MicroRNAs combined with the TLR4/TDAG8 mRNAs and proinflammatory cytokines are biomarkers for the rapid diagnosis of sepsis

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Received February 24, 2020; Accepted September 29, 2021

DOI: 10.3892/mmr.2022.12850

Abstract. The early diagnosis and treatment of sepsis are of particular importance to patient survival. To obtain novel biomarkers that serve as prompt indicators of sepsis, the current study screened the differentially expressed microRNAs (DEMs) that were associated with sepsis susceptibility. The correlation between the elucidated DEMs and the inflammatory response was also examined. The present study included 40 patients with sepsis and 40 healthy controls. RNA-sequencing technology and bioinformatics analysis were applied to screen the DEMs between the two cohorts. The expression of these DEMs was subsequently verified by performing reverse transcription-quantitative PCR (RT-qPCR). In addition, IL-6, IL-21, C-X-C motif chemokine ligand-8 (CXCL8) and monocyte chemoattractant protein-1 (MCP-1) levels, along with T-cell death-associated gene 8 (TDAG8) and toll-like receptor 4 (TLR4) mRNA expression levels were assessed. The association between microRNA (miRNA/miR)-3663-3p and the secretion of various proinflammatory cytokines or TDAG8 and TLR4 mRNA expressions were subsequently evaluated by linear correlation analysis. The results revealed 305 DEMs ($P<0.05$; fold change >2) between patients with sepsis and healthy controls. Among these, the top 18 up- and downregulated miRNAs were selected for RT-qPCR verification. In addition, the serum content of IL-6, IL-21, CXCL8 and MCP-1, and the expression of TDAG8 and TLR4 mRNAs were significantly increased in patients with sepsis compared with healthy controls. Moreover, in patients with sepsis, a positive correlation was identified between miR-3663-3p and the secretion of inflammatory cytokines or TDAG8 and TLR4 mRNA expression. A positive correlation was also elucidated between TDAG8 and TLR4 mRNA expression and proinflammatory cytokine/chemokine secretion. Receiver operating characteristic curve analysis of miR-3663-3p expression, IL-6, IL-21, CXCL8 and MCP-1 secretion and TDAG8 and TLR4 mRNA expression demonstrated that miRNA analysis may be invaluable for the diagnosis of sepsis. Collectively, the results determined that miR-3663-3p may be a potentially powerful diagnostic and predictive biomarker of sepsis and that the combined and simultaneous detection of several biomarkers, including proteins, miRNAs and mRNA may be a reliable approach for the fast diagnosis and early identification of sepsis.

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Key words: sepsis, differentially expressed microRNAs, T-cell death-associated gene 8 mRNA, toll-like receptor 4 mRNA, proinflammatory cytokines, chemokines, correlation analysis

Introduction

Severe sepsis is one of the most prevalent diseases in patients admitted to the intensive care unit of hospitals (1). It is a main cause of death in critically ill patients and affects millions of individuals around the world each year, with 11 million sepsis deaths and 48.9 million cases of sepsis reported in 2017 (2). Despite an overwhelming increase in knowledge regarding the pathogenesis of sepsis and subsequent advances in clinical care, the incidence of sepsis is still increasing in both adults and children, accounting for an unacceptably high mortality rate ranging between 25-30% depending on age and disease severity (3). It is often characterized by a systemic inflammatory response to infection that is typically bacterial in origin (4). Moreover, sepsis is defined as a documented or suspected infection in a subset of four findings (body temperature >38˚C or <36˚C; heart rate >90 beats/min; hyperventilation evidenced by breathing rate of >20 breaths/min or $PaCO_2$ <32 mmHg; white blood cell count >12,000 cells/μl or <4,000/μl) that describe systemic inflammatory response syndrome (4,5). Sepsis can progress rapidly, resulting in organ failure (severe sepsis) or impaired tissue perfusion (septic shock) (6). Although a number of biomarkers, such as procalcitonin and C-reactive protein, have been proposed as candidate markers for the diagnosis, prognosis and therapeutic guidance of sepsis, each have certain limitations; for example, low

specificity for early diagnosis, and the lack of definitive evaluation parameters for the severity of sepsis and the prognosis of patients, which make it difficult to diagnose sepsis with high sensitivity and specificity (7). Therefore, new biomarkers with high sensitivity and specificity are urgently required.

MicroRNAs (miRNAs/miRs) are a class of small noncoding RNAs that pair to sites in mRNAs to regulate gene expression in eukaryotes (8). To date, ~1,000 miRNAs have been identified in humans, which may directly regulate at least 30% of the genes in a cell, therefore serving important roles in a variety of cellular functions as well as in several diseases (9,10). Thus, miRNAs are involved in the regulation of almost all major cellular functions, including cell development, differentiation, proliferation and apoptosis (11). The abnormal expression of miRNAs may implicate changes in a wide array of cellular and developmental processes of disease initiation and progression that can lead to malignant phenotypes (12-14). This means that in various pathological conditions, such as inflammation, infection and sepsis, miRNA levels may change, which is a parameter that can be quickly detected (15). Thus, miRNAs isolated from the peripheral blood of patients with sepsis may be measured by performing genome-wide profiling microarrays in leukocytes in order to elucidate potential biomarkers of sepsis. Interestingly, serum miR-16 and miR-483-5p have been identified as prognostic predictors of patients with sepsis, as they are associated with sepsis-induced death (16).

To identify putative miRNA biomarkers involved in the process of sepsis, the current study analyzed the differentially expressed microRNAs (DEMs) via microarray analysis followed by verification via reverse transcription-quantitative PCR (RT-qPCR) and bioinformatics. Additionally, the correlations between the identified miRNAs and proinflammatory cytokines and chemokines were evaluated. The results of the present study may further clarify the roles of miRNAs in the diagnosis and treatment of sepsis, so as to elucidate a novel miRNA biomarker.

