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ORIGINAL RESEARCH

Interleukin-18 and miR-130a in severe sepsis patients with thrombocytopenia

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Background: Thrombocytopenia is one of the most common laboratory abnormalities encountered in patients with severe sepsis. It has been reported that thrombocytopenia is linked to mortality in patients with severe sepsis. However, the mechanism of thrombocytopenia in sepsis is unknown. We hypothesized that inflammatory cytokines and microRNAs (miRNAs) are not only involved in the pathogenesis of sepsis, but also are correlated with thrombocytopenia.

Patients and methods: Eligible patients with severe sepsis were prospectively recruited and treated at our hospital between June 2012 and May 2014. The miRNA and protein expression of interleukin (IL)-18 and IL-27 were detected by real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. The expression of miR-130a and miR-150 was detected by TaqMan real-time polymerase chain reaction.

Results: Sixty eligible patients were divided into two groups: 28 severe sepsis patients with thrombocytopenia and 32 severe sepsis patients without thrombocytopenia. The results demonstrated that the miRNA expression and plasma concentration of IL-18 in severe sepsis patients with thrombocytopenia were higher than those in severe sepsis patients without thrombocytopenia (P=0.015 and P=0.034, respectively), and miR-130a expression was significantly lower in severe sepsis patients with thrombocytopenia (P<0.003).

Conclusion: Our data demonstrate that severe sepsis patients with thrombocytopenia have increased plasma and miRNA expression levels of IL-18 and decreased expression of miR-130a, suggesting that IL-18 and miR-130a might be involved in the pathophysiological process of severe sepsis with thrombocytopenia.

Keywords: IL-18, miR-130a, severe sepsis, thrombocytopenia, IL-27, miR-150, pathophysiological process

Introduction

Severe sepsis is a major health care problem affecting millions of people around the world.[1] Sepsis is a complex clinical syndrome characterized by severe infection in the body and bloodstream that most commonly originates in the lung, urinary tract, or abdomen. Thrombocytopenia, which is defined as a platelet count (PLT) in the peripheral blood of less than 100×10^9/L, is one of the most common laboratory abnormalities encountered in patients with severe sepsis.[2,3] It has been reported that the incidence of thrombocytopenia in intensive care units varies from 23% to 41%.[4,5] Thrombocytopenia has been reported to be directly linked to the mortality of patients with severe sepsis.[5–7] Therefore, elucidating the mechanism of thrombocytopenia is very important for the identification of reliable inflammatory mediators and the development of a promising therapeutic approach for treating these patients.

Excess production of pro- and anti-inflammatory cytokines is frequently found in the circulation of septic patients. Interleukin (IL)-18, a proinflammatory cytokine,

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is produced by activated macrophages during endogenous inflammatory processes and plays a vital role in the pathophysiology of sepsis.[8] Among critically ill patients, urinary excretion of IL-18 is significantly higher in patients with sepsis than in patients without sepsis.[9–11] Several studies have shown that elevated serum levels of IL-18 are associated with poor clinical outcome in severe inflammatory and septic conditions.[12–14] IL-27, a novel heterodimeric cytokine of the IL-12 family, was shown to be rapidly induced during murine experimental peritonitis induced by cecal ligation and puncture. It has been reported that IL-27 is a key negative regulator of innate immune cell function in septic peritonitis. Furthermore, in vivo blockade of IL-27 is a novel potential therapeutic target for the treatment of sepsis.[15] It has been demonstrated that IL-18 and IL-27 play a role in the pathogenesis of idiopathic thrombocytopenic purpura, which is characterized by low platelet numbers in the peripheral blood.[16–20] Most importantly, several studies also demonstrated that IL-27 and IL-18 are novel prognostic cytokines in infection-induced sepsis.[8,21–24] Therefore, it was speculated that IL-18 and IL-27 are involved in severe sepsis with thrombocytopenia.

