Exploring the prognostic necroptosis-related genes and underlying mechanism in sepsis using bioinformatics

Jie Liu
The Second Affiliated Hospital of Xi’an Jiaotong University

Lin Li
The Second Affiliated Hospital of Xi’an Jiaotong University

Shuyang He
Queen mary school of Nanchang University

Xin Zheng
The Second Affiliated Hospital of Xi’an Jiaotong University

Dan Zhu
The Second Affiliated Hospital of Xi’an Jiaotong University

Guangyao Kong
The Second Affiliated Hospital of Xi’an Jiaotong University

Ping Li (✉ lipingjun@163.com)
The Second Affiliated Hospital of Xi’an Jiaotong University

Research Article

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Abstract

Sepsis is a life-threatening disease due to a dysregulated host response to infection, with an unknown regulatory mechanism for prognostic necroptosis-related genes (NRGs). Using GEO datasets GSE65682 and GSE134347, we identified six NRG biomarkers (ATRX, TSC1, CD40, BACH2, BCL2, and LEF1) with survival and diagnostic significance through Kaplan-Meier (KM) and ROC analyses. The ingenuity pathway analysis (IPA) highlighted enrichment in hepatic fibrosis pathways and BEX2 protein. We examined their regulatory targets and functional links with necroptotic signaling molecules via miRDB, TargetScan, Network analyst, and GeneMANIA.

Hsa-miR-5195-3p and hsa-miR-145-5p regulated ATRX, BACH2, and CD40, while TF YY1 showed strong connectivity, concurrently controlling LEF1, ATRX, BCL2, BACH2, and CD40. CD40 exhibited similar expression patterns to RIPK3 and MLKL, and LEF1 was functionally associated with MLKL. Additionally, DrugBank analysis identified Paclitaxel, Docetaxel, and Rasagiline as potential BCL2-targeting sepsis treatments. Real-Time Quantitative PCR confirmed ATRX, TSC1, and LEF1 down-regulation in sepsis samples, contrasting CD40’s increased expression in case samples. Variations in BACH2 and BCL2 expression between disease and normal samples may result from sample differences. In conclusion, ATRX, TSC1, CD40, BACH2, BCL2, and LEF1 may be critical regulatory targets of necroptosis in sepsis, providing a basis for further necroptosis-related studies in sepsis.

Introduction

According to the 2016 Third International Consensus on Sepsis and Septic Shock, sepsis is characterized by grievous organ dysfunction due to a dysregulated immune response to infection [44]. Sepsis can induce multiorgan failure and is the most widely recognized cause for death in intensive care units [54]. According to a study [12], 26.7% of hospitalized sepsis cases are estimated to die, while 41.9% of septic cases treated in ICU are expected to die. Currently, sepsis treatments include early antibacterial therapy, resuscitation, and other supportive treatments such as lung protective ventilation, fluid therapy, sedatives, nutrition, and blood glucose management [19]. These treatments can improve the prognosis of sepsis to varying degrees, but so far, no approved specific molecular therapy has proved effective. There are also some published bioinformatics studies on sepsis. For example, in 2023, research which aimed to identify novel therapeutic targets for sepsis based on machine learning and pyroptosis-related genes screened 13 hub genes and identified 8 key genes[6]. Another research in 2023 analyzed mitochondrion-related gene expression through bioinformatics, and constructed a novel diagnostic model containing six mitochondrion-related genes[21]. These studies enlightened us that bioinformatics research methods are promising in the field of sepsis.

Necroptosis is a kind of programmed cell death (PCD) [14]. When some triggers, such as toll-like receptors (TLR), tumor necrosis factor (TNF), lipopolysaccharide (LPS), Fast ligand (Fas L), etc., bind to cell membrane surface receptors, the activation of RIPK1 is initiated [48, 13]. Receptor-interacting serine-threonine kinase 1 (RIPK1) and receptor-interacting serine-threonine kinase 3 (RIPK3) proteins together
form a "necrosomes" that phosphorylate mix lineage kinase domain-like pseudo kinase (MLKL) [51], phosphorylated MLKL proteins further aggregate on the cell membrane to form ion channels that ultimately induce the occurrence of necroptosis [48]. Cells lyse after necroptosis and release many damage-associated molecular patterns (DAMPs), which are essential mediators of inflammation in vivo [23]. Moreover, some previous studies have also shown that necroptosis is a crucial step during systemic inflammatory response syndrome (SIRS) and sepsis [10]. In 2022, Yan du et al. [9] published a study that investigated the role that necroptosis-related genes (NRGs) play in Sepsis-induced myocardial dysfunction (SIMD). Through bioinformatics analysis, they found that in the mouse model of LPS-induced myocardial failure, there was a differential expression of 35 genes associated with necroptosis (including RIPK3 and MLKL). Exactly a year ago, Qipao Xu et al. [56] studied how necrotic apoptosis causes liver damage in a lipopolysaccharide-induced septic piglet model and found that Nec-23 is a well-described necroptosis inhibitor. Myocardial dysfunction and liver injury are common organ injuries in sepsis, and they are closely related to necroptosis, so it is vital to find the key genes of sepsis, especially those related to necroptosis.

