Identification of potential genes and miRNAs associated with sepsis based on microarray analysis

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Abstract. Sepsis is a type of systemic inflammatory response syndrome caused by infection. The present study aimed to examine key genes and microRNAs (miRNAs) involved in the pathogenesis of sepsis. The GSE13205 microarray dataset, downloaded from the Gene Expression Omnibus was analyzed using bioinformatics tools, and included muscle biopsy specimens of 13 patients with sepsis and eight healthy controls. The differentially expressed genes (DEGs) in samples from patients with sepsis were identified using the Linear Models for Microarray package in R language. Using the Database for Annotation, Visualization and Integration Discovery tool, functional and pathway enrichment analyses were performed to examine the potential functions of the DEGs. The protein-protein interaction (PPI) network was constructed with the DEGs using the Search Tool for the Retrieval of Interacting Genes, and the network topology was analyzed using CytoNCA. Subsequently, MCODE in Cytoscape was used to identify modules in the PPI network. Finally, the integrated regulatory network was constructed based on the DEGs, miRNAs and transcription factors (TFs). A total of 259 upregulated DEGs (*MYC* and *BYSL*) and 204 downregulated DEGs were identified in the patients with sepsis. *NOP14*, *NOP2*, *AATF*, *GTPBP4*, *BYSL* and *TRMT6* were key genes in the MCODE module. In the integrated DEG-miRNA-TF regulatory network, hsa-miR-150 (target gene *MYLK3*) and 21 TFs, comprising 14 upregulated DEGs (including *MYC*) and seven downregulated DEGs, were identified. The results suggested that *NOP14*, *NOP2*, *AATF*, *GTPBP4*, *BYSL*, *MYC*, *MYLK3* and miR-150 may be involved in the pathogenesis of sepsis.

Introduction

Sepsis, a type of systemic inflammatory response syndrome (SIRS), is a complication caused by the response of the whole body, and injured tissues and organs to an infection (1). In the last decade, the incidence rates of sepsis and severe sepsis in the population were 4.37 and 2.70 cases per 1,000 individuals, respectively, in high-income-countries (including seven countries on four continents); annually, the global mortality rate is as high as 5,300,000 (2). In China, a survey revealed that, 10 years ago, the incidence and mortality rates of sepsis were 8.68 and 48.7%, respectively (3). It is difficult to predict, diagnose and treat patients with sepsis (4). In addition, there are no effective drugs or medical interventions for treating this disease. Therefore, the investigation of target genes and microRNAs (miRNAs) in sepsis is urgently required.

Proinflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), have been implicated as key mediators in inflammation associated with sepsis (5). *Nrf2*, a basic leucine zipper transcription factor, is a regulatory factor for response to lipopolysaccharides (LPS) and TNF-α by activating the expression of nuclear factor-κB (NF-κB) during experimental sepsis (6). *PTP1B* is a target gene for the treatment of sepsis via the reduction of cardiovascular inflammation (7). In addition, miRNAs are small non-coding RNA molecules of 20-23 nucleotides present in almost all living things, ranging from viruses to higher animals (8). miRNAs have various biological functions and are involved in inflammation, metabolism and tumor processes through a series of signaling pathways (9). miRNA (miR)-34a (10), miR-150 (11) and miR-15a (10) are considered to be novel indicators for the early diagnosis and prognosis of sepsis. However, the role of target genes and miRNAs in sepsis requires further elucidation. To clarify the role of key genes and miRNAs in sepsis, the present study aimed to identify potential genes and miRNAs associated with sepsis, based on the GSE13205 dataset from the Gene Expression Omnibus (GEO) database (12).

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The present study identified differentially expressed genes (DEGs) in sepsis, and then analyzed the potential functions of these DEGs using Gene Ontology (GO) functional and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. Subsequently, a protein-protein interaction (PPI) network between the DEGs was constructed and GO-associated analysis of the PPI network module was performed. Finally, an integrated regulatory network was constructed based on the DEGs, miRNAs and transcription factors (TFs) for the prediction of key targets in sepsis.