MicroRNAs (miRNAs) are a type of small, noncoding, single-stranded RNAs that can posttranscriptionally downregulate specific genes by targeting miRNAs for cleavage or translational repression.[25] It was demonstrated that miR-146a expression in septic patients is significantly decreased compared to the levels in normal controls,[26,27] suggesting that miR-146a may be significantly associated with sepsis. Furthermore, serum miRNAs such as miR-223, miR-146a, and miR-15a are newly emerging biomarkers for sepsis,[28] and miR-223, miR-15a, miR-16, miR-122, miR-193b, and mR-483-5p can be used as predictors for mortality in patients with sepsis.[29] Most importantly, the plasma levels ratio for miR-150/IL-18 can be used for assessing the severity of the sepsis.[30] Collectively, these studies demonstrate that miRNAs play an important role in the pathophysiology of sepsis. In the present study, we aimed to identify the miRNAs that target IL-18 and/or IL-27 to further study new functional miRNAs for the treatment of sepsis with thrombocytopenia.

Patients and methods

Study design and eligible patients

Eligible patients with severe sepsis were prospectively recruited and treated at the Intensive Care Unit of Tianjin First Center Hospital between June 2012 and May 2014. The study protocol was in accordance with the ethical guidelines of the 1995 Declaration of Helsinki and was approved by the independent ethics committees at Tianjin First Center Hospital. In addition, informed written consent was obtained from each patient. Consistent with an earlier study,[31] the assessable patients were divided into two groups: sepsis patients with thrombocytopenia and sepsis patients without thrombocytopenia. Both groups received standard care and appropriate medical support based on the guidelines issued by the surviving sepsis campaign.[32]

Severe sepsis was defined as an inflammatory response with evidence or suspicion of microbial processes and accompanied by evidence of hypoperfusion or dysfunction of at least one organ system, which was established according to the International Sepsis Definitions Conference.[32] Patients younger than 18 years old; those suffering from diabetes, malignancies, cirrhosis, chronic heart failure, chronic respiratory failure, chronic renal failure, human immunodeficiency virus infection, autoimmune diseases, or acquired immune deficiency syndrome; patients who had undergone transplantation; and patients who were receiving immunosuppressive, steroid, or radiation therapy were excluded from this study.

Their peripheral blood samples were collected and analyzed within 24 hours from the diagnosis of severe sepsis. At the same time, we determined fibrin–fibrinogen degradation product, plasminogen activator inhibitor-1, fibrinogen, prothrombin time, C-reactive protein, creatinine, albumin, white blood cell count, PLT, the Acute Physiology and Chronic Health Evaluation II score, and sequential organ failure assessment score. In addition, the following clinical parameters were recorded for each patient: age, sex, site of infection, existence of shock, the number and kind of organ dysfunction, as well as 28-day mortality.

RNA isolation, reverse transcription, and real-time polymerase chain reaction

Peripheral blood was collected into ethylenediaminetetraacetic acid-anticoagulant vacuum tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Ficoll-Hypaque gradients. Total RNA was isolated from 2×10^6 PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and then the reverse transcription (RT) reactions were carried out using the Superscript First-Strand Synthesis System (Invitrogen) following the manufacturer's protocol. To detect the miRNA expression of IL-18 and IL-27 in PBMCs of sepsis patients, we performed real-time polymerase chain reaction (PCR) on an ABI PRISM-7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The sequences of primers were as follows: for IL-18: 5′-CCCCAATCCCTTTATTACCC-3′ (forward), 5′-CGAAGTGGTGGTCTTGTTGC-3′ (reverse);

and for IL-27: 5′-CTTGGCTGGCGTCTCAGCCT-3′ (forward), 5′-CGGAGAGCAGCTTCCTGGCG-3′ (reverse). For PCR amplification, an initial denaturation at 94°C for 10 minutes was followed by 40 cycles at 94°C for 15 seconds and at 60°C for 1 minute. After PCR, a melting curve analysis was performed by increasing the temperature from 60°C to 95°C with a temperature transition rate of 0.1°C/s. Relative gene expression levels were obtained by comparing the expression of each cytokine to that of β-actin using the $2^{-\Delta\Delta Ct}$ method ($Ct,_{\text{target gene}} - Ct,_{\beta\text{-actin}}$).

Enzyme-linked immunosorbent assay for cytokine concentrations

Plasma samples were collected after a short centrifugation and were stored at −80°C until they were analyzed. Plasma IL-18 and IL-27 concentrations were measured by using enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (NeoBioscience Technology Co., Ltd., Shenzhen, China).

