Identification of the TF-miRNA-mRNA Co-regulatory Networks Involved in Sepsis

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Abstract

Background: Sepsis is a life-threatening medical condition caused by a dysregulated host response to infection. Recent studies have found that the expression of miRNAs is associated with the pathogenesis of sepsis and septic shock. Our study aimed to reveal which miRNAs may be involved in the dysregulated immune response in sepsis and how these miRNAs interact with transcription factors (TFs) using a computational approach with in vitro validation studies.

Methods: To determine the network of TFs, miRNAs, and target genes involved in sepsis, GEO datasets GSE94717 and GSE131761 were used to identify differentially expressed miRNAs and DEGs. TargetScan and miRWalk databases were used to predict biological targets that overlap with the identified DEGs of differentially expressed miRNAs. The TransmiR database was used to predict the differential miRNA TFs that overlap with the identified DEGs. The TF-miRNA-mRNA network was constructed and visualized. Finally, qRT-PCR was used to verify the expression of TFs and miRNA in HUVECs.

Result: Between the healthy and sepsis groups, there were 146 upregulated and 98 downregulated DEGs in the GSE131761 dataset, and there were 1 upregulated and 183 downregulated DEMs in the GSE94717 dataset. A regulatory network of the TF-miRna target genes was established. According to the experimental results, RUNX3 was found to be downregulated while MAPK14 was upregulated, which corroborates the result of the computational expression analysis. In a HUVECs model, miR-19b-1-5p and miR-5009-5p were found to be significantly downregulated. Other TFs and miRNAs did not correlate with our bioinformatics expression analysis.

Conclusion: We constructed a TF-miRNA-target gene regulatory network and identified potential treatment targets RUNX3, MAPK14, miR-19b-1-5p, and miR-5009-5p. This information provides an initial basis for understanding the complex sepsis regulatory mechanisms.

Keywords: sepsis; miRNA; transcription factors

Introduction

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host immune response to an infection[1]. It is estimated that approximately 30 million people worldwide are diagnosed with sepsis every year [2]. The leading causes of sepsis in intensive care units are pneumonia, abdominal cavity infection, bloodstream infection, and urinary tract infections[3]. Although the research into sepsis and thus our understanding of the condition is increasing, the underlying pathological mechanisms of sepsis still need further study. The capacity to treat sepsis by supporting organ function and hemodynamics in intensive care units (ICUs) has increasingly strengthened, but sepsis-related organ damage and its associated mortality remain high[4]. Therefore, because there is a paucity in understanding the precise molecular mechanisms underlying sepsis, it is important to find biological markers for early diagnosis and novel treatment targets of sepsis.

MicroRNAs (miRNAs) are short, noncoding RNAs of 18-25 nucleotides in length that regulate the translation of mRNAs[5]. Mature miRNAs can bind to the complementary site of the 3' untranslated region (UTR) of their specific target mRNAs and regulate translation at the post-transcriptional level[6]. Recent studies have found that the expression of miRNAs is associated with the pathogenesis of sepsis and septic shock[7, 8]. Transcription factors are DNA-binding proteins that can play significant roles in regulating gene expression and sepsis-induced organ dysfunction[9]. Zhang et al. have found that MYC and STAT3 may be the key regulatory genes in the underlying pathology of sepsis-induced ARDS[10]. In a previous study conducted by Mussbacher et al., the transcription factor NF-κB is critically involved in these pathophysiological processes as it induces both inflammatory and thrombotic responses[11].

In this study, we investigated the comprehensive miRNA-TF co-regulatory network in sepsis. Firstly, we identified the potential targets of sepsis-related TFs and miRNAs. miRNAs were further predicted according to the TFs that targeted the differentially expressed genes (DEGs), and interactions between miRNAs and TFs were predicted with online tools. Based on these results, a TF-miRNA-mRNA network was then constructed, which can help us to reveal the complicated regulatory mechanisms underlying sepsis. Finally, RT-PCR was used to verify the expression of the identified miRNAs and TFs using bioinformatics.

Material & Methods

Dataset collection and identification of DEGs. The GEO (http://www.ncbi.nlm.nih.gov/geo) database is a public functional genomics data repository of high-throughput gene expression data, chips, and microarrays[12]. Two datasets, GSE131761[13] and GSE94717[14] , met our retrieval requirement for this study. The differentially expressed mRNAs and miRNAs between healthy controls and samples collected from patients who experienced sepsis were identified using the web tool named GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r). Adjusted P-values (adj. P) and the Benjamini and Hochberg procedure were applied to increase the reliability of the results. LogFC (fold change) values greater than 2 and adj. P-values less than 0.05 were considered statistically significant.

Functional analysis and pathway enrichment analysis of DEGs. The biological functions and signaling pathways of the identified differentially expressed genes were analyzed using the

Database for Annotation, Visualization, and Integrated Discovery (DAVID). DAVID (http://david.ncifcrf.gov) is a common tool used for GO (gene ontology) annotation and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways enrichment analysis[15]. It provides a comprehensive set of tools for functional annotation of genes and proteins[16]. $P<0.05$ was considered statistically significant.