Materials and methods

Microarray data. The gene expression profile of GSE13205 was downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/), which included muscle biopsy specimens from 13 patients with sepsis from the General Intensive Care Unit at Karolinska University Hospital (Huddinge, Sweden) and eight healthy controls from Ersta Hospital (Stockholm, Sweden). Muscle biopsy samples were obtained from the lateral portion of the vastus lateralis muscle (10-20 cm above the knee). The GSE13205 profile was deposited by Fredriksson *et al* according to the platform of GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. Their study was approved by the Ethical Committee of Karolinska Institutet (Stockholm, Sweden) and received local ethics approval for gene expression analysis at Heriot-Watt University (Edinburgh, Scotland) (13). Informed consent was obtained from all patients or close relatives.

Data preprocessing and identification of DEGs. The microarray data were normalized using the robust multi-array average function in the Affy package (version 1.52.0; http://bioconductor.org/packages/release/bioc/html/affy.html)of R software (version 3.3.2; https://cran.r-project.org/bin/windows/base/) (14). Subsequently, the Bayesian method of the Linear Models for Microarray (LIMMA; version 3.30.3; http://bioconductor.org/packages/release/bioc/html/limma.html) package in R was used for identifying the DEGs between the samples from patients with sepsis and those from the healthy controls (15). The threshold value of DEGs was set as |log fold change (FC)| >1 and P<0.05.

GO and KEGG pathway enrichment analyses. GO analysis (http://geneontology.org/page/go-enrichment-analysis) has become a predictable method for enrichment analysis to understand biological process (BP), cellular localization and molecular function (16). KEGG (http://www.genome.jp/kegg/) is a well-known database for the systematic analysis of gene function and genomic information, including the GENES and PATHWAY databases (17). The Database for Annotation, Visualization and Integration Discovery (DAVID; version 6.7; https://david-d.ncifcrf.gov/) is a public high-throughput functional annotation tool, which provides functional annotation bioinformatics microarray analysis for integrating data-mining environments and analyzing gene lists (18). The DEGs were input into DAVID online for GO and KEGG enrichment analyses. P<0.05 was considered to be statistically significant.

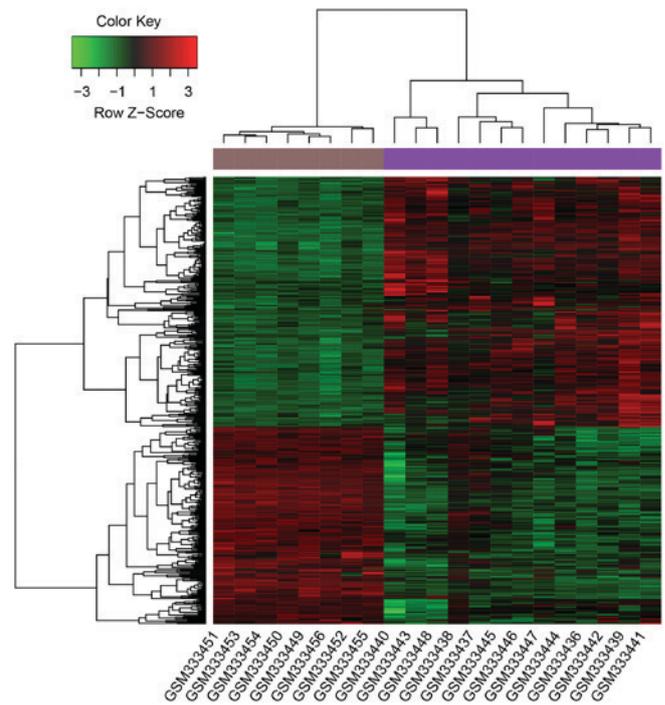Figure 1. Heatmap of clustering analysis. The heatmap showed all significant DEGs (|log fold change| >1 and P<0.05) identified from the comparison of samples from patients with sepsis, vs. those from healthy controls. GSM333436-GSM333448 (purple) indicates the sepsis group and GSM333449-GSM333456 (brown) indicates the healthy control group. The red color indicates upregulated DEGs and the green color indicates downregulated DEGs, compared with corresponding samples from healthy controls; DEGs, differentially expressed genes.

