

Original Article

Serum miR-126-3p level is down-regulated in sepsis patients

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Abstract: Background: Endothelial injury is part of the pathogenesis of sepsis. The microRNA-126 (miR-126) was previously identified as an endothelial biomarker and is known to play a critical role in preserving endothelial cell integrity. However, the role of miRNA-126 in sepsis is unclear. Method: Blood samples were collected from sepsis patients at the first Affiliated Hospital of Sun Yat-sen University within 24 h (n = 60) and on day 7 (n = 51) after diagnosis, and once from control subjects (n = 46). MiR-126-3p expression was evaluated by quantitative real-time PCR. The miR-126-3p level was correlated with clinical data and a set of routine and experimental biomarkers. The outcome of sepsis patients was determined by follow-up at 28 days after collection of blood samples on day 7. Result: MiR-126-3p level was significantly downregulated in sepsis patients 24 h after diagnosis compared with control subjects. Degree of downregulation of serum miR-126-3p correlated with the severity of sepsis. To determine the diagnostic accuracy of miR-126-3p, the receiver operating characteristic (ROC) was performed and the AUC of miR-126-3p was 0.735. Furthermore, serum miR-126-3p concentration at this time point was correlated with the expression markers of systemic inflammation, bacterial infection, and renal and hepatic dysfunction. However, serum miR-126-3p level on day 7 did not differ between surviving sepsis patients and those who died. Conclusion: These results indicate that miR-126-3p could be a diagnostic biomarker for sepsis.

Keywords: Sepsis, endothelial injury, miR-126-3p, diagnosis, prognosis

Introduction

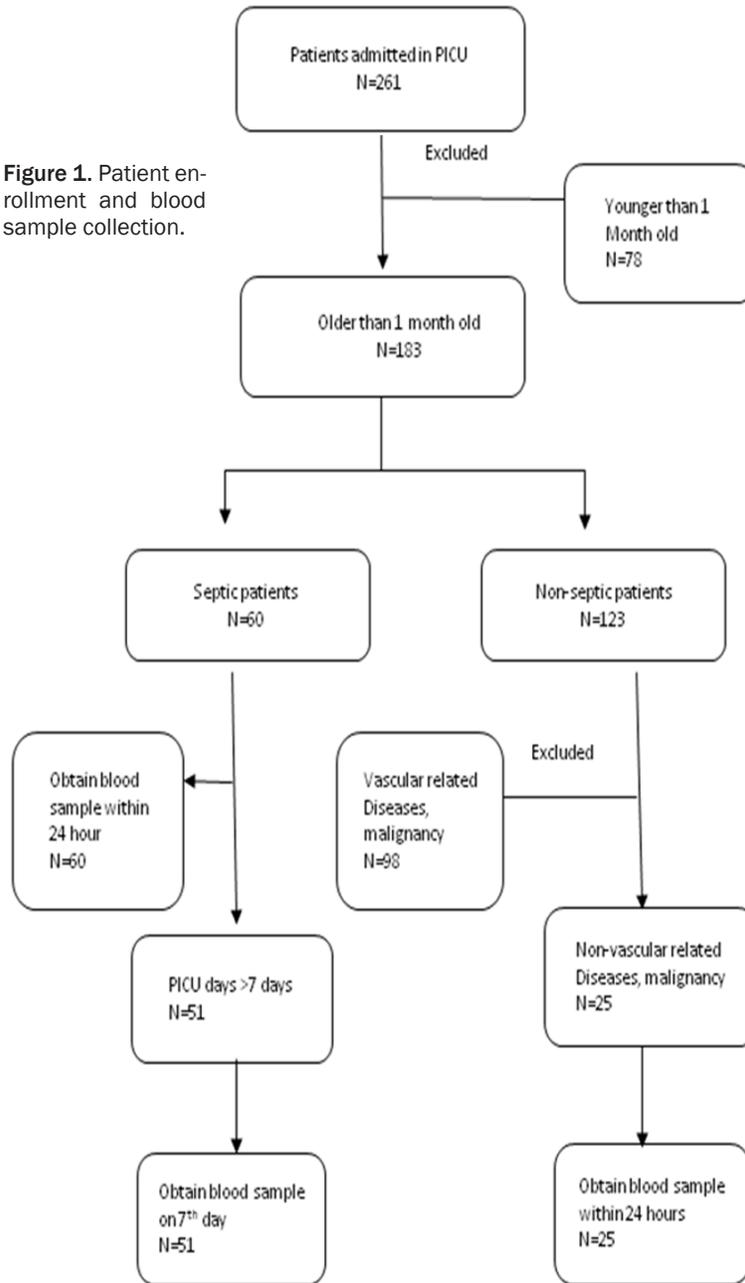
Sepsis is defined as life-threatening organ disorder caused by a dysregulation of the host response to infection [1, 2]. Pediatric sepsis is complicated by clinical symptoms such as fever, high or low white blood cell count, low true positive rate, and delays in blood culture results. Sepsis has a poor outcome and it is the main cause of death for patients in intensive care units (ICUs), with a 30-day mortality rate of 30%-50% worldwide. It is also a major public health burden, accounting for more than \$20 billion (5.2%) of total costs at U.S. hospitals in 2011 [3] and costing \$11,390 per hospitalized patient in China in 2005 [4]. With the ever-increasing incidence of sepsis [5, 6], pediatric intensive care unit (PICU) patients also face high morbidity and mortality rates and a high cost of treatment. The pathogenesis of sepsis is complex, involving perturbations in coagula-

