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Serum level of MicroRNA 15a and interleukin 6 as biomarkers for sepsis in critically Ill Patients

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Abstract

This study intended to evaluate the diagnostic efficacy of serum interleukin-6 (IL-6) and serum expression level of microRNA 15a (miRNA 15a) in sepsis patients admitted to the medical intensive care unit (ICU), and to correlate the results with the outcome of patients. This observational case-control study, was done from January 2022 to June 2022, included 75 adult ICU patients ≥18 years old, divided into 2 groups. Group 1: included 38 adults, critically ill sepsis patients and Group 2 (control group), included 37 adults, non-sepsis patients admitted to the ICU. Venous blood was withdrawn from cases and controls under aseptic conditions for assessment of serum IL-6 level by ELISA and serum expression level of miRNA 15a by quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR). Patient demographic data in addition to their characteristics including site of infection, blood culture results, length of stay and outcome of patients were included in the study. miRNA 15a was significantly higher in the sepsis group with mean of 3.99±1.61 compared to the controls (1.03±0.03, $p< 0.001$) while serum IL-6 levels were higher in the sepsis patients compared to the controls, however the difference did not reach statistical significance ($p= 0.92$). No difference was found in IL-6 and miRNA 15a levels between surviving and non-surviving sepsis patients, shocked and non-shocked sepsis patients. In conclusion, both IL-6 and miRNA 15a were up-regulated in ICU patients with sepsis. Both biomarkers did not show a significant difference regarding the outcome of patients. miRNA 15a could be considered a highly specific and sensitive marker in differentiating sepsis cases from controls, so it could be used as a diagnostic rather than a prognostic biomarker for sepsis.

Keywords: microRNA 15a; IL-6; Sepsis; qRT-PCR; ELISA.

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Introduction

Sepsis is defined as a life-threatening acute organ dysfunction induced by infection. In the United States of America and Australia, it affects around 19 million people each year. In-hospital mortality has fallen from 35% in 2000 to 18% in 2012, resulting in a large number of sepsis survivors.[1] Individuals who survive sepsis

frequently acquire long-term disability and worsening of preexisting health conditions.[2]

Sepsis must be diagnosed as soon as possible because of its high fatality rate, high risk of organ dysfunction and high healthcare costs.[3] There is a variety of biomarkers with elevated serum levels throughout the sepsis process, beneficial but not conclusive, in predicting sepsis severity and death. Furthermore, there is no single standard biomarker, or even a combination of biomarkers, is generally reliable for conclusive early identification of sepsis.[4] Moreover, early massive antibiotic therapy can cause microbial cultures to be negative, in addition to the extended delay for culture results.[5]

Because of its quick rise following endotoxin challenge, interleukin-6 (IL-6) has been suggested to function as an early indicator of sepsis. IL-6 can also drive T and B cell differentiation and boost the creation of acute phase response proteins in the liver. As a result, IL-6 detection was considered an excellent signal for detecting the early start of sepsis.[6]

MicroRNAs (miRNAs) are a category of noncoding single-stranded RNAs that regulates gene expression by binding to its target mRNA and causing either protein translation suppression or RNA destruction.[7] A single miRNA can target several genes, and multiple miRNAs can affect the same target gene. miRNAs control gene expression by binding to the 3' untranslated regions of the target mRNAs. miRNAs were shown to be crucial for immune responses and to be implicated in autoimmune disorders and malignancies due to their regulatory effects.[8]

Several miRNAs, including miRNA16, miRNA21, miRNA31, miRNA150 and miRNA122, were certified as sepsis biomarkers. According to Kingsley et al., 2017 and Kreth et al., 2018, miRNA 15a can be used as a diagnostic sepsis biomarker.[9,10] Several genes that promote vascular permeability during sepsis, such as vascular endothelial growth factor A and C are also known to be inhibited by miRNA 15a. Toll like receptor 4 signaling can be targeted by miRNA 15a. miRNA 15a can also trigger apoptosis and alter cytokine release, providing it an anti-cancer effect.[11,12]

The objective of this study was to assess the diagnostic efficacy of serum IL-6 levels, measured by an enzyme linked immunosorbent assay (ELISA) and serum expression levels of miRNA 15a, measured by quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR) in sepsis patients admitted to the medical intensive care unit (ICU), and to correlate the results with patient outcomes.