Materials and methods

Patients. A total of 40 patients (21 male patients and 19 female patients; age range, 20-80 years; mean age, 66.5±5.8 years) admitted to the Department of Respiratory and Critical Care Medicine of the Third Affiliated Hospital of Inner Mongolia Medical University (Baotou, China) between January 1st 2015 and October 31st 2015 were enrolled in the current study. The inclusion criteria were as follows: Patients with sepsis were diagnosed based on the Sepsis 3.0 guidelines, which can be simplified as follows: Sepsis = infection + sequential organ failure assessment (SOFA) ≥2 (17). The exclusion criteria were as follows: i) Patients <18 or >80 years of age; ii) patients with liver and kidney failure; iii) patients that were pregnant or lactating; iv) patients with tumors and hematological diseases; v) an agranulocytosis score of <0.5x109/l; vi) patients with human immunodeficiency virus-related complications; and vii) patients administered glucocorticoids up to 4 weeks before enrollment or that were receiving immunotherapy after organ transplantation. A total of 40 healthy individuals that donated samples following routine physical examinations were used as a control group (20 males, 20 females; aged 20-80 years). A further 80 samples (including 40 samples from patients with sepsis and 40 samples from healthy controls, whose details are listed in Table I) were obtained from individuals admitted to the same hospital that were enrolled to the present study between April 10th 2020 and January 31st 2021. The inclusion criteria were the same as aforementioned. There was no significant difference in the mean values of sex and age between the test group and the control group. All protocols were approved by the Local Ethics Committee of the Third Affiliated Hospital of Inner Mongolia Medical University (Baotou, China), and written informed consent was obtained from all subjects who were permitted to withdraw from clinical observation at any time for any reason.

Total RNA extraction from blood and quality control. To compare the DEMs in patients with sepsis and healthy controls, 2 ml whole blood samples were collected into sterilized Eppendorf tubes. Total RNA was extracted using the mirVana miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. Subsequently, the concentration and integrity of total RNA was determined using a spectrophotometer (NanoDrop™; Thermo Fisher Scientific, Inc.) and agarose gel electrophoresis. The conditions required for microarray analysis included: An average $A_{260}/_{280}$ ratio of total RNA ≥1.8, RNA content ≥1 μg/ml and clear 28S and 18S electrophoresis bands of total RNA (18).

Microarray assay and data analysis. Microarray analysis was performed by Sangon Biotech Co., Ltd. Briefly, total RNA (~200 ng) extracted from the aforementioned blood samples was subjected to dephosphorylation and labeling using the miRNA Complete Labeling and Hyb kit (Agilent Technologies, Inc.), where Cyanine3-pCp was connected to the 3' end of RNA using T4 RNA ligase. The labeled reaction products were concentrated using a vacuum concentrator for 3 h at 45-55˚C and subsequently underwent overnight hybridization using Agilent SureSelect Capture Technology (Agilent Technologies, Inc.). After washing with saline-sodium citrate at room temperature, slides were dried by air and scanned using the Agilent Scanner G2505C (Agilent Technologies, Inc.), after which the Agilent Feature Extraction (v10.7; Agilent technologies, Inc.) was utilized to extract data and analyze the hybridization results. Agilent GeneSpring (version 12.5; Agilent Technologies, Inc.) was used for quartile data normalization and to determine differences between groups. A Q-value of ≥5% and a fold change (FC) value of >2.0 or <0.5 were selected as cut-off points for DEMs. Statistical significance was determined using an unpaired t-test and was represented using a P-value. To reduce the risk of false positives, values were adjusted for multiple testing using the Benjamini-Hochberg False Discovery Rate (FDR) method. The corrected value was represented by FDR (19). FDR <0.05 was selected as the cut-off value for DEM screening. The possible miRNAs that target sepsis were predicted using TargetScan software (http://www.targetscan.org/vert_80/).

Identification of DEMs in the peripheral blood of patients with sepsis via RT-qPCR. According to microarray assay, 305 miRNAs with >2-fold expression changes (patients with sepsis vs. healthy people) were detected. Subsequently, the top 18 up- and downregulated miRNAs were selected for microarray analysis verification via RT-qPCR. The primers used for RT-qPCR were synthesized by Sangon Biotech Co., Ltd. and

Table I. Clinical characteristics of patients with sepsis and healthy controls (n=80).

A, Original samples

Characteristic	Sepsis	Normal control	P-value
Age (mean ± SD)	66.5±5.8	67.2±4.2	0.12
Male (n)	21	20	0.80
Female (n)	19	20	0.74

B, Expanded samples

Characteristic	Sepsis	Normal control	P-value
Age (mean ± SD)	63.1±1.8	61.5±9.4	0.17
Male (n)	18	21	0.56
Female (n)	22	19	0.43

are listed in Table II. Total RNA was extracted using RNAiso Plus (cat. no. 9108; Takara Bio, Inc.), and reverse transcribed into cDNA using miRNA First Strand cDNA Synthesis (Stem-loop Method) (cat. no. B532453; Sangon Biotech Co., Ltd.) according to the manufacturers' instructions. RT-qPCR was performed using SYBR Premix Ex Taq™ II (Takara Bio, Inc.) with U6 as an internal control for miRNA detection. The thermocycling conditions were as follows: Pre-denaturation at 95˚C for 10 min, followed by 40 cycles of denaturing at 95˚C for 5 sec, annealing at 60˚C for 20 sec and elongation at 70˚C for 10 sec. The melt curve conditions were as follows: 60˚C to 95˚C in increments of 0.3˚C; this was used to determine the melting temperature of the detected miRNAs and primer dimers. The relative expression of DEMs was calculated using the comparative $2^{-\Delta\Delta cq}$ method (20) and the data were analyzed using SPSS v19.0 software (IBM Corp.).

