signs and symptoms [8].

Therefore, we performed the present study to assess the role of miRNA 15b and miRNA 378a as diagnostic and prognostic biomarkers for neonatal sepsis.

With regard to clinical data, the mean age of the included patients was 1.72 ± 2.11; while the majority of them were females (52%). There was no statistically significant difference observed between both groups. In line with our findings, (Arowosegbe et al., 2017) [15] reported that the mean age of majority of neonates with sepsis was less than 3 days and also majority of them were females.

On contrary to our finding, (Guo et al., 2019) [16] concluded that the majority of patients with neonatal sepsis were males (66%) and had late-onset sepsis. The difference between our findings and (Guo et al., 2019) can be explained by the variations in the characteristics and demography of the included patients. The largest of sample size may be another cause.

According to maternal factors, It was found no significant difference between septic cases and controls in terms of maternal age, maternal diseases, maternal medications, maternal exposure to radiation or smoking, fever in pregnancy, previous abortion, still birth and family history (p > 0.05). These results were in accordance with (Husada et al., 2020) findings [17] and on the contrast with (Siakwa et al., 2014) [18] who showed that the maternal factors were significantly associated with neonatal sepsis.

In terms of natal history, our results showed a statistically significant association between resuscitation at birth and neonatal sepsis (p =0.001). Additionally, there were no statisitical significant differences between septic cases and controls in terms of mode of delivery, complications of deliver, and gestational age. In agreement with our findings, (Adatara et al., 2019) [19] demonstrated that neonatal risk factors, which suspect the incidence of sepsis, were low APGAR score indicating severe disease (p < 0.001) and resuscitation at birth (p < 0.004). Likewise, (Soman et al., 1985) [20] demonstrated a strong association between resuscitation at birth and higher incidence of neonatal sepsis (OR

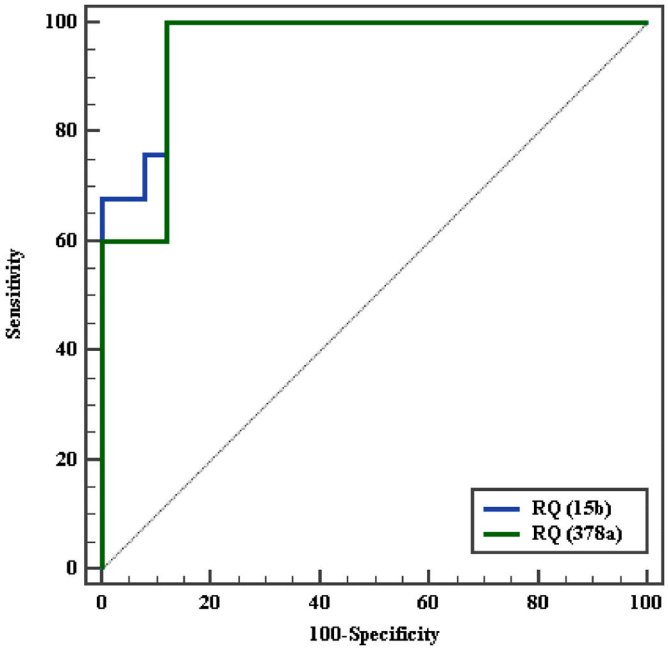

Fig. 3. ROC curve for different parameters to predict cases (vs.)control.

Table 6
Correlation between RQ (15b) and (378a) with different parameters in cases group (n = 25).

	RQ (15b) (Up)		RQ (378a) (Down)	
	rs	p	rs	p
Heart rate	-0.055	0.796	-0.132	0.529
Respiratory rate	0.415	0.039*	-0.451	0.024*
Temperature	0.375	0.065	0.027	0.899
Weight	0.367	0.071	0.305	0.138
RD downes score	0.099	0.638	0.181	0.388
WBC	0.408	0.043*	-0.442	0.027*
HB	-0.043	0.836	-0.056	0.792
PLT	-0.197	0.344	-0.321	0.118
CRP	0.407	0.043*	-0.459	0.021*

Table 5
Agreement (sensitivity, specificity) for different parameters to predict cases (vs control).

