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Serum miR-146a and miR-223 as potential new biomarkers for sepsis

Jia-feng Wang [a,1], Man-li Yu [b,1], Guang Yu [c,1], Jin-jun Bian [a], Xiao-ming Deng [a], Xiao-jian Wan [a], Ke-ming Zhu [a,*]

[a] Department of Anesthesiology and Intensive Care Medicine, Changhai Hospital, Second Military Medical University, Shanghai 200433, China
[b] Department of Cardiology, Changhai Hospital, Second Military Medical University, Shanghai 200433, China
[c] Department of Nephrology, Changhai Hospital, Second Military Medical University, Shanghai 200433, China

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ABSTRACT

Objective: Current biomarkers cannot completely distinguish sepsis from systemic inflammatory response syndrome (SIRS) caused by other non-infectious diseases. Circulating microRNAs (miRNAs) are promising biomarkers for several diseases, but their correlation with sepsis is not totally clarified.
Methods: Seven miRNAs related to inflammation or infection were included in the present study. Serum miRNA expression was investigated in 50 patients diagnosed with sepsis, 30 patients with SIRS and 20 healthy controls to evaluate the diagnostic and prognostic value. Expression levels of serum miRNAs were determined by quantitative PCR using the Qiagen miScript system. Serum CRP and IL-6 levels were determined by enzyme linked immunosorbent assay.
Results: Serum miR-146a and miR-223 were significantly reduced in septic patients compared with SIRS patients and healthy controls. The areas under the receiver operating characteristic curve of miR-146a, miR-223 and IL-6 were 0.858, 0.804 and 0.785, respectively.
Conclusion: Serum miR-146a and miR-223 might serve as new biomarkers for sepsis with high specificity and sensitivity. (ClinicalTrials.gov number, NCT00862290.)

1. Introduction

Sepsis is defined as the combination of infection and systemic inflammatory response syndrome (SIRS) [1]. Despite of the development in diagnostic and therapeutic techniques, the incidence and mortality remained high in the last 20 years [2]. Delayed diagnosis and intervention usually resulted in severe outcome, such as organ dysfunction and death [3], and the economic burden is extremely heavy [4,5]. Many studies have been performed to identify or evaluate an early biomarker of sepsis, including acute phase proteins (C-reactive protein), cytokines (IL-1, -6, -10 and TNF-α), chemokines (IL-8, MCP-1 and G-CSF), procalcitonin [6–9] and metabonomic approach [10,11]. Procalcitonin, which is the most promising biomarker of sepsis, has even been proposed as a diagnostic marker involved in the definition of sepsis [12]. However, a recent meta-analysis showed that procalcitonin cannot differentiate sepsis from SIRS caused by other non-infectious disease reliably [13]. Further studies on biomarkers for sepsis are still warranted.

microRNAs (miRNAs) are small non-coding RNAs that regulate gene expression and play important roles in a variety of cellular functions [14,15]. Recently, these miRNAs were also identified in serum and plasma as biomarkers for several diseases [16]. The circulating miRNAs were first investigated as biomarkers for cancer detection [17,18]. The area under the receiver operating characteristic curve of miR-141 in detecting prostate cancer was as high as 0.907. Diagnostic value of miRNAs was not only limited in cancer, but also in acute stage of diseases, such as drug induced liver injury [19]. Recently, miR-150, identified by miRNA microarray analysis of peripheral leukocyte, was demonstrated to be a potential biomarker of sepsis [20]. However, sepsis was a complicated syndrome involving multiple organs and tissues, but circulating microRNAs derived from other tissues and cells during sepsis were not evaluated. The present study was to investigate the serum levels of macrophages and inflammation related microRNAs during sepsis and SIRS.

2. Methods

2.1. Participants

Fifty septic patients with positive microbiological culture results were retrospectively chosen from an ongoing program on sepsis supported by the National Natural Science Foundation of China

* Corresponding author. Address: Department of Anesthesiology and Intensive Care Medicine, Changhai Hospital, Second Military Medical University, 168 Changhai Road, Shanghai 200433, China. Fax: +86 21 81873501.
E-mail address: kmzhu_md@yahoo.com.cn (K.-m. Zhu).
[1] These authors contributed equally to this work.

