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MicroRNAs as Potential Diagnostic New Biomarkers in Diagnosis of Sepsis in Pediatric Patients

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Abstract

Background: Sepsis is one of the most common causes of multiorgan failure. Sepsis requires the presence of infection with a resultant systemic inflammatory state. Organ dysfunction occurs from the combination of the two processes. Sepsis is the main cause of mortality at intensive care units, with 30-50% mortality rate for all septic episodes. MicroRNA (miRNA) profile data could be beneficial as a specific diagnostic biomarker for sepsis and systemic inflammatory response syndrome (SIRS).

Methods: Expression of miRNAs -122, -181b, -223 and -146a levels were assayed by quantitative real time polymerase chain reaction (qRT-PCR) in a prospective case control study, where forty septic cases were compared to 40 healthy controls of matched age and gender.

Results: miRNAs -122 and -181b were significantly upregulated during early septic conditions, indicating that they could be sensitive and specific biomarkers for diagnosing sepsis. miRNA-223 and miRNA-146a could also represent highly specific and sensitive diagnostic biomarkers, as they were found to be significantly down-regulated. Serum levels of miRNA-223 could be used to predict poor prognosis with 70% sensitivity and 75% specificity, whereas the other three miRNAs could not predict prognosis.

Conclusions: Our study shows that all tested miRNAs can be used for early detection of sepsis, with miRNA-223 being predictive of mortality, hence preventing multi-organ failure and reducing mortality, and predicting poor outcomes, thereby assisting in early categorization of ICU patients for rapid appropriate treatment and medico legal aspects.

Keywords: Micrna-223, Systemic inflammatory response syndrome (SIRS), Sepsis, Biomarker.

Introduction

Sepsis diagnosis requires the presence of infection with a resultant systemic inflammatory state. Organ dysfunction occurs from the combination of these two processes (1). Globally there are approximately 1.2 million cases of pediatric and 3 million cases of neonatal sepsis, annually, results in significant morbidity and mortality (2). Since 2017 the World Health Organization (WHO) recognized sepsis as a global health problem (3), and adopted a resolution on improving the prevention, diagnosis, and management of sepsis (4).

The sepsis criteria are not specific enough for prompt diagnosis and prediction of outcomes (5). The signs and symptoms of sepsis mimic those of non-infectious Systemic inflammatory response syndrome (SIRS), and conventional diagnostic tools are inadequate to differentiate between both conditions (6). Differentiating SIRS from infection is essential for an accurate and swift diagnosis of

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sepsis, hence prompt and appropriate treatment, (7) also avoiding the unnecessary random use of antibiotics and emergence of antibiotics resistant strains of bacteria (8). Specific tests for sepsis diagnosis and/or mortality prediction, lack sufficient sensitivity and specificity (9). Hence, identifying specific, sensitive, accurate, and rapid biomarkers for differentiating sepsis from noninfectious SIRS, improving the prediction of mortality, especially in the early phase of sepsis when levels of certain pro-inflammatory cytokines and proteins are elevated (7).

C-Reactive Protein (CRP) is an acute phase inflammatory protein synthesized in the liver in response to infection, commonly used diagnostic indicator of sepsis. The high sensitivity CRP (hsCRP) level in the serum is increased under stress conditions, thus accurately reflecting the inflammatory response state of the body. (10) hsCRP assays could detect lower grade of inflammation (11).

MicroRNAs (miRNAs) are single-stranded RNA molecules approximately 22 nucleotides (nt) in length and more than 1000 miRNA genes have been annotated for the human genome (12). miRNAs are expressed in different tissues and cell types and dysregulated expression of these small RNAs have significant impact on health and disease. Also, over 1900 miRNAs have been reported to have critical regulatory functions (13). miRNA transcripts are generated and form the precursor miRNA (pre-miRNA) in the nucleus. The pre-miRNA is transported to the cytoplasm by the nuclear transport receptor exportin-5 and the nuclear protein Ran-GTP. It is then cleaved by Dicer to produce a short duplex molecule of mature miRNA of about 22 nt. These molecules are loaded into a member of the Argonaute protein subfamily and form the miRNA-induced silencing complex (RISC) (14). miRNAs regulate the expression of at least half the human transcriptome. The silencing mechanism may be determined by the extent of base pairing between the miRNA and the target mRNA when miRNA binds to the complimentary target sites located in the 3'untranslated region of the target mRNA (15).

