Evidence for circulating microRNA hsa-let-7d-3p as a potential new biomarker for sepsis in human subject

Zhaorui Zhang
Chinese PLA General Hospital
Hailun Luo
liangxiang hospital
Chunsun Li
Chinese PLA General Hospital
Zhixin Liang (liangzhixin301@163.com)
PLA Army General Hospital

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Abstract

Background: Current biomarkers for early detection of sepsis have low sensitivity and specificity. Serum microRNAs (miRNAs) have been proposed as novel non-invasive biomarkers for various diseases. The aim of the present study was to discover a novel diagnostic biomarker for sepsis in human subjects.

Methods: miRNA expression profile was performed using peripheral blood from three sepsis patients in sepsis stage and condition improved stage using microarray screening. The differentially expressed miRNAs were primary validated by real-time quantitative polymerase chain reaction (qRT-PCR) in a further set of 20 sepsis patients in the sepsis stage and condition improved stage. We finally validate the different expressed miRNA in 95 sepsis patients and 66 non sepsis patients. The validated miRNAs along with patients' clinical indictors were analyzed in a multivariate logistic regression model. The diagnosis value of the changed miRNA in sepsis was determined and compared with CRP and WBC by analyzing the receiver operating characteristic (ROC) curves.

Results: According to the criteria we detected 3 miRNAs up regulated and 8 miRNAs down regulated by miRNA chip. qRT-PCR detection showed that the expression of hsa-let-7d-3p in sepsis patient was up regulated compared with non-sepsis patients. In a multiple logistic regression analysis, serum miRNA hsa-let-7d-3p was found to be independent predictor of sepsis. Receiver operating characteristic curve (ROC) analysis showed that the area under ROC curve of serum miRNA hsa-let-7d-3p was 0.696 (95% confidence interval [0.615, 0.778]).

Conclusion: miRNA hsa-let-7d-3p was identified as novel biomarkers for the early detection of sepsis.

Introduction

Sepsis should be defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Despite of the development in diagnostic and therapeutic techniques, sepsis is still the major cause of mortality in critical ill patients in the past 20 years[1 2]. Approximate 1/5 – 1/2 of the patients with sepsis dies of multi organ dysfunction syndrome [3 4]. Delayed diagnosis and accurate assessment of patients with sepsis usually increased heavy economic burden and mortality rate [5–7]. Current biomarkers such as C reactive protein (CRP), procalcitonin (PCT), have been widely applied in clinical to monitor sepsis. However, a meta-analysis showed only a low sensitivity and specificity for CRP to indicate an infection and was not recommend in the current sepsis guidelines as a sepsis biomarker [8]. PCT is better than CRP in the diagnosis of bacterial infection and but is still lack of specificity [9 10]. Biomarkers, which can be used to establish a diagnosis for sepsis, were still not well established [6].

miRNAs are a class of 18–25 nucleotides noncoding RNAs ,which regulate gene expression at the post-transcriptional level and play a role in diverse biomolecular processes, including inflammation and immunity[11 12]. Studies showed that miRNAs were present in a cell-free form (serum, plasma or other body fluids), and the levels of individual miRNA or specific miRNA signatures were linked to various cancers or other diseases [13–15]. Recently, previous studies reported that miR-25 and miR-122 may be

related to sepsis, however the diagnosis values of these miRNAs were unsatisfactory[8]. Novel biomarkers which can accurately guide diagnosis and treatment of sepsis were still in needed.

In this study, we hypothesized that the sepsis related miRNA in serum has the potential to be a diagnostic biomarker. Accordingly, the goal of our present study was to discover miRNAs which early diagnose sepsis in critically ill patients.

Methods

Ethics

This study was approved by the ethics committee of General Hospital of PLA, No.s2014-048-01. All subjects or their agents signed the informed consent.