Analysis of the PPI network. The PPI network was constructed with all the DEGs using the Search Tool for the Retrieval of Interacting Genes (STRING) from a well-known online server (version 10.0; http://www.string-db.org/) (19). PPI links with a combined score >0.4 were identified for constructing the PPI network. The PPI network was visualized using Cytoscape software (http://cytoscape.org/), and the network topology was analyzed using CytoNCA (version 2.1.6; http://apps.cytoscape.org/apps/cytonca) (20). The parameter was set as 'without weight'.

GO-associated analysis of the PPI network module. MCODE in Cytoscape was used to search for modules in the PPI network (21). The modules were identified when the degree of cut-off was 2, the node score cut-off was 0.2, the K-core was 2, and the maximum depth was 100. GO-associated analysis was performed on the module with the maximum score using Golorize (version: 1.0.0.beta1) (22). In addition, the top five GO terms in the module were analyzed.

Construction of the integrated regulatory network. The integrated regulatory network was constructed based on the DEGs, miRNAs and TFs. miRNAs associated with DEGs were identified from the mir2disease database (http://www.mir2disease.org/) and miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) (23,24). Finally, the miRNA-target gene links, which overlapped with DEGs, were considered to be closely associated with sepsis. TFs were selected from the integrated transcription factor platform

Table I. Results of GO and KEGG enrichment on upregulated differentially expressed genes.

Category	Term	Count	P-value
GO term BP	GO:0045768-Positive regulation of anti-apoptosis	5	6.2E-4
GO term BP	GO:0045767-Regulation of anti-apoptosis	5	1.5E-3
GO term BP	GO:0022613-Ribonucleoprotein complex biogenesis	9	2.7E-3
GO term BP	GO:0010033-Response to organic substance	20	3.2E-3
GO term BP	GO:0006366-Transcription from RNA polymerase II promoter	10	3.9E-3
KEGG pathway	Hsa04210: Apoptosis	5	1.3E-2
KEGG pathway	Hsa00480: Glutathione metabolism	4	1.6E-2

GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process.

Table II. Results of GO and KEGG enrichment on downregulated differentially expressed genes.

Category	Term	Count	P-value
GO term BP	GO:0007517-Muscle organ development	14	3.68E-8
GO term BP	GO:0006936-Muscle contraction	11	8.74E-7
GO term BP	GO:0003012-Muscle system process	11	2.05E-6
GO term BP	GO:0044057-Regulation of system process	13	1.60E-5
GO term BP	GO:0006942-Regulation of striated muscle contraction	5	3.56E-5
KEGG pathway	Hsa04020: Calcium signaling pathway	8	1.40E-3
KEGG pathway	Hsa04960: Aldosterone-regulated sodium reabsorption	4	7.30E-3
KEGG pathway	Hsa04270: Vascular smooth muscle contraction	5	2.36E-2
KEGG pathway	Hsa04920: Adipocytokine signaling pathway	4	2.75E-2
KEGG pathway	Hsa04260: Cardiac muscle contraction	4	4.06E-2

GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process.

(http://itfp.biosino.org/itfp) (25) and TRANSFAC (http://www.gene-regulation.com/pub/databases.html) among DEGs (26). The DEG-miRNA-TF network was constructed and visualized using Cytoscape software.

Results

DEGs in sepsis. A total of 463 DEGs were identified from patients with sepsis and healthy controls, which comprised 259 upregulated DEGs, including *MYC* and *BYSL*, and 204 downregulated DEGs, including *MYLK3*. The clustering heatmap showed that DEGs were well distinguished between samples from patients with sepsis and those from healthy controls (Fig. 1).

Results of GO and KEGG enrichment analysis. For the upregulated DEGs, all were enriched in 51 GO BP terms and two KEGG pathways. *MAP3K14* was enriched in the apoptosis pathway (Table I). Downregulated DEGs were enriched in 37 GO BP terms and five KEGG pathways, including the adipocytokine and calcium signaling pathways (Table II).

Construction of the PPI network. The PPI network was constructed using all DEGs and included 292 nodes (individual proteins) and 731 edges (interaction events), as shown in Fig. 2.