TaqMan real-time PCR for quantification of mature miRNAs

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Ten nanograms of total RNA was then used to synthesize complementary DNAs using miRNA-specific primers and an RT kit (Applied Biosystems). Quantitative RT-PCR assays were performed using a TaqMan® MicroRNA assay kit (Applied Biosystems) for miR-130a and miR-150, according to the manufacturer's instructions. Ribonucleic acid U6B (RNU6B) small nuclear RNA was quantified as a control to normalize differences in total RNA levels. PCR amplification reactions were performed on an ABI PRISM-7500 Sequence Detection System (Applied Biosystems). An initial denaturation at 94°C for 10 minutes was followed by 40 cycles of denaturation at 94°C for 15 seconds and extension at 60°C for 1 minute. The relative gene expression values were obtained by comparing the expression of target genes to that of RNU6B using the $2^{-\Delta\Delta Ct}$ method ($Ct,_{\text{target miRNA}} - Ct,_{\text{RNU6B}}$).

Sample size and statistical analysis

The all-cause mortality of severe sepsis varies in different patient populations. The 28-day mortality ranging from 20.8% to 61% has been reported.[33–35] Using the 28-day mortality, the sample size was calculated to detect a 10% difference in mortality between the two groups with a two-tailed test, a significance level (α) of 5%, and a power of 80%. Thus, 60 patients with severe sepsis were needed for this study.

The results are presented as median and range as well as mean ± standard deviation values. Quantitative data were analyzed using Student's *t*-test. Differences with values of $P<0.05$ were considered to be statistically significant. Statistical Package for the Social Sciences version 19.0 (IBM Corporation, Armonk, NY, USA) was used for statistical analysis.

Results

Demographic data and baseline characteristics

A total of 67 patients with severe sepsis were recruited from June 2012 to May 2014. Seven patients were excluded either because of acute gastrointestinal hemorrhage (two patients) or receiving platelet transfusion (three patients) or insufficient data (two patients). Ultimately, 60 eligible patients were divided into two groups: 28 severe sepsis patients with thrombocytopenia and 32 severe sepsis patients without thrombocytopenia. There were no significant differences in baseline characteristics in terms of sex, age, Acute Physiology and Chronic Health Evaluation II score, sequential organ failure assessment score, C-reactive protein, creatinine, albumin, white blood cell count, site of infection, the number and kind of organ dysfunction, fibrin–fibrinogen degradation product, plasminogen activator inhibitor-1, fibrinogen other than PLT, prothrombin time, existence of shock, and 28-day mortality (Tables 1 and 2).

IL-18 and IL-27 miRNA expression in PBMCs of sepsis patients

The expression of IL-18 miRNA in severe sepsis patients with thrombocytopenia was higher than that in those without thrombocytopenia (P=0.015) (Figure 1). There was no significant difference in the expression of IL-27 between the two groups.

Plasma concentrations of IL-18 and IL-27 in sepsis patients

As shown in Figure 2, the plasma concentration of IL-18 in severe sepsis patients with thrombocytopenia was significantly higher than that in sepsis patients without thrombocytopenia (P=0.034). No significant difference was found in the expression of IL-27 between the two groups.

miR-130a and miR-150 expression in PBMCs of sepsis patients

Because miR-130a and miR-150 have been reported to target IL-18,[18,25] we measured the expression of miR-130a and

Table 1 Clinical characteristics of patients

Variable	All (60 patients)	SST (28 patients)	SS (32 patients)
Sex (male/female)	29/31	13/15	16/16
Age, median (range), years	61 (19–82)	60 (19–74)	63 (23–82)
APACHE II score, median (range)	22 (2–45)	21 (2–43)	24 (3–45)
SOFA score, median (range)	7.2 (0–17)	7.0 (0–16)	8.0 (0–17)
CRP, mg/dL, median (range)	9.6 (2.3–34.6)	10 (2.3–34.6)	9.4 (3.0–29.8)
Creatinine, median (range), mg/dL	1.2 (0–14)	1.0 (0.4–14)	1.3 (0–10.9)
Albumin, median (range), mg/dL	27.3 (16.0–51.8)	28.6 (16.0–51.8)	26.4 (17.0–50.3)
WBC, median (range), $\times 10^3/\mu L$	11.0 (2.4–31.0)	10.1 (3.0–31.0)	11.8 (2.4–28.5)
PLT, median (range), $\times 10^3/\mu L$	–	20.0 (10.0–67.0)	124 (101.0–452.0)*
FDP (mean ± SD), μg/L	16.7±5.2	17.7±2.7	15.9±5.4
PAI-1 (mean ± SD), ng/mL	28.6±3.5	30.6±3.4	25.3±7.2
Fibrinogen (mean ± SD), g/L	4.9±1.6	5.1±2.2	4.8±1.2
PT (mean ± SD), seconds	–	13.97±2.86	16.57±2.98*

Note: *$P<0.001$.

