Regulatory mechanisms underlying sepsis progression in patients with tumor necrosis factor-α genetic variations

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Abstract. The present study aimed to investigate the regulatory mechanisms underlying sepsis progression in patients with tumor necrosis factor (TNF)-α genetic variations. The GSE5760 expression profile data, which was downloaded from the Gene Expression Omnibus database, contained 30 wild-type (WT) and 28 mutation (MUT) samples. Differentially expressed genes (DEGs) between the two types of samples were identified using the Student's t-test, and the corresponding microRNAs (miRNAs) were screened using WebGestalt software. An integrated miRNA-DEG network was constructed using the Cytoscape software, based on the interactions between the DEGs, as identified using the Search Tool for the Retrieval of Interacting Genes/Proteins database, and the correlation between miRNAs and their target genes. Furthermore, Gene Ontology and pathway enrichment analyses were conducted for the DEGs using the Database for Annotation, Visualization and Integrated Discovery and the KEGG Orthology Based Annotation System, respectively. A total of 390 DEGS between the WT and MUT samples, along with 11 -associated miRNAs, were identified. The integrated miRNA-DEG network consisted of 38 DEGs and 11 miRNAs. Within this network, *COPS2* was found to be associated with transcriptional functions, while *FUS* was found to be involved in mRNA metabolic processes. Other DEGs, including *FBXW7* and *CUL3*, were enriched in the ubiquitin-mediated proteolysis pathway. In addition, miR-15 was predicted to target *COPS2* and *CUL3*. The results of the present study suggested that *COPS2*, *FUS*, *FBXW7* and *CUL3* may be associated with sepsis in patients with TNF-α genetic variations. In the progression of sepsis, *FBXW7* and *CUL3* may participate in the ubiquitin-mediated proteolysis pathway, whereas *COPS2* may regulate the phosphorylation and ubiquitination of the FUS protein. Furthermore, *COPS2* and *CUL3* may be novel targets of miR-15.

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Introduction

Multiple trauma, which is commonly associated with severe injuries and multiple organ failure, may lead to various complications, including sepsis and septic shock, which are major healthcare problems worldwide (1-3). There are 400,000-500,000 cases of sepsis in the United States annually (4). Antimicrobial therapy may be applied for the management of sepsis; however, the mortality rate associated with sepsis has increased, and was reported to be as high as 40% in 2003 (5).

Tumor necrosis factor (TNF)-α, a cytokine that is predominantly secreted by macrophages, has been shown to be involved in the regulation of numerous biological processes, including cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation (6-8). However, the role of TNF-α in tumorigenesis remains unclear. This cytokine has been reported to induce tumor necrosis and apoptosis, as well as to promote tumor development (9). However, previous studies investigating the role of TNF-α in clinical sepsis syndrome or septic shock have reported conflicting results (10-12). TNF-α has been established as an effective marker in the diagnosis of neonatal sepsis (13); however, the mechanisms underlying the regulatory role of TNF-α in the development of sepsis syndrome remain undefined. Genetic variations have previously been implicated in the progression of numerous types of cancer (14,15). In addition, the clinical outcomes of sepsis have been associated with genetic polymorphisms in the genes encoding various inflammatory cytokines (16). Menges *et al* (17) demonstrated that common variants of the TNF-α gene were associated with sepsis syndrome and mortality following severe injury. Reportedly, the common TNF-α gene variant carrying the TNF rs1800629 A allele is correlated with higher TNF-α serum concentrations and alteration of genes strongly associated with proinflammatory and apoptosis (17). Furthermore, TNF rs1800629 A is closely associated with sepsis syndrome and mortality following multiple trauma (18).

The present study re-analyzed the GSE5760 microarray data deposited in the Gene Expression Omnibus (GEO) database by Menges *et al* (17), in order to detect genes that were differentially expressed between patients with and without TNF-α genetic variations, and to identify their potential functions and pathways. Furthermore, the regulatory associations

between differentially expressed genes (DEGs) and microRNAs (miRNAs) were analyzed in order to elucidate the regulatory mechanisms underlying sepsis in patients with TNF-α genetic variations, at the transcriptional and post transcriptional levels.