The dysregulated expression of miRNAs in the immune system can contribute to the disease pathogenesis and may be critical for immune responses to pathogens such as viruses and bacteria (16). Some serum miRNAs, including miRNAs-15a, 16, 223, 146a, 499-5p, 122, and 193b, were identified as the biomarkers for the diagnosis of adult sepsis (17). In addition, serum miRNAs-223, 15a, 16, 122, 193, 483-5p, and 574-5p were significantly differentially expressed between survivors and non-survivors, so they were identified as prognostic predictors for sepsis patients (18). miRNAs are considered perfect biomarkers, as they are stable in many body fluids like serum, can be measured by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), and have proved to be sensitive and specific biomarkers for the diagnosis of many disease conditions including sepsis (19).

miRNAs are increasingly recognized to play an important role in the post-transcriptional regulation of gene expression. They play key roles in a wide variety of biological processes, including cell differentiation and growth, development, metabolism, cell signaling, apoptosis, and disease processes linked to cancer and inflammation (20).

Materials and Methods

The study protocol was approved by the Ethical Committee of Peditarics' department, Faculty of Medicine, Kasr Alainy Hospital, Cairo University, Egypt, the approval was taken from patients' legal guardians, the written informed consent was voluntary obtained from each participant upon explanation of the study rules and procedures. The study was conducted following the ethical guidelines of the declaration of Helsinki, October 2013.

This is a prospective case control study which was conducted upon 40 critically ill pediatric patients (1 – 24 months) at Cairo University Children's hospital who were enrolled in this study with various grades of sepsis including sepsis, severe sepsis, septic shock, and multiple organ dysfunction syndrome (MODS)and 40 age matched healthy

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controls from pre-operative general pediatric surgery clinics. Patients included showed either clinical and/or laboratory evidence of sepsis; infection associated with at least two of the following abnormalities: Temperature (> 38.5 ºC or< 36 ºC), Tachycardia, (Mean heart rate> 2 SD for age), Tachypnea (Mean respiratory rate> 2 SD for age), Leucocytosis, leucopenia or bandemia (> 10%) or Severe sepsis defined as either evidence of end-organ dysfunction (e.g., altered mental status, episode of hypotension, elevated creatinine, or evidence of disseminated intravascular coagulopathy) or Septic shock (persistent hypotension despite adequate fluid resuscitation or tissue hypo perfusion), those who received any treatment were excluded. Blood samples were collected from eligible cases on day one of intensive care unit (ICU) admission

Blood sampling

Two mL peripheral blood sample were withdrawn from all cases and controls by venipuncture in dry sterile vacutainer tube then into plain tube for serum separation to be used in C-Reactive Protein (CRP) quantitation by ELISA technique, RNA extraction, and real time PCR for microRNAs.

Measurements of High Sensitivity C-Reactive Protein by ELISA Assay

A high Sensitivity CRP kit (apDia bvba, Belgium) was used. Microtiter strips coated with anti-CRP antibody were incubated with diluted standard sera and patient samples allowing CRP to bind, unbound proteins in sera were removed by washing. The antigen-antibody complex was detected with specific peroxidase-conjugated antibodies, a blue color developed in proportion to the amount of immunocomplex bound to the wells of the strips after incubation with a chromogen solution containing tetramethylbenzidine and hydrogen peroxide. The absorbance values were determined at 450 nm, then plotted versus the corresponding standard values, standard curve was obtained from which CRP concentrations were interpolated.