Abbreviations: APACHE II score, Acute Physiology and Chronic Health Evaluation II score; CRP, C-reactive protein; FDP, fibrin-fibrinogen degradation product; PAI-1, plasminogen activator inhibitor-1; PLT, platelet count; PT, prothrombin time; SOFA score, sequential organ failure assessment score; SS, severe sepsis without thrombocytopenia; SST, severe sepsis with thrombocytopenia; WBC, white blood cell count.

Table 2 Baseline characteristics and 28-day mortality of patients

Variable	All (60 patients)	SST (28 patients)	SS (32 patients)
Primary infection site			
Respiratory	30 (50%)	14 (50%)	16 (50%)
Abdominal	10 (16.7%)	5 (17.9%)	5 (15.6%)
Urinary tract	7 (11.7%)	3 (10.7%)	4 (12.4%)
Bloodstream	6 (10%)	3 (10.7%)	3 (9.4%)
Others	4 (6.6%)	2 (7.1%)	2 (6.3%)
Unknown	3 (5%)	1 (3.6%)	2 (6.3%)
Microorganism identified			
Gram-negative bacilli	25 (41.6%)	13 (46.4%)	12 (37.5%)
Gram-positive cocci	20 (33.3%)	9 (32.1%)	11 (34.5%)
Fungus	2 (3.3%)	1 (3.6%)	1 (3.1%)
Intracellular germs	2 (3.3%)	1 (3.6%)	1 (3.1%)
Others	10 (6.5%)	4 (14.3%)	6 (18.8%)
Existence of shock			
Yes	15 (25%)	11 (39.3%)*	4 (12.5%)*
No	45 (75%)	17 (60.7%)*	28 (87.5%)*
The number of organ dysfunction			
1	20 (33.3%)	8 (28.6%)	12 (37.4%)
2	20 (33.3%)	10 (35.7%)	10 (31.3%)
≥3	20 (33.4%)	10 (35.7%)	10 (31.3%)
The kind of organ dysfunction			
Arterial hypoxemia	14 (23.3%)	7 (25%)	7 (21.9%)
Arterial hypotension	49 (81.7%)	22 (78.6%)	27 (84.4%)
Acute respiratory failure	14 (23.3%)	6 (21.4%)	8 (25%)
Acute renal failure	21 (35%)	9 (32.1%)	12 (37.5%)
Impaired neurological status	13 (21.7%)	7 (25%)	6 (18.8%)
Acute liver failure	10 (16.7%)	5 (17.9%)	5 (15.6%)
Others	11 (18.3%)	5 (17.9%)	6 (18.8%)
None	0	0	0
Twenty-eight days survival			
Alive	34 (56.7%)	12 (42.9%)*	22 (68.8%)*
Dead	26 (43.3%)	16 (57.1%)*	10 (31.2%)*

Note: *$P<0.05$.

Abbreviations: SS, severe sepsis without thrombocytopenia; SST, severe sepsis with thrombocytopenia.