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(30872454/C160203) and their serum samples were collected within 24 h after admitted to ICU during that program. Cause of sepsis included major abdominal surgery, intestinal obstruction, intestinal perforation, mesenteric vein thrombosis, abdominal or pelvic abscess and multiple trauma. These patients were all admitted to the intensive care unit (ICU) of our hospital from August 2008 to May 2009. Thirty patients undergoing cardiac surgery with cardiopulmonary bypass with SIRS but not organ dysfunction and sepsis on the second day after surgery were defined as a SIRS control group. These patients were recruited in the department of cardiothoracic surgery from August 2008 to June 2009. Another 20 healthy volunteers aged 18–60 years were included as a healthy control group. This study was approved by the Specialty Committee on Ethics of Biomedicine Research of Second Military Medical University and all subjects provided their informed consent.

2.2. Sampling and serum miRNA isolation

In septic group, coagulated blood sample were collected within 24 h after the patients were admitted in ICU. As for the SIRS group, sample were collected on the second day after cardiac surgery. Meanwhile, drainage fluid, sputum, urine and blood were collected for microbiological to screening the potential infection foci. After incubating the whole blood at 37 °C for 1 h and centrifuging it at 2500 rpm for 10 min, serum was extracted. Serum miRNA was isolated by TRIzol reagent (Invitrogen, USA) and a miRcute miRNA extraction kit (TIANGEN, China). In general, 500 μl TRIzol reagent and 100 fmol mmu-miR-295 mimics (Qiagen, USA) were added into 200 μl serum and incubated for 5 min. Then 200 μl chloroform was added and the mixture was centrifuged at 12,000 rpm for 15 min. The supernatant was transferred to an absorption column and Solution C in the miRNA extraction kit of the same volume was added to precipitate RNA for 3 min. RNA was absorbed in the column and the waste solution was removed by centrifuge. Then the column was washed with wash solution in the kit for twice and finally the RNA was dissolved in 20 μl eluent.

2.3. Candidate miRNAs selection

The potential sepsis-related microRNAs were screened according to 2 microRNA microarray studies, in which monocytes and monocyte-derived dentritic cells were stimulated with lipopolysaccharide (LPS) [21,22]. These two microarray studies identified four main upregulated microRNAs: miR-132, miR-146a, miR-155 and miR-223. In order to extend the screening range, miR-15b [23,24], miR-126 [25], let-7i [26] were also included because of their correlation to BCL2 related apoptosis, VCAM1 related cell adhesion and Toll-like receptor-4, respectively. All these target genes have close relationship with pathogenesis of inflammation and sepsis; therefore, the 7 miRNAs mentioned above were included as the candidate miRNAs.

2.4. Quantitative RT-PCR

Quantitative real-time PCR (qRT-PCR) was performed with a miScript System (Qiagen, USA), which included specific primers for miRNAs. All procedures were performed according to the instructions provided by the manufacturer. These procedures were performed by the investigator who was blinded to the grouping. The reverse transcription (RT) reaction system contained 1 μl miScript Reverse Transcriptase Mix, 4 μl 5∗ miScript RT Buffer and 15 μl RNase-free water. The 20 μl RT product was diluted into 100 μl. The procedures was 37 °C for 60 min and 95 °C for 5 min. Reaction system of quantitative Real-time PCR contained 10 μl SYBR Green PCR Master Mix, 2 μl miScript universal primer, 2 μl specific primer, 1 μl cDNA and 5 μl RNase-free water. The procedure for PCR was 95 °C 15 min; 94 °C 15 s, 55 °C 30 s, 70 °C 30 s, 45 cycles. RT reaction was performed in ABI 9700 PCR system (ABI, USA) and Real-time PCR was performed in Rotor-gene 6000 PCR system (Corbett, Australia). Each reaction was run in duplicate and performed at least twice. Negative control reactions without RT reaction and template were also performed. To confirm the absence of mmu-miR-295 in normal human serum, qRT-PCR was performed with primer for mmu-miR-295 in serum sample without spiked-in mmu-miR-295.