Estimation of miRNA-122, miRNA-181b, miRNA-223 and miRNA-146a in serum RNA Extraction

Total RNA with preserved micro-RNAs were extracted using miRNeasy extraction kit (Qiagen, USA) following manufacturer's protocol. The extracted micro-RNAs were stored at -80 °C until use. RNA purity and quantity was assessed by using the NanoDrop® (ND)-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA) following manufacturer's protocol.

Reverse transcription (RT) and real-time quantitative PCR (qPCR)

Reverse transcription was carried out on total RNA in a final volume of 20 µL RT reactions (incubated for 60 min at 37 °C, followed by 5 min at 95 °C) using the miScript II RT kit (Qiagen, USA Cat. No. 218161) according to the manufacturer's instructions.

Real-time qPCR was performed using a MiScript SYBR Green PCR kit (Qiagen, USA Cat. No. 218073) and miScript primer assay miRNAs-122 F: 5'-GTGACAATGGTGGAATGTGG-3', R: 5'-AAA GCAAACGATGCCAAGAC-3', -181b F: 5′-TGCGGAACATTCATTGCTGTC-3′ R: 5′-CCAGTGCAGGGTCCGAGGT-3′, -223 F: 5′-AGC CGT GTCAGTTTG TCAAAT-3′; R: 5′-GTGCAGGGTCCGAGGTC-3′ and -146a F: 5′-TGAGAACTGAATTCCATGGGTT-3′ and R: 5′-TGAGCTGAGAACTGAATTCCATG-3′. 20 ng of cDNA were used as a template in a total volume of 20 µL reaction with the following conditions: denaturation at 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 34 s, in which fluorescence was acquired and detected by Rotor-gene Q Real-time PCR system (Qiagen, USA). After the PCR cycles, melting curves analyses were performed to validate the specific generation of the expected PCR product. SNORD F: 5'-ATCACTGTAAAACCGTTCCA-3' R: 5'-GAGCAGGGTCCGAGGT-3' was used as an

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endogenous control. The expression levels of miRNAs -122, -181b, -223 and -146a were evaluated using the ΔCt method. ΔCt was calculated by subtracting the Ct values of SNORD from those of target micro-RNAs. ΔΔCt was calculated by subtracting the ΔCt of the control samples from the ΔCt of the samples. The fold change in miRNAs -122, -181b, -223 and -146a expression was calculated by the equation 2–ΔΔCt.

Statistical analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 24. Data was summarized using mean, standard deviation, median, minimum, and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests. For comparing categorical data, Chi square ($\chi 2$) test was performed. Exact test was used instead when the expected frequency is less than 5. Correlations between quantitative variables were done using Spearman correlation coefficient. ROC curve was constructed with area under curve analysis performed to detect best cutoff value of miRNAs and CRP for detection of sepsis and mortality.

Results

Description and analysis of demographic data

Forty cases diagnosed with sepsis were recruited from the ICU at Kasr Alainy children's hospital (Abou Elrish), Cairo University with ages ranged from 1-24 months, 24 males (60%) and 16 females (40%) compared to 40 healthy controls from preoperative general pediatric surgery clinics aged 2-24 months, 27 males (67.5%) and 13 females (32.5%), there was no significant difference between the studied groups according to gender and sex.

Analysis of clinical data in correlation to sepsis

About half of the septic cases (45%) were caused by pneumonia, 2nd cause was encephalitis (17.5%), other causes ranged between gastroenteritis, postoperative, disorders of metabolism, and heart failure. 60% of cases needed inotropic support and 72.5% of cases were on mechanical ventilation, 45% of the cases had acidosis, 37.5% had elevated liver functions, 30% of the cases had prolonged bleeding profile, and 20% had elevated renal functions. 80% of cases showed leukocytosis. 35% had thrombocytosis and 32.5% had anemia. Different cultures (blood, sputum, CSF, stool, wound swab) that had been collected from cases revealed *Klebsiella* as the causative pathogen in 30% of cases, *Staphylococcus aureus* in 27.5 %, *pseudomonas* in 15% and, Candida in 12.5%.