In the PPI network, MYC interacted with MAP3K14 and GTPBP4. The interacting proteins of BYSL included GTPBP4, NOP14, NOP2, NOB1, AATF and TRMT6. Following analysis of the network topology, eight DEGs (degree >20) were identified (Table III), including MYC (degree=29), NOP2 (degree=29), GTPBP4 (degree=27), BYSL (degree=25) and AATF (degree=22). The degree represents the number of interactions with other proteins.

Function of the PPI network module. The MCODE module with the highest score (14.286) in the PPI network was identified; it included 15 upregulated DEGs and 100 edges. The function of this module was enriched in ribosome biogenesis following GO analysis (Fig. 3). The top five GO terms in the module were analyzed using GO analysis, and the results showed that NOP14, NOP2, AATF, GNL2, GTPBP4, BYSL and TRMT6 were key genes in the module (Fig. 4).

Construction of the integrated DEG-miRNA-TF regulatory network. The integrated regulatory network was constructed with the target DEGs, miRNAs and TFs (Fig. 5). In the network, hsa-miR-150 was identified, which was the only miRNA associated with sepsis. Its target DEGs, including *MYLK3*, are shown in Table IV. A total of 21 TFs were identified in the

Table III. Eight differentially expressed genes (degree >20) in the network topology.

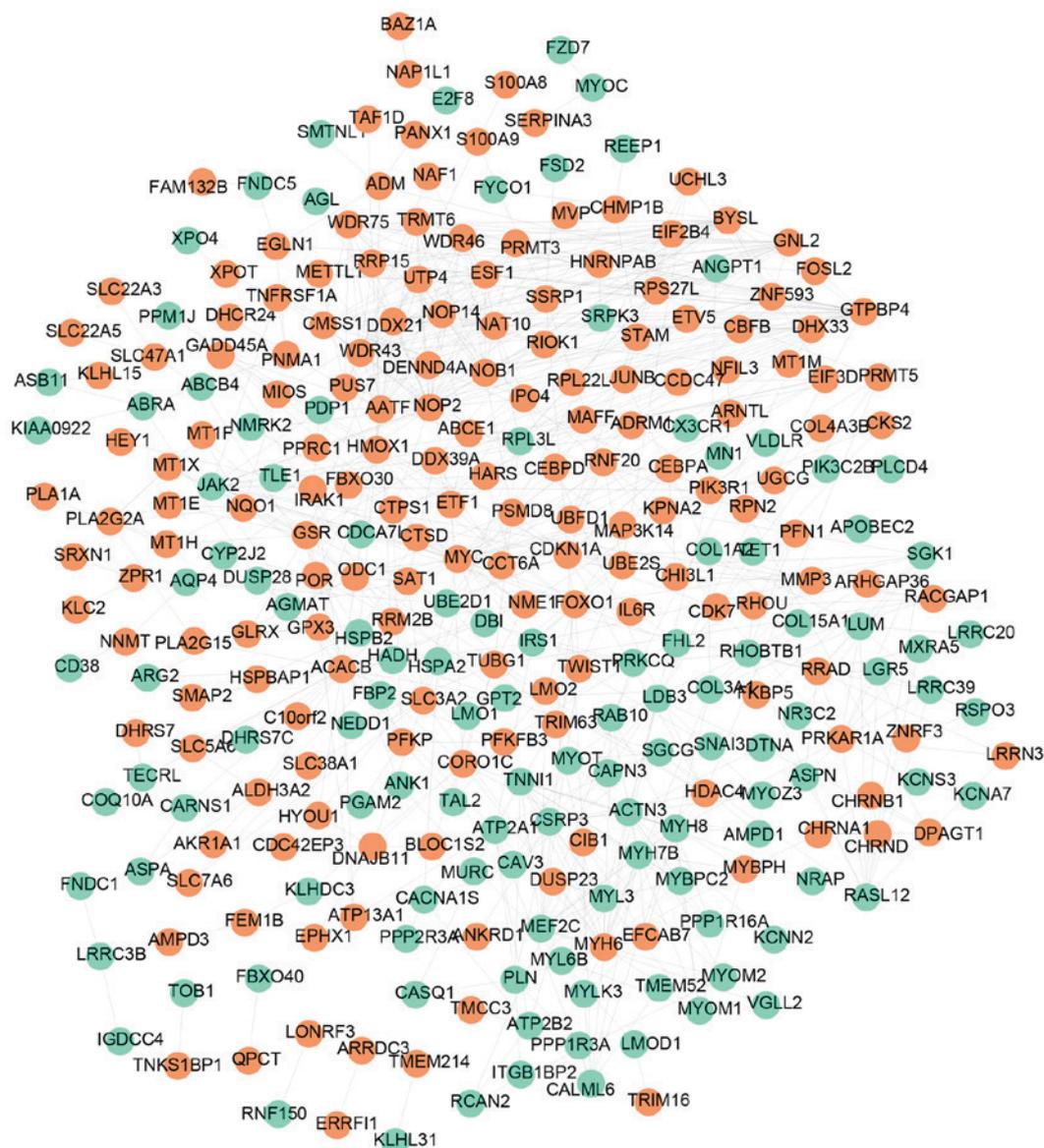| Gene	Degree	Betweenness	Closeness
MYC	29.0	14,147.05	0.054
NOP2	29.0	4,228.61	0.053
GTPBP4	27.0	5,434.96	0.053
ACACB	25.0	12,036.69	0.053
BYSL	25.0	1,432.08	0.052
MYH6	23.0	5,115.25	0.053
AATF	22.0	4,423.36	0.053
NAT10	22.0	1,173.44	0.052