Patients and sepsis definition

The study population consisted of 161 patients with 95 sepsis patients and 66 no sepsis patients during the period from April 2012 to May 2014. Patients newly hospitalized in the ICU of the PLA Hospital were screened for candidate. Patients were included if met two of the following criteria: 1) core temperature > 38 °C or < 36 °C; 2) heart rate > 90 beats/minute; 3) respiratory rate > 20 breaths/min or a $PaCO_2$ < 32 mmHg; 4) white blood cell count > 12×10^9/L or < 4×10^9/L or > 10% immature neutrophils [16]. Patients were excluded according to the following criteria 1) age < 18 years; 2) pregnancy or lactation;3) HIV infection; and 4) unwillingness to participate. Patients who died or discharged within 24 h after admission into ICU were excluded from the study. According to the International Sepsis Definitions Conference sponsored by SCCM/ESICM/ACCP/ATS/SIS in 2001[17], patients were classified to none sepsis and sepsis, at the time of admission by two intensivists independently. Agreement concerning the diagnosis was achieved in all cases. In clinical practice, antimicrobial therapy was prescribed according to the usual practice of the ICU when infection was suspected by the attending physician without interference by this study. The age, gender, sequential organ failure assessment (SOFA) score were recorded, also, the levels of C - reactive protein (CRP) and procalcitonin (PCT) in serum were analyzed routinely. The condition improved of sepsis patients was defined as 1) core temperature < 38 °C and > 36 °C), 2) the white blood cell return to < 12×10^9/L and > 4×10^9/L. Three patients in the condition improved sepsis patients were selected for miRNA screening and 20 patients with condition improved were selected for primary validation.

RNA isolation and quality assessment

Total RNA from peripheral blood was isolated and purified with TRI Reagent BD kit according to manufacturer's instructions. RNA quality and quantity was measured by using nanodrop spectrophotometer (ND-1000, Nanodrop Technologies). RNA integrity was assessed by agarose gel electrophoresis.

miRNA gene chip analysis

The serum of 3 sepsis patients in the sepsis stage and condition improved stage were collected. The samples underwent microarray analysis using the 7th generation of the miRCURY LNA™ miRNA Array (Exiqon A/S, Vedbaek, Denmark). Genes with Benjamini–Hochberg false discovery rate (FDR) corrected p values of 0.05 and threshold values of fold ≥ 2 or < 0.5 change were considered to be differentially expressed.

Real-time quantitative polymerase chain reaction

The serum of 20 sepsis patients in the sepsis stage and condition improved stage were collected. Primary qRT-PCR analysis was conducted to detect the expression level of the selected miRNAs in the patients with sepsis for validation.

Final qRT-PCR analysis was conducted to detect the expression levels of the primary validate target miRNAs in patients with sepsis and patients without sepsis.

Total RNA was retrotranscribed to cDNA using a PrimeScript™ RT Reagent Kit with a gDNA Eraser according to the manufacturer's instructions. (TIANScript RT Kit, Beijing,China). 20 μl volume reactions was used in real time quantitative polymerase chain reaction(qRT-PCR) containing 4 μl RNA (50 μl purified serum) ,2 μl Bulge-Loop™ RT primer(500 nM), 2 μl Super Pure dNTPs, 6.5 μl nuclease-free H_2O, 4 μl 5 × First-Strand Buffer, 0.5 μl RNasin, 1 μl TIANScript M-MLV. Reaction setting consisted of a predenaturation step for 1 min at 95 ℃ followed by 40 cycles denaturation step for 10 s at 95℃, 20 s at 60℃ and 10 s at 70℃. Samples were heated for 1 min at 95℃ and 1 min at 60℃ and then heated from 55℃ to 100℃ for melt curve. Expression level of each miRNA was analyzed by the comparative CT method ($2^{-\triangle CT}$). The cel-miR-39-3p was used as reference gene.

Statistical Analysis

Data were statistically analyzed using SPSS 17.0. In the univariate analysis, data with normal and non-normal distributions and the count data were evaluated through t-test, Mann–Whitney test, and χ2 test, respectively. $p < 0.05$ indicated significant differences. A multivariable logistic regression analysis was also used to evaluate the predictive values and odds ratios of these miRNAs. The specificity and sensitivity of miRNAs in the diagnosis of sepsis were calculated in terms of the area under the receiver operating characteristic (ROC) curve.