Analysis of studied miRNAs in correlation to clinical data

We found a significant statistical correlation a miRNA-122 and miRNA-181b and the age of the patients where their serum levels decrease with as age increases and vice versa (Fig. 1).

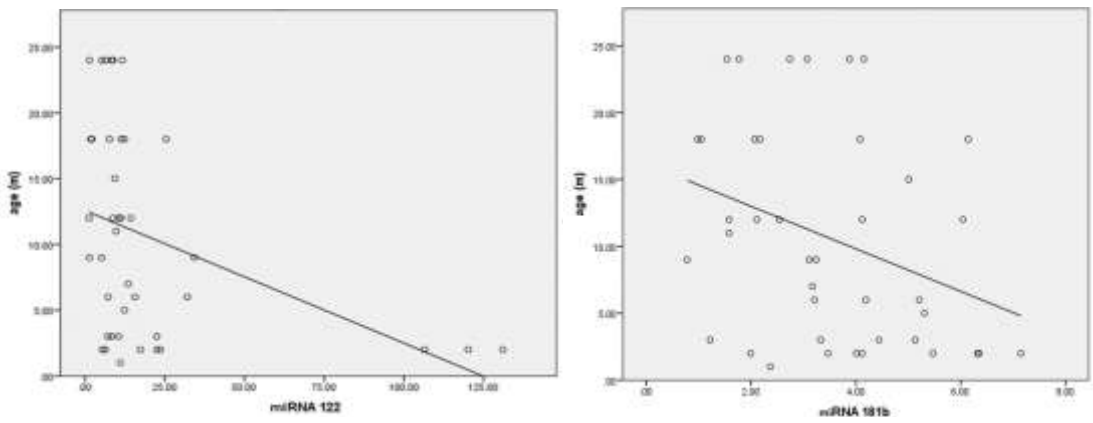

Fig. 1. Reverse correlation between miRNA-122 and miRNA-181b and age, *m: month.

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On the other hand, there was no statistical correlation between levels of the four studied miRNAs and CRP level with neither temperature nor the length of stay in ICU. Only miRNA-181b showed significant relationship with gender, where its level was higher in females than males. The serum levels of hsCRP and the four miRNAs showed no correlation with the different primary diagnoses, causative organisms, inotropic support, mechanical ventilation, hypotension, disturbed conscious level, PT, PTT, INR, leukocytosis, thrombocytosis, and anaemia. miRNA-188b was the only miRNA significantly higher in patients with acidosis and those with hepatic impaired functions, while miRNA-122 was the only miRNA that showed significant lower levels in impaired renal functions cases. Mortality in cases was 60% while 40% improved and discharged, there was no relation between the outcome and the length of stay in ICU, miRNA-223 level was significantly lower in patients who died later.

High Sensitivity CRP (hsCRP) was significantly higher in cases than control with median= 30 (1.19-96). miRNAs-122, miRNA-181b, miRNA-223 and miRNA-146a were significantly higher in cases compared to controls with medians= 10.56 (1.23-13.1),= 3.28 (0.78-7.15),= 0.48 (0.05-1.10) and= 0.15 (0.01-0.47), respectively, (Fig. 2).

