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ORIGINAL ARTICLE

Neutrophil Cytosolic Factor 1 Contributes to the Development of Sepsis

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Abstract— To identify differentially expressed genes in sepsis and potential key role of reactive oxygen species (ROS) genes associated with sepsis. Gene expression dataset was available from GSE46599. Firstly, we screened the differentially expressed genes between sepsis and healthy samples. Then, the Database for Annotation, Visualization and Integrated Discovery (DAVID) online tools were utilized to perform gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses at the functional level. Differentially expressed genes mediating ROS levels were validated in the next investigation and analysis. We identified 1094 genes expressed differentially between normal and sepsis samples, including 655 upregulated genes and 439 downregulated genes. At the functional level, GO and KEGG pathway enrichment analysis showed that those differentially expressed genes were majorly associated with the immune response and metabolic process in sepsis. Further analysis revealed that neutrophil cytosolic factor 1(NCF1), a critical gene in the ROS system, upregulated in THP-1 cell and monocytes under lipopolysaccharides stimulation. Moreover, we identified the upregulation of NCF1 in a sepsis model. We screened the differentially expressed genes from the global level and identified NCF1 might be a critical target gene in sepsis.

KEY WORDS: NCF1; sepsis; bioinformatics analysis; ROS system.

INTRODUCTION

Sepsis is a complex pathology that arises from dysregulated host inflammatory responses to systemic bacterial infection [16]. Acute overwhelming sepsis with accompanying organ dysfunction remains one of the leading causes of morbidity and mortality in intensive care units (ICUs) [1]. In the past years, major advances have been obtained in our understanding of the underlying biologic features of sepsis. However, we lack a fundamental grasp of its pathophysiology. The overall mortality rate ranges from 26% in patients with systemic inflammatory response syndrome (SIRS) to 82% in patients with septic shock [10, 15]. So understanding the molecular mechanism development of sepsis was of utmost importance for more effective diagnostic and therapeutic strategies.

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Interestingly, overwhelming evidence implicated an underlying role of oxidative stress in the pathogenesis of sepsis [1, 2]. Under normal physiologic conditions, redox balance exists through a complex interplay of genes that mediate oxidant generation and antioxidant responses. An imbalance between the production of reactive oxygen species (ROS) and the capacity for detoxification of their reactive intermediates results in oxidative stress [5, 11]. Numerous studies demonstrate an association between sepsis and elevated oxidative stress levels [1, 2]. Cellular redox status plays a complex and dynamic role in host innate immune regulation and survival in sepsis and 137 ROS genes had been identified in the previous study [2]. While excessive ROS can contribute to cell and tissue injury, free oxygen radicals and their oxidized substrates are key signaling molecules involved in pathogen recognition and clearance [11, 12]. Therefore, investigation of ROS-related genes in sepsis may contribute to understanding the development of sepsis.

In the current study, we downloaded the original data from a public dataset and identified differentially expressed genes in sepsis patients compared with the control group by microarray technology. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were adopted to investigate differentially expressed genes at the functional level. Furthermore, we hypothesized ROS-related genes might play a vital role in the development of sepsis. We mined the most essential ROS genes as potential molecular targets and predictive or diagnostic biomarkers for treatment of sepsis.

MATERIAL AND METHOD

Bioinformatics Analysis

In the present study, we downloaded the original data (GSE46955) from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) [14]. Limma package of R language was used to identify differentially expressed genes between sepsis and non-sepsis samples. Fold change (FC) > 2 and false discovery rate (FDR) < 0.05 were used as the threshold to determine the significance of gene expression difference. The heat map was used to ensure that the screened genes have significant differences. In order to analyze the different genes at the functional level, GO enrichment and KEGG pathway analysis were performed using DAVID online tool. FDR < 0.05 was considered statistically significant. The STRING database was deemed to evaluate the protein-protein interaction (PPI) formation. What's more, the relationship between genes and genes was visualized by Cytoscape software.

Monocyte and THP-1 Cell Culture and Stimulation

Five-milliliter whole blood was obtained from three healthy donors. Magnetic-activated cell sorting (MACS, Miltenyi) was carried out to separate CD14+ monocytes according to the manufacturer's instructions. All monocytes were resuspended at a concentration of 1×10^6/ml in 500 μl of prewarmed RPMI 1640 complete medium, supplemented with 10% fetal calf serum and penicillin/streptomycin. THP-1 cells were cultured in the same conditions. For stimulation assays, 4×10^5 cells were exposed to lipopolysaccharides (LPS) at the final concentration of 100 ng/ml in a 37 °C humidified incubator with 5% CO_2. The treated cells and the non-treated control cells were harvested 4 h after the stimulus.