RT-qPCR determination of T-cell death-associated gene 8 (TDAG8) and toll-like receptor 4 (TLR4) mRNA expression. It has been reported that TDAG8 is involved in the maintenance of lysosomal function, particularly during pathogen defense (21). In addition, TLR4 can be activated by lipopolysaccharide (LPS) to induce the production of proinflammatory mediators to eradicate bacteria or other pathogens (22). Dysregulation of the host response to LPS can lead to sepsis, which is a systemic inflammatory condition (23). The current study therefore detected the expression levels of TDAG8 and TLR4 mRNA in the peripheral blood of patients with sepsis. Total RNA was extracted using RNAiso Plus, and reverse transcribed into cDNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (cat. no. R047A; Takara Bio, Inc.) according to the manufacturer instructions. The primers used for this reaction are listed in Table II. Amplification was performed using the SYBR Premix Ex Taq™ II under the following thermocycling conditions: Initial denaturation at 95˚C for 30 sec; followed by 50 cycles of denaturation at 95°C for 5 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. The expression of TDAG8 and TLR4 mRNA was calculated using the $2^{-\Delta\Delta Cq}$ method and GAPDH was used as an internal control.

TargetScan software assay. The current results revealed that the expression levels of miR-3663-3p and miR-6881-3p in patients with sepsis were significantly increased compared with healthy controls. In addition, our pre-experiment determined that TDAG8 was closely related to the regulation of the inflammatory response in patients with sepsis. That is, in the sepsis group, the correlation coefficients (r) of TDAG8 mRNA expression and IL-6 or CXCL8 concentration were 0.8455 or 0.7117, respectively, indicating that TDAG8 mRNA expression was positively correlated with IL-6 and CXCL8 levels. In the present study, TargetScan software (www.targetscan.org/vert_72/; TargetScanHuman 7.2) was applied to predict whether TDAG8 was the biological target of miR-3663 and miR-6881. After selecting 'human' as the species, the human gene TDAG8 (GPR65) was entered. The database was subsequently searched for miR-3663-3p and miR-6881-3p.

ELISA. Sepsis often leads to multiple organ failure as a result of an uncontrolled inflammatory response (24). In the present study, ELISA was performed to detect the concentrations of various proinflammatory cytokines, including IL-6 (cat. no. CSB-E04638h; Cusabio Technology LLC), IL-21 (cat. no. CSB-E11707h; Cusabio Technology LLC), C-X-C motif chemokine ligand-8 (CXCL8) (cat. no. CSB-E04641h; Cusabio Technology LLC) and monocyte chemoattractant protein-1 (MCP-1) (cat. no. CSB-E04655h; Cusabio Technology LLC) in the peripheral blood of patients with sepsis. All procedures were performed in accordance with the respective protocols of commercially available ELISA kits (R&D Systems, Inc.). Each sample was measured three times and an average value was calculated.

Statistical analysis. Data were obtained from three independent experiments and were expressed as the mean ± SD. All statistical analyses were performed using SPSS v19.0 software. The statistical differences between two groups were detected using unpaired Student's t-test. The correlation between inflammatory response and miRNA-3663-3p or TDAG8/TLR4 mRNA expression was determined using Pearson's correlation

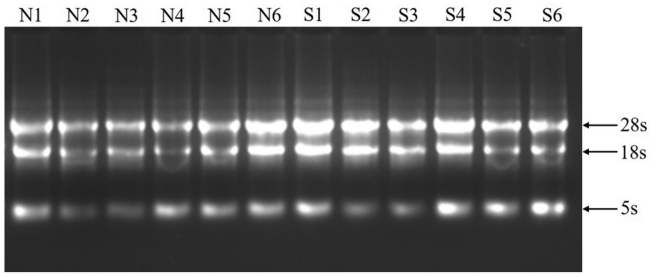

Figure 1. Denaturing agarose gel electrophoresis of total RNA extracted from the peripheral blood of patients with sepsis and healthy controls. N, normal healthy controls; S, sepsis.

analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of clinical characteristics between patients with sepsis and healthy controls. There were 21 males and 19 females in the sepsis group, aged between 20-80 years, with a mean age of 66.5±5.8 years. A total of 40 healthy individuals that donated samples following routine physical examinations were selected as the control group (20 males; 20 females; age range, 20-80 years; mean age, 67.2±4.2 years). There were no significant differences between the mean values of sex and age between the sepsis and healthy control groups (Table I).

Total RNA extracted from peripheral blood demonstrates good integrity. The integrity of total RNA was assessed using agarose gel electrophoresis and subsequent 18S and 28S RNA band staining for visualization. As presented in Fig. 1, the electrophoretic bands of 28S and 18S RNA were clear. In addition, the ratio of A260 to A280 in the extracted RNA was 1.8-2.0. Therefore, total RNA samples with high purity and good integrity were used for microarray analysis.

Scatter plot maps and DEM hierarchical clustering. To clarify the potential function of DEMs, microarray analysis was performed by Sangon Biotech Co., Ltd. Agilent GeneSpring was used for the analysis of DEM expression between patients with sepsis and healthy controls in order to identify characteristic DEMs. The significance of microarray analysis was denoted by FDR (FDR <0.05). As presented in Fig. 2A, the results revealed 305 miRNAs that demonstrated a >2-fold change in expression in patients with sepsis compared with in healthy individuals, including 212 upregulated and 93 downregulated DEMs. Among these, the top 18 up- or downregulated miRNAs, which exhibited significant differential expression (P<0.05; FC >2.0), were selected for further analysis. A total of 9 miRNAs demonstrated an increased expression, while 9 miRNAs exhibited a decreased expression (Table III). Hierarchical clustering of the top 18 DEMs was subsequently performed, the results of which are presented in Fig. 2B.