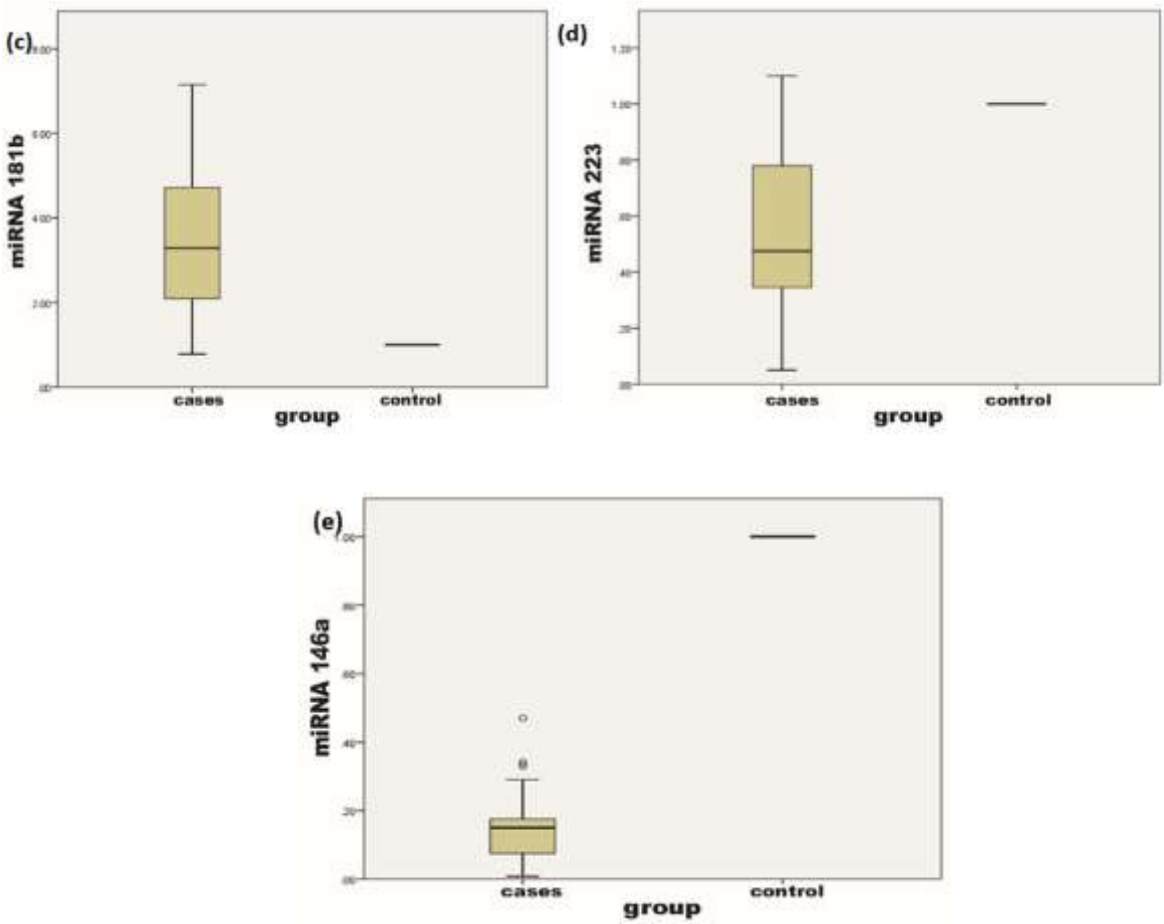

Fig. 2. (a) hsCRP,(b) miRNA-122, (c) miRNA-181b, (d) miRNA-223 and (e) miRNA-146a *cases: patients compared to controls.

In relation to each other, there were high significant positive correlations between miRNAs -223 and -146a serum levels r= 0.415 (p< 0.008) and between miRNAs -181b and -122 serum levels r= 0.728 (p< 0.001), while miRNA-181b increases with increase serum level of CRP, r= 0.329, (p< 0.0038) (Fig. 3).