Real-time PCR

Cells were lysed in Trizol (Life Technologies, Invitrogen), and total RNA was prepared using the RNeasy kit (QIAGEN) as per manufacturer's instructions. First-strand cDNA was synthesized with 2 μg of RNA samples. Real-time PCR was performed on a 7900HT real-time PCR system. The relative quantification method was used per the manufacturer's instructions. Samples were executed in triplicate and then normalized to the housekeeping gene GAPDH. At least three independent samples per group were analyzed.

Statistical Analysis

Student's *t* test was used to determine the significance of the differences between groups. A value of $P < 0.05$ was considered statistically significant.

Abbreviations: ROS, reactive oxygen species; NCF1, neutrophil cytosolic factor 1; FC, fold change; FDR, false discovery rate; BP, biological processes; MF, molecular function; *CF*, component function; NADPH, nicotinamide adenine dinucleotide phosphate; SIRS, systemic inflammatory response syndrome; DAVID, the Database for Annotation, Visualization and Integrated Discovery; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes pathway; ICUs, intensive care units; GEO, Gene Expression Omnibus; PPI, protein-protein interaction; LPS, lipopolysaccharides; DEGs, differentially expressed genes; CLP, cecal ligation and puncture.

RESULTS

Identification of Differentially Expressed Genes in Sepsis Patients

After preprocessing, a total of eight sepsis monocytes samples and five normal samples were considered in our analysis. We finally identified 1094 genes were differentially expressed between sepsis and normal samples (FDR < 0. 05 and FC ≥ 2.0), including 655 upregulated and 439 downregulated genes (Fig. 1a). Especially, the IDO1 ranked at the first with a 6.13-fold change in all upregulated genes and FABP4 ranked at the first with a 7.30-fold change in all downregulated genes. The heat map of top 50 upregulated and downregulated genes were shown in Fig. 1b.

GO and KEGG Pathway Analysis

All up- and down-expressed genes were uploaded to DAVID dataset to perform GO and KEGG pathways analysis. For biological processes (BP), overexpressed genes were significantly enriched in the immune process, defense response, and immune system process. Under-expressed genes were significantly enriched in including small molecule metabolic process, carboxylic acid metabolic process, and oxoacid metabolic process (Table 1). For molecular function (MF), the upregulated differentially expressed genes (DEGs) were enriched in molecular transducer activity, chemokine receptor binding, and chemokine activity. The downregulated DEGs were enriched in catalytic activity, lyase activity, and oxidoreductase activity (Table 1). As showed in Table 2, the most significantly enriched pathways of the upregulated DEGs were enriched in cytokine-cytokine receptor interaction, chemokine signaling pathway, and NF-kappa B signaling pathway, while the downregulated DEGs were enriched in metabolic pathways, fatty acid elongation, and carbon metabolism. Both GO and KEGG analysis results suggested upregulated genes were rich in the immune system and downregulated genes were rich in the metabolic process (Table 3).

Differentially Expressed Genes in the ROS System

In order to assess the ROS genes in the progress of sepsis, we next investigated ROS genes in the upregulated genes. The previous study had identified 137 genes related to ROS response (Table S1); we found 5 genes (CCL5, HIF1A, NCF1, PDLIM1, PTGS2) were upregulated in our study (Fig. 1c). Particularly, NCF1 with a 2.65-fold change was chosen for the next investigation. Then, we uploaded all the upregulated genes in the STRING database; PPI network was constructed by Cytoscape software. We found that NCF1 had a relationship with NFKB1, IL6, HMOX1, and MAPK13 (Fig. 1d). Therefore, we considered NCF1 might affect the expression of some immune-related genes and play an important role in sepsis.

Upregulation of NCF1 Was Validated in Cells and Sepsis Animal

In order to ensure the overexpression of NCF1 in the sepsis, we examined its expression at the transcription level in monocytes and THP-1 cell after 4 h under LPS stimulation. As shown in Fig. 2a, transcriptional expression of NCF1 upregulated 1.84-fold in treated THP-1 cells compared with non-treated cells ($P < 0.05$). Figure 2b revealed NCF1 expression increased 4.08-fold after LPS stimulation ($P < 0.001$) in monocytes. Furthermore, we wonder whether elevated NCF1 levels were present in septic mice; we searched the expression in the GSE69345 dataset. There were four mice for each group. Unique transcriptomic response after cecal ligation and puncture (CLP) at 0 h and 2 h and 1 and 3 days was adopted for analysis. The results revealed that the NCF1 upregulated in CLP models (Fig. 2c). Particularly, the NCF1 reached the top at 1 day after CLP with 2.93-fold changes ($P < 0.001$).