tion, the systemic inflammatory response, and microvascular embolism [7]. It is linked to severe endothelial dysfunction and injury leading to systemic vascular leakage and irreversible multiple organ dysfunction. However, the molecular mechanism responsible for sepsis-induced loss of vascular integrity remains unclear.

MicroRNAs (miRNAs) are a class of endogenous, non-coding, small RNAs approximately 22 nucleotides in length [8] that primarily function as post-transcriptional regulators, inducing mRNA degradation or translational repression [9]. Numerous studies indicate that miRNAs are stably expressed in human serum or plasma [10]. They are tissue-specific [11-13] and played an important role in many diseases, such as cancers, acute coronary syndrome, and diabetes [14-18]. MiRNAs such as MiR-126, miR-15a and miR-155 are endothelial cell-enriched or

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Figure 1. Patient enrollment and blood sample collection.



endothelial-specific miRNAs and play an important role in vascular function [19-21]. Al-Kafaji G et al showed circulating miR-126 could be a biomarker for patients with diabetic nephropathy [22]. Liu et al found miR-15a was involved in the pathogenesis of acute coronary syndrome [23]. Moreover, miR-15a affected angiogenesis in many diseases and miR-146 impacted endothelial cell function via involvement in the inflammatory response [24]. Vascular dysfunction was closely related to occurrence of sepsis. Therefore, we wanted to determine the role

of endothelial cell-enriched or endothelial-specific miRNAs in sepsis.

To determine the relationship between endothelial cell-enriched or endothelial-specific miRNAs and sepsis, some of those miRNAs were measured. Among them, miRNA-126-3p was significantly decreased in septic patients in our preliminary studies. In present study, we were able to further investigate the correlation between miR-126-3p and sepsis.

Materials and methods

Blood sample collection from sepsis patients and control subject

In total, 60 sepsis patients (median age: 2.5 years; range: 1 month-13 years) and 46 control subjects (median age: 6.0 years; range: 1 month-13 years) were enrolled in the study. Control subjects included 25 non-sepsis patients (median age: 3.0 years; range: 1 month-12 years) in the PICU and 21 healthy children (median age: 8 years; range: 5-13 years) who were admitted to the First Affiliated Hospital of Sun Yat-sen University for post-hemotomy and with no other disease. Blood samples were collected from sepsis patients (n = 60) within