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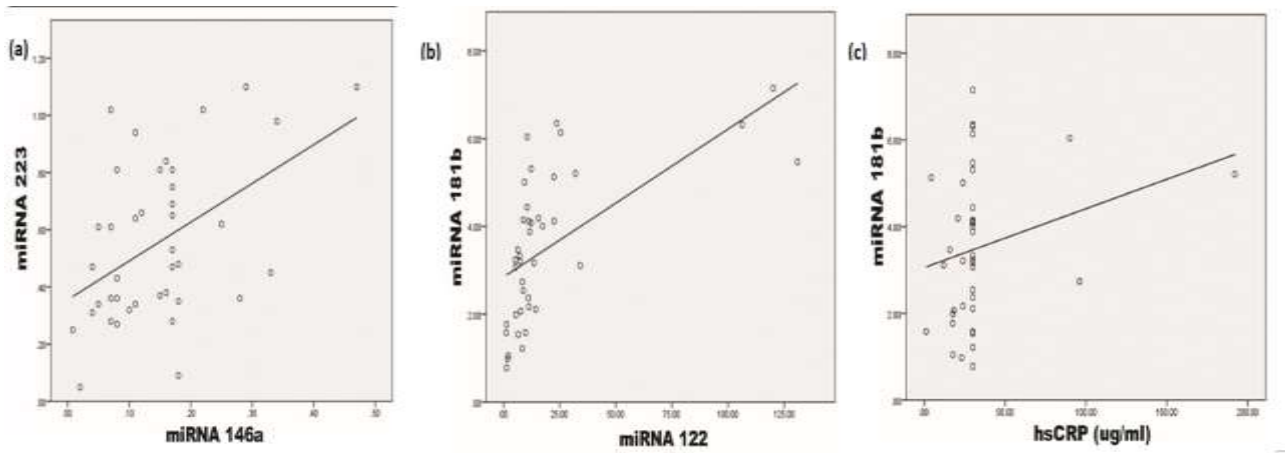

Fig. 3. Correlation between **(a)** miRNAs -223 and -146a, **(b)** miRNAs -181b and -122 serum, and between **(c)** hsCRP and miRNA 181b.

Analysis of studied miRNAs in sepsis diagnostics

hsCRP showed sensitivity 92.5%, specificity 92.5% and AUC was 0.95, cut off= 15.04. miRNA-122: AUC: 1.00, cut off 1.29, sensitivity 97.5% and specificity 100%, miRNA- 181b: AUC 0.95, cut off 1.38, sensitivity 92.5% and specificity 100%. miRNA-223: AUC 0.9, cut off 0.89, sensitivity 85% and specificity 100%. miRNA-146a: AUC 1.00, cut off 0.73, sensitivity= 100% and specificity 100% (Fig. 4).