Results

Clinical characters of the subjects

The clinical characteristics of the patients were shown in Table 1. Exactly 161 patients with 95 patients with sepsis and 66 non-sepsis, including 105 males (65.2%) and 56 females (34.8%) with an average age of 64.0 (47.5–78.0) years old, were enrolled in this study. Exactly 95 patients with sepsis including 68 males (71.6%) and 27 females (28.4%) with an average age of 67.0(53.0–81.0) years old, were diagnosed by sepsis criteria. The comorbidities and platelet between sepsis patients and non sepsis

patients were comparable without significant difference. As anticipated, sepsis patients had higher SOFA score, APACHE Ⅱscore, WBC counts and mortality rate($p < 0.05$). The details of 3 patients for miRNA screening and 20 patients for primary validation were shown in supplement materials.

number	Time interval of serum collection	SOFA scors	SOFA scors(condition improved)	PCT,ng/ml (sepsis)	PCT,ng/ml (conditionimproved)
1	37	9	0	6.60	0.08
2	11	12	4	35.18	0.24
3	10	8	1	12.09	0.34

Table 1 The characterist of the three patients using for mi-RNA chip

Overview of differentially expressed miRNA in sepsis

11 miRNAs, namely hsa-miR-373-5p, hsa-miR-495-3p, hsa-miR-642a-3p, hsa-miR-5584-3p, hsa-miR-2682-3p, hsa-miR-381-5p, hsa-miR-3591-5p, hsa-miR-3685, hsa-miR-501-5p, hsa-miR-4795-3p, hsa-let-7d-3p satisfied the screening criteria of $p < 0.05$, fold change ≥ 2 or ≤ 0.5. Among these miRNAs, hsa-miR-373-5p, hsa-miR-495-3p, hsa-miR-642a-3p expression were up regulated and hsa-miR-5584-3p, hsa-miR-2682-3p, hsa-miR-381-5p, hsa-miR-3591-5p, hsa-miR-3685, hsa-miR-501-5p, hsa-miR-4795-3p, hsa-let-7d-3p were down regulated. The fold change of the miRNAs were shown in Table 2. The change of hsa-miR-3591-5p、hsa-miR-501-5p were down regulated without significant difference ($p > 0.05$). The fold change of hsa-let-7d-3p was 6.49 with significant difference ($p < 0.05$).

Table 2
Differentially expressed miRNAs in gene chip analysis

miRNA	Regulation	Fold change
hsa-miR-373-5p	Up	3.248689
hsa-miR-495-3p	Up	2.86105
hsa-miR-642a-3p	Up	4.738026
hsa-miR-5584-3p	down	0.220295
hsa-miR-2682-3p	down	0.144371
hsa-miR-381-5p	down	0.179186
hsa-miR-3591-5p	down	0.136297
hsa-miR-3685	down	0.147137
hsa-miR-501-5p	down	0.110752
hsa-miR-4795-3p	down	0.108122
hsa-let-7d-3p	down	0.194955

Primary miRNA confirmation and selection by qRT-PCR

In this regard, relative data regarding miRNAs in sepsis were reviewed, we selected hsa-miR-3591-5p□hsa-miR-501-5p□hsa-let-7d-3p for primary validation. The serum of the patients were collected in sepsis stage and condition improved stage. The interval between the serum collection of was from 6 to 37 days. The median SOFA scores and CRP level were significantly reduced when the condition of the patients improved(Fig. 1). The results of qRT-PCR also indicated that the expression levels of hsa-let-7d-3p was significantly increased when the condition of the patients improved (Fig. 2). The fold changes of the selected miRNAs were shown in Table 3. The expression of hsa-let-7d-3p was significantly higher than control group($p < 0.05$). 95% confidence interval was 6.49(4.33,9.22). The other two miRNAs were lower than control without significant difference($p > 0.05$).

Table 3
Fold change of the miRNA by self-control comparison in 20 patients

microRNA	Fold change(95%CI)	p-value
hsa-miR-3591-5p	0.72(0.42,1.13)	0.18
hsa-miR-501-5p	0.68(0.44,1.02)	0.08
hsa-let-7d-3p	6.49(4.33,9.22)	0.00

Final validation of hsa-let-7d-3p detected by qRT-PCR

In this step, 161 patients, including 95 sepsis patients and 66 non-sepsis patients were used for final qRT-PCR validation. The relative expression levels of hsa-let-7d-3p in sepsis patients were significantly higher than in non-sepsis patients ($p < 0.01$) (Fig. 3). In addition, the miRNA altered expression patterns in the validation set were consistent with the results from the primary validation set.