Figure 2. PPI network of DEGs. All upregulated and downregulated DEGs were used to construct a PPI network. Orange nodes represent upregulated DEGs and green nodes represent downregulated DEGs. The edges between them represent PPIs. DEGs, differentially expressed genes; PPI, protein-protein interaction.

network, comprising 14 upregulated DEGs, including *MYC*, *WDR46*, *IPO4* and *ZNF593*, and seven downregulated DEGs (Table V).

Discussion

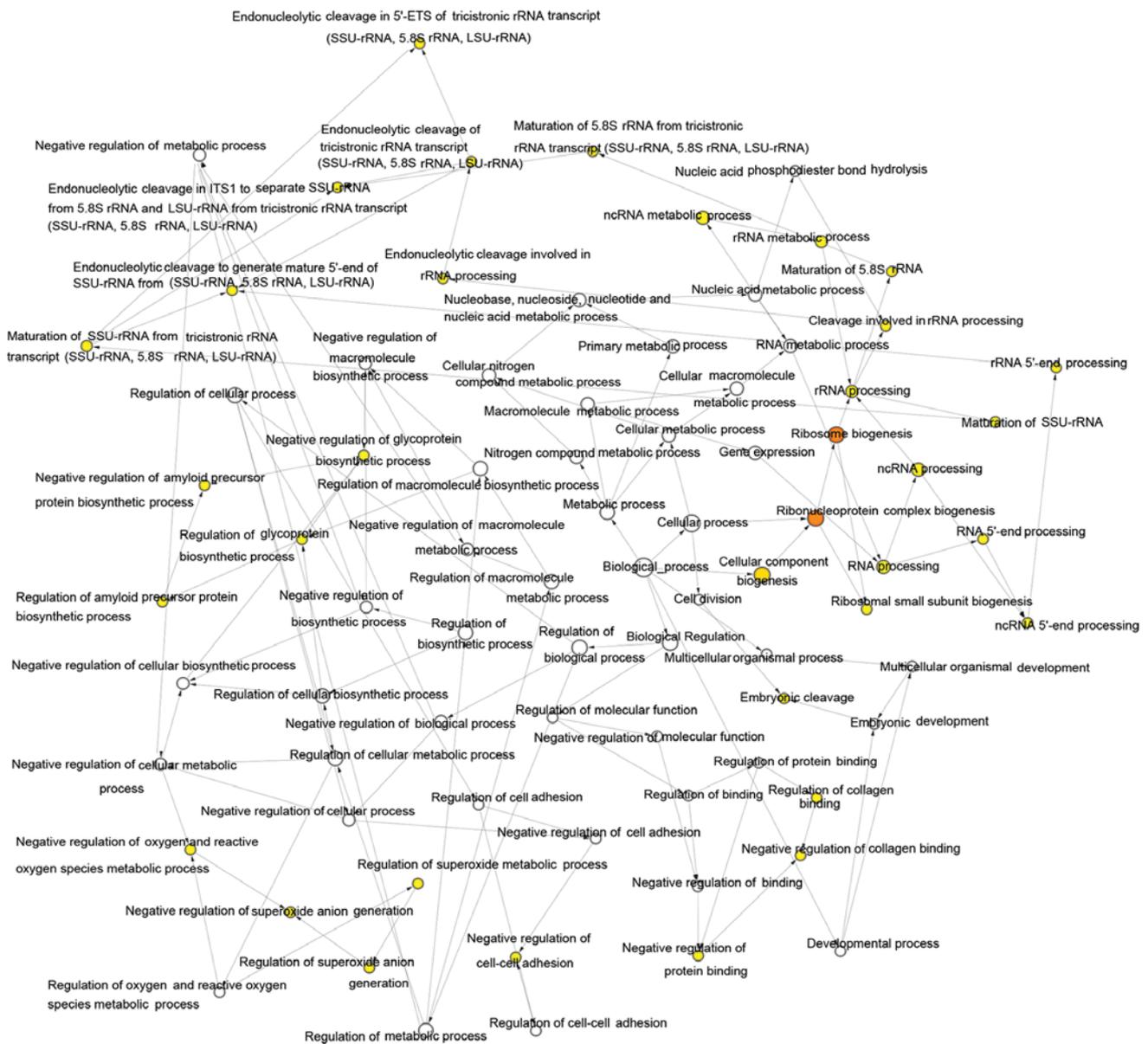In the present study, there were 259 upregulated and 204