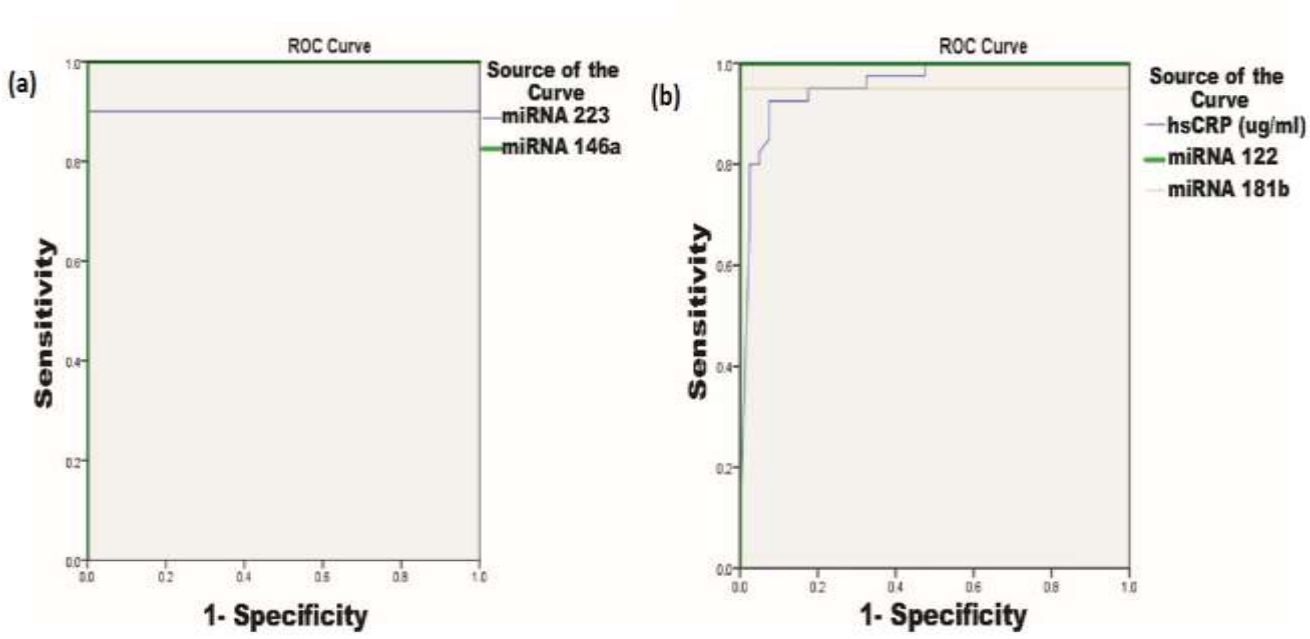

Fig. 4. Specificity and sensitivity of **(a)** miRNAs -223 and -146a**, (b)**miRNAs -181b and -122 as per diagnosis of sepsis.

Analysis of studied miRNAs in sepsis prognostics

hsCRP: AUC= 0.448, (p value= 0.58), miRNA-122: AUC= 0.54, (p value 0.619) and miRNA-181b: AUC= 0.658, (p value= 0.095), therefore they are not capable of prediction of poor prognosis or mortality in sepsis condition, while miRNA- 223 showed AUC 0.73, (p value= 0.015, cut off 0.50, sensitivity 70.8% and specificity 75%. miRNA-146a showed AUC 0.53, (p value= 0.699). Therefore, serum levels of miRNA-223 can predict mortality in sepsis with sensitivity 70% and specificity 75%, whilst level of miRNA-146a cannot predict poor prognosis or mortality (Fig. 5).

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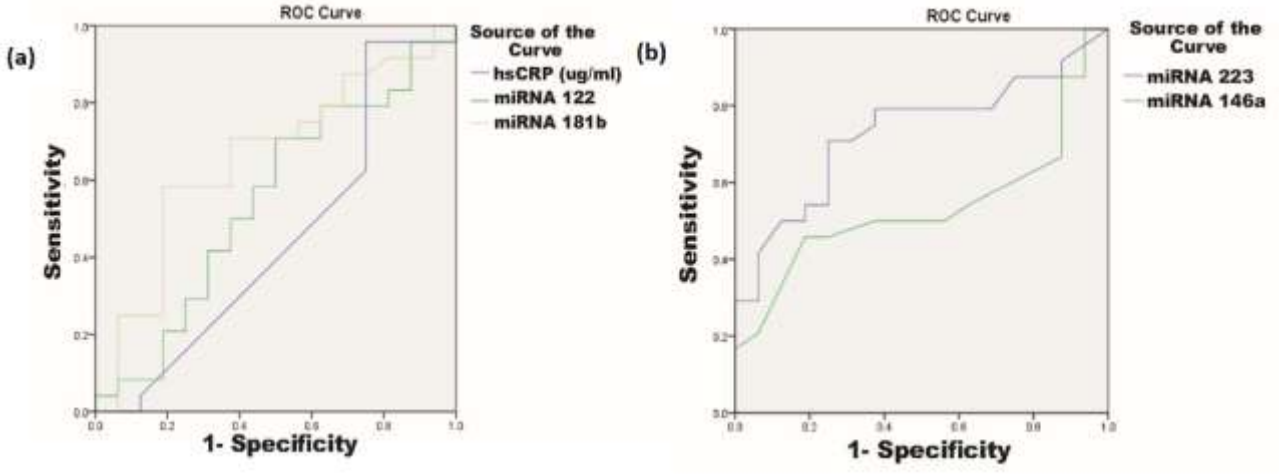

Fig. 5. Specificity and sensitivity of (a) miRNAs -223 and -146a, (b) miRNAs -181b and -122 as per prognosis of sepsis.