2.5. Enzyme linked immunosorbent assay

Serum levels of C-reactive protein (CRP) and interleukin-6 (IL-6) were determined by enzyme linked immunosorbent assay (ELISA) with specific kit (CRP: Biosource, USA; IL-6: eBioscience, USA). They were also performed by the investigator who was blinded to the grouping.

2.6. Statistical analysis

Quantitative data was expressed as mean ± standard deviation. Expression level of miRNAs was analyzed using the $2^{-\Delta\Delta C_T}$ method. Spiked-in mmu-miR-295 was chosen as the normalized internal control. The miRNA level of healthy controls was chosen as the basal level and level of SIRS and sepsis group was expressed as the relative changing fold compared with basal level. Quantitative data was compared with *t*-test between two groups and ANOVA between three groups. In order to evaluate the predictive value of miRNAs, receiver operating characteristic (ROC) curve analysis was applied and area under curve (AUC) was compared. $p < 0.05$ was considered as statistical significant.

3. Results

3.1. General characteristics of the patients with sepsis and SIRS

General data of the 50 septic patients was listed in Table 1. The leading causes of sepsis in these patients were major abdominal surgery, intestinal obstruction and intestinal perforation. All patients were admitted into ICU after surgical treatment. Abdomen and lung were the main infection foci and the main infection types

Table 1
General data of septic patients.

Variable	Sepsis group ($n = 50$)
Age (years)	53.1 ± 15.5
Male/female	34/16
Primary diagnosis (%)	
Major surgery	21 (42.0)
Intestinal obstruction	15 (30.0)
Intestinal perforation	6 (12.0)
Mesenteric vein thrombosis	3 (6.0)
Abdominal or pelvic abscess	3 (8.0)
Multiple trauma	2 (4.0)
Infection site (%)	
Abdomen	21 (42.0)
Lung	17 (34.0)
Blood	10 (20.0)
Urinary tract	7 (14.0)
Others	13 (26.0)
Pathogen type (%)	
Gram-negative infection	19 (38.0)
Gram-positive infection	8 (16.0)
Mixed infection	23 (46.0)
Fungus infection	12 (24.0)
APACHE || score	15.7 ± 6.3
28-Day mortality (%)	18 (36.0%)

were mixed infection and Gram-negative infection. A total of 18 patients died within 28 days after admitted into ICU. The 30 SIRS patients, aged 47.9 ± 11.6 years, included 21 patients underwent valvular surgery and 9 patients underwent coronary artery bypass graft. Nineteen of them were males while the rest were females. All of them developed SIRS on the second day of surgery and none of them developed sepsis during the following 1 weeks.

3.2. Expression levels of miRNAs and inflammatory factors in serum

The qRT-PCR of RNA without spiked-in mmu-miR-295 revealed that mmu-miR-295 was absent in normal human serum and mmu-miR-295 could be applied as internal control for quantitative Real-time PCR. Quantitative RT-PCR revealed that expression levels of miR-146a and miR-223 were significantly reduced in serum of septic patients compared with SIRS patients and normal controls. miR-146a was also significantly reduced in SIRS patients compared to normal controls, while miR-223 was comparable between SIRS patients and normal controls. Level of miR-126 was reduced in septic and SIRS patients, and no significant difference of miR-126 level was detected between septic and SIRS patients. Expression of miR-15b, miR-132, miR-155 and let-7i were similar among septic patients, SIRS patients and normal controls (Fig. 1). Serum IL-6 level in serum of septic patients was much higher than SIRS patients, but serum CRP level was comparable between SIRS and sepsis groups (Fig. 2).

3.3. Predictive value of miRNAs

In order to compare the predictive value of miRNAs and inflammatory factors, ROC curve analysis was performed. The results revealed that miR-223 had a highest AUC of 0.858 (0.748–0.968), followed by miR-146a with an AUC of 0.804 (0.679, 0.928). AUC of IL-6 was 0.785 (0.669, 0.901), lower than the two miRNAs (Fig. 3 and Table 2). At a cut-off point set at −1.89, miR-223 yielded a specificity of 100% and a sensitivity of 80%, while at a cut-off point set at −2.98, miR-146a yielded a specificity of 100% and a sensitivity of 63.3% (Fig. 4).