PPI network construction and identification of hub genes. In order to identify hub genes among the identified DEGs, the STRING database (Search Tool for the Retrieval of Interacting Genes; version 10.0; http://string-db.org) was used to obtain the predicted interactions[17]. Cytoscape (version 3.6.1) is an open source bioinformatics software platform that visualizes a PPI (protein-protein interaction) network[18]. The MCODE (Molecular Complex Detection) plugin in Cytoscape can identify highly connected proteins and significant modules from the network analyzed by STRING. Genes with degree connectivity>8 were defined as hub genes.

Prediction and construction network of the TF-miRNA-mRNA network. TargetScan (http://www.targetscan.org/vert_72/) and miRWalk (http://mirwalk.umm.uni-heidelberg.de) were used to predict the biological targets of differentially expressed miRNAs (DEMs), and those that had overlap with the targets of DEGs were identified. The TransmiR database (http://www.cuilab.cn/transmir) was used to predict TF- miRNA relations. Based on the regulation relationship between TFs, miRNAs, and mRNAs, the TF-miRNA-mRNA network was constructed and visualized using Cytoscape (version 3.6.1).

Cell culture and treatment. Human Umbilical Vein Endothelial Cells **(**HUVECs) were obtained from the Cell Resource Center of the Shanghai Institute of Life Science (Shanghai, China). The cells were cultured in endothelial cell medium (ECM) (ScienCell, San Diego, CA) and were maintained at 37°C in 5% CO2 using standard cell culture methods. After reaching a confluence of approximately 80%, HUVECs were detached using 0.25% trypsin in ethylenediaminetetraacetic acid (EDTA). HUVECs were treated with 1 μ g/ml lipopolysaccharide (LPS) for 24 hours.

Cell viability assay. Cell Count Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was performed to measure cell viability. Approximately 5×10^3 transfected cells were seeded into each well of a 96-well plate. 10 μl CCK-8 was added to each well every 24 h for 30-minute incubation. Subsequently, the absorbance values of the experimental wells were analyzed by a microplate reader at 490 nm. For 5-ethynyl-2′-deoxyuridine (EdU) staining, 200 μL 2×10^4/mL cells were incubated with 50-μM EdU for 8 h and then fixed in 70% alcohol for 15 min. After permeabilization with Triton X-100 for 20min, the cells were incubated with Apollo staining reaction liquid (Ribobio, Guangzhou, China).

Total RNA extraction, reverse transcription, and quantitative real-time PCR. Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. A reverse transcription reagent was added to the RNA sample according to the commercial kit instructions, and the resulting cDNA was used for quantitative real-time PCR (qRT-PCR). QRT-PCR was accomplished using the FastStart Universal SYBR Green Master (Rox) (Roche) in the ABI PRISM® 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). We used melting curves to monitor nonspecific amplifications. Relative expression level was

computed using 2-ΔΔCt method.

Statistical analysis. Results were reported as mean± standard deviation (Mean ±SD). When comparing two groups of data, the unpaired t-test or Student's t-test was used to analyze real-time gene expression analysis. One-way ANOVA was used when comparing multiple groups of data. $P<0.05$ was considered statistically significant. All data and graphs were constructed and analyzed using SPSS software (version 19.0, US) and GraphPad Prism software (version 8.0, US).

Result

Identification of DEGs and DEMs in patients with sepsis. A total of 146 upregulated and 62 downregulated DEGs were identified in the GSE131761 dataset, while 1 upregulated and 183 downregulated DEMs were identified in the GSE94717 dataset. The volcano plots and heatmap are shown in Figure1.

Gene ontology enrichment analysis and KEGG pathway analysis. GO functional enrichment analysis of the identified DEGs showed that the biological processes of these DEGs were significantly enriched in immune response, innate immune response, defense response to fungi, defense response to bacteria, and response to lipopolysaccharide (Figure 2A). The cellular component was significantly enriched in the extracellular exosome, T-cell receptor complex, extracellular space, extracellular region, and plasma membrane (Figure 2B). The molecular function was significantly enriched in MHC class II receptor activity, RAGE receptor binding, and peptide antigen binding (Figure 2C). These DEGs were also significantly associated with the pathway involved in asthma, graft-versus-host disease, systemic lupus erythematosus, and allograft rejection (Figure 2D).

PPI network construction and hub gene selection. The PPI network was obtained and visualized using Cytoscape. Genes with connectivity degree >8 were identified as hub genes. A total of 48 genes were identified as hub genes (Figure 3).