She et al have preliminarily explored that the potential necroptosis-biomarkers with good prognostic value in sepsis using several machine learning algorithms [42]. This study was conducted to further explore the underlying functions and regulatory networks targeting biomarkers as well as the functionality correlations of which with key necroptotic signaling pathway molecules, such as RIPK1, RIPK3 and MLKL. Gene Expression Omnibus (GEO) datasets GSE65682 and GSE134347 were downloaded and analyzed to find key necroptosis-related genes (NRGs). Further, independent prognostic analysis, Ingenuity Pathway Analysis (IPA), immune microenvironment analysis, drug prediction and molecular docking analysis, and construction of the Transcription factors (TF)-mRNA-miRNA network and gene-gene functional interaction network were carried out, providing inspiration for new personalized prediction and treatment of patients with sepsis.

Materials and methods

2.1. Data Acquisition

The datasets GSE65682 (training set) and GSE134347 (validation set) were downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). The GSE65682 is the source of clinical data for sepsis patients in this study for survival analysis and contains 802 blood-derived RNA-seq from 760 sepsis patients (479 patients with survival information) and 42 HC. The GSE134347 includes 239 blood leukocyte-derived RNA-seq from 156 sepsis patients and 83 HC. In addition, 63 NRGs were obtained from the previous studies [22].

2.2 Identification of DENRGs

First, the limma package (version 3.52.2) [39] based on |log2 fold-change (FC)| > 0.5 and p.value < 0.05 was used in the training set to screen for DEGs in sepsis patients and HC. The Volcano plots and
pheatmap of DEGs were plotted by the ggplot2 (version 3.3.6) package [17] and heatmap (version 1.0.12) package [58]. Venn diagrams were used to illustrate the intersection between DEGs and NRGs.

### 2.3 Screening and validation of biomarkers

According to the median value of the expression level of DENRGs, the sepsis patients in the training set were split into two groups: high expression and low expression. To find the DENRGs related to sepsis survival, the overall survival (OS) Kaplan-Meier (KM) curves of DENRGs were plotted by the R package survminer (version 0.4.9) [28]. We also used the pROC (version 1.18.0) package [40] to perform ROC analysis on DENRGs to get DENRGs that distinguish between septic patients and HC. Venn plots were used to depict NRGs that had both diagnostic and survival importance and to define them as biomarkers.

According to the amount of biomarker expression, 144 sepsis patients with complete clinical data were divided into two groups: high expression and low expression. Using the chi-square test, the variations in biomarkers between the two groups were evaluated. By Wilcox.test, we tested the consistency of expression levels and trends of biomarkers in training and validation sets. Box line plots were drawn with the R package ggpubr (version 0.4.0) [53] for visualization.

### 2.4 Independent prognostic analysis

To identify prognostic factors that affect sepsis patients independently among clinical data and biomarkers from 479 sepsis patients with survival information, univariate and multivariate Cox analyses were conducted. Based on the independent prognostic factors, the nomogram model was drawn for sepsis patients at 1, 2, and 4 weeks using the rms software package [34]. Finally, calibration curves were used to verify the reliability of the nomogram model.

### 2.5 Ingenuity pathway analysis (IPA)

IPA [25] was used to probe biomarker-enriched disease and functional pathways, where a Z-score > 2 is a significantly activated state and a Z-score <-2 is a significantly suppressed state. In addition, heatmaps were further used to visualize activated or inhibited pathways using biomarker expression matrices.