Materials and methods

Microarray data. The GSE5760 gene expression profile data was downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). Based on the description provided by Menges *et al* (17), the profile data consisted of 30 wild-type (WT) peripheral blood samples from 12 injured patients without the TNF-α rs1800629 A variant and 28 mutation (MUT) peripheral blood samples from 10 injured patients carrying the TNF-α rs1800629 A variant. The technical replicate numbers for the WT samples were three replicates for 6 patients and two replicates for the other 6 patients. The technical replicate numbers for the MUT samples were three replicates for 8 patients and two replicates for 2 patients. Menges *et al* (17) had used the GPL4204 platform (GE Healthcare/Amersham Biosciences CodeLink UniSet Human I Bioarray; GE Healthcare, Little Chalfont, UK). The annotation information in the platform was also downloaded.

Data preprocessing and identification of DEGs. The gene expression value was calculated from raw microarray data of the probe value. In the case that multiple probes corresponded to one gene, the average value was calculated as the expression level of this gene, whereas in the case that one probe corresponded to multiple genes, the probe value was removed. Following transformation of the data by $\log_2$ and normalization using the median method (19), DEGs between the WT and MUT samples were identified using the Limma package in R software (20) and Student's t-test. The Benjamini-Hochberg procedure (21) was applied, in order to control the false discovery rate (FDR). The threshold criteria for the DEGs were FDR<0.05 and $|\log_2$ fold change (FC)| of >0.05.

Selection of miRNAs targeted to DEGs. Following identification of the DEGs, WebGestal software (version 2.0; Vanderbilt University, Nashville, TN, USA; http://bioinfo.vanderbilt.edu/webgestalt/) (22) was used in order to identify miRNAs that were associated with the DEGs. A threshold of adjusted P<0.05 was used.

Construction of an integrated regulatory network between miRNAs and their target DEGs. The DEGs were mapped using the Search Tool for the Retrieval of Interacting Genes/Proteins database (http://string-db.org/) (23), in order to identify potential protein-protein interactions between the DEGs. Interaction pairs were identified using the default parameter of a combined score of ≥0.6. The integrated regulatory miRNA-DEG network was constructed and visualized using the Cytoscape software (24), based on the DEG interaction pairs and the interactions between the 11 miRNAs and their target DEGs.

Function and pathway enrichment analyses for the identified DEGs. The biological functions of the DEGs in the established network were investigated by Gene Ontology (GO) enrichment analysis, using the Database for Annotation, Visualization and Integrated Discovery online software (https://david.ncifcrf.gov/) (25). P<0.05 was considered to indicate a significantly enriched GO term. In addition, pathway enrichment analyses were conducted using the KEGG Orthology Based Annotation System (KOBAS, version 2; http://kobas.cbi.pku.edu.cn/home.do), in order to identify the pathways in which the DEGs were involved. In addition, the statistical method of cumulative hypergeometric distribution was applied and P<0.05 was considered to indicate a significantly enriched pathway.

Results

Identification of DEGs between the WT and MUT samples, and the associated miRNAs. Based on the preset criteria of FDR<0.05 and |log2FC|>0.05, a total of 390 genes were shown to be differentially expressed between the WT and MUT samples, including 238 genes that were upregulated and 152 genes that were downregulated. Based on an established threshold for miRNA searching, 11 miRNAs, including miR-141, miR-374, miR-204, miR-23, miR-182, miR-26, miR-15, miR-30, miR-34, miR-181 and miR-130, were significantly associated with the identified DEGs, and were selected for inclusion in the integrated miRNA-DEG regulatory network (Table I).

Construction of the integrated miRNA-DEG regulatory network. A total of 36 DEG interaction pairs were identified, according to their predicted protein-protein interactions. Combined with the identified DEGs targeted by miRNAs, the integrated miRNA-DEG regulatory network was constructed. This network comprised 49 nodes and 88 edges, involving 11 miRNAs and 38 DEGs, including 19 upregulated and 19 downregulated genes (Fig. 1).

Biological function and pathway annotation of the DEGs. In order to investigate the functions of the DEGs associated with TNF-α genetic variations, the identified DEGs were subjected to GO analysis. As presented in Table II, the DEGs were predominantly enriched in seven GO terms associated with transcriptional events, phosphorylation and RNA functions. DEGs associated with transcriptional regulation were as follows: *COPS2*, *THRB*, *SFPQ*, *SALL1*, *PNRC2*, *CTNND2*, *PIM1*, *SF1*, *MAP3K10*, *TGFBR3*, *HOXA9* and *WHSC1*. DEGs associated with transcription were as follows: *COPS2*, *PAPOLA*, *THRB*, *SFPQ*, *SALL1*, *PNRC2*, *CTNND2*, *SF1*, *HOXA9* and *WHSC1*. In addition, DEGs associated with GO terms such as phosphorylation and protein amino acid phosphorylation were *NTRK2*, *PIM1*, *MAP3K10*, *TGFBR3*, *BRD4* and *PRKG1*.The six DEGs associated with RNA processing were *FUS*, *DDX17*, *PAPOLA*, *SFPQ*, *SF1* and *CPSF6*. Those involved in mRNA metabolic processes, which was the most significantly enriched GO term (P=0.001632), included *FUS*, *PAPOLA*, *SFPQ*, *PNRC2*, *SF1* and *CPSF6*. DEGs, including *NTRK2*, *PIM1*, *MAP3K10*, *TGFBR3*, *BRD4* and *GAP43,* were significantly associated with positive regulation of molecular function.