Figure 3. GO-enriched terms in the MCODE module. The module contains the highest number of genes (15 upregulated differentially expressed genes) of all modules identified via MCODE from the protein-protein interaction network and has the highest MCODE score (14.286). The yellow color indicates GO terms with a significant difference; white indicates GO terms for which the difference is not significant. Larger nodes and darker colors represent higher degrees of difference. The arrows indicate that the hierarchical structure of GO terms. GO, Gene Ontology.

downregulated DEGs in the samples from patients with sepsis, compared with those in samples from healthy controls. The upregulated DEGs were enriched in the apoptosis pathway and in the metabolism of glutathione. The downregulated DEGs were enriched in pathways, including the calcium signaling pathway and adipocytokine signaling pathway. A total of 292 nodes and 731 edges were identified in the PPI network, including eight DEGs with higher degrees (degree >20). Subsequently, key genes in the module with the maximum score (14.286; 15 upregulated DEGs and 100 edges) in the PPI network were identified. Finally, target DEGs, miRNAs and TFs were identified in samples from patients with sepsis.

In the PPI network, MYC, NOP2, GTPBP4, BYSL and AATF had a higher degree (>20). The MYC protein is encoded by the *MYC* gene, which is associated with cell cycle,

transformation and apoptosis (27). There is no direct evidence to confirm the role of *MYC* in sepsis. However, proinflammatory cytokines, including TNF-α, are closely associated with sepsis characterized by SIRS (28), which may have important implications on the treatment of sepsis (29). For example, TNF-α protects against microvascular endothelial dysfunction in patients with sepsis by activating NF-B and p38 in endothelial cells (30). In addition, *MYC* promotes the expression of TNF-α in chronic liver diseases (31). Therefore, MYC