Multivariable logistic regression analysis

Furthermore, a logistic regression model was used to evaluate these variables associations including hsa-let-7d-3p along with age, sofa score, WBC counts, CRP and trauma (Table 4). From the analysis, only hsa-let-7d-3p and SOFA score showed statistically significant differences ($p < 0.05$). The results indicated that hsa-let-7d-3p and SOFA score were independent predictors for the diagnosis of sepsis.

Table 4
Multivariate logistic regression analyses of hsa-let-7d-3p and the clinical indicators between sepsis patients and non-sepsis patient

Variable	Coefficient	Standard Error	Wald	P Value	Odds Ratio
Age	0.025	0.011	5.320	0.021	1.026
SOFA score	0.217	0.068	10.170	0.001	1.242
WBC counts	0.069	0.039	3.021	0.082	1.071
CRP	0.059	0.032	3.424	0.064	1.061
hsa-let-7d-3p	0.143	0.062	5.336	0.021	1.153
Trauma	-1.107	0.614	3.254	0.071	0.331

Comparisons of the diagnostic values of the miRNA,CRP and WBC counts

The predictive capability of three variables (CRP, hsa-let-7d-3p, WBC counts) were analyzed using ROC curve. ROC analyses of the biomarkers to predict sepsis showed areas under the curve were 0.742 (95% CI, 0.662–0.822) for CRP, 0.696 (95% CI, 0.615–0.778) for hsa-let-7d-3p, and 0.627 (95% CI, 0.541–0.713) for WBC counts (Fig. 4).

The results suggest that serum hsa-let-7d-3p level count be used to early diagnose patients with sepsis. The best cutoff value of has-let-7d-3p for sepsis diagnosis was 2.6, with a sensitivity of 60% and a specificity of 75%.

Discussion

miRNAs have been used as biomarkers for various type of disease since discovery of circulating miRNAs in human peripheral sera. Our study demonstrated that hsa-let-7d-3p had the potential to be a novel diagnostic biomarker of sepsis, with high sensitivity and specificity. Previous studies have found several miRNA as diagnostic and prognostic biomarkers for sepsis[8]. Yao and colleagues found that miR-25 displayed a superior diagnostic accuracy for sepsis compared to well established markers such as CRP and PCT according to ROC curve analysis in a well characterized cohort of 70 patients with sepsis and 30 patients with non-infectious SIRS[18]. They further found that decreased miRNA-25 level was related to the level of oxidative stress indicators in sepsis patients. Xie et al found that circulating miR-122 as a potential biomarker of critical illness and sepsis in a sample of 214 patients with sepsis (117 survivor and 97 non-survivors), Mir-122 predicted patients' short and long term survival with high accuracy[19 20]. The hsa-let-7d-3p is one of the tumor suppressive let-7d family members. Let-7d is down regulated in numerous types of cancer, including ovarian cancer and directly targets various oncogenes. Gunal have reported that has-let-7d-3p could down regulated HMGA2 and KRAS gene and the loss of has-let-7d-3p expression led to the progression of epithelial ovarian cancer related to the tumorigenesis, invasion, and metastasis[21]. However, it have not been reported to be associated with the early diagnosis of sepsis. To the best of our knowledge hsa-let-7d-3p was the first time be identified as a biomarker of sepsis.

In our study, gene chip analysis revealed that 11 miRNAs satisfied the screening criteria of $p < 0.05$, fold change fold change ≥ 2 or < 0.5. Limited data has been presented regarding the relationship of these 11 miRNAs with sepsis because of the following factors: 1) Sepsis was a complicated syndrome with multi organ failure in pathogenic microorganism, severity and comorbidities. The complexity of sepsis lead to the complexity of sepsis biomarker, it is difficult to find a single biomarker of sepsis with high sensitivity and specificity. The miRNAs which can early diagnosis the sepsis various with different studies.

In our study, the number of samples in the gene chip analysis was limited to the serum of 3 patients, but we further verified the results of the gene chip through qRT-PCR analysis to compensate for the limitations of our study. The regulating of the hsa-let-7d-3p was down regulated in the gene chip analysis, and it was up regulated in the further validation in the sepsis patients. The reason of the opposite results may be we only enrolled three samples of patients for screening. However the results of the qRT-PCR validation were more convincing than PCR screening.