DEM expression from RNA-sequencing data is validated by RT-qPCR. To validate microarray analysis data, DEM expression levels were detected by RT-qPCR. As presented in Fig. 2C, the results of RT-qPCR were almost consistent with those obtained through microarray analysis. Similar trends in the following miRNAs were detected: miR-625-5p, miR-6786-5p, miR-17-3p, miR-501-3p, miR-6756-5p, miR-501-5p, miR-6786-5p, miR-3663-3p, miR-4694-5p, miR-6881-3p, miR-592, miR-491-5p, miR-1260b, miR-3591-3p, miR-6514-3p and miR-491-3p. However, the expression levels of miR-892b and miR-362-5p differed to that of microarray analysis; therefore, a larger sample size for RT-qPCR analysis is required to validate these results. Integrated analysis of miRNA and mRNA expression profiles indicated that among the highly expressed miRNAs, the expression levels of miR-3663-3p, miR-6881-3p, miR-625-5p, miR-3591-3p and miR-6514-3p were significantly different compared with the control group [FC (abs) >10]. Notably, the expression levels of miR-3663-3p were significantly increased in the peripheral blood of patients

Table II. All primer sequences used in the current study.

Primer	Sequence (5' to 3')
hsa-miR-3663-3p	TGAGCACCACACAGGCCG
hsa-miR-625-5p	AGGGGGAAAGTTCTATAGTCC
hsa-miR-892b	CACTGGCTCCTTTCTGGGT
hsa-miR-491-5p	AGTGGGGAACCCTTCCATGA
hsa-miR-592	GATTGTGTCAATATGCGATGATGT
hsa-miR-3591-3p	AAACACCATTGTCACACTCCAC
hsa-miR-6514-3p	CTGCCTGTTCTTCCACTCC
hsa-miR-4694-5p	ATCCCGTAUCTCTTCGTCTTCGA
hsa-miR-362-5p	AATCCTTGGAACCTAGGTGTGA
hsa-miR-1260b	ATCCCACCACTGCCACCAT
hsa-miR-491-3p	CTTATGCAAGATTCCCTTCTA
hsa-miR-501-3p	GCGGCGGAATGCACCCGGGCAAG
hsa-miR-17-3p	GCCGCAAAGTGCTTACAGTG
hsa-miR-6881-3p	TCTGGCTTGTATCTAGCGTATGA
hsa-miR-501-5p	AATCCTTTGTCCCTGGGTGAGA
hsa-miR-6756-5p	TTTTTCCGATTATTGCTCCTGACC
hsa-miR-6752-5p	TACTGCCCTGACCTGTCCTGTCC
hsa-miR-6786-5p	TAACCGCACTGTCTGGTAAAGAT
Universal reverse miRNA primer	GTGCAGGGTCCGAGGT
Human-TDAG8	
Forward	TTCCTGGGCTACGCAATACC
Reverse	CCGTAGCTTGGTTGTGCTTC
Human TLR4	
Forward	TTCCTGGGCTACGCAATACC
Reverse	CCGTAGCTTGGTTGTGCTTC
GAPDH	
Forward	CAGGAGGCATTGCTGATGAT
Reverse	GAAGGCTGGGGCTCATTT
U6	
Forward	GCTTCGGCAGCACATATACTAAAAT
Reverse	CGCTTCACGAATTTGCGTGTCAT

hsa, *Homo sapiens*; miR, microRNA; TDAG8, T-cell death-associated gene 8; TLR4, toll-like receptor 4.