Predicted target mRNA analysis and miRNA-mRNA network construction. The top 16 significantly differentially expressed miRNAs of GSE94717 were selected for further analysis. The target genes of these miRNAs were intersected with the differentially expressed mRNAs of GSE131761. The miRNA-mRNA network was constructed and visualized using Cytoscape software (version 3.6.1) based on the negative regulation relationship between miRNAs and mRNAs. The constructed miRNA-mRNA network is shown in Figure 4A. The intersection of 48 hub genes obtained from the above analysis and 16 target genes of differentially expressed miRNAs was taken to obtain 25 hub genes. Additionally for the dataset GSE131761 differentially expressed 208 genes and 454 potential transcription factors predicted by the TransmiR database for differentially expressed 16 miRNAs, the intersection set was taken to obtain 4 transcription factors including: LEF1, MAPK14, RUNX3 and TBX21 (Figure 4B). There are six miRNAs (miR-150-3p, miR-19b-1-5p, miR-5009-5p, miR-2362, miR4659a-5p and miR-3681-5p) corresponding to these four transcription factors. In figure 4A, there are 17 hub genes that have regulatory relationships with these six miRNAs (ELANE, CCR7, MPO, CHI3L1, STOM, CEACAM8, FCAR, MMP8,

CZMK, LCK, CD3G, CD8B, CEACAM1, KLRB1, MMP9, CD5 and CXCR3).

Construction of the TF- miRNA-mRNA regulatory network and qRT-PCR validation. The regulatory network of TF-miRNA-mRNA was established, as shown in Figure 4C, involving 4 Transcription factors (LEF1, MAPK14, RUNX3, and TBX21), 6 miRNAs (miR-150-3p, miR-19b-1-5p, miR-5009-5p, miR-2362, miR4659a-5p, and miR-3681-5p), and 17 hub-genes (ELANE, CCR7, MPO, CHI3L1, STOM, CEACAM8, FCAR, MMP8, CZMK, LCK, CD3G, CD8B, CEACAM1, KLRB1, MMP9, CD5 and CXCR3).

To confirm the reliability of our findings, we validated the identified TFs and miRNAs in our well-established LPS-HUVECs model by qRT-PCR. HUVECs were administrated with different concentrations of LPS (0, 0.5, or 1 μ g/mL) at different time points (6, 12, and 24 hours). As shown in Fig 5A, incubation of HUVECs with LPS induced a dose-dependent reduction in cell viability ($p<0.05$). Figure 5B showed that the expression levels of inflammatory cytokines were markedly increased upon LPS stimulation ($P < 0.05$). According to our experimental results, RUNX3 expression was downregulated while MAPK14 expression was upregulated, in agreement with our computational expression analysis (Figure 6A). In the HUVECs model, miR-19b-1-5p and miR-5009-5p was found to be significantly downregulated (Figure 6B). Other TFs and miRNAs did not correlate with our bioinformatics expression analysis.

Discussion

Sepsis is a complex disorder that develops as a dysregulated host response to an infection, and is associated with acute organ dysfunction and a high risk of death[19]. Despite advances in medical treatment, there is still no treatment method containing the dysregulated host response. Therefore, the molecular mechanisms that regulate the progression of sepsis are important discoveries that might lead to the prevention of this potentially life-threatening disease.

Transcription factors (TFs) are DNA-binding proteins that can play significant roles in regulating gene expression and in sepsis-induced organ dysfunction by inducing the expression of pro-inflammatory genes. Meanwhile, miRNAs play an important role in regulating the immune response and molecular pathology in sepsis[20, 21]. Our study aimed to identify the potential network of TFs, miRNA, and mRNAs involved in the dysregulated immune response using computational bioinformatics.

We established TF-miRNA-mRNA regulation loops, such as module RUNX3-miR5009-5p-MMP8, MAPK14-miR19b-1-5P-STOM, TBX21-miR3681-5p-CD5, and LEF1-miR150-3p-MPO, that might play important roles in sepsis. Seventeen hub-genes (ELANE, CCR7, MPO, CHI3L1, STOM, CEACAM8, FCAR, MMP8, CZMK, LCK, CD3G, CD8B, CEACAM1, KLRB1, MMP9, CD5 and CXCR3), four TFs (LEF1, MAPK14, RUNX3 and TBX21) and 6 miRNAs (miR-150-3p, miR-19b-1-5p, miR-5009-5p, miR-2362, miR4659a-5p and miR-3681-5p) were selected out. Of the four transcription factors, MAPK14 and RUNX3 were experimentally confirmed by qRT-PCR to have significantly different expressions in LPS-treated HUVECs. Additionally, of the miRNAs associated with MAPK14 and RUNX3, miR-19b-1-5p and miR-5009-5p were confirmed to have a significantly different expression level.

RUNX3 is one of the three mammalian Runt-domain transcription factors[22]. A previous study has shown that RUNX3 might be involved in the development of hematopoietic malignancies and

epithelial malignancies[23]. Other studies have shown that RUNX3 plays an important function in controlling immunity and inflammation. Dicken et al. and Yin et al. found that RUNX3 and its downstream target genes in immune and inflammatory cells act as protectors against a range of immune-related diseases[24, 25]. Currently, there are a few reports on RUNX3 in the field of sepsis research. The downregulated expression of RUNX3 is consistent with the findings of these previous studies, suggesting that RUNX3 may protect against the development of sepsis.