KOBAS analysis was also conducted in order to identify significantly enriched pathways. Two significantly enriched pathways were identified, including the glycosphingolipid biosynthesis (including *B3GNT5* and *B3GALT2*) and ubiquitin-mediated proteolysis, which was the most significantly enriched pathway with three DEGs (including *CUL3*,

Table I. miRNAs associated with DEGs.

miRNA	ID	DEG counts	Raw P-value	Adjusted P-value
Has_CAGTGTT, miR-141	DB_ID:690	15	$7.29x10^{-8}$	$1.39x10^{-6}$
Has_TATTATA, miR-374	DB_ID:727	14	$1.72x10^{-7}$	$1.63x10^{-6}$
Has_AAAGGGA, miR-204	DB_ID:682	12	$4.49x10^{-7}$	$2.84x10^{-6}$
Has_AATGTGA, miR-23	DB_ID:683	16	$6.71x10^{-7}$	$3.19x10^{-6}$
Has_TTGCCAA, miR-182	DB_ID:757	13	$4.62x10^{-6}$	$1.76x10^{-5}$
Has_TACTTGA, miR-26	DB_ID:687	12	$9.99x10^{-6}$	$3.16x10^{-5}$
Has_TGCTGCT, miR-15	DB_ID:666	17	$1.48x10^{-5}$	$4.02x10^{-5}$
Has_TGTTTAC, miR-30	DB_ID:667	16	$3.52x10^{-5}$	$7.43x10^{-5}$
Has_CACTGCC, miR-34	DB_ID:673	10	0.0001	0.0002
Has_TGAATGT, miR-181	DB_ID:669	13	0.0003	0.0005
Has_TTGCACT, miR-130	DB_ID:676	11	0.0006	0.0009

miR/miRNA, microRNA; DEG, differentially expressed gene.

Table II. GO enrichment analysis of the DEGs in the integrated regulatory network.

Term	DEGs	P-value
GO:0016071 - mRNA metabolic process	*FUS,PAPOLA, SFPQ, PNRC2, SF1, CPSF6*	0.001632
GO:0006396 - RNA processing	*FUS, DDX17, PAPOLA, SFPQ, SF1, CPSF6*	0.008649
GO:0044093 - positive regulation of molecular function	*NTRK2, PIM1, MAP3K10, TGFBR3, BRD4, GAP43*	0.011448
GO:0006468 - protein amino acid phosphorylation	*NTRK2, PIM1, MAP3K10, TGFBR3, BRD4, PRKG1*	0.019140
GO:0045449 - regulation of transcription	*COPS2, THRB, SFPQ, SALL1, PNRC2, CTNND2, PIM1, SF1, MAP3K10, TGFBR3, HOXA9, WHSC1*	0.031480
GO:0016310 - phosphorylation	*NTRK2, PIM1, MAP3K10, TGFBR3, BRD4, PRKG1*	0.038131
GO:0006350 - transcription	*COPS2, PAPOLA, THRB, SFPQ, SALL1, PNRC2, CTNND2, SF1, HOXA9, WHSC1*	0.049845

GO, gene ontology; DEG, differentially expressed gene.

Table III. Enriched pathways of the differentially expressed genes in the integrated regulatory network.

ID	Pathway	P-value	Genes
hsa04120	Ubiquitin mediated proteolysis	0.02628	*CUL3, FBXW7, KLHL13*
hsa00601	Glycosphingolipid biosynthesis	0.04213	*B3GNT5, B3GALT2*

FBXW7 and *KLHL13*) (Table III). Based on the information from the integrated regulatory network (Fig. 1), both *COPS2* and *CUL3* were found to be targets of miR-15.