### 2.6 Immune microenvironment analysis

The proportion of 22 immune cells per sample in the training set was calculated using the CIBERSORT algorithm (version 1.03) to investigate the differences in the immune microenvironment between sepsis patients and HC. In addition, we also used Wilcox.test to identify differential immune cells of different types and the vioplot package (version 0.3.7) [46] to draw violin plots to visualize the comparison results. Finally, the Spearman correlation was used to analyze the correlation of differential immune cells with biomarkers.

The correlations between biomarkers and immunosuppressive and immunostimulatory factors were further analyzed to explore the way biomarkers regulate the immune microenvironment. We gathered the 24 immunosuppressives and 46 immune activating factors from the previous study [55] and calculated the Spearman correlations between them and biomarkers.
2.7 Drug prediction

The DrugBank database (https://go.drugbank.com/) searches for biomarkers available for therapeutic agents, thus investigating potential therapeutic agents for sepsis. Cytoscape software visualizes drug-gene interactions. Protein structures of biomarkers were downloaded from the PDB database, and protein hydrogenation and charge calculation were done by AutoDock Tools (version 1.5.6) [31]. Then download active ingredient structures from the PubChem database and perform charge-balanced, rotatable bond checks on small molecules using AutoDock Tools. Docking simulations were performed by AutoDock Vina [49] to generate docking energies. Finally, PyMol (version 2.4.1) software [3] was used to visualize the docked complexes.

2.8 Construction of regulatory network of biomarkers

TargetScan (https://www.targetscan.org) database was used to screen biomarker-associated miRNAs with the restriction of context + score − 0.1. Biomarker-associated miRNAs were screened by the miRDB database (http://www.mirdb.org/) according to the criterion of Target Score > 80. The predicted duplicate miRNAs from both databases were de-duplicated, and Cytoscape was used to display the miRNA-mRNA connection pairs. Similarly, an analysis was conducted using NetworkAnalyst 3.0 (https://www.networkanalyst.ca/) to predict transcription factors (TFs) and construct a regulatory network of TF-mRNAs. Finally, based on the mRNA-miRNA network and mRNA-TFs regulatory network, the miRNA-mRNA-TF regulatory network was constructed. Besides, GeneMANIA (version 3.6.0) was implemented to identify the interaction network of classical necroptotic signaling pathway molecules (RIPK1/RIPK3/MLKL) and biomarkers.

2.10 RNA Extraction and Real-Time Quantitative PCR

A total of twenty samples (Supplementary Table 1) were tested individually for each of the six biomarkers identified using bioinformatics tools. Total RNA was extracted by using TRIzol reagent (Ambion). SureScript-First-strand-cDNA-synthesis-kit (Servicebio) were used for reverse transcription. Real-time quantitative PCR (qPCR) experiments were performed on CFX Connect (BIO-RAD) Real-Time PCR System. We used the $2^{-\Delta\Delta CT}$ method to calculate the relative mRNA expression level. The primer sequences of biomarkers were displayed in Table 1.
Table 1
The primer sequences of biomarkers.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATRX F</td>
<td>GCGGAGAAGATGGGCTTCAT</td>
</tr>
<tr>
<td>ATRX R</td>
<td>CTTCCGCACACCACCTACAT</td>
</tr>
<tr>
<td>TSC1 F</td>
<td>CCGTGGCCCTATGCTTGTAAG</td>
</tr>
<tr>
<td>TSC1 R</td>
<td>CGGCTTTGCCCACATAATTCG</td>
</tr>
<tr>
<td>CD40 F</td>
<td>GACCAAGACCTGGTTGTGC</td>
</tr>
<tr>
<td>CD40 R</td>
<td>CTGAGGACTCACTGATAAGACCAG</td>
</tr>
<tr>
<td>BACH2 F</td>
<td>GCGGAAAGAGGACGCAAAGTT</td>
</tr>
<tr>
<td>BACH2 R</td>
<td>AAGGGCTCATCAGCTTGGTC</td>
</tr>
<tr>
<td>BCL2 F</td>
<td>TGGTGATGTGAGTCTGGGCT</td>
</tr>
<tr>
<td>BCL2 R</td>
<td>GATTTTATTTTCGCCGGCTCCAC</td>
</tr>
<tr>
<td>LEF1 F</td>
<td>TGCATCAGGTACAGGTCCCAA</td>
</tr>
<tr>
<td>LEF1 R</td>
<td>ACGTTGGGAATGAGCTTCGT</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>CGAAGGTGGAGTCAACGGATT</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>ATGGGTGGAATCATAATTTGAAC</td>
</tr>
</tbody>
</table>

Result

3.1 Identification of DENRGs between Sepsis patients and HC

The principal component analysis (PCA) results of GSE65682 showed that sepsis patients and HC could be well distinguished (Fig. 1A). Out of the 4439 DEGs analyzed from patients with sepsis and HC, 1397 genes were up-regulated in the former, while 3042 were down-regulated (Fig. 1B-1C)(Supplementary Table 2). 23 NRGs were differentially expressed (Fig. 1D) (Supplementary Table 3).