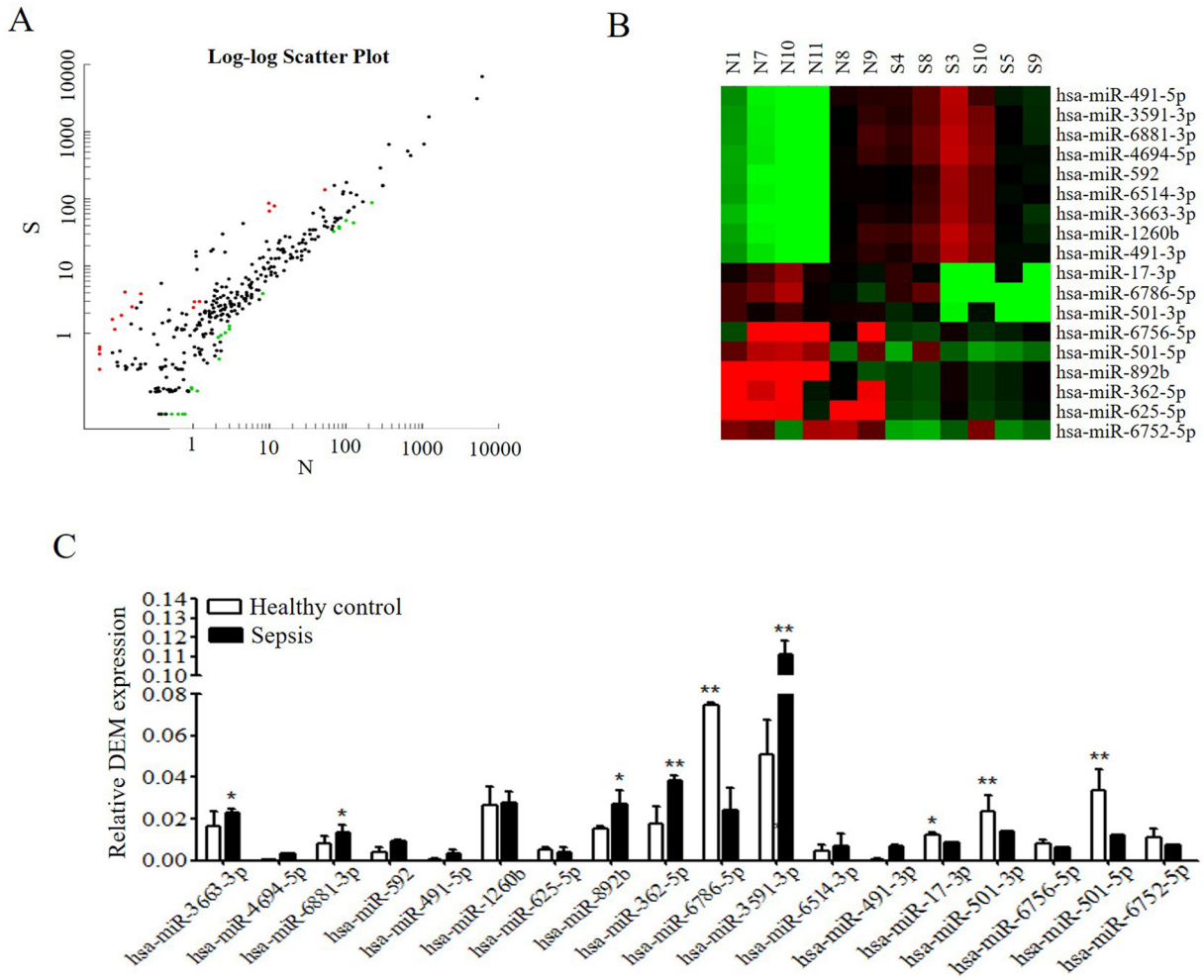

Figure 2. Detection of DEMs between patients with sepsis and healthy controls. (A) Scatter plot of microarray analysis presents the gene expression levels in patients with sepsis compared with healthy controls. The x-axis demonstrates the fluorescence intensity of healthy controls, whereas the y-axis demonstrates the fluorescence intensity of patients with sepsis. Red and green dots indicate the up-(ratio >2) and downregulated DEMs (ratio <0.5), respectively (false discovery rate <0.05). Black coloration indicates the DEMs that demonstrated no difference between the two groups (0.5 < ratio <2). (B) Hierarchical clustering of DEMs in the peripheral blood of patients with sepsis. Red represents high expression while green represents low expression. (C) Reverse transcription-quantitative PCR validation of DEMs in the peripheral blood of patients with sepsis. *P<0.05 and **P<0.01 vs. the healthy control group. DEMs, differentially expressed microRNAs; N, normal healthy controls (n=80); S, patients with sepsis (n=80); miR, microRNA; hsa, *Homo sapiens*.

with sepsis according to RT-qPCR. TargetScan software was used to evaluate the putative target genes of miR-3663-3p. The results revealed that TDAG8 was predicated to be a potential target gene of miR-3663-3p (data not shown), as evolutionary conservation was demonstrated. As the TLR4 signaling pathway has been reported to serve a role in sepsis (25,26) and TDAG8 has been reported to be involved in regulation of cell functions associated with airway inflammation (27), TDAG8 and TLR4 were selected for further experimentation.

Sepsis upregulates the expression of TDAG8 and TLR4 mRNA. To further clarify whether TDAG8 and TLR4 are involved in the occurrence and development of sepsis, the mRNA expression levels of TDAG8 and TLR4 were determined in human blood samples via RT-qPCR. As presented in Fig. 3, significant differences were demonstrated in both TDAG8 and TLR4 mRNA levels between healthy controls and patients with sepsis. More specifically, the expression levels of serum TDAG8 (1.0±0.02 vs. 2.7±0.13) and TLR4 (1.0±0.08 vs. 1.5±0.10) mRNA in patients with sepsis were significantly higher compared with healthy controls. The results indicated that TDAG8 and TLR4 mRNA expression is upregulated in patients with sepsis.

Serum IL-6, IL-21, CXCL8 and MCP-1 content in patients with sepsis. Given the consideration that high expression levels of TLR4 can result in the production of chemokines and proinflammatory cytokines (28), serum IL-6, IL-21, CXCL8 and MCP-1 levels were determined in the current study by performing ELISA. As presented in Fig. 4, serum IL-6, IL-21, CXCL8 and MCP-1 levels in the healthy control group were 2.12±0.18, 48.50±7.9, 0.15±0.04 and 8.25±1.45 ng/l, while levels in patients with sepsis were 6.24±0.92, 137.0±29.20, 0.28±0.05 and 30.40±5.30 ng/l, respectively. The results demonstrated that proinflammatory cytokines IL-6 and IL-21 and CXCL8 and MCP-1 chemokines were significantly increased in patients with sepsis (t=6.89, 6.07, 11.8 and 9.03, respectively), suggesting an increased inflammatory response.

Correlation between the inflammatory response and miR-3663-3p or TDAG8/TLR4 mRNA expression. To clarify

Table III. Differentially expressed microRNAs obtained from microarray analysis.

Systematic name	P-value (Corr)	P-value	FC (abs)	Expression
hsa-miR-3663-3p	0.000472	1.320987	16.258206	Upregulated
hsa-miR-4694-5p	0.372410	0.020556	9.608086	Upregulated
hsa-miR-6881-3p	0.086800	0.001669	22.589320	Upregulated
hsa-miR-592	0.452431	0.050030	9.550354	Upregulated
hsa-miR-491-5p	0.353573	0.008057	9.395253	Upregulated
hsa-miR-1260b	0.372410	0.022981	5.556636	Upregulated
hsa-miR-625-5p	0.238966	0.000756	10.871430	Downregulated
hsa-miR-892b	0.452431	0.055429	5.355109	Downregulated
hsa-miR-362-5p	0.490820	0.099653	3.538647	Downregulated
hsa-miR-6786-5p	0.519619	0.115105	2.160317	Downregulated
hsa-miR-3591-3p	0.354531	0.015007	12.917550	Upregulated
hsa-miR-6514-3p	0.372414	0.025927	10.691710	Upregulated
hsa-miR-491-3p	0.402106	0.031632	8.345849	Upregulated
hsa-miR-17-3p	0.321635	0.015670	4.457891	Downregulated
hsa-miR-501-3p	0.217634	0.024245	2.678529	Downregulated
hsa-miR-6756-5p	0.032456	0.007564	3.678321	Downregulated
hsa-miR-501-5p	0.213489	0.012359	6.765479	Downregulated
hsa-miR-6752-5p	0.316784	0.045679	7.120635	Downregulated

miR, microRNA; hsa, *Homo sapiens*; FC (abs), fold change (absolute); (Corr), corrected.