Subjects and Methods

Sample size calculation

The Power Analysis & Sample Size (PASS) 11 sample size calculator was used to calculate the minimal sample size needed to detect test specificity and sensitivity of IL-6 and miRNA15a, with 0.05 alpha error and power of the study 0.80. The sample size of 75 patients was calculated based on assuming a disease prevalence of 0.4, null hypothesis 0.6 and the alternative hypothesis was 0.8 for the specificity.

Study design

This study was an observational case-control study, done during the period from January 2022 to June 2022. It included 75 sepsis patients admitted to the medical ICU at Kasr Al-Ainy hospitals. The selected patients enrolled in this study were divided into two groups. Group 1 included 38 adults, critically ill septic patients who were clinically diagnosed with sepsis or septic shock within the last 24 hours according to the sequential organ failure assessment score.[13] And, group 2 (a control group), included 37 adults, non-sepsis patients admitted to the ICU. Patients with history of blood transfusion in the previous three months before admission, autoimmune diseases, primary immuno-deficiency, treatment with corticosteroids or immunomodulators in the previous six months and malignancies were excluded from the study.[14]

Demographic data and patients' characteristics

Patient demographic data (age and sex) were recorded in the study. Other data were collected from patients' hospital records, including site of infection, result of blood

culture, ICU length of stay and outcome of patients within 28 days (either discharge with clinical improvement, septic shock, or death).

Ethical considerations

The study protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Cairo University (approval code MD-39-2019). The study was explained to all participants or their relatives (if unconscious), and a verbal informed consent was taken from each before being enrolled in the study.

Specimen collection

A volume of 5 ml of peripheral or central venous blood was withdrawn from cases and controls under aseptic conditions. All samples were allowed to clot spontaneously at room temperature before being centrifuged at 1000 × g for 10 min. Then serum samples were separated and divided into 2 portions. The first portion was used for determination of IL-6 level by an ELISA technique. The second portion was used for qRT-PCR of miRNA 15a. Serum samples were stored at -80°C till used in processing.[15]

Detection of IL-6 serum levels by ELISA

Quantitative determination of serum IL-6 in both cases and controls was performed using commercial Human IL-6 ELISA Kits (Elabscience, USA) according to the manufacturer's instructions. The optical density was read at wave length 450 nm using a Micro ELISA reader (Auto reader State Fax-2100, GMI, Germany). The detection range of the kit is from 7.81 to 500 pg/ml.

Detection of Serum miRNA 15a

-Purification of RNA from serum samples

Isolation of total RNA from each sample was performed using a commercial kit (miRNeasy Mini®, Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

-Reverse Transcription

The isolated RNA was assayed by qRT-PCR using commercial kits (miScript II RT ®kit, Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

-Amplification and quantification of miRNA 15a

Amplification of the target cDNA was carried out using Maxima SYBR Green qRT-PCR Master Mix (2X) (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The used sequence-specific primers for miRNA 15a are shown in Table 1.[16]

The components of qRT-PCR mixture, as shown in Table 2, were prepared in polypropylene tubes. The PCR tubes were placed and processed in a thermal cycler (Step One thermal cycler with software version 3.1, Applied Biosystems, USA) for amplification and analysis. The cycling conditions are shown in Table 3.

Table 1. Primer Sequences for qRT-PCR.

Target	Primer Sequence
miRNA-15a	F: 5'-TAGCAGCACATAATGGTTTGT-3' R: 5'-GCGAGCACAGAATTAATACGAC-3'
U6 (Endogenous control)	F: 5'-CAGCACATATACTAAAATTGGAACG-3' R: 5'-ACGAATTTGCGTGTCATCC-3'

Table 2. Components of Polymerase Chain Reaction.