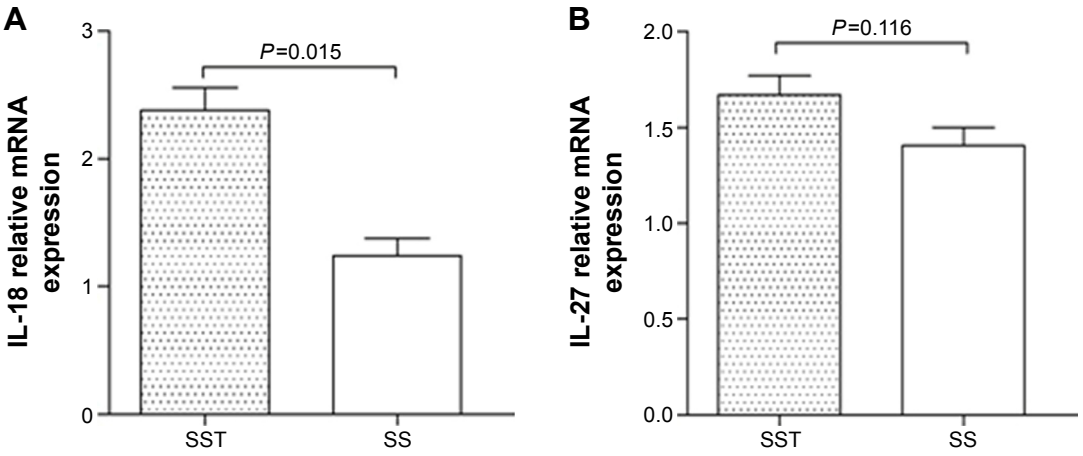

Figure 1 miRNA expression of IL-18 and IL-27 in PBMCs of sepsis patients.
Notes: (**A**) miRNA expression of IL-18 in severe sepsis patients with thrombocytopenia was greater than that in those without thrombocytopenia (P=0.015). (**B**) There was no significant difference in the expression of IL-27 between the two groups (P=0.116).
Abbreviations: IL, interleukin; miRNA, micro RNA; PBMCs, peripheral blood mononuclear cells; SS, severe sepsis without thrombocytopenia; SST, severe sepsis with thrombocytopenia.

miR-150 in PBMCs of sepsis patients by TaqMan real-time PCR. The results demonstrated that miR-130a expression was significantly lower in severe sepsis patients with thrombocytopenia than in those without thrombocytopenia ($P<0.003$) (Figure 3), whereas miR-150 expression was not significantly different between the two groups (data not shown).

Discussion

Sepsis is a systemic disease characterized by microbial infection and systemic inflammatory response syndrome, and is associated with high morbidity and mortality, especially in severe cases.[32] Thrombocytopenia is common among severe sepsis patients,[36] and a low PLT is predictive of a poor outcome.[6] To date, the mechanism of severe sepsis with thrombocytopenia is still unknown. In the present study, we selected two cytokines linked to sepsis and thrombocytopenia, IL-18 and IL-27, to study the mechanism of the development of thrombocytopenia in severe sepsis patients.

It has been reported that the serum IL-18 level in sepsis patients is significantly greater than that in healthy volunteers.[37] It has also been reported that a high blood IL-18 level may be an early predictor of mortality.[38] The results of the present study showed that the miRNA expression and plasma concentration of IL-18 in severe sepsis patients with thrombocytopenia were higher than those in sepsis patients without thrombocytopenia (P=0.015 and P=0.034,

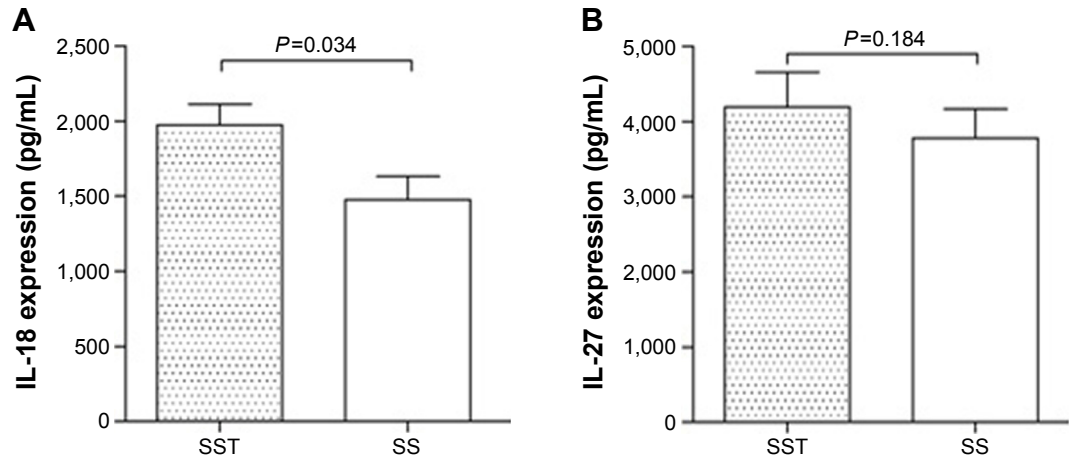

Figure 2 Plasma concentrations of IL-18 and IL-27 in sepsis patients.
Notes: (**A**) The plasma concentration of IL-18 in severe sepsis patients with thrombocytopenia was found to be significantly higher than that in those without thrombocytopenia (P=0.034). (**B**) No significant difference in the expression of IL-27 was found between the two groups (P=0.184).
Abbreviations: IL, interleukin; SS, severe sepsis without thrombocytopenia; SST, severe sepsis with thrombocytopenia.