4. Discussion

Early diagnosis and evaluation of sepsis are crucial for timely correction of this complicated syndrome. Microbiological culture is the gold standard to distinguish sepsis from other non-infectious diseases, but this technique always consumes a long time, and treatment would be delayed. It also lacks sensitivity and specificity

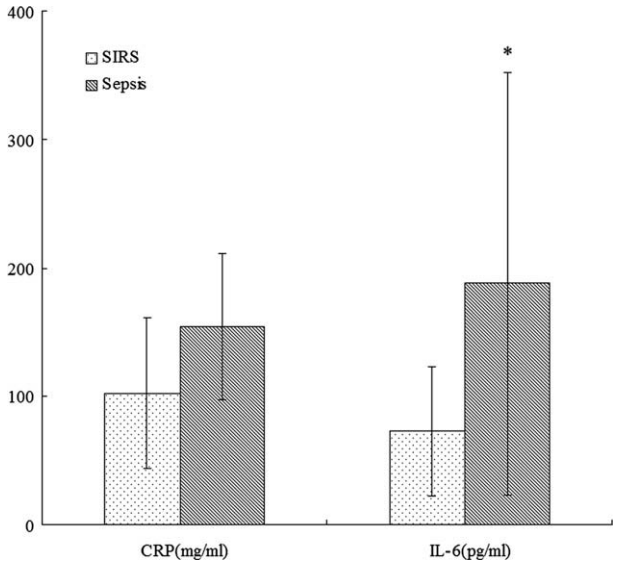

Fig. 2. Serum level of inflammatory factors in sepsis and SIRS patients. *$p < 0.001$.

[27]. Therefore, many studies have been carried out to identify an ideal biomarker for sepsis. The potential biomarkers for sepsis include acute phase protein, cytokines and chemokines, all of which are not specific enough due to an overlap with other inflammatory diseases [28]. In the past 15 years, a variety of studies were performed to evaluate the diagnostic and prognostic value of procalcitonin in sepsis. In the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference, procalcitonin level was even chosen as one of the diagnostic criteria for sepsis. However, capacity of procalcitonin in distinguishing sepsis from SIRS caused by other non-infectious disease was not definite up to now, and procalcitonin could not be used widely in critical care settings [29,30]. Therefore, biomarkers with higher specificity and sensitivity remained to be identified.

The present study revealed that serum microRNAs might be used as biomarkers for sepsis. Levels of miR-146a and miR-223 in sepsis group were significantly lower than in SIRS group and normal control. These two miRNAs might serve as new biomarkers for sepsis. Similar to CRP, serum miR-126 level in SIRS and sepsis groups was pretty different from that of normal controls, but it could not distinguish sepsis from SIRS. Serum miR-146a level was similar to IL-6, with significantly different level between SIRS,