The other differentially expressed TF, MAPK14, plays an important role in initiating numerous conditions, including inflammation, cardiovascular diseases, and cancer[26]. MAPK14 can be activated by environmental stress and by various proinflammatory cytokines. Therefore, with the progression of sepsis and septic shock, MAPK14 would be upregulated. Li et al. reported that miR-128-3p could enhance the protective effect of dexmedetomidine on acute lung injury (ALI) in a septic mouse model by inhibiting MAPK14 expression[27]. Pan et al. have also found that miR-124 could alleviate acute lung injury symptoms of by inhibiting the activation of the MAPK signaling pathway via inhibiting MAPK14 expression[28]. A previous bioinformatics study has already speculated that MAPK14 may be a hub-gene of sepsis[29]. In the TransmiR v2.0 database, MAPK14 is a transcription factor that can regulate several miRNAs, but this needs to be further verified experimentally.

At present, there is not much information available on miR-19b-1-5p. miR-19b-1-5p is one of the microRNAs from the miR-17-92 cluster, which can regulate the development of cardiovascular diseases[30]. Additionally, it was found that the low expression of miR-19b-1-5p in platelets can lead to sustained platelet aggregation and increase the risk of future adverse cardio-cerebrovascular events. Kok et al. found that the expression of miR-19b-1-5p was also associated with aspirin insensitivity after aspirin use[31]. In the TransmiR v2.0 database, the relationships between MAPK14 and miR-19b-1-5p are listed as regulation and feedback, which means the action type is unclear, and TF may be a target gene for miRNA. In our PCR assay, the expression of MAPK14 was high, while the expression of miR-19b-1-5p is low in the LPS group. This may indicate a negative regulatory relationship between miR-19b-1-5p and MAPK14, though this would require further experimental proof. Previous reports of miR-5009-5p could not be found after an extensive literature search. However, its downregulation in the LPS-treated group suggests that it may play a role in regulating the sepsis response.

While these results are promising, they are still inconclusive. Verification experiments confirming the relevance of the hub genes were also not performed. Therefore, future in vitro and in vivo experiments are required to investigate the function and pathway of these genes in sepsis pathology. Studies with larger cohorts of patients with sepsis are also required to confirm the diagnostic and therapeutic value of the identified genes, transcription factors, and miRNAs.

Conclusions

A TF-miRNA-mRNA regulation network was constructed through the bioinformatics analysis of mRNA and miRNA expression data. After experimental validation, it was found that the TFs MAPK14 and RUNX3 and the miRNAs miR-19b-1-5p and miR-5009-5p potentially play important roles in the development of sepsis. These results contribute to a deeper understanding of the molecular mechanisms of sepsis and present potential targets for clinical treatment.

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Figure legend

Figure 1 Expression profiles of distinct mRNAs. (A)The volcano diagram of GSE131761 showed the differential expression of mRNAs between the normal and sepsis groups. (B) The volcano diagram of GSE94717 showed the differential expression of mRNAs between the normal and sepsis groups (C) The heatmap based on GSE131761 showed the differential expression patterns of several mRNAs.

