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ORIGINAL ARTICLE

Diagnostic value of microRNA-150 and microRNA-146a in critically ill patients with suspected sepsis

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ABSTRACT

Background: *Sepsis is a major cause of death; therefore, early diagnosis is crucial. A promising method for diagnosis is the use of specific miRNA as biomarkers for sepsis.* ***Objective:*** *This study aimed to validate the diagnostic value of miRNA-150 and 146-a as biomarkers for sepsis patients in different intensive care units in Alexandria, Egypt.* ***Methodology:*** *Blood samples from fifty sepsis patients from different intensive care units and twenty healthy volunteers were collected, inoculated onto aerobic culture bottle for culture and sensitivity, and sera was separated for quantification of miRNA 146-a and 150 using real-time PCR.* ***Results:*** *56% and 52% showed overexpression of miRNA 146-a and 150 respectively in sepsis patients. Their co-expression showed a moderate positive correlation in the studied group which was found to be statistically significant.* ***Conclusion:*** *Up to our Knowledge, this is the first study to associate between type of organism and expression of miRNA 146-a and 150.*

INTRODUCTION

Sepsis, a syndrome of physiologic, pathologic, and biochemical abnormalities induced by infection, is a major public health concern[1]. The European Society of Intensive Care Medicine guidelines in 2016 redefined sepsis as 'a life-threatening organ dysfunction caused by a dysregulated host response to infection[2]. It is clinically diagnosed by the presence of infection with signs/and symptoms of the systemic inflammatory response syndrome (SIRS)[3]. Although the true incidence is unknown, it remains a major lethal healthcare problem, with a reported mortality of >25%[4]. It is amongst the most common reasons for admission to intensive care units (ICUs) throughout the world [5]. The causative pathogens of sepsis include Gram-positive and Gram-negative bacteria, anaerobes, fungi, and parasites[6].

The increased percentage of patients suffering from sepsis imposed developing new rapid inexpensive methods with high specificity and selectivity for evaluation and monitoring treatment. At the moment there are a number of biomarkers for sepsis, mainly used in clinical laboratory analysis. The most used biomarkers for sepsis are procalcitonin (PCT) [7], C-reactive protein (CRP), and interleukin 6 (IL-6) [8]. The problem with these biomarkers is given by their low selectivity and specificity. Microbiological culture still represents the gold standard in distinguishing sepsis from other non-infectious diseases[9]; however, this technique is time-consuming and often related to false negative results. Recent studies call into question the use of new biomarkers for sepsis, such as microRNAs (miRNAs) [10]. The properties that miRNAs have in addition to the conventional biomarkers are their higher stability, selectivity, and specificity (11). Therefore, the aim of the present work was to validate the diagnostic and prognostic value of miRNA-150 and miRNA-146 a as biomarkers for patients with sepsis.

METHODOLOGY

Patients:

Fifty patients suffering from sepsis selected from the ICUs from Alexandria University hospitals and Hepatology ICU in Alexandria fever hospital were included in this study. Twenty control healthy volunteers matched by age and sex were also included in the study.

Sepsis was diagnosed by two or more of the following criteria: heart rate >90 beats/min, respiratory rate >20 breaths/min, temperature >38 °C or <36 °C, white blood cell >12000 cells/mm³ or <4000 cells/mm³.

Patients <10 years old, pregnant females, patients with traumatic and burn injuries, patients with cardiac dysrhythmias, acute and active gastrointestinal bleeding and acute drug overdose were excluded from this study.

The research study was approved by the Ethical committee of the Medical Research Institute,

Alexandria University. All patients were informed about the study and signed an informed consent.

Medical History:

All relevant information was collected from patients' hospital charts including personal data (age, sex) medical diagnosis at admission, lengths of stay in the hospital, antibiotics taken by the patients as well as any comorbidities (hepatic failure, active malignancy, diabetes mellitus) and immunodeficiency (AIDS, immunosuppressive drugs, chemotherapy, steroids).