Discussion

The present study identified 390 genes that were differentially expressed between the WT and MUT (carrying TNF-α rs1800629 A variant) samples, and established a regulatory network containing 38 DEGs and 11 miRNAs. This network suggested that *COPS2* was predominantly associated with transcriptional functions, whereas *FUS* was primarily involved in mRNA metabolic processes. Other DEGs, including *FBXW7* and *CUL3*, were enriched in the ubiquitin-mediated proteolysis pathway. Furthermore, miR-15 was predicted to target both *COPS2* and *CUL3*.

COPS2, also known as COP9 signalosome subunit 2 or *CSN2*, is an essential component of the COP9 signalosome complex, which is involved in diverse cellular processes and acts as a critical regulator of the ubiquitin conjugation pathway (26). In a previous study it was demonstrated that TNF-α was able to promote the stabilization of Snail (the most important transcriptional repressor of E-cadherin) and β-catenin, by inhibiting GSK-3β-mediated phosphorylation of these proteins via the nuclear factor-κB and Akt signalling pathways, which in turn promoted tumor cell invasion.

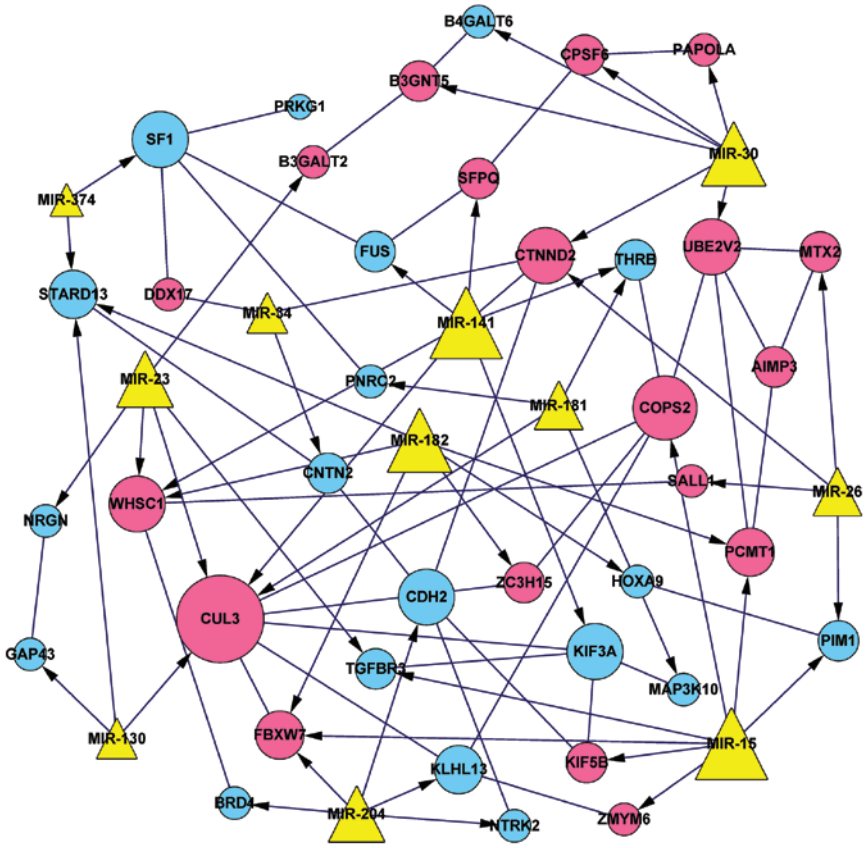

Figure 1. Integrated microRNA (miRNA)-differentially expressed gene (DEG) network. Circular nodes represent DEGs (pink circles, upregulated genes; blue circles, downregulated genes). Yellow triangles represent the miRNAs, with edges with arrowheads depicting associations between miRNAs and DEGs, and edges with lines depicting protein-protein interactions between the DEGs.

Notably, *COPS2* had a crucial role in this process since it was able to inhibit the phosphorylation and ubiquitination of Snail by preventing the binding of Snail to its receptors (27).

The protein encoded by *FUS* is a multifunctional component of the heterogeneous nuclear ribonucleoprotein complex, which is involved in pre-mRNA splicing and the export of fully processed mRNA to the cytoplasm (28). *FUS*, which contains multiple RNA binding domains and an N-terminal glutamine-rich domain, acts as a mediator of RNA binding and as a potent transcriptional activator (29). In addition, *FUS* has previously been associated with familial amyotrophic lateral sclerosis (30) and the pathogenesis of myxoid liposarcoma (31); however, to the best of our knowledge, no previous studies have investigated the association between *FUS* and sepsis. In the present study, *COPS2* was shown to be upregulated in the MUT samples, and was strongly associated with transcriptional functions, whereas *FUS* was downregulated in the TNF-α rs1800629 A allele variant samples, and was associated with mRNA metabolic processes. Furthermore, *COPS2* and *FUS* were shown to interact in the integrated regulatory network. These results suggested that *COPS2* may play a vital role in the pathogenesis of sepsis in patients with TNF-α rs1800629 A allele variation by regulating the phosphorylation and ubiquitination of the protein encoded by *FUS* at the transcriptional level.