24 h of diagnosis and again on day 7 (n = 51) from those receiving long-term (>7 days after diagnosis) treatment. We obtained 25 blood samples from non-sepsis patients on the first day after their admission to the PICU and 21 samples from healthy individuals. Patients with vascular disease, malignancy, and those younger than 1 month old were excluded (**Figure 1**). Patient characteristics are shown in **Table 1**. Patients were admitted to the PICU between January and October 2016. The outcome of sepsis patients was recorded after 28 days by

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Table 1. Baseline patient characteristics

Parameter	All patients	Non-sepsis	Sepsis	P-values
Number	85	25	60	n.a.
Sex (male/female)	50/35	14/11	36/24	n.s.
Age median (range) [years]	2.67 (1/12-13)	3 (1/12-12)	2.5 (1/12-13)	n.s.
PCIS score median (range)	86 (50-96)	88 (82-96)	86 (50-96)	0.033
Prism score median (range)	15 (5-27)	12 (10-27)	16 (12-27)	0.034
ICU day median (range) [day]	14 (7-364)	12 (7-364)	20 (7-27)	n.s.
Death during ICU or follow-up (%)	27%	16%	31.6%	n.s.
Blood urea nitrogen median (range) [mmol/L]	5.55 (2.4-24)	5.3 (3.4-8.6)	5.9 (2.4-24)	0.18
Creatinine median (range) [μ mol/L]	68.5 (12-421)	92 (68-113)	46 (12-421)	n.s.
Albumin median (range)	35.6 (23.1-54.1)	36 (23.1-44)	28 (22-52.4)	n.s.
Procalcitonin median (range) [μ g/l]	0.76 (0.11-161.7)	0.62 (0.14-4.32)	1.2 (0.11-161.7)	n.s.
CRP median (range) [mg/dl]	8.05 (0.06-155)	6.9 (1.2-13.6)	26 (0.06-155)	<0.01
Interleukin-6 median (range) [pg/ml]	55.1 (2.92-480.4)	17 (47-102)	89.4 (2.92-480.4)	0.03
Total bilirubin median (range) [mg/dl]	10.4 (1.8-28)	15.6 (7.4-26.7)	9.6 (1.8-28)	0.018
Alanine transaminase median (range) [U/L]	32.5 (4-1015)	34 (12-88)	32 (4-1015)	n.s.
Aspartate transaminase median (range) [U/L]	41.5 (8-2241)	37 (17-46)	49 (8-2241)	n.s.

Abbreviation: PCIS, Pediatric Critical Illness Score; ICU, intensive care unit; CRP, C-reactive protein.

contacting patients' relatives after blood withdrawal on day 7. Sepsis was diagnosed according to Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock (2012) [25]. Informed consent was obtained from all participants or their guardians and the study protocol was approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University.

RNA isolation

Peripheral blood samples were collected in tubes (BD Biosciences, Franklin Lakes, NJ, USA) with dipotassium ethylenediaminetetraacetic acid, and centrifuged at $6857 \times g$ for 15 min at room temperature. The supernatant was collected and centrifuged at $17,125 \times g$ for 30 min. An 800- μ l volume of TRIzol (Takara Bio, Dalian, China) was added to 400 μ l supernatant along with 200 μ l chloroform (Tianjin Damao Chemical Reagent Factory, Tianjin, China), with vigorous mixing for 15 s followed by incubation at room temperature for 10 min. Samples were centrifuged for 10 min at $13,700 \times g$ at 4°C to precipitate cell debris. The aqueous phase containing total RNA was precipitated by adding an equivalent volume of 100% isopropanol (Guangzhou Chemical Reagent Factory, Guangzhou, China) followed by incubation at room temperature for 20 min. After centrifugation at 4°C for 15 min at $13,700 \times g$, the

pellets were washed once with 75% ethanol (Guangzhou Chemical Reagent Factory) and precipitated RNA was resuspended in 30 μ l RNase-free water (Sigma-Aldrich, St. Louis, MO, USA). RNA quality and quantity were evaluated by agarose gel electrophoresis [26].