There were several advantages of our study. First, we choose self-control comparison to primary validate the different expressed miRNAs between sepsis stage and condition improved stage. Self-control study can maximum reduce the influence factors: such as age, gender, illness severity, comorbidities and so on. So the results of our study were more convincing than other studies. Second, reference gene cel-mirRNA-39-3p was chosen as reference gene in our study as previously reported[13 22]. Currently, there is no consensus on what reference gene should be used[13 22]. Reference gene such as U6, 5S rRNA in the serum or plasma had been used in previous study, however they were found to be unstable in other studies[14 23]. Fabian Benz have reported that levels of U6 was significantly up-regulated in sera of patients with critical illness and sepsis and was correlated with inflammation. Third, the selection of the control group in our study was better. Compared with healthy control in other studies control group in our study included patients with COPD, hypertension, diabetes malignant tumor, trauma and so on. There were no significant difference of comorbidity between sepsis group and control group. The selection of control group in our study were more similar to real world condition. Distinguish sepsis patients from healthy control is easier than distinguish sepsis patient from patients with infectious disease and other comorbidities. So the results of our studies were more convincing than other studies which using normal healthy control.

There were several limitations of our study. First, in the RT-PCR validation of miRNAs, we found that 11 miRNA meeting our screening criteria, however we only enrolled 3 miRNAs for primary validation for shortage of funds. Second, the total number of cases enrolled in the study was limited. Hence, the value of hsa-let-7d-3p in predicting early sepsis should be further evaluated in large samples.

Conclusion:

We revealed for the first time that hsa-let-7d-3p may be a novel biomarker for the early diagnosis of sepsis. Further study is needed to verify the connection and to explore the mechanism.

Abbreviations

miRNA: microRNA; qRT-PCR: real-time quantitative polymerase chain reaction; ROC: receiver operating characteristic curve; CRP: C reactive protein; PCT: procalcitonin; HIV: human immunodeficiency virus; SOFA: sequential organ failure assessment score; SIRS: Systemic Inflammatory Response Syndrome, COPD: chronic obstructive pulmonary disease.

Declarations

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Not applicable

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Availability of data and materials

The datasets used and/or analyzed are available from corresponding author upon reasonable request.

Authors' contributions

ZL conceived and designed the experiments. HL performed the experiments. CL analyzed the data. ZZ wrote the article. All authors have read and approved the final manuscript.

There is no conflict of interest.

Ethics approval and consent to participate

The medical ethics committee of the Chinese PLA general hospital approved the study protocol. All subjects were approached for written informed consent to participate in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