Component	Volume/20-µL Reaction
Maxima SYBR Green qPCR Master Mix (2x)	10 µL
Forward Primer	1 µL
Reverse primer	1 µL
cDNA template	3µ L
Nuclease-free water	5 µL
Total Volume	20 µL

Table 3. Cycling conditions for quantitative RT-PCR

System	Stage	Temperature	Time
One cycle	Hold	50 °C	2 min.
	Hold	95 °C	10 min.
40 cycles	Denaturation	95 °C	15 sec.
	Annealing and Extension	60 °C	1 min.

Statistical Analysis

The statistical package for the social sciences (SPSS) version 28 was used to code and input the data (IBM Corp., Armonk, NY, USA). The mean, standard deviation, median, minimum, and maximum values were used to describe quantitative data. Categorical statistics were tabulated using frequency (count) and relative frequency (percentage). The non-parametric Mann-Whitney test was used to compare quantifiable factors.[17] The Chi square ($\chi 2$) test was used to compare categorial data. When the expected frequency is less than 5, the exact test was used instead.[18] The Spearman correlation coefficient was used to calculate correlations between quantitative variables.[19] To determine the optimal cutoff value of important parameters for detection of sepsis, a receiver operating characteristic (ROC) curve was designed and area under curve analysis was conducted. Statistical significance was defined as *p*-values less than 0.05.

Results

The demographic data of sepsis patients and control subjects are shown in Table 4. Ages of sepsis patients ranged from 21-90 years with a mean of 64.32±14.78 years. Meanwhile, ages of the control group ranged from 21 to 88 years with a mean of 57.81±15.3 years. Out of the 38 sepsis patients, 22 (57.9%) were males and 16 (42.1%) were females. Of the 37 controls, 23 (62.2%) were males and 14 (37.8%) were females.

Table 4. Demographic data of patients and control subjects.

Variable		Sepsis patients	Controls	*p* value
Age	Range	21- 90	21- 88	0.033
	Mean±SD	64.32±14.78	57.81±15.3	
Sex	Males	22 (57.9%)	23 (62.2%)	NS
	Females	16 (42.1%)	14 (37.8%)	

P > 0.05 is not significant (NS).

Chest infection was the most prominent accounting for 68.4 % followed by combined chest and skin infection (10.5%). Regarding the causative organism, *Klebsiella* spp. were isolated in 34.2 % of cases, others included *Escherichia coli*, *Enterococcus* spp., coagulase negative staphylococci (CoNS) and *Candida*. (Table 5)

The outcome of sepsis cases is shown in Table 6. There was 50 % survival within 28 days and 73 % cases with progression to septic shock.

Table 5. Descriptive Analysis of Sepsis Patients.

Variables		Cases (n=38)	
		Count	%
Site of infection	Chest	26	68.4%
	Chest & skin	4	10.5%
	Skin	3	7.9%
	Central nervous system	1	2.6%
	Mediastinum	1	2.6%
	Pyothorax	1	2.6%
	Abdominal	1	2.6%
	Urinary tract infection	1	2.6%
Culture Results	*Klebsiella* spp	13	34.2%
	E. coli	2	5.3%
	Candida	2	5.3%
	Enterococci	1	2.6%
	E. coli & CoNS	1	2.6%
	No growth	14	36.8%
	Not done	5	13.2%

CoNS: Coagulase negative *staphylococci*

Table 6. Outcome of the 38 sepsis cases.

28-day mortality	Death	19	50.0%
	Survival	19	50.0%
Septic shock	Yes	28	73.7%
	No	10	26.3%

Serum IL-6 levels were higher in the sepsis patients (203±542) compared to the controls (101 ± 123.6), but the difference did not reach statistical significance (p= 0.92). Meanwhile, the expression levels of miRNA 15a were significantly higher in sepsis patients (3.99±1.61) compared to the controls (1.03±0.03) (p< 0.001) (Table 7).