DISCUSSION

Sepsis syndromes remain a major cause of morbidity and mortality in ICUs, so understanding the mechanism of sepsis is of great important [4, 6]. In our current study, we deeply mined differentially expressed genes between sepsis and healthy people. GO and KEGG analysis showed that immune-related biological process and metabolic process were crucial to the development of sepsis. Those results were consistent with previous reports. Finally, we discovered NCF1 was differentially expressed in the sepsis monocytes. Our findings revealed a role for NCF-1 in the innate immune response to LPS in the THP-1 and monocyte cells. Furthermore, the NCF-1 was also differentially expressed in the sepsis animal models.

Presently, several papers have reported that oxidative stress plays a major role in the pathogenesis of

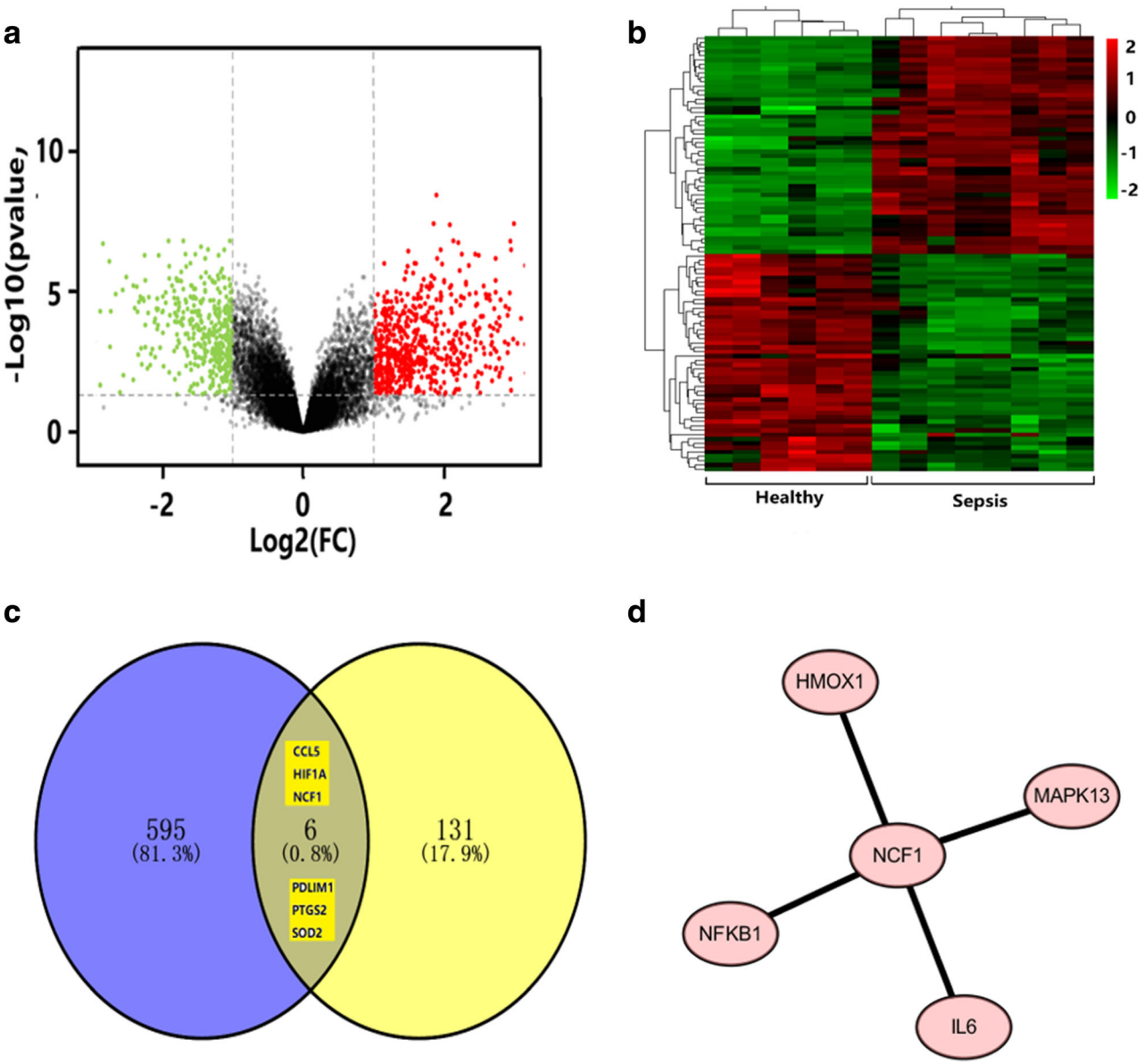

Fig. 1. **a** The volcano plot of differentially expressed genes (8 sepsis patients *versus* 5 normal people). The abscissa was log2 FC and the ordinates were −log10 (FDR value). The red dots stood for the upregulated genes while the green dots represented downregulated genes and blue dots represented not differentially expressed genes. **b** Heat map of the top 100 differentially expressed genes (50 upregulated genes and 50 downregulated genes). Red: upregulation; green: downregulation. **c** Schematic diagram representing genome-wide and ROS-associated overlapping genes. Six overlapping genes were identified. **d** NCF1 is related to several immune genes.

sepsis [1, 2, 14]. Generation of ROS (specifically superoxide) constitutes a fundamental pathway in pathogen clearance [1]. Phagocyte-produced ROS have a well-established role in antimicrobial defense and major ROS producers such as neutrophils and macrophages are naturally well equipped to respond to microorganisms and PAMPs. ROS are generated by membrane-localized nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX). The activity of NOX 2 is regulated by the neutrophil cytosolic factor 1 (NCF1), or p47phox [8, 9]. So we can hypothesis NCF1 might be an important target for the diagnosis and treatment of sepsis. However, no study revealed how the NCF1 affected the development of sepsis, and the deep mechanism needs

Table 1. GO Enrichment Analysis for Upregulated Genes

ID	Pathway description	Gene count	FDR
Biological processes			
GO.0006955	Immune response	153	7.76E-53
GO.0006952	Defense response	149	2.88E-46