may be crucial in the progress of sepsis by promoting the expression of TNF-α. Qiao *et al* found that *NOP2* and *GTPBP4* may be differentially expressed in neonatal sepsis (32), which is consistent with the results of the present study. Therefore, NOP2 and GTPBP4 may be associated with the progress of sepsis. The AATF protein, also known as Che-1, is encoded by the AATF gene, which is involved in cell apoptosis (33).
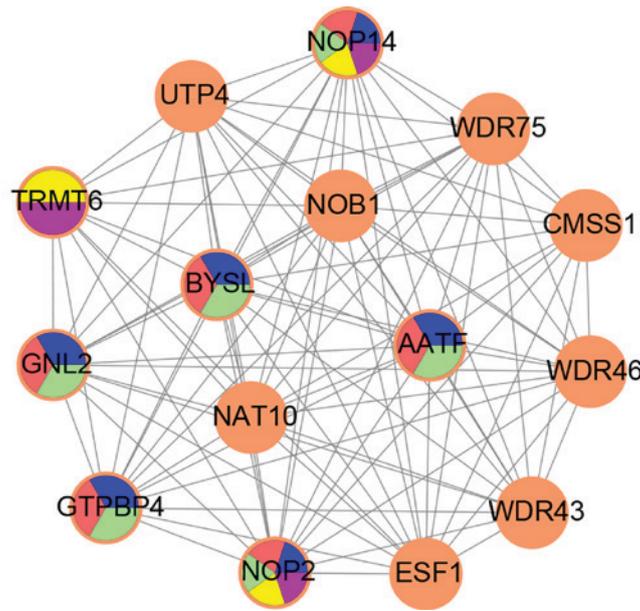
Figure 4. Genes in the top five terms in the MCODE module. Orange indicates upregulated DEGs, blue indicates biogenesis of the ribonucleoprotein complex, red indicates biogenesis of ribosomes, green indicates biogenesis of cellular components, yellow indicates ncRNA metabolic processes, purple indicates ncRNA processing. The lines indicate there are interacted with the two nodes in the network.
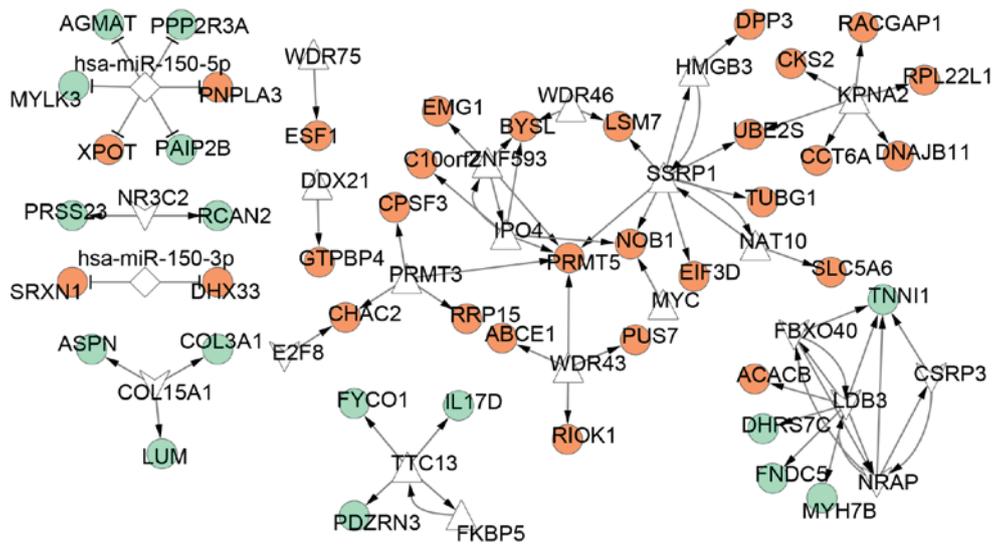

Figure 5. Integrated regulatory network. Circles represent DEGs; orange represents upregulated DEGs and green represents downregulated DEGs. The triangles represent upregulated TFs, inverted triangles represent downregulated TFs and diamonds represent microRNAs. Lines without arrows indicate regulation associations of microRNAs. The arrowed lines indicate that the regulation associations of TFs. DEGs, differentially expressed genes; TFs, transcription factors; miR, microRNA.

The overexpression of AATF interferes with MAP3K12/DLK (a protein kinase)-induced apoptosis (34,35), which forms heterodimers with a leucine zipper containing TFs, including MYC, and has a regulatory role (36). There is no evidence to confirm the function of *AATF* in sepsis. However, *AATF* represents an immune- or inflammation-associated gene and encodes miRNA-2909, which is responsible for the regulation of genes involved in inflammation (37,38). Therefore, AATF may be associated with inflammation in sepsis by regulating the expression of miRNA-2909.