Table 7. Serum IL-6 and miRNA 15a in cases and control groups.

Biomarker	Cases (n=38)			Control (n=37)			*p* value
	Mean±SD	Median	Range	Mean±SD	Median	Range	
IL-6(pg/ml)	203.13±542.96	56.80	8-3270	101.00±123.58	62.00	6-604	NS
miRNA 15a	3.99±1.61	3.95	1.30-6.9	1.03±0.03	1.02	0.97-1.09	< 0.001

P > 0.05 is not significant (NS).

Concerning serum levels of IL-6 and miRNA 15a, no difference was found between surviving and non-surviving sepsis patients, shocked and non-shocked sepsis patients (p>0.05). ROC curve analysis revealed that miRNA 15a, could differentiate between sepsis patients and controls with an area under the curve (AUC) = 1 (sensitivity = 100 %, specificity = 100 % at a cutoff expression value of 1.195, p<0.001). However, serum IL-6 could not discriminate between sepsis and controls with AUC = 0.507 (p= 0.92) (Table 8 and Figure 1).

Table 8. Sensitivity and Specificity of IL-6 and miRNA 15a.

Biomarker	Area Under the Curve	*p* value	95% Confidence Interval		Cut off	Sensitivity (%)	Specificity (%)
			Lower Bound	Upper Bound			
miRNA 15a	1.000	< 0.001	1.000	1.000	1.195	100	100
IL-6	0.507	NS	0.374	0.639	-----	-----	-----

P > 0.05 is not significant (NS).

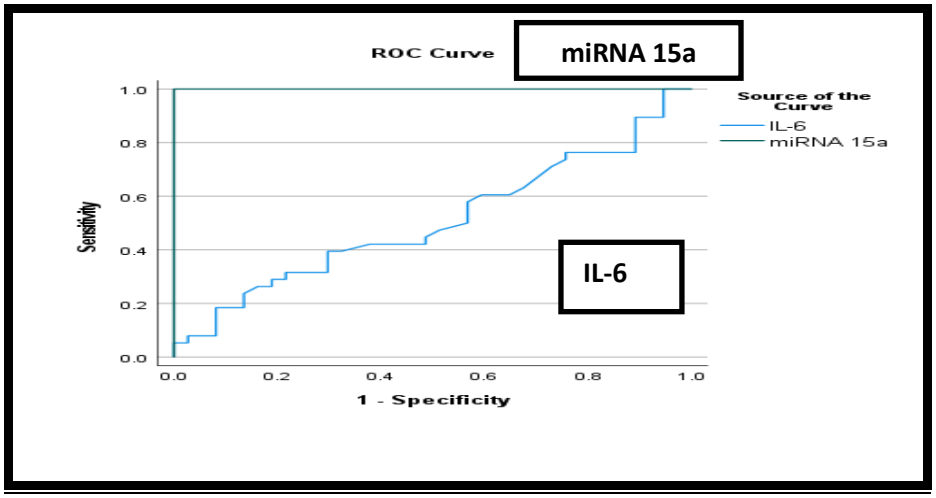

Figure 1. Receiver operating characteristic curve for diagnosis of sepsis using serum level of microRNA 15a and IL-6.

Discussion

This study aimed to assess the diagnostic efficacy of serum IL-6 levels, and serum levels of expressed miRNA 15a, in sepsis patients admitted to ICUs, and to correlate the results with patient outcomes. In the conducted study, miRNA 15a was significantly higher in sepsis group compared to control group with (p<0.001). This goes in line with findings reported by Wang et al., 2012[15] and Wang et al., 2015.[20] In a study done by Lou and Huang, 2020, it was stated that miRNA15a is a vital regulator and involved in the inflammatory process in sepsis by inhibiting TNIP2 which is a negative regulator of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. Thus, miRNA 15a over expression can augment NF-κB pathway aiding in the inflammatory response in sepsis.[21]