3.2 Identification and validation of biomarkers

The results in Fig. 2A showed that 8 DENRGs, which survived significantly different between two groups with different levels of expression (p < 0.05), were all protective factors (Supplementary Table 4). We defined the genes with AUC > 0.8 as diagnostic genes (Fig. 2B) (Supplementary Table 5). The results in Fig. 2C show that six NRGs (ATRX, TSC1, CD40, BACH2, BCL2, and LEF1) with survival and diagnostic
significance were defined as biomarkers. Each biomarker expression level was used to divide Sepsis patients into two groups, and chi-square tests were used to identify differences in clinical factors between the high and low expression groups. Detailed results are shown in Supplementary Table 6. There were differences in Pneumonia in the high and low expression groups for BACH2 and BCL2, and significant differences in Pneumonia, Gender, and ICU Acquired Infection in the high and low expression groups for LEF1. Six biomarkers showed consistent expression levels and trends in the training and validation sets (Fig. 2D). In the results, these six biomarkers were found to be stable and sensitive.

### 3.3 Nomogram model construction

Using the clinical characteristics and biomarkers of 479 sepsis patients in conjunction with survival information, a univariate Cox analysis was conducted (Fig. 3A). The results showed that ATRX, TSC1, BCL2, and LEF1 were statistically significant (P < 0.05). Based on a multifactorial Cox analysis, only ATRX and BCL2 were found to be independent prognostic factors (Fig. 3B). Using these two independent prognostic factors, we constructed a nomogram model of survival at 1, 2, and 4 weeks for patients with sepsis (Fig. 3C). The c-index = 0.6212 in the calibration curve indicated that the nomogram model was a good predictor (Fig. 3D).

### 3.4 IPA based on biomarkers

The canonical pathway analysis of IPA showed the enrichment of biomarkers in typical signaling pathways. The results showed significant enrichment of 106 pathways (Supplementary Table 7), mainly in BEX2 Signaling Pathway, Hepatic Fibrosis Signaling Pathway, and Prostate Cancer Signaling (P < 0.01) (Fig. 4). The BEX2 Signaling Pathway inhibited the expression of BCL2 (Supplementary Fig. 1). Expression of RNA, RNA transcription, and Migration of cells were inhibited. Necrosis and Apoptosis of tumor cell lines were activated (Supplementary Table 8).

### 3.5 Differences of immune infiltrating cells in sepsis

The CIBERSORT algorithm was used in each sample to calculate the percentage of immune cells in 22 categories (Fig. 5A) (Supplementary Table 9). Subsequently, those immune cells with zero percentage of immune cells were excluded and a heatmap of the remaining immune cell abundance in the samples was shown (Fig. 5B). As shown in Fig. 5C, B cells memory, T cells CD4 naive, T cells regulatory, and Mast cells resting were significantly higher in HC than in sepsis patients, while Plasma cells, T cells CD4 memory activated, T cells γδ, NK cells resting, Macrophages M0, Macrophages M1, and Neutrophils were significantly lower than those of sepsis patients (Supplementary Table 10). Spearman correlation analysis of biomarkers with differential immune cells showed that BACH2 correlated with T cells γδ; BCL2 with T cells γδ, Macrophages M0, Macrophages M1; LEF1 with T cells γδ, and Macrophages M0 all had high correlation (cor > 0.3 & P < 0.05) (Fig. 5D) (Supplementary Table 11). Except for HAVCR2 and
3.6 Drug prediction and molecular docking analysis