Quantitative real-time (qRT)-PCR analysis of miR-126-3p expression

RNA levels were determined by qRT-PCR. Total RNA (1 μ g) was used to synthesize cDNA using the Reverse Transcriptase kit (DBI, Shanghai, China) according to the manufacturer's instructions. The cDNA samples (1 μ l) were used for qRT-PCR in a total reaction volume of 20 μ l containing 10 μ l SYBRGreen qPCR Master Mix (DBI), 0.5 μ l each forward (5'-CTCGCTTCGG-CAGCACA-3') and reverse (5'-AACGCTTCACGA-ATTTGCGT-3') primers (BDI), and 8 μ l RNase-free water (Sigma-Aldrich, St. Louis, MO, USA). The reaction was carried out on an ABI 9700 Biometra (Applied Biosystems, Foster City, CA, USA) detection system at 94°C for 2 min, followed by 40 cycles of 94°C for 20 s, 58°C for 20 s, and 72°C for 20 s. Reactions were prepared in triplicate. Fluorescence quantitative PCR performed on a Stratagene Mx3000P Real-time qPCR system (Agilent Technologies, Santa Clara, CA, USA). Relative gene expression levels were determined with the $2^{-\Delta\Delta\text{CT}}$ method [27].

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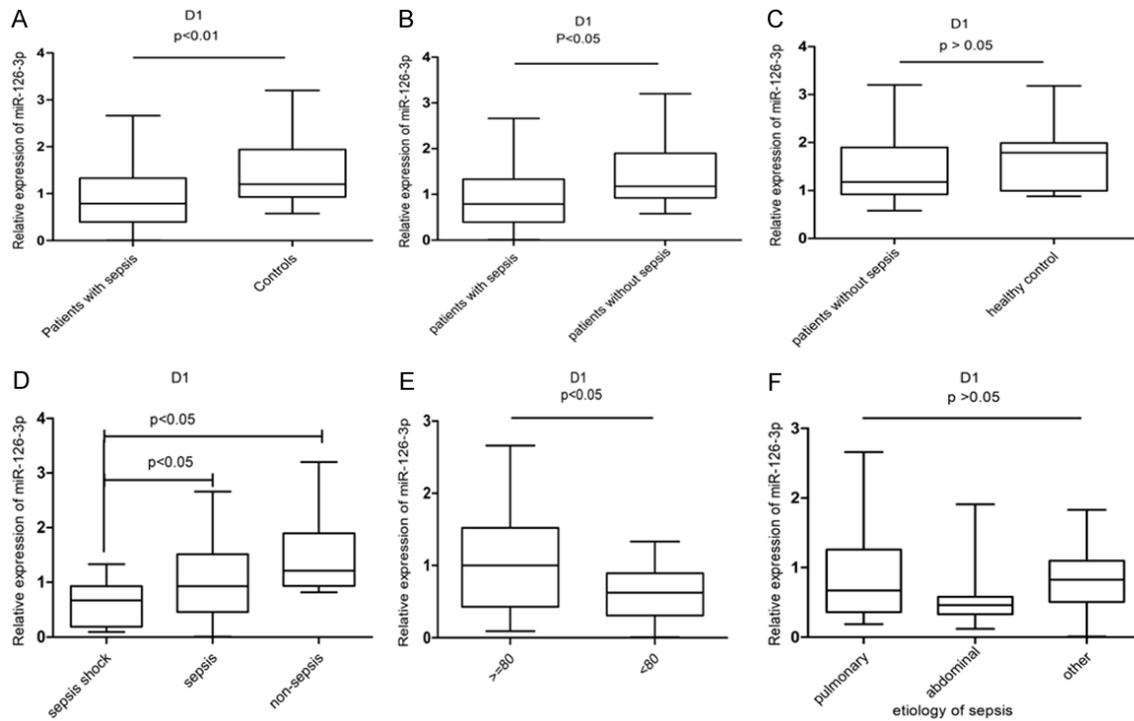