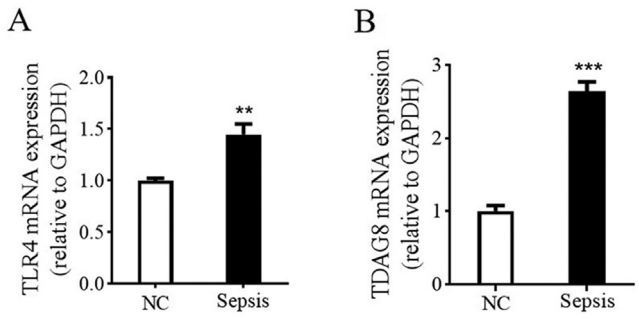

Figure 3. Expression levels of *TLR4* and *TDAG8*. Reverse transcription-quantitative PCR determination of (A) TLR4 and (B) TDAG8 mRNA expression in the serum of patients with sepsis and healthy controls. **P<0.01 and ***P<0.001 vs. healthy controls. TLR4, toll-like receptor 4; TDAG8, T-cell death-associated gene 8; NC, normal healthy controls.

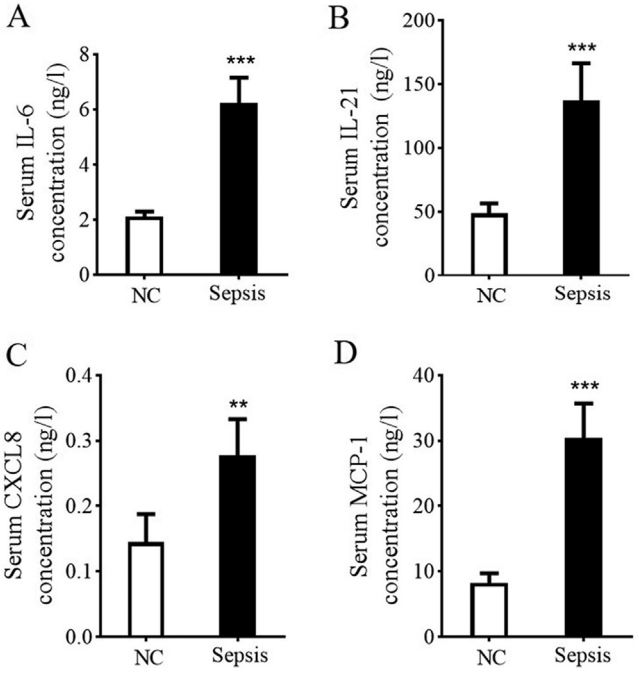

Figure 4. Levels of serum cytokines/chemokines. ELISA determination of serum (A) IL-6, (B) IL-21, (C) CXCL8 and (D) MCP-1 content in patients with sepsis and healthy controls. **P<0.01 and ***P<0.001 vs. healthy controls. CXCL8, C-X-C motif chemokine ligand-8; MCP-1, monocyte chemoattractant protein-1; NC, normal healthy controls.

the relationship between miR-3663-3p and the secretion of proinflammatory cytokines, linear correlation analysis was performed. The results revealed that, in patients with sepsis, the correlation coefficients (r) of miR-3663-3p expression and IL-6, IL-21, CXCL8 and MCP-1 levels were 0.8352, 0.8976, 0.6633 and 0.7661, respectively. Furthermore, the correlation coefficients (r) of the same miRNA with TDAG8 and TLR4 mRNA expression levels were 0.7895 and 0.8622, respectively (Fig. 5A). The results indicated a positive correlation between miRNA-3663-3p and the inflammatory response. As the secretion of inflammatory factors is closely associated with the activation of TLR4 (29), correlation analysis was performed between the expression levels of TDAG8/TLR4 mRNA and the production of IL-6, IL-21, CXCL8 and MCP-1. As presented in Fig. 5B and C, the correlation coefficients (r) of TDAG8 mRNA with the contents of IL-6, IL-21, CXCL8 and MCP-1 were 0.856, 0.914, 0.515 and 0.902, respectively. Additionally, the correlation coefficients (r) of TLR4 mRNA with the contents of IL-6, CXCL8, IL-21 and MCP-1 were