GO.0002376	Immune system process	176	2.01E-45
GO.0002684	Positive regulation of immune system process	107	9.79E-40
GO.0002682	Regulation of the immune system process	134	6.90E-38
molecular function			
GO.0060089	Molecular transducer activity	99	3.15E-09
GO.0042379	Chemokine receptor binding	14	2.19E-07
GO.0008009	Chemokine activity	13	2.34E-07
GO.0003674	Molecular function	407	1.47E-06
GO.0004872	Receptor activity	78	1.47E-06
Component function			
GO.0009897	External side of plasma membrane	35	3.06E-13
GO.0098552	Side of membrane	43	4.13E-11
GO.0005886	Plasma membrane	196	5.15E-10
GO.0071944	Cell periphery	198	5.15E-10
GO.0005576	Extracellular region	187	1.53E-08

to be studied. Previous studies suggested ROS killed bacteria in professional phagocytes (such as macrophages and neutrophils with highly efficient phagocytosis) and damages cells and tissues during inflammation [3, 7, 17]. Defects in NCF1 lead to lower production of ROS. So we can image that NCF1 may play a significant role in sepsis by regulated the production of ROS. Furthermore, the crucial role of NCF1 for macrophage activation was previously shown where NCF1 deleted specifically in macrophages regulated chronic inflammation [3, 13]. Moreover, NCF1 polymorphism reveals oxidative regulation of autoimmune chronic inflammation [8]. From the above, NCF1 could affect the process of sepsis.

None had studied the role of NCF1 in the development of sepsis. Our study firstly reported the upregulation of NCF1 in sepsis. However, it is a pity for us not to

Table 2. GO Enrichment Analysis for Downregulated Genes

ID	Pathway description	Gene count	FDR
Biological processes			
GO.0044281	Small molecule metabolic process	92	4.07E-11
GO.0019752	Carboxylic acid metabolic process	48	2.87E-09
GO.0043436	Oxoacid metabolic process	52	2.87E-09
GO.1901564	Organonitrogen compound metabolic process	72	3.04E-09
GO.0044711	Single-organism biosynthetic process	60	1.34E-07
Molecular function			
GO.0003824	Catalytic activity	141	0.00164
GO.0016829	Lyase activity	15	0.00167
GO.0016614	Oxidoreductase activity, acting on CH–OH group of donors	12	0.00223
GO.0016616	Oxidoreductase activity, NAD, or NADP as acceptor	11	0.00223
GO.0048037	Cofactor binding	18	0.00223
Component function			
GO.0031988	Membrane-bounded vesicle	144	4.96E-19
GO.0070062	Extracellular exosome	124	2.65E-18
GO.0044421	Extracellular region part	133	1.35E-12
GO.0044444	Cytoplasmic part	208	3.37E-12
GO.0005576	Extracellular region	142	3.58E-10