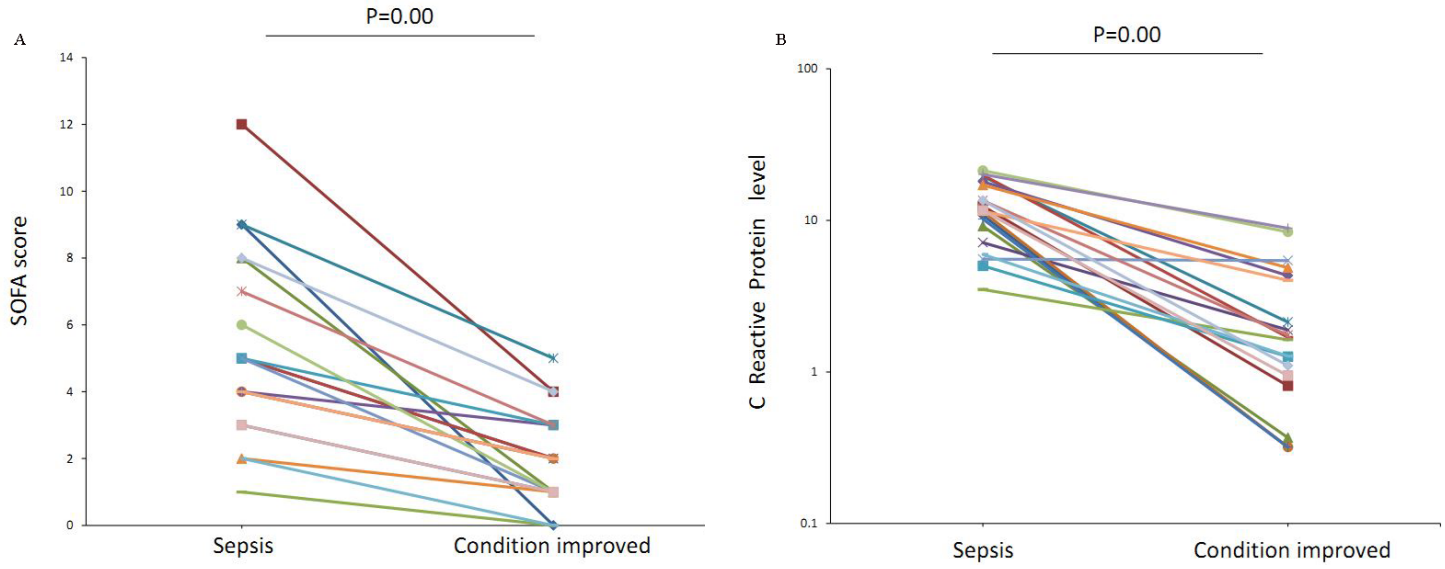

Figure 1

SOFA scores and CRP level in the sepsis patients in sepsis stage and condition improved stage. A) the SOFA scores; B) the C Reactive Protein level(mg/L)