Figure 2. Serum miR-126-3p levels in pediatric sepsis patients and controls. A. Serum miR-126-3p level in sepsis patients (n = 60) and control subjects (n = 46) were significantly different on the first day after diagnosis. B. MiR-126-3p level on the first day was down-regulated in pediatric patients with sepsis as compared to those without sepsis. C. MiR-126-3p levels in patients without sepsis (n = 25) were similar to those in healthy controls (n = 21). D. The serum miR-126-3p level was associated with the degree of sepsis. E. Serum miR-126-3p level of sepsis patients with PCIS scores ≥ 80 was significantly higher than those with scores < 80 . F. Serum miR-126-3p levels did not vary according to the source of sepsis.

Statistical analysis

Continuous variables were tested for normality with the Kolmogorov-Smirnov test. Comparisons between two groups were done by Mann-Whitney U test or student-t-test. Comparisons between groups were performed by analysis of variance (ANOVA). Correlations between variables were analyzed with Spearman's correlation test. Receiver operating characteristic (ROC) curve analysis was performed and area under the curve (AUC) was estimated for diagnostic accuracy of miR-126-3p. Statistical significance was accepted at $P < 0.05$. Statistical analysis was performed using SPSS version 20.0 software and figures were performed by using Prism 5 software (Graph Pad Inc., La Jolla, CA, USA).

Results

Clinical characteristics of sepsis patients and control subjects

A total of 60 pediatric sepsis patients and 25 control subjects without sepsis were enrolled in

the study. There were no differences in age, gender, ICU day, procalcitonin level, or hepatic (albumin, ALT) or renal (blood urea nitrogen, creatinine) function between the two groups. However, C-reactive protein, interleukin-6, total bilirubin content, pediatric risk of mortality (PRISM) score and Pediatric Critical Illness Score (PCIS) differed significantly (**Table 1**).

Serum miR-126-3p levels were reduced in pediatric sepsis patients

Serum miR-126-3p concentrations in sepsis patients on days 1 and 7 after diagnosis and in control subjects were measured by qRT-PCR. Serum miR-126-3p levels in sepsis patients were decreased on the first day after diagnosis compared with control subjects (**Figure 2A**, $P < 0.01$) and patients without sepsis (**Figure 2B**, $P < 0.05$). However, there was no difference in serum miR-126-3p level between the critical illness patients without sepsis and healthy controls at 24 h (**Figure 2C**, $P > 0.05$). Moreover, degree of reduction of the miR-126-3p level correlated with the severity of sepsis (**Figure**

Table 2. Correlation of miR-126-3p serum concentrations of septic patients at ICU admission with other laboratory markers

Parameter	Septic patients	
	R	P
Makers of liver function		
Total bilirubin	-0.501	0.01
Albumin	-0.013	n.s.
ALT	0.077	n.s.
Makers of inflammation		
C-reactive protein	-0.365	0.019
Procalcitonin	-0.337	0.031
IL-6	-0.244	n.s.
WBC	-0.416	0.006
Makers of renal function		
Creatinine	0.038	n.s.
Blood urea nitrogen	-0.838	<0.001
Other variables		
Lactate	-0.588	<0.001
BE	-0.365	0.019
Platelets	-0.041	n.s.
Glucose	-0.063	n.s.
Clinical scoring		
Prism	-0.125	n.s.
PCIS	-0.117	n.s.
SOFA	-0.086	n.s.

2D, $P < 0.05$). We also analyzed serum miR-126-3p levels in sepsis patients with PCIS scores < 80 and ≥ 80 on day 1 and found the miR-126-3p level was significantly higher in the group of PCIS score ≥ 80 (Figure 2E, $P < 0.05$). Subgroup analyses were carried out to determine whether serum miR-126-3p level at 24 h was altered due to disease. However, there were no significant differences in terms of the incidence of pulmonary, abdominal, and other diseases (Figure 2F, $P > 0.05$).