Sample collection:

Blood (15 ml) was withdrawn from each patient using aseptic technique. An aerobic Bact/Alert blood culture bottle was inoculated by 10 ml of blood. The remaining 5 ml were allowed to clot, centrifuged, serum was separated, aliquoted and stored at -80ºC.

Identification and antimicrobial susceptibility testing of microorganisms:

Blood culture bottles were processed in Bact/ALERT 3D (bioMérieux SA, Marcy L'Etoile, France) automatic system at 35°C for at least seven days. Samples were monitored for bacterial growth by detection of fluorescent change[12]. Samples from positive blood culture bottles were withdrawn, sub-cultured on blood agar, MacConkey agar and Sabouraud Dextrose agar (SDA) plates which were incubated for 24 hours up to 48 hours. The isolated colonies were then further identified by Gram staining and biochemical tests. Antimicrobial susceptibility testing was carried out by Kirby-Bauer method. Antibiotics were chosen according to the Clinical and Laboratory Standards Institute [13].

Quantification of microRNA 150 and 146a using real-time PCR:

miRNA extraction was performed using miRNeasy Mini Kit (Qiagen) according to manufacturer's instructions. The concentration of RNA was determined using Nano Drop Thermo Spectrophotometry (Thermo Scientific, USA)

miRNA was reverse transcribed into cDNA using Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) according to manufacturer's instructions.

Real-time polymerase chain reaction (PCR) reaction mixture included 10 µl PCR master mix, 1 µl probe (146a or 150 and U6), 4 µl cDNA to a total volume of 20 µl. The reaction took place under the following thermal profile; initial incubation at 95°C for 10 min to activate the DNA polymerase, followed by 40 cycles of 2 PCR-step amplification, denaturation at 95° C for 15 sec, followed by annealing and extension at 60° C for 1 min, with real time fluorescence detection.

Relative expression of miRNA 150 and 146a calculated using the comparative CT method:

The method describes the change in expression of the target gene relative to reference gene. In the current study, the relative expression of the 'target genes" (miRNA 146a and 150) in sepsis patients are compared to a housekeeping gene (U6). The design is to use the $\mathbf{C_t}$ of samples of healthy controls as "calibrator", the C_t of samples from sepsis patients as "target" compared to the housekeeping gene (U6) as "reference". The relative gene expression is usually set to 1 for control group because ΔΔCT is equal to 0 and therefore 2^0 is equal to 1. Accordingly, a value less than 1 is considered down-expression while more than 1 is overexpression.

RESULTS

The present study included fifty patients suffering from sepsis and twenty control healthy volunteers matched by age and sex. Males represented 39 (78%) of patients while females were 11 (22%). The patients' age ranged from 34–79 years with a mean of 66.26. Patients stayed in the hospital for at least 4 days and a maximum of 16 with a mean of 9.28± 2.92 days.

As regards the patients' vital signs, the mean temperature was 38.84±1.54°C. Mean heart rate was 110.90±31.09 beat/minute and that of respiratory rate was 25.30±7.07 breath/minute. The mean white blood cell count was 35380± 8410 with a minimum of 20000 and a maximum of 55000 cells/mm^3.

Majority of patients 44(88%) were admitted to the ICU for medical reasons while only 6 (12%) were admitted after surgical interventions. Out of the 50 patients; 18 (36%) suffered from gastrointestinal tract infection and 16 (32%) suffered from blood stream infection, 7 (14%) from respiratory tract infections, skin and soft tissue infection in only 5 (10%) of patients and the least source of infection was from urinary tract infection in 4 (8%) of patients. Blood culture results are summarized in Table 1.

Table 1: Distribution of causative agents of sepsis among the studies cases

Organism	No.	%
E.coli	13	26.0
Candida	9	18.0
Staphylococcus aureus	8	16.0
Pseudomonas	6	12.0
Klebsiella	6	12.0
Enterococcus	4	8.0
Staphylococcus epidermidis	3	6.0
Streptococcus pneumoniae	1	2.0
Total	**50**	**100.0**

Antimicrobial susceptibility testing was performed for each organism according to the CLSI guidelines. Levels of resistance is shown in Table 2.