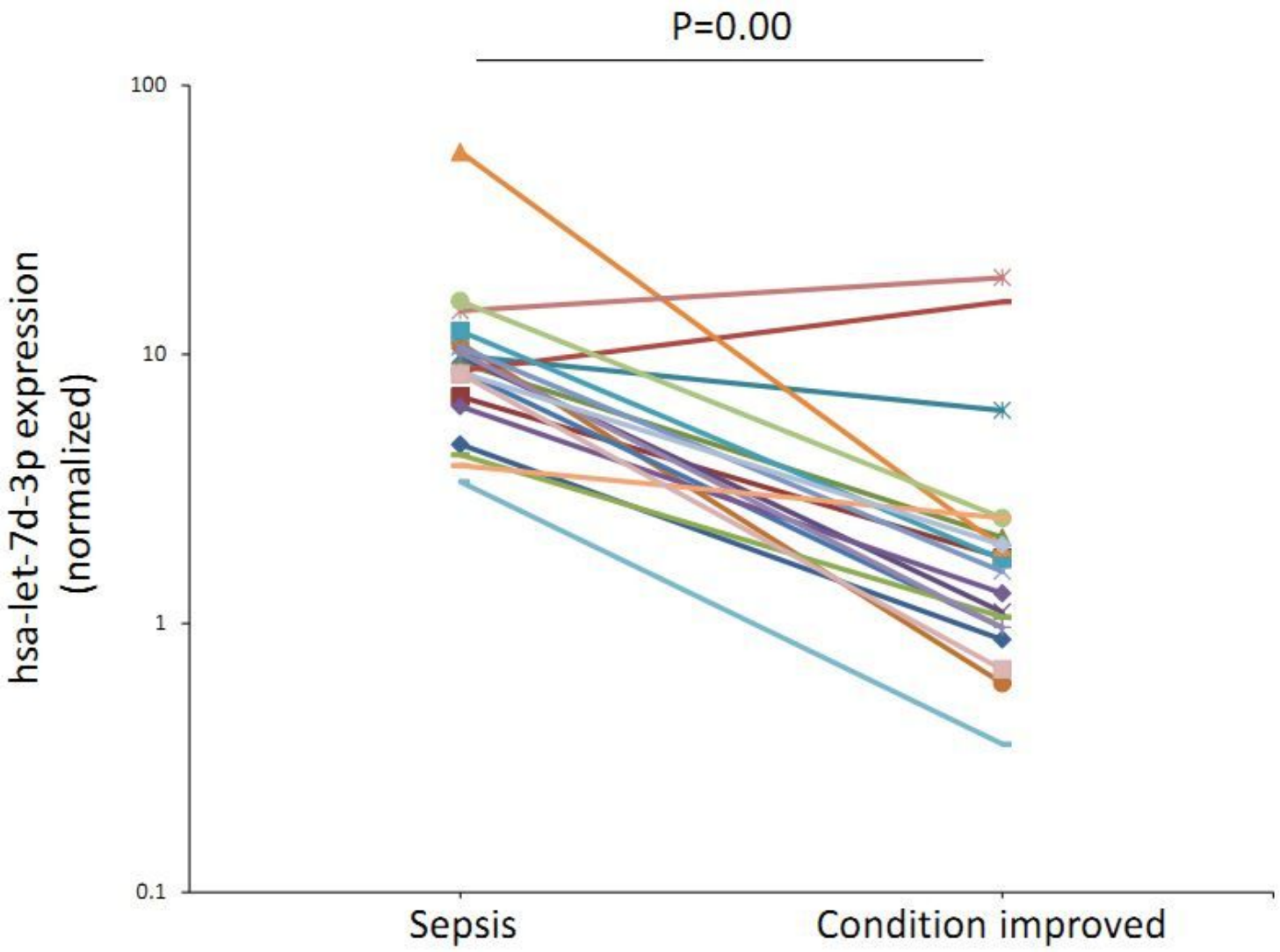

Figure 2

The hsa-let-7d-3p expression in the sepsis patients in sepsis stage and condition improved stage.

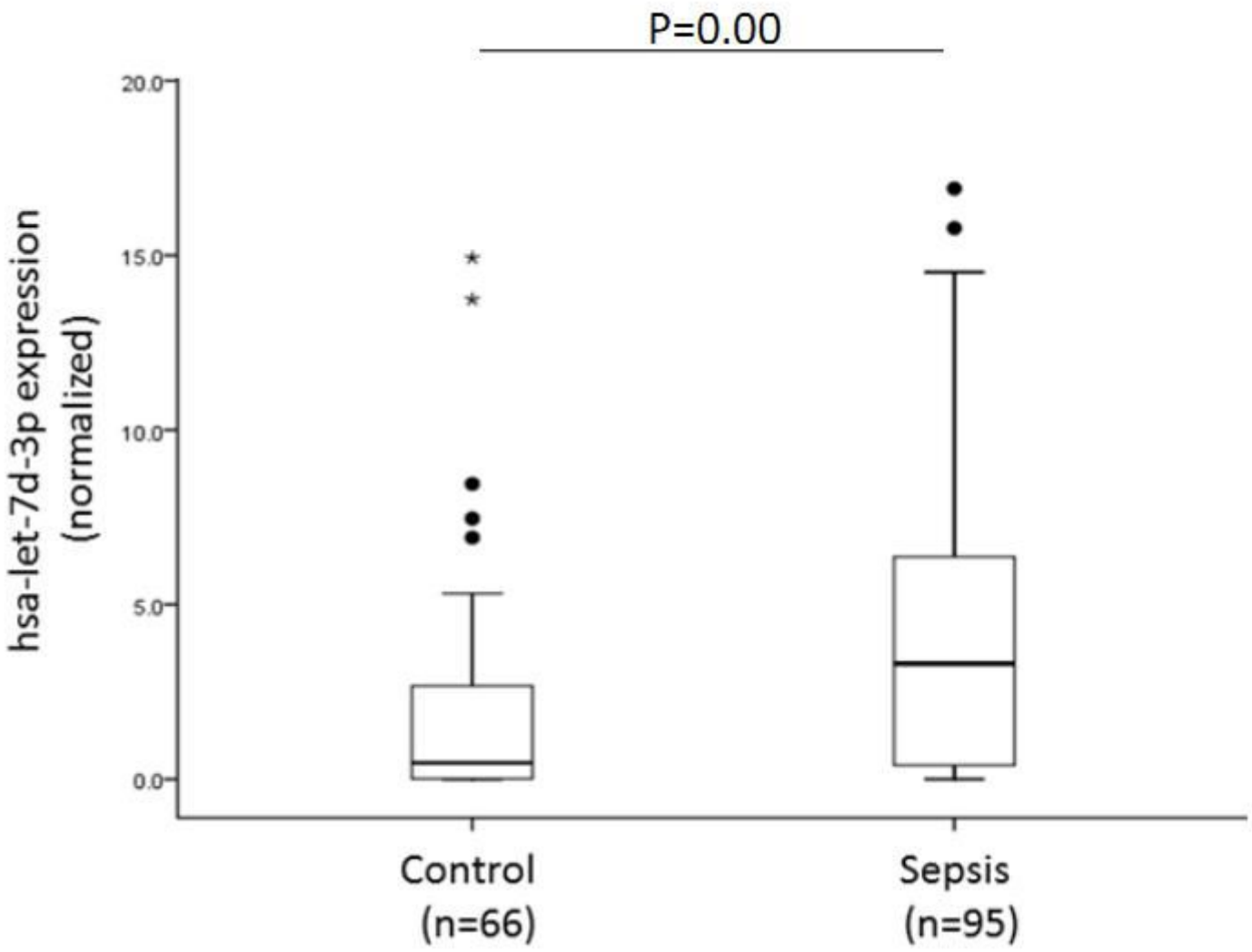

Figure 3

The ΔCT value of hsa-let-7d-3p in the sepsis and control groups.