Correlation between serum miR-126-3p and clinical experimental parameters

To evaluate the significance of serum miR-126-3p level in sepsis patients, the correlation between serum miR-126-3p level and clinical experimental parameters was analyzed by correlation analyses. The results revealed that serum miR-126-3p level at 24 h was strongly associated with the expression of markers of systemic inflammation and bacterial infection such as white blood cell count ($r = -0.416$,

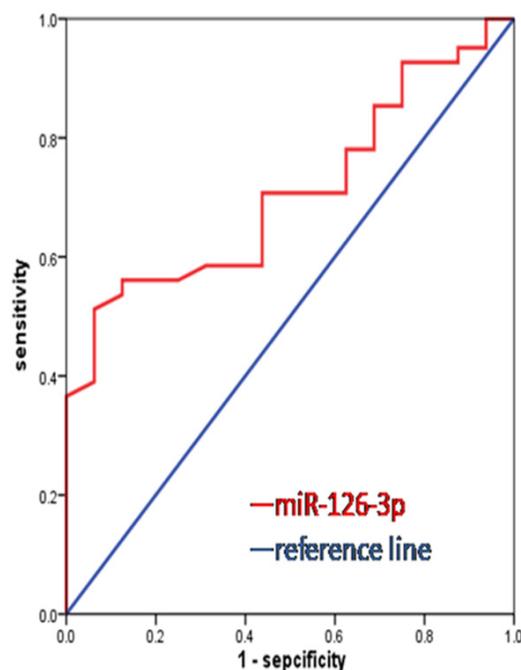


Figure 3. ROC curve analysis of the diagnostic accuracy of serum miR-126-3p levels.

$P < 0.001$), C-reactive protein concentration ($r = -0.365$, $P < 0.05$), and procalcitonin concentration ($r = -0.337$, $P < 0.05$) (Table 2). We also found that serum miR-126-3p concentration was strongly correlated with base excess ($r = -0.365$, $P < 0.05$), blood lactate ($r = -0.588$, $P < 0.001$), urea ($r = -0.838$, $P < 0.001$), and total bilirubin ($r = -0.501$, $P < 0.05$) concentrations (Table 2), suggesting that serum miR-126-3p may play an important role in sepsis-induced organ dysfunction.

Serum miR-126-3p level as a potential predictor of sepsis

To assess the diagnostic value of miR-126-3p, an ROC curve was performed and AUC was calculated. The AUC for miR-126-3p was 0.735 (95% CI, 0.618-0.852) (Figure 3), indicating that circulating miR-126-3p level differs between sepsis patients and control subjects within 24 h of diagnosis and could be a diagnostic biomarker for sepsis.

Serum miR-126-3p level on day 7 was unrelated to outcome in sepsis patients

Serum miR-126-3p concentrations were measured on days 1 and 7 in pediatric patients. There was a significant difference in the level

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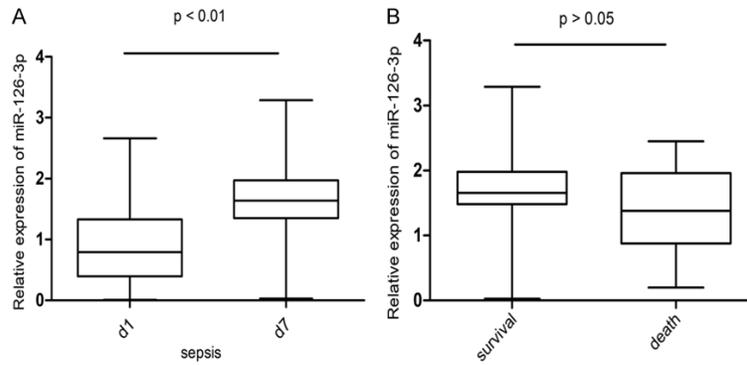


Figure 4. A. MiR-126-3p level of sepsis patients on day 7 was significantly higher than on day 1. B. MiR-126-3p level on day 7 in sepsis patients did not differ between patients who survived and those who had died after a 28 day follow-up visit.

between the two time points (**Figure 4A**, $P < 0.001$). Importantly, circulating serum miR-126-3p levels were low on the first day of treatment in the ICU but were increased on day 7.