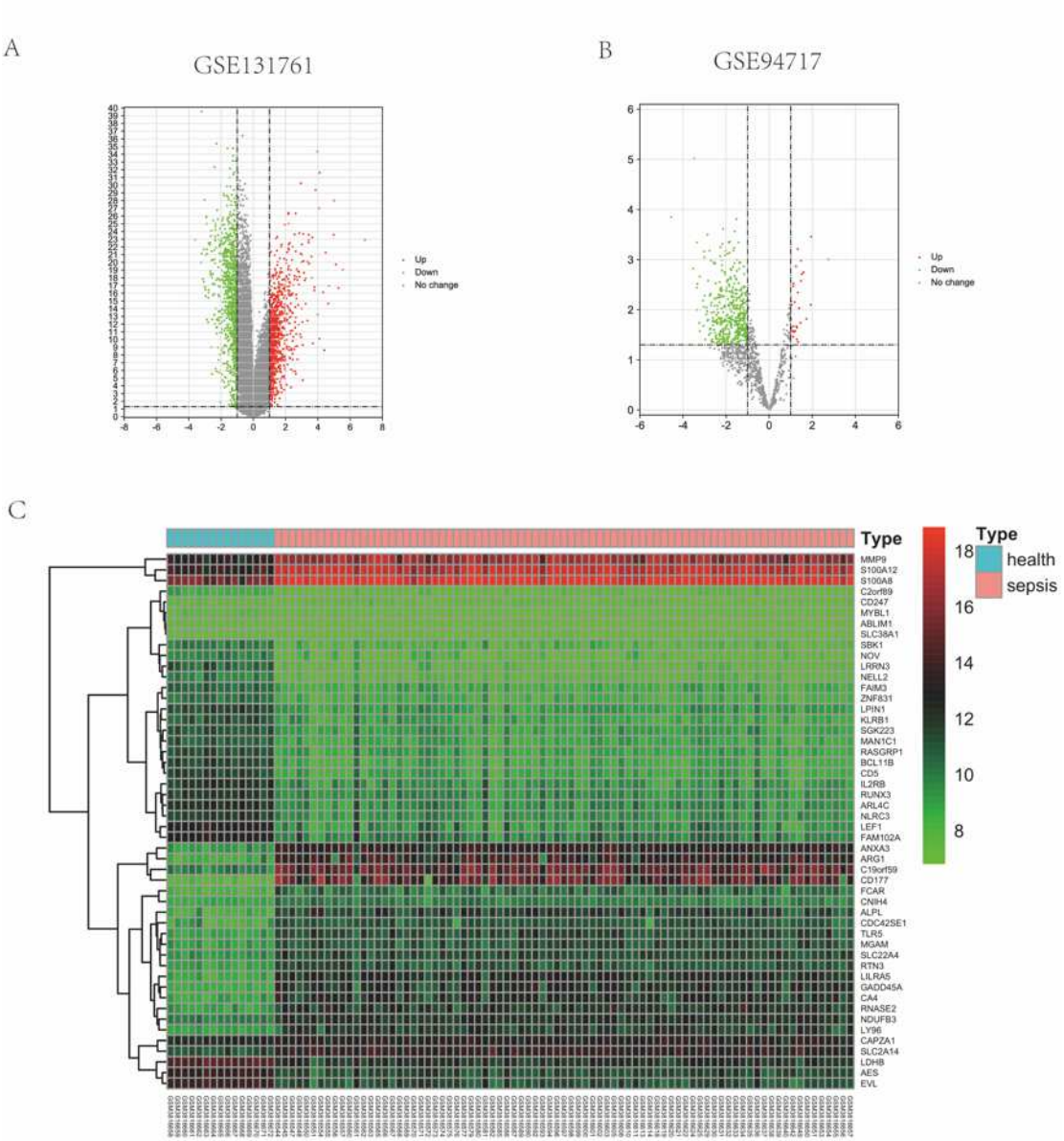


Figure 2 GO enrichment annotation and KEGG pathway analysis of the DEGs. (A) Top 20 BP (biological process) terms of DEGs. (B) Top 20 CC (cellular component) terms of DEGs. (C) Top 10 MF (Molecular Function) terms of DEGs. (D) Top 20 KEGG terms of DEGs. The analysis was conducted using the DAVID (https://david.ncifcrf.gov/summary.jsp) database. GO, Gene Ontology; KEGG, the Kyoto Encyclopedia of Genes and Genome.

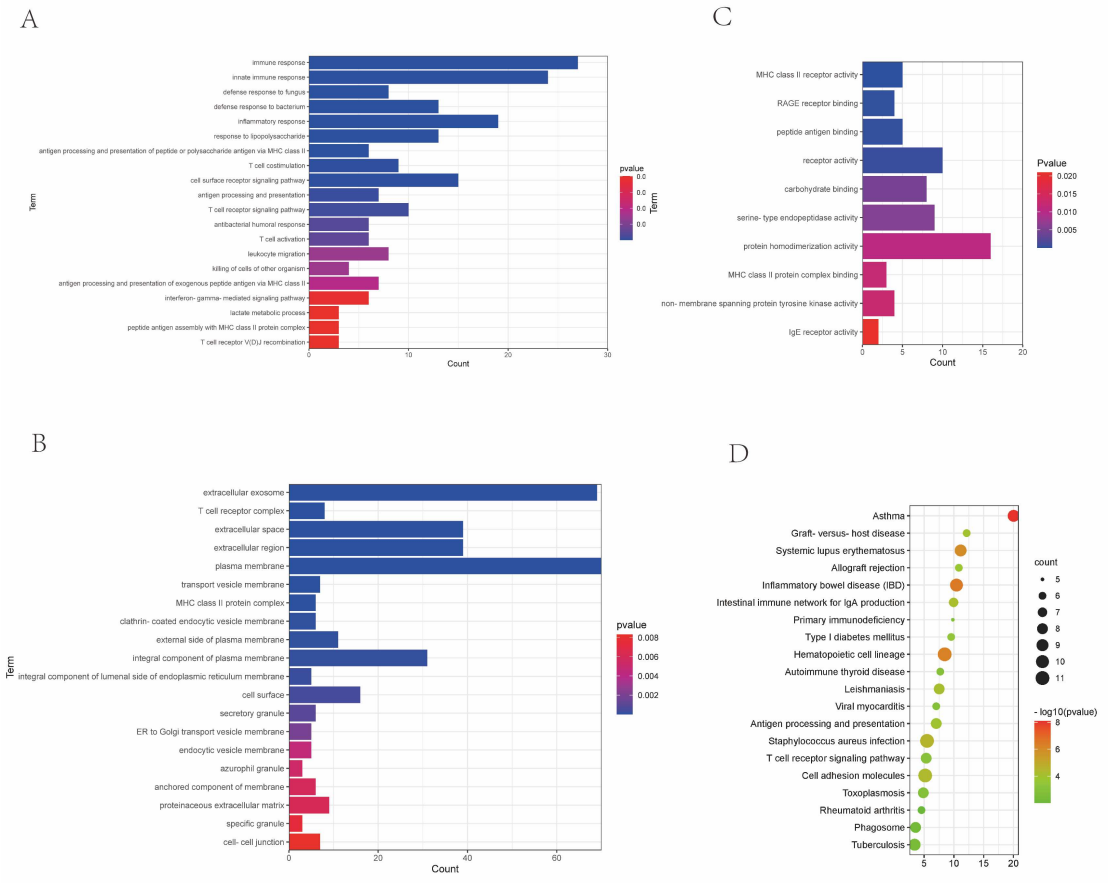


Figure 3 Protein-protein interaction networks of the DEGs in GSE131761. The most significant module was obtained from the PPI network using Cytoscape. Hub genes were identified as genes with connectivity degree > 8.