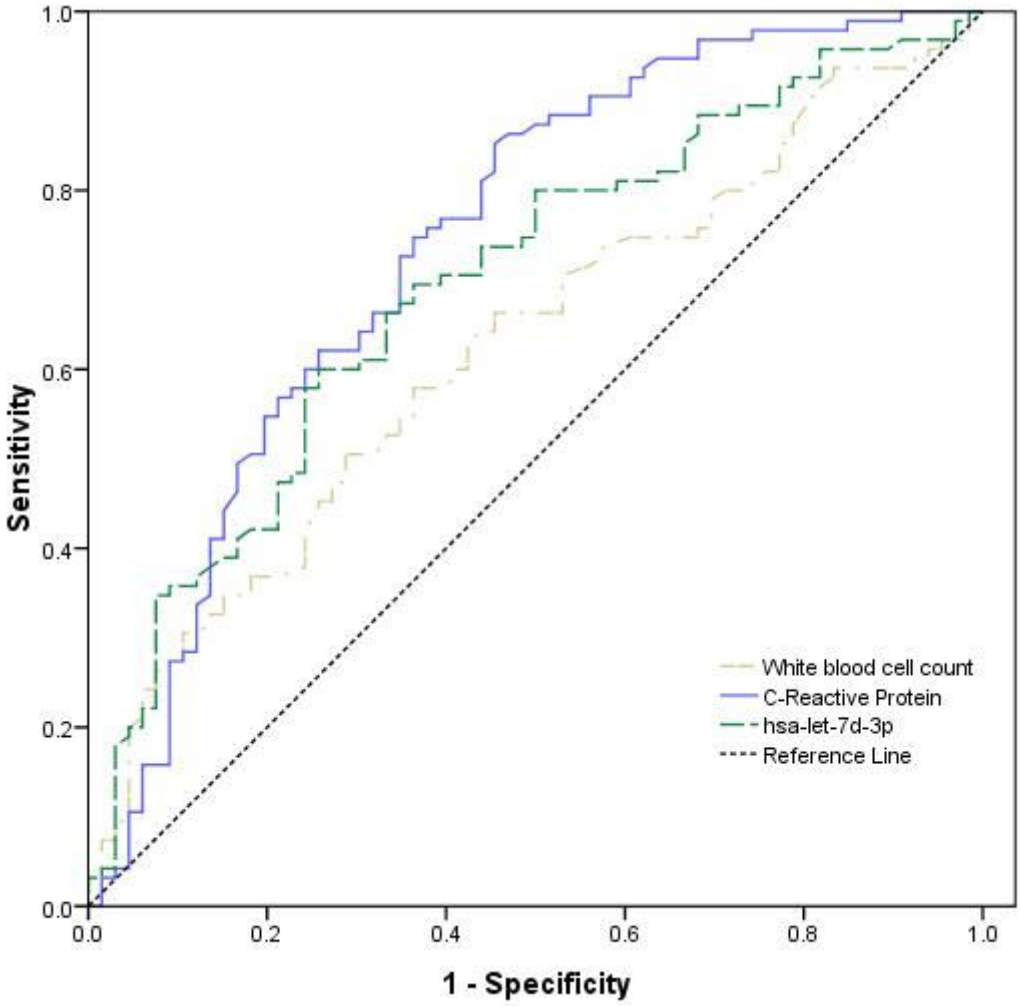

Figure 4

ROC curves of has-let-3p-d, CRP and WBC count. The green dotted line represents hsa-letp-7d-3p; the purple solid line represents the C-reactive protein; the white dotted line represents the white blood cell counts.

Supplementary Files

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- table2.docx
- table1.docx