Discussion

Sepsis is a significant health problem with high mortality rate and long-term morbidity (21). Since the discovery of circulating miRNAs in human peripheral serum, these are used as biomarkers of various cancer types (22). MicroRNAs are type of biomarkers, which were found to act as post -transcriptional regulators of gene expression (23). We investigated the expression levels of miRNAs (122, 181b, 223 and 146a) profiles in serum samples in septic and healthy children from a developing country, Egypt. A different subset of miRNAs is dysregulated in neonatal sepsis compared to reports of adult sepsis patients. Thus, comparing miRNA data arising from different sample types or measurement platforms must be considered cautiously (24). A study done in 2010, found that the serum levels of miRNA-146a and miRNA-223 were significantly reduced in septic patients compared with SIRS patients and healthy control subjects, then stated that those miRNAs can potentially serve as novel, highly sensitive, and specific biomarkers for sepsis (25). They had another study in 2012 where they screened 12 miRNAs, from which miRNA-122 was up-regulated, on the other hand, miR-223 was down-regulated in the non-surviving group compared to the surviving group, which goes along with our results that miRNA-223 and miRNA-146a were down regulated in septic patients (26). In contrary to our results a third study, described higher levels of miRNA-223 and lower levels of miRNA-122 in septic patients and suggested a direct association between high miRNA-223 serum levels and patient's outcome (17). Then, in 2014(14) a study recognized that myocardial dysfunction in septic patients was a major manifestation in severe sepsis and finally lead to death in critically ill patients. They performed a miRNA array analysis in mice hearts and found that 19 miRNAs were dysregulated, among which miRNA-223 (3p) and miRNA 223 (5p) were most significantly down regulated. They also validated that miRNA 223 negatively regulate the expression of STAT-3 and IL-6 in mice hearts. Collectively, loss of miRNA-223 caused an aggravation of sepsis induced inflammation, myocardial dysfunction, and mortality (14).

Another study showed in a cohort of 116 patients (43 with mild sepsis and 73 with severe sepsis/septic shock) that elevated miRNA-223 levels were indicative of the presence of septic disease and correlate with an impaired prognosis in these patients. On the other hand, in a cohort of 80 patients (30 with SIRS and 50 with sepsis) miRNA-223 levels were decreased in patients that fulfilled criteria of sepsis compared to healthy controls. They stated that despite the large number of samples analyzed, they failed to demonstrate significant alterations in serum miRNA-223

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concentrations in critical illness and sepsis and that might partly be explained by differences of experimental procedures in the different studies (27). Like our results, another study, 2015 reported that macrophages were stimulated during sepsis to up-regulate the expression of miRNA-181b. These findings coupled with the improved survival observed in miRNA-181b "rescue" studies in septic mice. They explained miRNA-181b upregulated levels by attributing it to the immune tolerance of IL-6. It may be due to down-regulation of IL-6, which might be an important regulatory mechanism for controlling pro-inflammatory immune response and induction of endotoxin tolerance (28). Against our results in 2013, another study identified patients with sepsis having reduced circulating plasma levels for miRNA-181b compared with control patients without sepsis and identified miRNA-181b as a cytokine-responsive miRNA which regulates the endothelial response to inflammation by regulating the NF-κB signaling pathway (29). As confirming to our results, a study in 2016 stated that miRNA-223 was down-regulated during sepsis which resulted in the promotion of pro-inflammatory cytokines, including IL-6 and IL-1b. In turn, IL-6 (triggered by TLR stimulation), was responsible for the downregulation of miRNA-223, thus forming a positive regulatory loop for pro-inflammatory cytokine production. miRNA-223 function in immune response has been studied as a potent negative regulator of inflammatory response (30). Discrepancies between previously published studies and our study could be explained by the differences in age, study design, sample size, geographic/ethnicity, by gene-gene or gene-environmental interactions.

Our data brought a novel observation of genetic determination for sepsis and finding a way for new biomarkers in diagnosing sepsis. Further studies are needed to identify new biomarkers for detection of severity and prediction of mortality in sepsis. It is supposed that genetic information will be used in the future by clinicians to define different subtypes of the diseases and to stratify patients according to the severity and outcome of the disease. Genotyping will be also used to determine optimal drugs and dosage for treating patients while minimizing adverse effects.

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