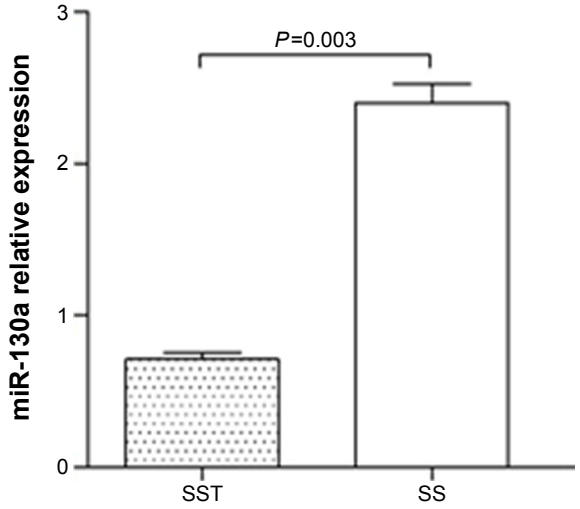

Figure 3 miR-130a expression in PBMCs of sepsis patients. miR-130a expression was significantly lower in severe sepsis patients with thrombocytopenia than in severe sepsis patients without thrombocytopenia ($P<0.003$).
Abbreviations: PBMCs, peripheral blood mononuclear cells; SS, severe sepsis without thrombocytopenia; SST, severe sepsis with thrombocytopenia.

respectively), whereas there was no significant difference in IL-27 expression between the two groups, indicating that IL-18 is involved in the development of thrombocytopenia in severe sepsis patients.

The discovery of miRNAs had led to the discovery of novel regulatory mechanisms for development of sepsis.[26–30] It has been reported that IL-18 is the target gene of miR-130a and miR-150.[18,30] Therefore, in this study, we also detected the expression of miR-130a and miR-150 by TaqMan real-time PCR, and the results showed that miR-130a expression was significantly lower in severe sepsis patients with thrombocytopenia, compared to that in sepsis patients without thrombocytopenia ($P<0.003$), indicating that miR-130a plays an important role in the pathogenesis of thrombocytopenia in severe sepsis patients.

Indeed, it is clear that knowing the primary infection site, identifying the microorganism, knowing the existence of shock, the number and kind of organ dysfunction, and clinical management of severe sepsis, including initial volemic resuscitation with goal-directed fluid challenge, diagnosis of infection with microbiological sampling coupled with imaging studies, treatment of infection with antibiotics, and so on, are very important. Even though severe sepsis is a syndrome involving the whole body, identification of these potential confounders causes important subsequent actions. For example, a severe sepsis patient with thrombocytopenia was associated with 28-day mortality, which may be due to the induction of shock (Table 2). Therefore, presence or absence of thrombocytopenia should be considered along with sepsis when approaching the evaluation or treatment of patients with severe sepsis.[31]

This study had potential weaknesses. First, the all-cause mortality of sepsis varies in different patient populations, which could have also affected outcome. Moreover, it involved a relatively small number of patients who were treated with heterogeneous regimens and therapies. Although the results demonstrate that severe sepsis patients with thrombocytopenia have increased plasma and miRNA expression levels of IL-18 and reduced expression of miR-130a, suggesting that IL-18 and miR-130a are involved in the pathophysiological process of thrombocytopenia in severe sepsis, further investigation is warranted to identify who would benefit most from analysis of these laboratory parameters. Taken together, these findings suggest that direct neutralization of IL-18 and upregulation of miR-130a to inhibit IL-18 indirectly may be promising therapeutic approaches of treating severe sepsis patients with thrombocytopenia.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors have no financial disclosures and are not using any copyrighted information in this paper. No text, text boxes, or figures in this article have been previously published or owned by another party. The authors report no conflicts of interest in this work.

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