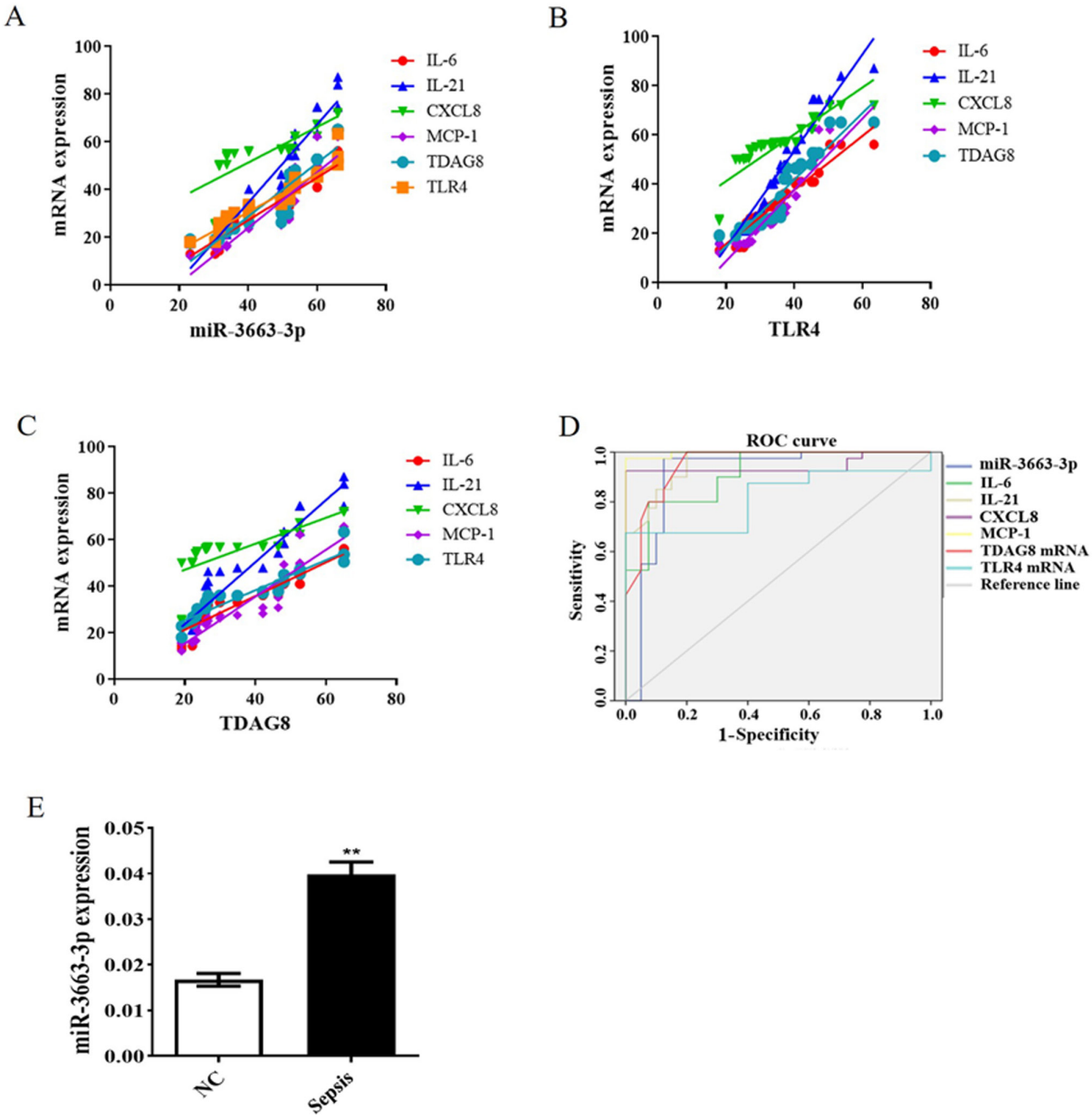

Figure 5. Relationship between miR-3663-3p expression and inflammation in sepsis. Correlation analysis of (A) miR-3663-3p expression with the serum levels of IL-6, IL-21, CXCL8 and MCP-1 and TDAG8 and TLR4 mRNA. Correlation analysis of (B) TLR4 and (C) TDGA8 mRNA expression with the serum contents of IL-6, IL-21, CXCL8 and MCP-1. (D) ROC curve analysis of miR-3663-3p expression, total serum IL-6, IL-21, CXCL8 and MCP-1 concentration and TDAG8 and TLR4 mRNA expression in patients with sepsis. (E) Expression levels of miR-3663-3p in human peripheral blood samples detected by reverse transcription-quantitative PCR. **P<0.01 vs. healthy controls. miR, microRNA; CXCL8, C-X-C motif chemokine ligand-8; MCP-1, monocyte chemoattractant protein-1; TDAG8, T-cell death-associated gene 8; TLR4, toll-like receptor 4; ROC, receiver operating characteristic; NC, normal control.

0.940, 0.946, 0.715 and 0.890, respectively. The data indicated a positive correlation between TDAG8/TLR4 mRNA expression and proinflammatory cytokine/chemokine secretion. TDAG8 mRNA expression also revealed a positive correlation with TLR4 mRNA expression, with a correlation coefficient value (r) of 0.878).

Significance of miRNA analysis in the diagnosis of sepsis. In the current study, miR-3663-3p and the expression of various cytokines/chemokines (IL-6, IL-21, CXCL8 and MCP-1) were selected for sensitivity and specificity analysis using a receiver operating characteristic (ROC) curve. ROC curves revealed that miR-3663-3p, IL-6, IL-21, CXCL8 and MCP-1 levels, along with TDAG8 and TLR4 mRNA had area under the curve values of 0.908, 0.912, 0.959, 0.944, 0.996, 0.952 and 0.815, respectively, when distinguishing patients with sepsis from healthy controls (Fig. 5D). When selecting a cut-off value of 0.02 for miR-3663-3p, as determined via ROC curve analysis, the diagnostic sensitivity and specificity were determined to be 100%. The cut-off points, diagnostic sensitivities and diagnostic specificities of IL-6, IL-21, CXCL8 and MCP-1 are listed in Table IV. The area under the ROC curve of IL-6, IL-21, CXCL8 and MCP-1 were >0.9, indicating that they may be used to provide an earlier warning of sepsis when combined with miR-3663-3p.To validate these findings, an additional 80 samples (40 samples for healthy controls and 40 samples

Table IV. Diagnostic value of miR-3663-3p, IL-6, IL-21, CXCL8 and MCP-1 in patients with sepsis.

Parameter	AUC	Cut-off value	Sensitivity (%)	Specificity (%)
miR-3663-3p	0.908	0.02	100.0	100.0
IL-6	0.912	2.84	67.7	91.7
IL-21	0.959	57.48	79.2	100.0
CXCL8	0.944	14.28	100.0	95.8
MCP-1	0.996	14.28	97.5	100.0
TDAG8 mRNA	0.952	91.29	100.0	80.0
TLR4 mRNA	0.815	999.00	67.5	100.0

miR, microRNA; CXCL8, C-X-C motif chemokine ligand-8; MCP-1, monocyte chemoattractant protein-1; TDAG8, T-cell death-associated gene 8; TLR4, toll-like receptor 4; AUC, area under the curve. P<0.001 for all analyses.

for patients with sepsis) were obtained for RT-qRCR analysis. The results revealed that the expression levels of miR-3663-3p were significantly increased in patients with sepsis compared with healthy controls (P<0.01; Fig. 5E). The data indicated that miR-3663-3p could serve as a potentially powerful diagnostic and predictive biomarker for sepsis, and that miRNA analysis combined with the measurement of inflammatory cytokine secretion may be a reliable approach for the fast diagnosis and early identification of sepsis.