	AUC	P	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
RQ (15b)	0.965	<0.001*	0.920 – 1.0	>3.24	76.0	88.0	86.4	78.6
RQ (378a)	0.952	<0.001*	0.895 – 1.0	≤0.361	60.0	88.0	83.3	68.7

AUC: Area Under a Curve. CI: Confidence Intervals NPV: Negative predictive value PPV: Positive predictive value.

= 36.25, p less than 0.001).

Our study found that patients with sepsis had statistically significant higher heart rate (tachycardia), respiratory rate (tachypnea), respiratory distress and temperature (fever). In addition, patients with sepsis were more likely to have cyanosis and pallor. In line with these findings by (Khassawneh et al., 2009) [21] who reported that the respiratory distress, metabolic acidosis and requirement of ventilation were found in 74.7%, 40.5%, and 58.2% of patients with neonatal sepsis, respectively. Hypotension was found in 22.9% of patients. (Mai et al., 2010) [22] showed that the clinical presentations of neonatal sepsis included fever, tachypnea, apnea and feeding intolerance.

In the present study, we found that statistically significant difference between septic cases and control in term of Downe score ($p < 0.001$). In accordance with our results, (Buch et al., 2013) [23] reported that neonatal chest examination, which predicted the occurrence of sepsis were Downe score >6 (p=0.0003).

Traditional blood markers for pediatric sepsis includes white blood cells (WBC), neutrophils count, CRP, and PCT (Lanziotti et al., 2016) [24]. On the other hand, low platelet count is a manifestation of sepsis and the thrombocytopenia 's magnitude is normally commensurate with that of infectious disease (Bhat et al., 2018) [25].

Our results reported that patients with sepsis had significantly lower platelet count. In addition, septic cases had statistically significant higher CRP levels. Similar to our findings, (Choudhary et al., 2018) [26]. They demonstrated that sepsis/bacteremia group patients had lower platelets count and higher CRP. This was also in agreement with similar studies done by (Sindhura and Reddy, 2017) [27] and (Mondal et al., 2012) [28].

With regard to study outcomes, our results showed that the patients had significantly higher level of MiRNA-15b than the healthy volunteers. On the other hand, patients with sepsis had significantly lower level of MiRNA-378a than the healthy volunteers. In line with our finding, (wang et al.,.2012) [29]. Investigated in adult population, the diagnostic role of serum MIRNAs in sepsis patients. They found that the expression level of miR-15b were significantly higher in patients with mild sepsis ($p < 0.01$) and patients with severe sepsis and septic shock ($p < 0.05$) than in normal control.

On the contrary to our findings, (Wang et al., 2015) [30] demonstrated that there was no statistical difference in the miRNA-15b level between septic neonates and healthy ones. However, the authors were in line with our finding that miR-378a was down regulated significantly with statistical difference. The difference between our findings and (Wang et al., 2015) can be explained by the variations in the characteristics and demography of the included patients, the largest sample in their study is another cause. Also, The difference in healthy status of control patients between two study may be another cause. In which they enrolled 41 control patients (17 patients with supper respiratory infection and 24 patients with pneumonia). While, our control study enrolled 25 full term neonates with no evidence of neonatal sepsis.

As regard to our study outcomes, It was found that the miRNA 15b expression had statistically significant positive correlation with respiratory rate (rs= 0.415, p=0.039), WBC (rs= 0.408, p=0.043) and CRP (rs=0.407,p=0.043). Likewise, miRNA 378a expression had statistically significant negative correlation with respiratory rate (rs= -0.451, p=0.024), WBC (rs= -0.442,p= 0.027) and CRP (rs= -0.459,p= 0.021).

Up to our knowledge, no previous studies made correlation between miR-15b, miR-378a and different variables related to neonatal sepsis.

Our research has some drawbacks including a comparatively limited number of patients and a single-center sample. A broader multi-centered research is also proposed for potential studies in future.

6. Conclusion

Both MiRNA 15b and 378a are promising biomarker for neonatal sepsis. The present study showed, In sepsis patients MiRNA 15b raised and revealed a further significant elevation in extreme sepsis and septic shocks patients. . On the other hand, the MiRNA 378a appears to be decreased in neonates with sepsis. However, more large-scale research are also needed to validate our results. It will be much more relevant to research the kinetics of the expression levels of these two miRNAs in septic therapy.

Funding source

Nil.

Declaration of competing interest

Nil.

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