Table 2: Antimicrobial susceptibility testing of isolated organisms

	E. coli (n=13)	*Klebsiella spp (n=6)*	*Pseudomonas spp (n=6)*	*Staphylococcus aureus (n=8)*	*Staphylococcus epidermidis (n=3)*	*Enterococcus spp* (n=4)	*Streptococcus pneumoniae (n=1)*
Ampicillin	100%	100%	-	-	-	100%	-
Amoxicillin/ clavulanate	100%	100%	-	-	-	-	0%
Ampicillin/ sulbactam	100%	100%	-	-	-	-	-
Piperacillin/ Tazobactam	33.3%	50%	0%	-	-	-	-
Cefoxitin	66.7%	75%	-	80%	50%	-	-
Cefotaxime	66.7%	75%	-	-	-	-	0%
Ceftriaxone	66.7%	66.7%	-	-	-	-	0%
Ceftazidime	66.7%	75%	0%	-	-	-	-
Cefuroxime	75%	100%	-	-	-	-	0%
Cefepime	66.7%	66.7%	50%	-	-	-	0%
Meropenem	25%	50%	50%	-	-	-	0%
Imipenem	25%	50%	50%	-	-	-	0%
Amikacin	66.7%	75%	0%	-	-	-	-
Gentamicin	80%	100%	50%	100%	25%	100%	-
Tobramycin	80%	100%	50%	-	-	-	-
Ciprofloxacin	66.7%	75%	50%	33.3%	25%	100%	-
Levofloxacin	66.7%	100%	100%	20%	40%	100%	100%
Trimethoprim/ sulfamethoxazole	100%	75%	-	33.3%	100%	-	100%
Erythromycin	-	-	-	40%	60%	-	50%
Clarithromycin	-	-	-	40%	60%	-	50%
Clindamycin	-	-	-	40%	33.3%	-	33.3%
Oxacillin	-	-	-	80%	50%	-	0%
Linezolid	-	-	-	16.7%	0%	0%	0%
Rifampin	-	-	-	33.3%	0%	-	0%

As regards the antifungal sensitivity pattern among the 9 patients diagnosed with Candida infection. All (100%) of isolates were sensitive to the 3 antifungal agents used, namely Fluconazole, voriconazole and amphotericin B.

The main interest of the present study was to measure the relative gene expression of miRNA 146a and 150 in sepsis patients using real time PCR. U6 was used as a positive internal control (housekeeping) for comparison of amount and stability of miRNA between specimens. The healthy controls were considered as the control group while sepsis patients were considered the target group.

As regards to the relative expression of miRNA 146a, 56% (28/50) of the cases showed overexpression in sepsis patients, while 44% (22/50) of the cases showed downregulated expression with a range between 0.08 – 10.30, a mean and S.D. of 1.52 ± 1.71 represented in Table 3 and Fig.(1)

As regards to the relative expression of miRNA 150, 52% (26/50) of the cases showed downregulated expression in sepsis patients, while 48% (24/50) of the cases showed overexpression with a range between 0.06 – 11.10, a mean and S.D. of 1.70 ± 2.46 represented Table 3 and Fig (2). The co-expression of miRNA 146a and miRNA 150 showed a moderate positive correlation in the studied group which was found to be statistically significant (r = 0.489, p=0.001) (Table4 and Figure 3 & 4). There is also a statistically significant correlation between the relative expression of miRNA 146a and 150 (Table5).