Discussion

The understanding of sepsis and its pathobiology has improved in recent years, which may improve the definition of sepsis (17). Severe stage blood-infection is characterized as sepsis, which may result in tissue damage, organ failure and death (6). Therefore, the fast diagnosis and early identification of sepsis (including sepsis, severe sepsis or septic shock) is crucial for the patient's survival and may be beneficial when applying the most appropriate treatment protocol. The regulatory roles of miRNAs make them suitable disease biomarkers, indicating that they may contribute to the improved prediction of survival in patients with sepsis (9,10,30).

Previous studies have demonstrated the association between the expression levels of miRNAs and the mortality of patients with sepsis (16,31,32). Furthermore, the altered levels of a miRNA may serve as a potentially powerful diagnostic and predictive biomarker of sepsis (33). The present study investigated the expression of various miRNAs in the context of sepsis. Considering the rapidity and accessibility of sampling miRNAs in liquid biopsies, total RNA was extracted from the peripheral blood of 40 patients with sepsis and 40 healthy controls to identify novel blood-specific biomarkers of sepsis. A total of 305 DEMs were identified in patients with sepsis, including 212 upregulated and 93 downregulated DEMs. Among these, the top 18 up- and downregulated miRNAs were selected and validated via RT-qPCR.

TLR4 plays a key role in the innate immune system and regulates the secretion of various proinflammatory cytokines, including TNF-α and IL-6 (29). The results of the present study confirmed that a significantly increased expression of TLR4 was detected in patients with sepsis. Once the TLR4 receptor is activated, its downstream signaling pathways, including NF-κB, MAPK and STAT, are subsequently activated. Following TLR4-NF-κB/MAPK/STAT signaling pathway activation, the abnormal secretion of certain proinflammatory cytokines, such as IL-6, CXCL8 and MCP-1, is observed (34-36). In the current study, serum IL-6, IL-21, CXCL8 and MCP-1 levels in patients with sepsis were significantly increased compared with healthy controls. TDAG8, which regulates macrophage extracellular acidification-induced inflammatory cytokine production, is a receptor with a pronounced immune cell-specific (macrophages, T cells and microglia) expression profile (37,38). In the present study, the highly upregulated expression of TLR4 and TDAG8 mRNA was detected in the peripheral blood of patients with sepsis, suggesting that TDAG8 and TLR4 expression was closely associated with the occurrence and development of sepsis. Given that the current results demonstrated an overproduction of certain proinflammatory cytokines and chemokines, including IL-6, IL-21, CXCL8 and MCP-1, accompanied by increased mRNA expressions of TDAG8 and TLR4, the correlation between the inflammatory response and miR-3663-3p or TDAG8/TLR4 mRNA expression was further analyzed. The results revealed a positive correlation between miR-3663-3p and the inflammatory response or TDAG8/TLR4 mRNA. In addition, the expression of TDAG8/TLR4 mRNA was also positively correlated with the secretion of proinflammatory cytokines and chemokines. Furthermore, ROC curve analysis demonstrated that miR-3663-3p had an area under the curve value of 0.908. With a cut-off point of 0.02, the diagnostic sensitivity and specificity of miR-3663-3p were 100%, indicating that miR-3663-3p is a potentially powerful diagnostic and predictive biomarker of sepsis. The ROC curve analysis of other biomarkers, such as IL-6, CXCL8 and MCP-1, also demonstrated that miRNA analysis combined with inflammatory cytokine secretion may be a reliable approach for the fast diagnosis and early identification of sepsis.

Taken together, the results of the current study focused on three structurally different types of biomarkers: Proteins (IL-6, IL-21 and CXCL-1, MCP-1), miRNAs (miR-3663-3p as an example) and mRNAs (TDAG8 and TLR4), as the combined detection of several biomarkers in a timely, specific and simultaneous way could ensure a more accurate diagnosis. The present study has certain limitations. For

instance, further experiments should be conducted to verify whether miR-3663-3p or other identified DEMs serve a role in sepsis. Furthermore, Gene Ontology enrichment analysis of biological processes, cellular components and molecular functions should be investigated in future studies. However, the validity of the present results are not affected by these limitations.

The present study elucidated multiple blood-specific, highly regulated miRNAs that, to the best of our knowledge, have not yet been associated with sepsis. Most importantly, the current data demonstrated three structurally different types of biomarkers (proteins, miRNAs and mRNAs), the simultaneous and combined detection of which may provide a more accurate diagnosis for the occurrence and development of sepsis. In addition, the practicality and applicability of sampling miRNAs in liquid biopsies will enhance biomarker research and eventually the clinical management of sepsis.

Acknowledgements

Not applicable.

Funding

This study was supported by the Natural Science Research Foundation of Inner Mongolia (grant no. 2016ms0810).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The microarray datasets generated and/or analyzed during the current study are available in the NCBI GEO repository under accession no. GSE174507 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE174507).

Authors' contributions

XX, BB and HT performed the experiments. XX and BB analyzed the data and wrote the manuscript. RW and JY designed the present study and provided experimental materials. JY and XX confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocols of the current study were approved by the Local Ethics Committee of the Third Affiliated Hospital of Inner Mongolia Medical University (Baotou, China), and written informed consent was obtained from all subjects, who were permitted to withdraw from clinical observation at any time for any reason.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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