Based on biomarkers, we predicted 18 potential drugs for the treatment of sepsis using the DrugBank database (Supplementary Table 14). Three drugs based on BCL2, Paclitaxel, Docetaxel, and Rasagiline, have been shown to have an effect, while the remaining 15 of them can only be used as potential drugs and need to be further investigated. The relationship network between biomarkers and drugs shows that BCL2 predicts 15 drugs, and Paclitaxel, Docetaxel, and Rasagiline are already approved drugs (Fig. 6A). The molecular docking results of BCL2 with Paclitaxel, Docetaxel, and Rasagiline are shown in Fig. 6B-6D (Supplementary Table 15). Three hydrogen bonds connected BCL2 to Paclitaxel, which had an energy of -7.0 kcal/mol, while BCL2 interacted with residued GLU-136 and ARG-146 on Docetaxel with a binding energy of -7.1 kcal/mol. Rasagiline had the largest binding energy with BCL2. Its docking results with drugs is shown in Supplementary Fig. 3.

3.7 Development of a TF-mRNA-miRNA network based on biomarkers

For prediction accuracy, there were 67 shared miRNAs predicted by TargetScan and the miRDB database (Fig. 7A) (Supplementary Table 16). miRNA-mRNA regulatory network constructed by six mRNAs and 67 miRNAs included 73 nodes and 101 relationship pairs (Fig. 7B). We found that hsa-miR-5195-3p and hsa-miR-145-5p had the highest connectivity, and they both regulated ATRX, BACH2, and CD40. Based on the biomarkers, a total of 58 TFs were predicted using NetworkAnalyst. After removing the duplicates, the regulatory network of TF-mRNA constructed by 31 TFs and 6 mRNAs consisted of 37 nodes and 58 relationship pairs (Fig. 7C). YY1 had the highest degree of connectivity with biomarkers, and it could regulate LEF1, ATRX, BCL2, BACH2, and CD40 simultaneously. Based on the mRNA-miRNA regulatory network and mRNA-TFs regulatory network obtained from the above analysis, a 67miRNA-6mRNA-31TF regulatory network was constructed using Cytoscape (Fig. 7D) (Supplementary Table 17). Through this network, we found that BACH2 had the highest number of connections to miRNAs and TF. In contrast, YY1 had the least, but it regulated five biomarkers, which showed its importance.

3.8 Interaction of key necroptotic signaling pathway molecules and six biomarkers.

Considering the activation of the RIPK1-RIPK3-MLKL pathway in necrotic cell death, GeneMANIA website were used to explore the interaction of six necroptosis-related biomarkers and these key necroptotic molecules. As shown in Fig. 8, CD40 was linked with RIPK3 and MLKL as the similar expression patterns. LEF1 was functionally associated with MLKL. In addition, the co-expression patterns of ATRX and TSC1
as well as the co-expression and functional association of BACH2 and BCL2 were observed (Supplementary Table 18).

3.9 Real-Time Quantitative PCR of six biomarkers.

The expression of the identified genes was detected and compared using a Real-Time Quantitative PCR (RT-qPCR) (Fig. 9). As a result of our validation, ATRX, TSC1, and LEF1 showed decreased levels of expression in patients with sepsis, as did in the public datasets (Fig. 9A-B, F). Whereas, there is an increasing trend in CD40 expression in sepsis (Fig. 9C), and the change of BACH2 and BCL2 mRNA levels was not distinct in case samples compared to controls (Fig. 9D-E).

Discussion

As a result of a dysregulated immune response to infection, sepsis results in multiple organ dysfunction and high morbidity and mortality rates. So far biomarker evaluation has been used for multiple applications of sepsis patients, including prediction, diagnosis, assessment of sepsis response to therapy, and guidance of antibiotic therapy [36]. According to the International Guidelines for Management of Sepsis and Septic Shock [38], sepsis biomarkers can supplement clinical evaluation. At present, in the diagnosis and treatment of sepsis, CRP and PCT are the two most widely used biomarkers, but their clinical significance is more focused on excluding non-sepsis diseases [35] rather than confirming sepsis. Therefore, it is impossible to make a judgment on the diagnosis. If a sepsis patient can’t be diagnosed as soon as possible, the antibiotic therapy on him will be delayed and improper, leading to poor outcomes and more likely to die [24]. Therefore, it is very important to find biomarkers that can diagnose sepsis early and help judge the diagnosis.