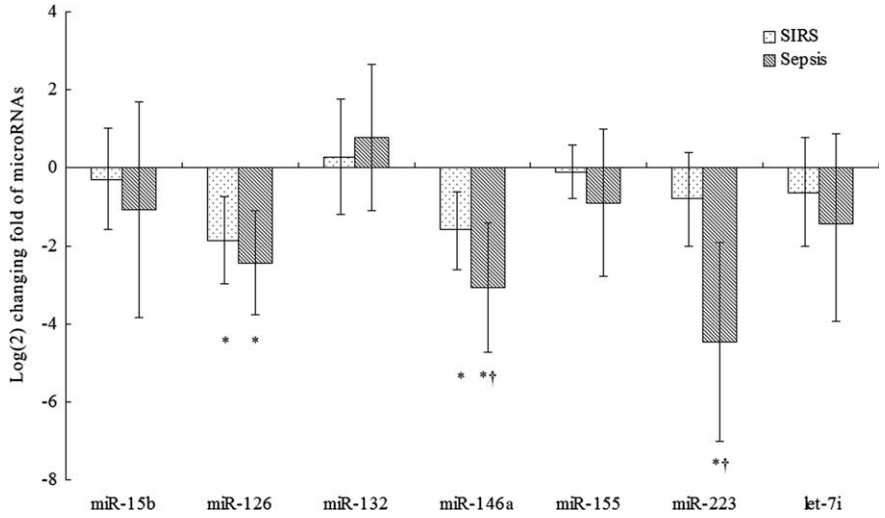

Fig. 1. Expression levels of microRNAs in serum of sepsis and SIRS patients. *Compared with normal control, $p < 0.01$; †compared with SIRS patients, $p < 0.01$.

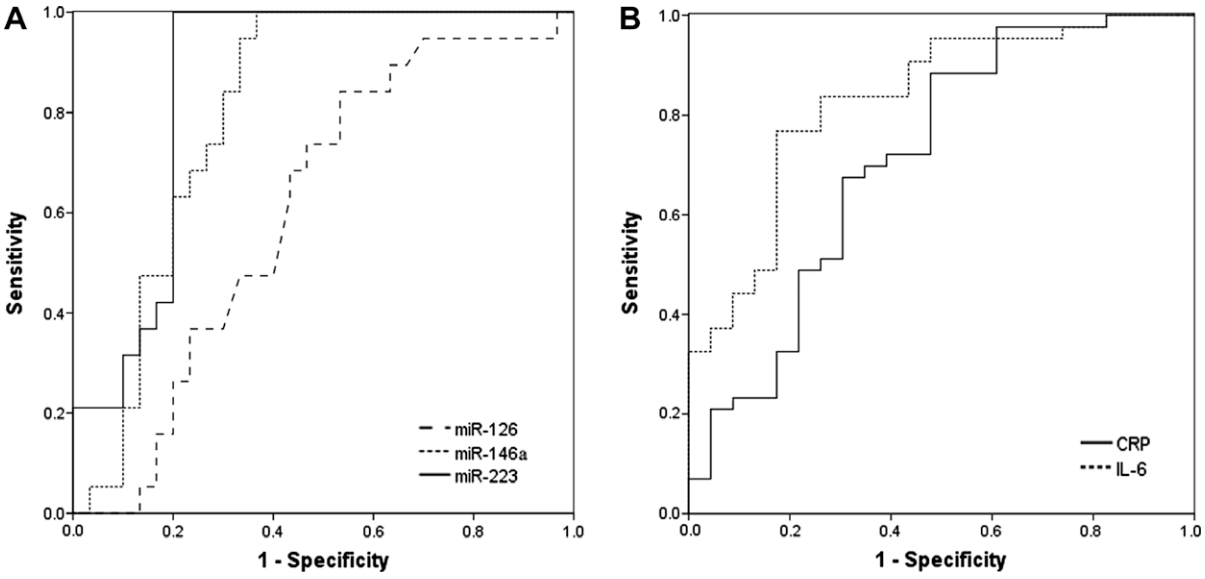

Fig. 3. Receiver operating characteristic curves of microRNAs (A) and inflammatory factors (B).

Table 2
Areas under the receiver operating characteristic curve of microRNAs and inflammatory factors.

Variable	AUC (95% CI)	*p*
miR-126	0.607 (0.448, 0.766)	0.211
miR-146	0.804 (0.679, 0.928)	<0.001
miR-223	0.858 (0.748, 0.968)	<0.001
CRP	0.589 (0.427, 0.751)	0.267
IL-6	0.785 (0.669, 0.901)	<0.001

AUC, area under curve.

sepsis patients and normal controls. What is more important, it could distinguish sepsis from SIRS. Serum miR-223 might be the optimal biomarkers for sepsis, because its level was reduced just in septic patients but not SIRS patients. The ROC curve analysis revealed that miR-223 had the highest AUC, followed by miR-146a and IL-6. The cut-off point of the miRNAs was also investigated. At a cut-off point set at −1.89, miR-223 had both high value of specificity and sensitivity. Therefore, miR-223 might be the optimal biomarkers for sepsis, because it can distinguish sepsis from SIRS more clearly. Correlation of miR-146a and miR-223 levels in serum with disease severity was not analyzed because of the relatively small sample size.