We examined the relationship between serum miR-126-3p level on day 7 and patient outcome on day 28 after blood sample collection. Interestingly, patients who survived tended to have higher miR-126-3p levels. However, the difference between these individuals and those who died was not statistically significant (**Figure 4B**, $P > 0.05$), demonstrating that miR-126-3p was unable to determine pediatric sepsis patient prognosis.

Discussion

Sepsis is a serious clinical syndrome with high morbidity and mortality in spite of the development of diagnosis and treatment. Diagnosis of sepsis is difficult in early stage because of non-specific clinical symptoms and delays in blood culture result. Thus, a specific diagnostic biomarker for sepsis is urgently needed. MiRNAs are small non-coding RNAs that directly regulate >30% of genes and indirectly regulate about 70% of genes in a cell, including those related to cell proliferation, migration, and apoptosis. Recent evidence showed that miRNAs played an important role in sepsis. For example, Vasilescu et al identified plasma miR-150 levels were significantly reduced and might be a prognostic biomarker for sepsis patients [28]. Wang et al found that circulating miR-146a were significantly downregulated in sepsis patients [16]. Wang revealed that miR-574-

5p were correlated with death of sepsis patients and might act as a prognostic predictor for sepsis patients [29].

Recent studies showed that miR-126-3p plays an important role in vascular endothelial cell proliferation, migration, and apoptosis, and regulates angiogenesis in cardiovascular diseases such as atherosclerosis and hypertension [30, 31]. Depletion of miR-126 leads to loss of vascular integrity and suppression of endothelial proliferation and migration, resulting

in defective angiogenesis in animal models of mouse and zebrafish [32]. Fish et al showed that miR-126 may decrease vascular permeability and leakage via down-regulating SPRED 1 and PIK3R and Harris et al found miR-126 could suppress endothelial cell adhesion molecular-1 expression and leukocyte adhesion to endothelial cells [33].

In the present study, we found that serum miR-126-3p levels were downregulated significantly in pediatric sepsis patients compared with control subjects on day 1 after diagnosis. The more serious the patients were, the lower the serum miR-126-3p. Moreover, we investigated the relationship between miR-126-3p with clinical data including inflammatory markers, markers of renal function. We detected a significant correlation between serum miR-126-3p level and white blood cell count, and C-reactive protein, procalcitonin, base excess, and lactate concentrations as well as other indicators of hepatic and renal failure. An ROC curve of miR-126-3p was performed to investigate the value for sepsis diagnosis. The result demonstrated the AUC of serum miR-126-3p for sepsis diagnosis was 0.735 (95% CI, 0.618-0.852). These findings suggested miR-126-3p is a potential diagnostic biomarker for pediatric sepsis patients. Also, a significant negative correlation between miR-126-3p and lactate and uric demonstrated that miR-126-3p might be associated with microcirculation disorder and organ dysfunction observed in sepsis.

We also found that serum miR-126-3p levels in patients with sepsis were higher on day 7 than

on day 1 after diagnosis, implying that PICU treatment relieves vascular injury and leads to miR-126-3p up-regulation in patients. However, our study found that miR-126-3p serum levels in sepsis patients on day 7 were not significantly different in survivals and the deaths after a follow-up visit in 28 days. Thus, serum miR-126-3p expression cannot predict patient survival.

Our study had some limitations. First, the number of subjects was relatively small. Second, we did not examine the functional significance of serum miR-126-3p levels in sepsis patients, which would require more detailed studies in animal models. A follow-up period longer than 28 days may provide more insight into the relationship between serum miR-126-3p level and patient outcome.

In summary, we found that miR-126-3p expression was down-regulated in the serum of patients with sepsis within 24 h of diagnosis as compared to control subjects. Thus, miR-126-3p might be a biomarker for the diagnosis of sepsis.

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Disclosure of conflict of interest

None.

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