Table 3. KEGG Pathway Analysis for Differentially Expressed Genes

Pathway ID	Pathway description	Gene count	FDR
Upregulated			
4060	Cytokine-cytokine receptor interaction	40	2.01E-15
4062	Chemokine signaling pathway	25	1.27E-08
4064	NF-kappa B signaling pathway	17	5.96E-08
5152	Tuberculosis	23	7.66E-08
4668	TNF signaling pathway	18	1.01E-07
Downregulated			
1100	Metabolic pathways	61	1.18E-09
62	Fatty acid elongation	6	0.000669
1200	Carbon metabolism	10	0.00233
290	Valine, leucine, and isoleucine biosynthesis	3	0.00233
1040	Biosynthesis of unsaturated fatty acids	5	0.00233

investigate how NCF1 affects the development of sepsis. For the *in vivo* experiment, if we could construct the NCF1−/− mice, we could directly understand whether NCF1 influences the outcome of sepsis models and detect how NCF1 affect the organ function of sepsis animals. For the *in vitro* experiment, we need to investigate the molecule mechanism of NCF1, causing sepsis. Moreover, we need to add some sepsis patients in our future study. On the one hand, we could confirm the upregulation of NCF1 in sepsis. On the other hand, we could identify whether NCF1 predict the development of sepsis or prognosis of sepsis patients.

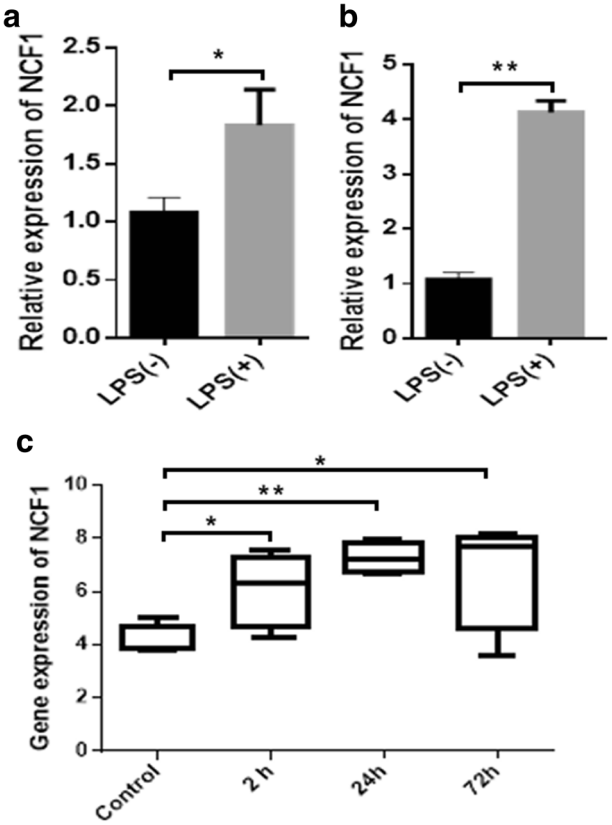

Fig. 2. **a** The expression of NCF1 elevated 1.84-fold in THP-1 cell stimulated 4 h by LPS at 100 ng/ml (*t* test, $*P < 0.05$ *versus* control). **b** NCF1 elevated 4.08-fold in monocyte cell stimulated by LPS at 100 ng/ml ($n = 5$) (*t* test, $**P < 0.001$ *versus* control). **c** In the GSE69345 dataset, NCF1 was folded elevated sepsis model ($n = 4$ for each group) (*t* test, $*P < 0.05$, $**P < 0.001$ *versus* 0 h).

CONCLUSION

In conclusion, our data provided a comprehensive bioinformatics analysis of differentially expressed genes in sepsis. We considered NCF1 could be a potential target gene for future investigation in the development of sepsis. However, further molecular biological experiments are required to confirm the function of NCF1 genes in sepsis.

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COMPLIANCE WITH ETHICAL STANDARDS

Competing Interests. The authors declare that they have no competing interests.

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