Cell death can be classified according to the morphological appearance of the lethal process, enzymological criteria, functional aspects, or immunological characteristics. As early as 2005, the editors of Cell Death and Differentiation established the Nomenclature Committee on Cell Death (NCCD), which for the first time, classified cell death "officially" into three types, namely apoptosis, autophagy, and necrosis. [26] Nevertheless, as scientists have studied cell death more and more, NCCD believes that this classification method is oversimplified and misleading. For example, ‘necroptosis' cells have both apoptosis and necrotic morphological characteristics [15]. So NCCD has developed a set of recommendations according to the molecular mechanism. Accidental cell death (ACD) and regulated cell death (RCD) are two types of cell death. The process of ACD occurs when unexpected noxious stimuli overwhelm a cell’s ability to adjust, resulting in cell death. RCD involves a signaling cascade involving effector molecules with unique biochemical characteristics, morphological characteristics, and immunological consequences. Physiological RCDs are called programmed cell deaths (PCDs) [16].

Necroptosis is a form of cell lysis of PCD that, unlike apoptosis and other forms of programmed cell necrosis, does not depend on caspase activity but requires RIPK3-regulated MLKL phosphorylation. This phosphorylation event allows MLKL to create pore complexes on the plasma membrane, resulting in DAMP secretion, cell swelling, and membrane rupture [2]. At the same time, sepsis is associated with
strong activation of the innate immune system, whereas necroptosis activates PRRs (pattern recognition receptors) through the release of DAMPs (endogenous molecules), thereby triggering an inflammatory response. As a consequence, targeting the molecular mediators of necroptosis may prove to be an effective therapeutic approach for sepsis. Additionally, a clinical trial (NCT04169412) demonstrated that necroptosis could predict mortality in patients with sepsis and that RIPK3 levels could also be used as a marker to evaluate necroptosis [37]. As a result, we conducted our study in order to determine the role of necroptosis genes in sepsis.

Prognostic analysis showed that patients with high expression of six NRGs (ATRX, TSC1, CD40, BACH2, BCL2, and LEF1) had a higher survival rate, and the expression of these six NRGs was reduced in disease samples both in the training and validation sets. It is worth that the PCR experiments confirmed the down-regulation of ATRX, TSC1, and LEF1 in sepsis samples, whereas the expression level of CD40 in case samples increased in reverse, and the expression difference of BACH2 and BCL2 between disease and normal samples was not significant.

To be specific, there are few studies on the direct relationship between ATRX/TSC1 and sepsis. According to the 2016 World Health Organization classification of central nervous system tumors, ATRX gene status can be a crucial marker for classifying central nervous system tumors, such as glioma [29]. Tuberous sclerosis complex 1 (TSC1) plays an important role in the function of macrophage M1 and M2 phenotypes, and mice with myeloid cell-specific TSC1 deficiency spontaneously develop an autoimmune syndrome that responds to endotoxin stress Hypersensitivity and resistance to OVA-induced allergic asthma [59]. Moreover, TSC1 is a negative regulator of mTOR signaling. In an LPS-induced experiment with septic acute kidney injury (AKI), TSC1 fl/fl mice showed an increase in apoptotic cells in the renal tubules [43], which also supports our view in this paper that TSC1 may be a protective factor for sepsis. In this study, the mRNA expression of ATRX and TSC1 was reduced in clinical sepsis samples, and they could be conducted as independent prognostic factors for clinical utilize based on the nomogram. Moreover, the co-expression pattern of ATRX and TSC1 was revealed using the GeneMANIA website, providing some novel ideas for sepsis.

Previous studies have revealed that the down-regulation of LEF1, BACH2, and BCL2 have good prognostic value in sepsis through bioinformatics analysis based on machine algorithms [42], as we found in the public data. LEF1 can be used as a biomarker in hematologic tumors, oral cancer, and colorectal cancer. There are also some tumor treatments targeting LEF1 [41]. Another research which studied the association between the extent of organ failure and the transcriptomic response of septic patients found that LEF1 is associated with T cell infiltration[1]. It was revealed in our study that LEF1 might be down-regulated and functionally associated with MLKL in sepsis. BACH2 is a key factor in B cell regulation, and low expression of BACHA2 can predict poor outcomes in chronic lymphocytic leukemia [7] It has been demonstrated that increased expression of BCL-2 contributes to tumor expansion, and impairment of BCL-2, considered an oncogene, has been implicated in the development of a wide range of cancerous manifestations, including lymphomas [11]. In the field of sepsis, BCL2 is closely associated with multiple diseases caused by sepsis. BCL-2 expression in liver tissues can be suppressed in lipopolysaccharide-
induced acute liver injury[5]. In another study in 2020, researchers found downregulated Bcl-2 expression and upregulated Bad expression in LPS-induced acute lung injury tissues. When BCL-2 was overexpressed, the found that Bcl-2 reduces the damage caused by LPS by preventing mitophagy[57]. In this study, the independent prognostic value of LEF1 and BCL2 was detected, and the decrease of three necroptosis hub genes was confirmed in online data. Further, the co-expression and functional association of BACH2 and BCL2 were observed in the GeneMANIA network. However, the expression difference of BACH2 and BCL2 between disease and normal samples was not significant, may be caused by sample differences.