FBXW7 and *CUL3* are two critical DEGs, which were found to be upregulated in the TNF-α rs1800629 A allele variant samples. *CUL3* (also known as cullin 3) encodes a member of the cullin protein family, and is the core component and scaffold protein of an E3 ubiquitin-protein ligase complex, which mediates the ubiquitiation and subsequent proteasomal degradation of target proteins (32). As major subunits of the cullin-RING ligase (CRL) family, cullins are active components of ~50% of human E3 ubiquitin ligases and are responsible for one fifth of all cellular proteasome-dependent protein degradation (33). *FBXW7* (also known as F-box and WD repeat domain-containing 7) is a substrate receptor for CRL1, and facilitates the ubiquitination and degradation of numerous proteins (34). Cyclin E, a member of the cyclin family, is a positive regulator of proliferation in mammalian fibroblasts. *CUL1* and *CUL3* have previously been implicated in the degradation of cyclin E via two distinct pathways (35). In comparison with the *CUL1*/*FBXW7*-based E3 ligase-dependent pathway, cyclin E does not require phosphorylation at threonine 380, and may be recognized as a substrate by *CUL3* in the *CUL3*-based E3 ligase pathway (36), which may be essential for the maintenance of quiescence in mammalian cells (35). Therefore, consistent with the findings of the present study, previous reports have suggested that *FBXW7* and *CUL3* may be involved in the ubiquitin-mediated proteolysis pathway (37,38).

Based on a novel mechanism of autophagy, CaMKIV (also known as calcium/calmodulin-dependent protein kinase IV) may inhibit ubiquitin-mediated mTOR degradation through the inhibition of *FBXW7* recruitment. However, in sepsis, mTOR expression is required for autophagy (39), indicating that *FBXW7* may regulate mTOR. Although Qiao *et al* (40) did not identify *CUL3* as a DEG in sepsis, it is associated with DEGs

in the PPI network, suggesting their potential involvement in sepsis. However, no study has previously reported a direct interaction between *FBXW7* and *CUL3*. The aforementioned findings collectively suggest that *FBXW7* and *CUL3* may exert proteolytic functions via ubiquitination in sepsis progression in patients with TNF-α genetic variations. In addition, there may be regulators between *FBXW7* and *CUL3* that facilitate the functions of the two genes.

miRNAs have a critical role in the regulation of gene expression at the post-transcriptional level (41). miR-15 has been demonstrated to be associated with apoptosis in various types of cancer. For instance, a previous study identified that miR-15 was closely associated with apoptosis in chronic lymphocytic leukemia, and it was demonstrated to induce apoptosis by negatively regulating the expression of B-cell lymphoma 2 (42). Furthermore, miR-15(a/b) was reported to have inhibited the expression of cyclin E and was identified as a novel transcriptional target of E2F1, which is a vital transcription factor that induces proliferation and cell death (43). Thus, miR-15 and *CUL3* may co-regulate the expression of cyclin E. Up-regulation of miR-15 has been found in the serum of neonatal sepsis patients, and this miRNA is proposed as a potential biomarker for neonatal sepsis prognosis (44). Notably, the present study identified *CUL3* as a potential target of miR-15, which is consistent with this hypothesis. In addition, although previous investigations have been unable to uncover a regulatory association between miR-15 and *COPS2*, the present study predicted that *COPS2* was a target of miR-15, thus suggesting that *COPS2* may be a novel target gene for miR-15 in the progression of sepsis.

In conclusion, in the present study, *COPS2*, *FUS*, *FBXW7* and *CUL3* were found to be associated with the progression of sepsis in patients with the TNF-α rs1800629 A variant. Of these genes, *FBXW7* and *CUL3* may exert regulatory roles in the ubiquitin-mediated proteolysis pathway, whereas *COPS2* may affect the development of sepsis by regulating the phosphorylation and ubiquitination of the FUS protein. In addition, *COPS2* and *CUL3* may be novel targets of miR-15. The aforementioned genes and miRNAs may be used as therapeutic biomarkers for sepsis diagnosis in patients with the TNF-α rs1800629 A variant.

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