BYSL, an adhesion molecule in mammalian preimplantation embryos (39), is crucial in pre-ribosomal RNA (rRNA)

processing, and its depletion leads to the accumulation of an unusual 18S rRNA precursor (40). There is no evidence to confirming its association with sepsis. In the present study, BYSL was found to closely interact with GTPBP4, NOP14, NOP2, NOB1 and AATF in the PPI network. In addition, the *NOP14* gene encodes a NOP14 protein, which is involved in pre-18S rRNA processing and small ribosomal subunit assembly (41). *NOB1* is involved in pre-rRNA processing and produces a mature 18S rRNA in a late cytoplasmic processing step (42). Studies have reported that 16S rRNA gene amplification by real-time polymerase chain reaction and sequencing rapidly and accurately diagnose neonatal sepsis (43-45).

Table IV. miRNA-target DEGs in the integrated DEG-miRNA-TF regulatory network.

miRNA	Target genes
Hsa-miR-150-3p	*SRXN1, DHX33*
Hsa-miR-150-5p	*AGMAT, PPP2R3A, MYLK3, PNPLA3, XPOT, PAIP2B*

miRNA/miR, microRNA.

Table V. TFs in the integrated DEG-miRNA-TF regulatory network.

DEGs	TFs
Upregulated	DDX21, FKBP5, HMGB3, IPO4, KPNA2, MYC, NAT10, PRMT3, SSRP1, TTC13, WDR43, WDR46, WDR75, ZNF593
Downregulated	COL15A1, CSRP3, E2F8, FBXO40, LDB3, NR3C2, NRAP

DEGs, differentially expressed genes; TFs, transcription factors.

Therefore, *BYSL*, *NOB1* and *NOP14* are involved in pre-18S rRNA processing in sepsis.

In the present study, hsa-miR-150, including its target genes (*MYLK3*) was identified in the integrated DEG-miRNA-TF regulatory network. Zhao *et al* also found that the serum levels of miR-150 decreased in a rat sepsis model, providing a valuable tool in evaluating the prognosis of sepsis (11). Similarly, the expression of miR-150 was reported to be decreased in a patient with sepsis (46). A previous study found that *MLCK*, including *MYLK1*, *MYLK2* and *MYLK3*, may not upregulate endothelial permeability induced by LPS exposure, whereas hyper-permeability was observed in sepsis and other inflammatory situations (47). TNF-α, LPS and 18% cyclic stretch each increased the activity of a *MYLK* gene 3'untranslated region luciferase reporter, and induction was reduced by mimics of each miRNA (48). As reported in the above study, a negative correlation was found between the expression of miR-150 and white blood cell counts, and the levels of IL-6, C-reactive protein and TNF-α (11). In the present study, *MYLK3* was identified as a downregulated DEG in sepsis and one of the target genes of miR-150. These findings suggested that the expression of miR-150 is increased by upregulating *MYLK3*, which provides a direction for sepsis therapy by developing a novel drug targeting *MYLK3* in miR-150.

There were a number of limitations in the present study, with the exception of the above finding. All predicted results require confirmation by laboratory data, however, there are currently insufficient samples from patients with sepsis. In future investigations, the expression of the DEGs discussed above requires validation using large-scale samples. In addition, future investigations aim to confirm the interactions of DEGs, regulatory associations between TFs and DEGs, and possible pathways underlying these gene alterations.

In conclusion, a total of 259 upregulated DEGs, including *MYC*, *AATF* and *BYSL*, and 204 downregulated DEGs, including *MYLK3*, were identified from patients with sepsis. *NOP14*, *NOP2*, *AATF*, *GTPBP4*, *BYSL*, *MYC* and *MYLK3* were key genes in sepsis, which may be involved in pre-18S rRNA processing in sepsis or may be associated with inflammation in sepsis by regulating the expression of miRNA-2909. In addition, miR-150 and its target genes, including *MYLK3*, were found to be key in sepsis. The findings may provide further understanding of the pathogenesis of sepsis and assist in developing a novel target drug for the treatment of sepsis.

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