It is noteworthy that the expression level of CD40 in clinical case samples increased in reverse, different from the down-regulated trends in online data. CD40L is a type of II transmembrane protein. CD40/CD40L pathway activation participates in the immunomodulation of sepsis by regulating the activation, apoptosis, and function of T cells, B cells, NK cells, and macrophages. As early as 2003, Sugimoto et al. discovered that the expression of CD40 on peripheral blood monocytes in patients with sepsis increased, and it played a protective role during severe sepsis [45]. Another study reported an improved survival rate for CLP-induced sepsis mice by combined inhibition of CD40/CD40L. CD40/CD40L expression was found to be a biomarker for sepsis patients [32], which coincides with our result that CD40 is one of the six biomarkers screened in our study. Combining the results of univariate and multivariate Cox analyses, and CD40 was linked with RIPK3 and MLKL as the similar expression patterns in the GeneMANIA network, the complex correlation of CD40 expressions and sepsis prognosis remains to be explored.

Our results showed that four types of immune cells (B cell memory, T cell CD4 naive, T cell regulatory, and resting mast cells) were significantly higher in HC than those in sepsis patients. Seven types of immune cells (plasma cells, T cell CD4 memory activation, T cell γδ, NK cell quiescence, macrophage M0, macrophage M1, and neutrophils) were significantly lower in HC than those in sepsis patients. Among them, BACH2 and T cells γδ, BCL2 and T cells γδ, MacrophagesM0, MacrophagesM1, LEF1 and T cells γδ, MacrophagesM0 all have a high correlation, so they may assist in establishing the immune microenvironment of sepsis. Neutrophils are necessary for long-term survival in sepsis. Monocytes and macrophages contribute to the pathophysiology of sepsis and inflammation [33]. Macrophages play a crucial role during the early and late stages of sepsis. At the beginning of an infection, macrophages secrete a large amount of pro-inflammatory factors and chemokines that aggravate the infection-related response and the ensuing inflammation [20, 27]. When sepsis progresses, macrophages undergo apoptosis and the immune system becomes deranged, organs are damaged and death occurs [52, 50]. Our results showed that sepsis patients had significantly more macrophages than healthy controls, probably because they were at the early stage of an infection. NK cells prevent the development of pneumonia by overproducing INF-, which inhibits bacterial growth, and therefore has a significant impact on the severity of sepsis [4]. It is consistent with our findings that NK cells have been found to increase in absolute numbers in patients with sepsis caused by community-acquired pneumonia [18]. Lymphocyte apoptosis is an essential step in the pathogenesis of sepsis [33]. According to previous studies, elderly patients with severe sepsis had fewer immunoreactive B cells and more secondary infections [47]. Additionally, sepsis patients with depleted B cell memory have a poor prognosis [8]. Early in the course of
sepsis, lymphocytes undergo apoptosis before gradually recovering later on [30]. It is therefore possible for the number of lymphocytes to fluctuate.

It should be noted that this study has several limitations. First, the expression of six NRGs (ATRX, TSC1, CD40, BACH2, BCL2, and LEF1) was significantly reduced, and we defined them as biomarkers. However, we have not yet validated them clinically and functionally to confirm how the identified genes specifically play a role in sepsis and how valuable they are in diagnosing sepsis. In addition, the data set we used was limited in the number of cases, and our results would be more convincing if a larger cohort of patients with sepsis could be studied.