Serum miRNA 15a levels showed no difference according to mortality and incidence of septic shock. In a study done by Goodwin et al., 2015, it was demonstrated that miRNA 15a decreased with development of septic shock.[12] On the other hand, it was increased in non-surviving sepsis patients in a study done by Wang et al, 2012.[15] Additionally, there was no significant correlation between miRNA 15a and ICU duration of stay, which means that it could have no impact on patient discharge. Serum levels of IL-6 are well known to be involved in sepsis pathogenesis and take part in the systemic response to infection.[22] In this study, there was no difference in serum level of IL-6 in sepsis cases (mean= 203 pg/ml) compared to control group (mean= 101 pg/ml). In contrary, Song et al., 2019 and Smok et al., 2020 found a significant increase in IL-6 in sepsis patients.[23, 24] Concerning outcome of sepsis patients in association with IL-6, it was observed that there was no difference in serum IL-6 concentrations among surviving versus non-surviving group, and in shocked versus non-shocked sepsis patients. In line with these results, Vivas et al.,

2021 did not find a significant correlation between serum levels of IL-6 and sepsis outcome in terms of mortality.[22] Takahashi et al., 2016 showed that serum IL-6 levels were significant predictors of 28-day mortality and their prognostic value increased over time up to 7 days.[25] On the contrary, Carta et al., 2016 showed elevated serum IL-6 was associated with non-survivors.[26]

Concerning the diagnostic efficacy of miRNA 15a, it was found that miRNA 15a discriminated early, within 24h of admission, between sepsis patients and non-sepsis patient as AUC=1, at a cutoff expression value of 1.195, $p < 0.001$. This is consistent with Goodwin et al., 2015 who found increased miRNA 15a in sepsis with AUC=0.7.[12] These results suggest that miRNA 15a, in one hand, can be proposed as a diagnostic biomarker in sepsis with high sensitivity and specificity.

On the other hand, in this study, serum IL-6 concentrations were not higher in sepsis patients versus controls. This resemblance can be explained by the presence of various variables that could have influenced serum IL-6 results. First of all, samples were withdrawn within 24 hours, meaning that management of patients with antibiotic therapy and intravenous fluids could have affected serum IL-6 concentration. Other studies assessed IL-6 concentration within one hour after the initial presentation of sepsis patients before management, with serially withdrawn samples then after to assess whether it increases or decreases with management of patients. This was illustrated in Vivas et al., 2021 who found that patients with sepsis had a significant decline in IL-6 levels after 48 hours when compared to that on admission.[22]

Secondly, the mean IL-6 concentration in the control group was high (101 pg/ml). The control group included critical patients in medical ICUs, most of them were cardiac patients having either congestive heart failure, or myocardial infarction and planned for cardiac catheterization. Tollefsen et al., 2021 and Groot et al., 2019 pointed to the high IL-6 concentration among myocardial infarction patients.[27&28] These data could help to explain the relatively high IL-6 concentration obtained within the control group in the current study.

Gentile et al., 2013 found that the levels of IL-6 in patients with sepsis caused by Gram-positive bacteria are greater than in patients with sepsis caused by Gram-negative bacteria.[29] This means that IL-6 concentrations within sepsis patients included in this study might have been higher if the incidence of Gram-positives was higher than the obtained with our investigation.

In conclusion, miRNA 15a was up regulated in ICU patients with sepsis compared to the control group. Furthermore, miRNA 15a was specific and sensitive in differentiating sepsis cases from controls. Hence, it can be proposed as a potential diagnostic biomarker in sepsis rather than a prognostic one. This would help to avoid the time cost of blood cultures with the high false negatives, thus aiding rapid prompt identification and management of sepsis.

Author Contributions

DN, MA, NS; Performed the lab work. DN, NS; made the statistical analysis. RA; examined the patients. DN, RA; collected samples. All authors participated in writing and reviewing the paper.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Cairo University (approval code MD-39-2019).

Informed consent

The study was explained to all participants or their relatives (if unconscious), and a verbal informed consent was taken from each before being enrolled in the study.

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