Table 3: Relative gene expression of miRNA 146a and 150 (n = 50)

microRNA	No.	%
146a		
Over expressed	28	56.0
Under expressed	22	44.0
Min. – Max.	0.08 – 10.30	
Mean ± SD.	1.52 ± 1.71	
Median	1.33	
150		
Over expressed	24	48.0
Under expressed	26	52.0
Min. – Max.	0.06 – 11.10	
Mean ± SD.	1.70 ± 2.46	
Median	0.92	

Table 4: Co-expression of miRNA 146a and miRNA 150 in the studied group

microRNA	146a	
	r_s	P
150	0.489*	<0.001*

r_s: Spearman coefficient
*: Statistically significant at $p \leq 0.05$

Table (5): Relation between the relative expression of miRNA 146a and 150 in the studied group

microRNA	146a				χ^2	p
	Under expressed		Over expressed			
	No.	%	No.	%		
150						
Under expressed	16	72.7	10	35.7	6.762*	0.009*
Over expressed	6	27.3	18	64.3		

χ^2: **Chi square test**
*: Statistically significant at $p \leq 0.05$
p: p value for comparing between the studied groups

DISCUSSION

Sepsis, the association of organ dysfunction with an infection, is a complex multifactorial disease that has severe health and economic burden on both the patient and healthcare systems worldwide [14]. Owing to the fact that sepsis presents highly variable clinical manifestations, it is often difficult to provide an accurate early diagnosis [9,15]. Late diagnosis and delayed therapy increase mortality considerably [16].

A promising method in this regard is the use of specific miRNA for the detection of microbiological species. It is demonstrated that miRNAs are stable in patients' sera. miRNAs are hard to degrade by RNases, and hence can be used as invaluable biomarkers to detect bacterial infection at earlier stages[17].

Numerous studies have highlighted the role of miRNAs in the pathogenesis of viruses and bacteria. The activity of bacteria in the human body significantly alters the expression of miRNAs, and these changes are considerably noticeable. Moreover, the expression of microRNAs is modified according to different immune responses in bacterial infections [18,19].

In this study real-time PCR was used to detect the expression of miRNA 146a and 150. Women have a lower incidence of sepsis [20, 21]. In the present study, 39 (78%) of sepsis cases were males and 11 (22%) were females. This result was in agreement with Nasir et al who reported the predominance of sepsis in males [22]. The causes of this sex difference remain unexplained but may involve the effect of sex hormones on innate and adaptive immunity and on the cardiovascular response to cytokine signaling[23].

Originally sepsis was described, and strongly considered to be, a disease specifically related to Gram-negative bacteria. This is because sepsis was considered to be a response to endotoxin - a molecule thought to be relatively specific for Gram-negative bacteria. However, it is now recognized that sepsis may occur from any bacteria, as well as from fungal and viral organisms[24].

In the present study, *E. coli* was found to be the most common causative agent of sepsis detected in 13 (26%) of cases followed by Candida in 9 (18%) of cases. *Staphylococcus aureus* and MRSA together were detected in 8 (16%) of cases. How et al [25] as well reported that the most common organism isolated among their 22 urosepsis patients was *E. coli* in 18 (81.8) and *Klebsiella pneumoniae* in 4 (18.2) patients. Sakr et al [26] reported that two thirds of their patients were infected by Gram-negative organisms and one half with Gram-positive organisms. The most common Gram-negative microorganisms recovered were *E. coli* in 470(22.7%), *Klebsiella spp in* 356(17.2%), *Pseudomonas spp* in 337 (16.3%) of cases and *Acinetobacter* spp in 243 (11.7%) cases. Fungi were reported in 266 (12.9%) of cases; a finding that also underline the importance of fungal infections. Guzman et al [27] concluded that Candida induced septic shock is a nearly fatal condition with mortality rate that triples that of bacterial septic shock. In contrary to the present study, Abebaw et al [28] showed that Gram-positive bacteria were predominant compared with Gram-negative bacteria.

Early diagnosis and evaluation of sepsis are crucial for timely correction of this complicated state. Wang et al [10] suggested the use of new biomarkers for sepsis, such as miRNAs which are small (18–24 nucleotides) non-coding RNAs, that function to inhibit protein synthesis by degrading or inhibiting translation of messenger RNA (mRNA). They have been proposed as possible biomarkers because of the research evidence that shows that changes in a range of cellular microRNAs correlate with various pathophysiological conditions, including inflammation, oxidative stress, sepsis diabetes and different types of cancer. These molecules have also been known for their low complexity, simple detection and amplification, tissue-restricted expression profiles, and sequence conservation between human and model organisms [29].