Conclusions

In summary, we used bioinformatics to analyze the data in this study and found differences in expression profiles between sepsis patients and normal individuals. Furthermore, necroptosis-related biomarkers, survival differences in biomarker high and low groups, biomarker Biomarker-related pathways and diseases, as well as the differences between sepsis patients and normal controls when it comes to immune infiltration, and the relationship between biomarkers and immune cells. We identified six biomarkers and found that the pathways were mainly enriched in the BEX2 Signaling Pathway, Hepatic Fibrosis Signaling Pathway, and Prostate Cancer Signaling through IPA functional enrichment analysis. Furthermore, we use IPA to predict the activation and inhibition of specific biological functions, including drug resistance of cells, expression of RNA, RNA transcription, cell viability, and migration of cells are inhibited; necrosis and apoptosis of tumor cell lines are activated. Finally, we also identified targeted therapeutic agents for biomarkers in patients with sepsis, and revealed the functionality correlations of which with key necroptotic signaling pathway molecules, providing a research basis for further understanding of the pathological mechanism and treatment of sepsis.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Jie Liu], [Lin Li], [Shuyang He], [Xin Zheng] and [Dan Zhu]. The first draft of the manuscript was written by [Lin Li] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.”

**Ethics approval**

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Xi’an Jiaotong University (Date 2022.06.07/No2022139).

**References**


Figures
Figure 1

Identification of differently expressed necroptosis-related genes (DE-NRGs). (A) PCA for sepsis and healthy control samples. (B) Volcano plot showing DE-NRGs between sepsis and control samples. (C) Heatmap of the expression levels of up-regulated and down-regulated genes in the sepsis and control groups. Red and blue squares indicate activation and suppression, respectively. (D) Venn diagram showing the intersection of DEGs and NRGs in the dataset.
Figure 2

Identification and validation of biomarkers. (A) DE-NRGs with significant differences in survival. Red and blue curves indicate high and low expression groups, respectively (P<0.05). (B) ROC curve of diagnostic genes (AUC > 0.8). (C) Venn diagram showing the intersection of ROC-genes and Survival-genes. The 8 DE-NRGs with significant differences in the survival analysis between high and low groups, and the 14 DE-NRGs with AUC values greater than 0.8 in both disease and regular ROC analysis. Those were
intersected to obtain genes with survival and diagnostic significance, as the biomarker of this study, 6 biomarkers were obtained. (D) Expression of 6 biomarkers in the sepsis and control samples in GSE65682 and GSE134347.
Construction of the nomogram model. (A) Univariate Cox analysis. (B) Multivariate Cox analysis. (C) Nomogram model of survival probability. The total score of each factor is added to correspond to the total score. According to the total score, the 1-week, 2-week, and 4-week survival rate are predicted. The higher the score, the higher the survival probability of patients. (D) Calibration curve (c-index = 0.6212). Based on the above prediction model, draw a correction curve, the closer the slope is to 1, the more accurate the prediction is.

![Nomogram model](image)

Figure 4

Significant classical pathway enrichment results of biomarkers based on Ingenuity Pathway Analysis (IPA) (P<0.01).
Figure 5

Immune cell infiltration analysis. **(A)** Bar plot showing percentage infiltration of 22 immune cells in each sample. **(B)** Heatmap showing the abundance of immune cell infiltration. **(C)** Violin plot showing differential infiltration of the 11 immune cells between different groups (sepsis and control) in GSE65682. **(D)** Heatmap of the correlation analysis between the 6 biomarkers and the percentage of immune cells. **(E)** Correlation of biomarkers and immune suppressive factors. Blue squares represent the negative
correlation; red squares represent the positive correlation. (F) Correlation of biomarkers and immune-activating factors.

Figure 6

Drug prediction and molecular docking analysis. (A) The relationship network between biomarkers and drugs. Green pentagons represent drugs; red circles represent biomarkers; orange edges represent
approved drugs; yellow-green edges represent experimental drugs; and purple edges represent review drugs. (B-D) The molecular docking results of BCL2 with Paclitaxel, Docetaxel, and Rasagiline.

Figure 7
Development of a TF-mRNA-miRNA network. (A) Venn diagram showing the intersection of miRNAs predicted by two datasets. (B) Regulatory relationship network between intersection mRNAs biomarkers.
Blue rectangles represent miRNAs; red ovals represent biomarkers. (C) TF-mRNA regulatory network. Cyan diamonds represent transcription factors; red ovals represent biomarkers. (D) miRNA-mRNA-TF regulatory network.

**Figure 9**
Results of Real-time Quantitative PCR experiments for the six biomarkers (*<0.05, **<0.01, ***<0.001).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.xlsx
- SupplementaryTable2.xlsx
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