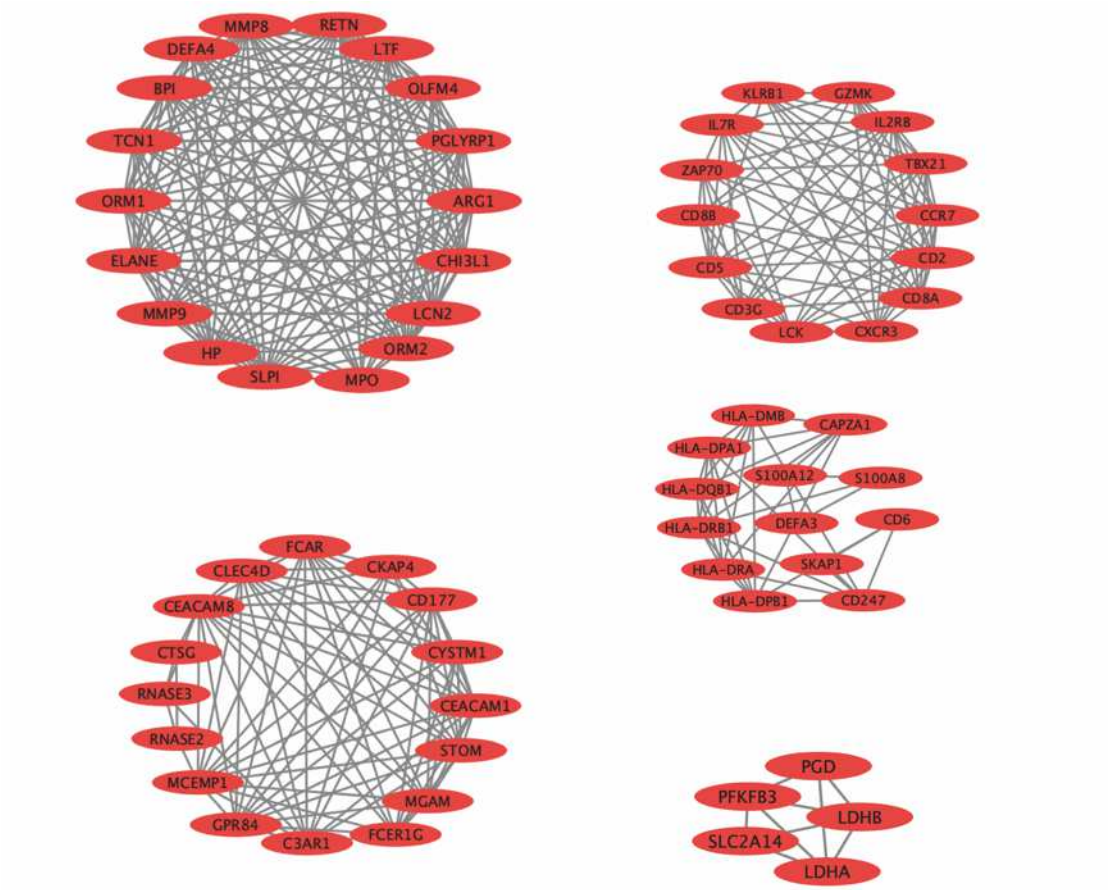


Figure 4, Construction of the TF-miRNA-mRNA regulatory networks. (A) Diagram of the miRNA-mRNA network based on DEMs, DEM target genes, and their inside regulations. Triangles represent mRNA, while diamonds represent miRNA. Red means upregulation, while blue means downregulation. (B) The overlap of DEGs identified the transcription factors of interest, and the top 20 miRNAs predicted TFs from TransmiR database. The hub genes were intersected with the target genes of the top 20 miRNAs from TargetScan and miRWalk databases. (C) TF-miRNA-mRNA regulatory networks were constructed for sepsis based on the above analysis and their inside interaction relationships. The red color represents TFs. The orange color represents miRNAs. The green color represents mRNAs.