Our selection of candidate miRNAs was based on their association with sepsis. In Vasilescu's study [20], they selected leukocyte derived miRNAs as candidate biomarkers for sepsis. But during sepsis many tissues and cells were activated, such as monocyte in different organs, endothelial cells in lung. Stimulation of LPS on monocytes and monocyte-derived dentritic cells was common during sepsis and circulating levels of miRNAs originating from these cells might be changed [21,22]. Lymphocyte apoptosis was common during sepsis and miR-15b was an important miRNA regulating apoptosis [23,24]. miR-126 was rich in lung, brain and kidney, and endothelial cell was the main kind of cells expressing miR-126. Endothelial injury and activation would affect the expression level of miR-126 [25], thus we chosen it as another potential biomarker for sepsis. Let-7i was included because its level might be changed by microbial infection to regulate expression of Toll-like receptor-4 [26]. Finally, the present study identified that levels of miR-146a and miR-223 were lower in septic patients, which might be related to the monocyte and monocyte derived dendritic cells [21,22].

Normalization of circulating miRNAs was crucial for objective evaluation of their expression level. In the first study investigating circulating miRNAs, spiked-in *Caenorhabditis elegans* miRNAs were chosen as internal control because these miRNAs were not present in mice [17]. In the present study, mmu-miR-295 was chosen as internal control due to its absence in rats. We also detected this miRNA in rat serum without spiked-in mmu-miR-295 and no amplification was observed by real-time PCR.

Although all studies on serum miRNAs demonstrate that serum miRNAs are promising biomarkers for several diseases. But our knowledge on serum miRNAs is still at a primary stage. As proposed by Jackson [31], circulating miRNAs might be a critical com-

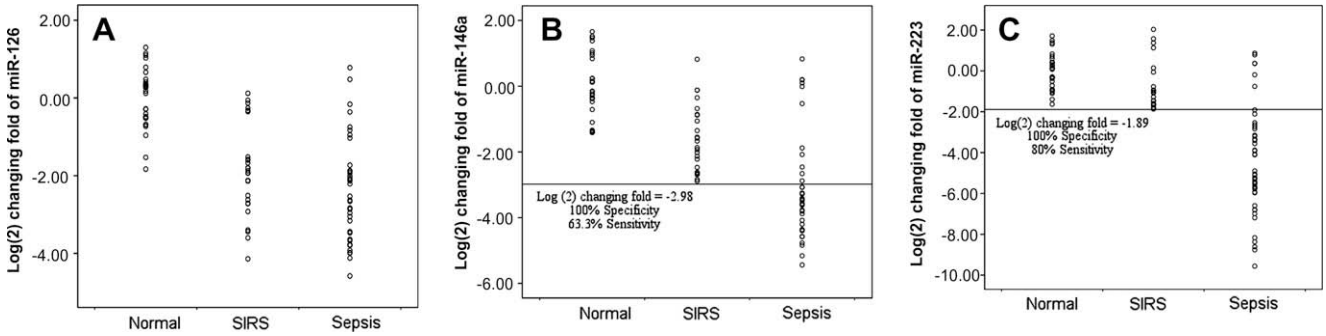

Fig. 4. Expression profiles of miR-126 (A), miR-146a (B) and miR-223 (C) in serum of septic patients. Cut-off points, specificity and sensitivity of miR-146a and miR-223 were listed in the figures.

ponent during pathogenesis of diseases like other biomarkers such as BCR-ABL and HER2. Thus future studies should be performed to clarify the physiological role of circulating miRNAs. It might also be helpful for further intervention on circulating miRNAs and treatment of sepsis. Besides, expression level of circulating miRNAs at different stage of sepsis and their potential correlation with injured organs should also be investigated for complete recognition of these new biomarkers of sepsis.

In general, the present study revealed that level of serum miR-146a and miR-223 were significantly reduced in septic patients compared with SIRS patients and healthy controls. These two miRNAs might serve as new biomarkers for sepsis with both high specificity and sensitivity.

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