The role of miRNAs is vital in biological processes such as cell growth, development, activities and metabolic pathways. They also function as key regulators at different stages of host immune response. Thus, expression of miRNAs at normal levels is crucial for maintaining homeostasis in all eukaryotes. microRNA-146 has important implications for endothelial damage[30].

The main interest of the present study was to measure the relative expression of miRNA -146 a and 150 in sepsis patients using real time PCR. miRNA -146a was overexpressed in 28 (56%) of sepsis patients, while 22 (44%) of the cases showed downregulated expression with a range between 0.08 – 10.30, a mean and S.D. of 1.52 ± 1.71. As regards miRNA-150, it was overexpressed in 26 (52%) of the cases, while 24 (48%) of the cases showed downregulated expression with a range between 0.06 – 11.10, a mean and S.D. of 1.70 ± 2.46.

Dhas et al [31] in 2018 reported that the expression of miRNA 146a among other microRNAs was downregulated in their 25 sepsis cases. In a similar study by Wang et al [32] in 2010, miR-146a was also found to be significantly low in septic cases with a specificity of 100% and a sensitivity of 63.3%. They confirmed these results by highlighting an increased expression of miRNA-146a in healthy patients. Downregulation of this miRNA may cause aberrant changes in inflammation associated with infection.

In a study by Wang et al [33] in 2013, they proposed that low serum expression of miR-146a and miR-223 distinguished patients with sepsis from those with SIRS. On the other hand, increased levels of miR-146 in human monocytic cell lines has been reported by Taganov et al[34] and O'Connell et al [35].

miRNA -150 was among the first miRNAs that were examined in patients with critical illness and sepsis. It was formerly identified as a key regulator of immune cell differentiation and activation [36]. During the maturation of B- and T-cells, miR-150 expression is down-regulated.

Vasilescu et al.[37] found lower miR-150 concentrations in a cohort of 16 patients with abdominal sepsis. In line, Ma et al [38] reported lower levels of miR-150 in two independent cohorts of patients with sepsis compared to patients with non-infectious SIRS or healthy controls. In addition, Roderburg et al [39] demonstrated that miRNA -150 serum levels appear slightly reduced in patients with critical illness; however, despite the very high number of tested patients, this regulation failed statistical significance. A study by How et al [25] in 2015 from Taiwan confirmed that miRNA -150 was significantly downregulated in Gram-negative bacteria urosepsis patients compared with those of healthy controls. Nonetheless, Puskarich et al [40] observed a strong correlation between low miRNA -150 levels and an impaired patients' prognosis in critical illness, suggesting that miRNA -150 rather has a role as a prognostic rather than diagnostic tool. Roderburg et al [39] as well, highlighted that high expression of miRNA -150 did correlate with enhanced survival whereas low expression was associated with increased risk of organ dysfunction and mortality.

How et al [25] concluded that the use of miRNAs as diagnostic biomarkers may represent a new perspective in the differential diagnosis between Gram-positive and Gram-negative bacteria.

CONCLUSION

The present study revealed that serum miRNA might be used as biomarkers for sepsis. miRNA expression is a strong candidate for the future of intensive care because of the early diagnosis opportunity and because of its capacity to interact with certain key points of the biochemical pathways. Moreover, these miRNA species can be determined in different body fluids, such as serum, plasma, and blood, widening the range of options for the determination of sepsis in critically ill patients. Using miRNAs as circulating biomarker for sepsis is still in its infancy and additional studies are required to increase the specificity and selectivity of this method. Further larger scale studies are necessary to identify the correct context for miRNA expression.

Strengthening a broader range of specific miRNAs for sepsis is required. In conclusion, we can affirm that it is necessary to improve detection and validation methods of specific miRNAs for sepsis.

Conflicts of interest:
The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
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