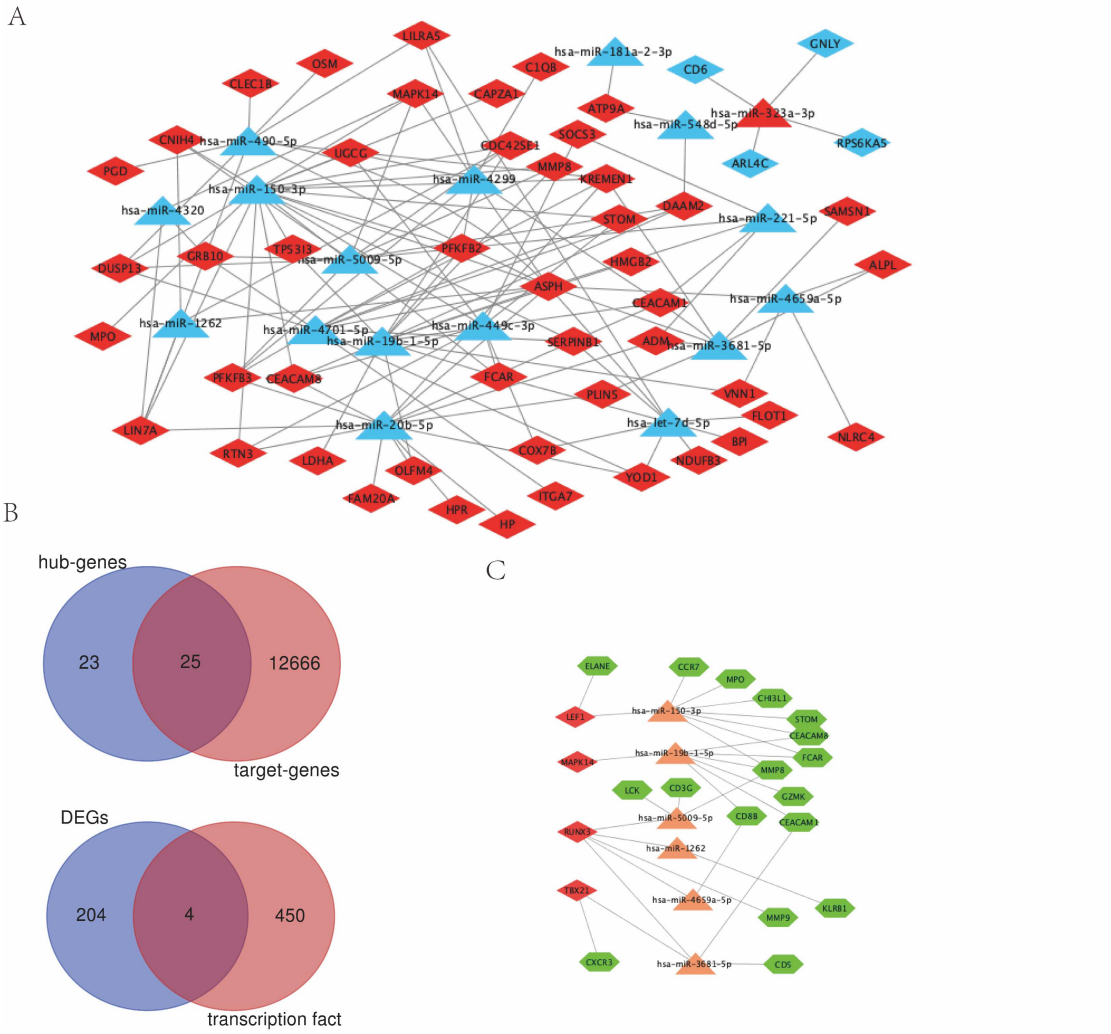


Figure 5 HUVECs were administrated with different concentrations of LPS (0, 0.5, or 1 μ g/mL) at different time points (6, 12, and 24 hours). (A) HUVECs with LPS induced a dose-dependent reduction in cell viability(p<0.05). (B) The expression level of inflammatory cytokines (IL-6, IFN-γ， IL-1β, and TNF-α) by qRT-PCR. The results showed that the expression levels of inflammatory cytokines were markedly increased upon LPS stimulation ($P < 0.05$).

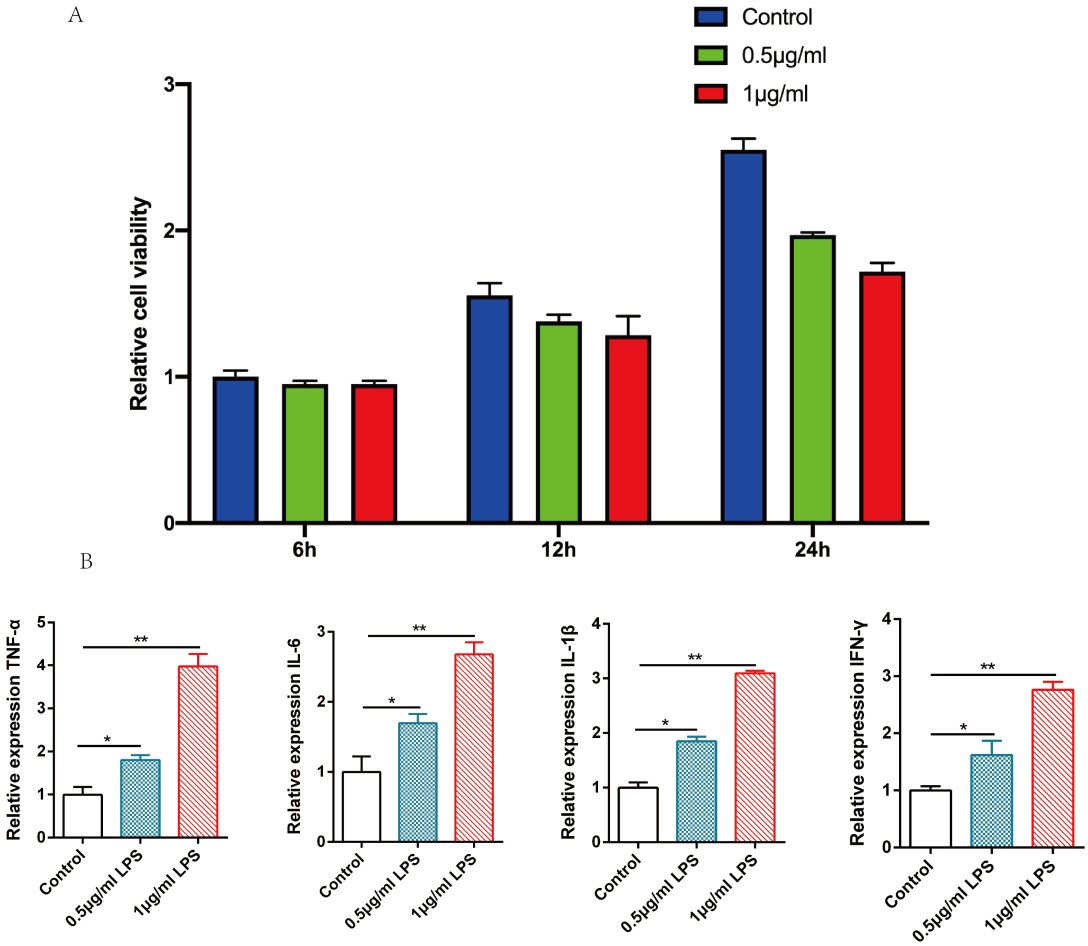
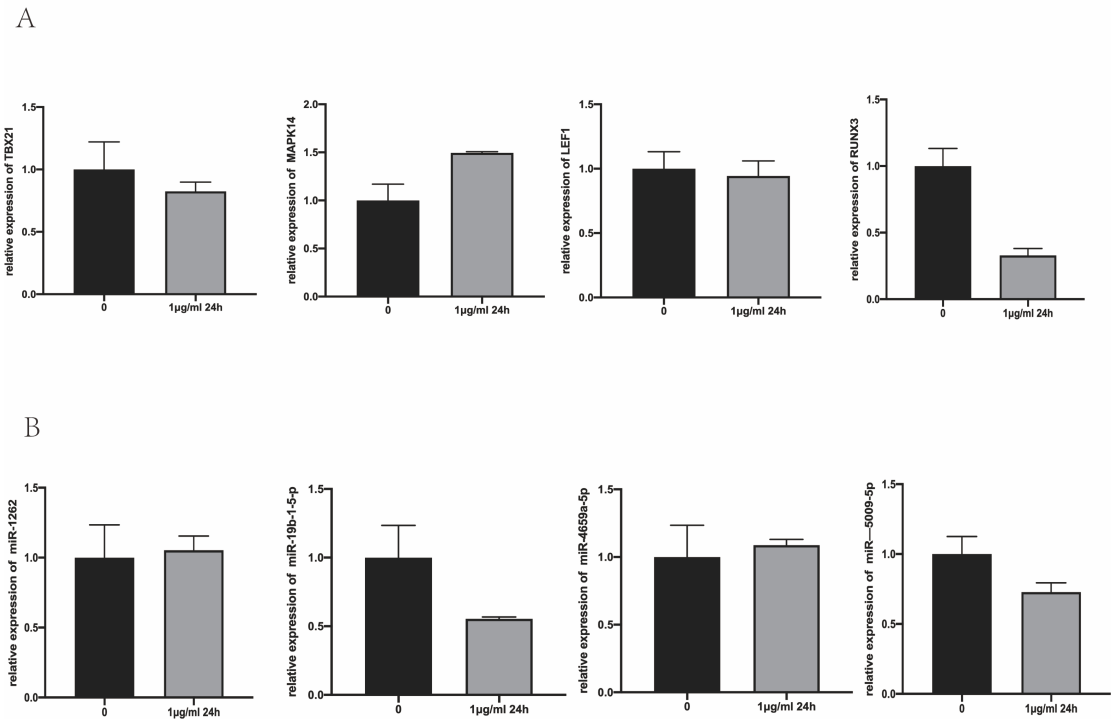


Figure 6 Network validation by qRT-PCR. (A) Validation results by RT-PCR of TBX21, MAPK14, LEF1, and RUNX3 expression. (B) Validation results by RT-PCR of miR-1262, miR-19b-1-5p, miR-4659a-5p, and miR-5009-5p expressions.



Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors have consented this study to publication

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Competing interests

There was no conflict of interest between each author

Authors' contributions

Qinghui Fu conceived this study. Qinghui Fu designed the study. Xiaoqian Luo and Jun Hu acquired and analyzed the data. Enjiang Chen and Weina Lu contributed analysis tools. Xiaoqian Luo wrote the paper. Shi Fu and Jianfeng Zhao were of immense help in the preparation of the manuscript. All authors read and approved the final manuscript.

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