The Role of Ferroptosis-Related Gene in the Immune Activity and Prognosis of Sepsis

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Keywords
sepsis, prognostic evaluation, ferroptosis, immune cell infiltration

Abstract
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Sepsis is a leading cause of mortality in intensive care units worldwide. Ferroptosis, a form of regulated cell-death–related iron, has been proven to be altered during sepsis, including increased iron transport and uptake into cells and decreased iron export. A better understanding of the role of ferroptosis in sepsis should expedite the identification of biomarkers for prognostic evaluation and therapeutic interventions.

Material and methods
We used the mRNA expression profiles of sepsis patients from Gene Expression Omnibus (GEO) to analyze the expression level of ferroptosis-related genes and construct molecular subtypes.

Results
Two distinct ferroptosis patterns were determined, and the overall survival of the two clusters was highly significantly different. Gene comparison analysis was performed on these two groups, and there were a total of 106 differentially expressed genes (DEGs). Pathway enrichment analysis of these genes showed that ferroptosis and immune-related pathways were enriched, suggesting that immune pathways might be critically involved in sepsis. To systematically predict the prognosis of sepsis, we constructed a nomogram model, the calibration plot nomogram showed excellent concordance for the 7-, 14-, and 28-days predicted and actual overall survival probabilities. Finally, the results of bioinformatics analysis were validated in animal and cell models.

Conclusions
In this study, we construct a ferroptosis-related nomogram that can be used for prognostic prediction in sepsis. In addition, we revealed the ferroptosis played a non-negligible role in immune cell infiltration and guiding more effective immunotherapy strategies.
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Introduction

Sepsis is a life-threatening organ dysfunction caused by the disorder of the body's response to infection. Sepsis has become one of the top 10 causes of death worldwide, and is a common cause of complications and death among patients in intensive care units (ICU). For many years, sepsis was thought to result from a highly inflammatory immune response in the host to
Cytokines produced by severe inflammatory responses activate neutrophils and cause excessive production of reactive oxygen species (ROS), which can lead to tissue and organ damage and further development of organ dysfunction and failure. However, the mechanism and biological role of sepsis induction are still not clear. Therefore, it is necessary to explore the role of immunity in sepsis from the perspective of molecular genetics.

In recent years, more and more attention has been paid to the role of abnormal metabolism of trace elements in sepsis. Iron is an important trace element that is needed for many basic processes, including DNA synthesis, energy production and immune function. Ferroptosis is a novel iron-dependent mode of cell death discovered in recent years, its main characteristics are iron metabolism changes and lipid peroxidation. Ferroptosis is a death pathway different from necrosis, apoptosis and other typical characteristics of cell death. The ultrastructure of ferroptosis showed that mitochondria became smaller, mitochondria cristae disappeared, membrane density increased, outer membrane rupture and cell size decreased. The main mechanism of iron divalent induced intracellular lipid peroxidation and decreased expression of glutathione peroxidase (GPX) and glutathione peroxidase 4 (GPX4). Recent studies have shown that ferroptosis in sepsis can be alleviated by improving oxidative stress. In this study, we explored the molecular mechanism of ferroptosis during sepsis, in order to provide ideas for prevention and treatment of sepsis.

The pathological mechanism of sepsis is complex, and many cell death modes are involved. With the development of research on the mechanism of ferroptosis over the last decade, ferroptosis has been proved to play a role in the pathogenesis of diseases caused by microbial infection. At present, a large number of studies have shown that ferroptosis plays an important role in inflammation. In addition, immune mechanisms can also regulate the sensitivity of cells to ferroptosis.
Many studies have studied the underlying mechanisms of the occurrence and development of various diseases from the perspective of ferroptosis, aiming to construct prognostic models based on bioinformatics analysis of ferroptosis.\(^9,10,16,18\) With the development of high-throughput sequencing technology, many studies have successfully constructed prognostic models based on ferroptosis-related genes.\(^19\) It shows potential clinical significance for prognostic prediction and development of molecular targeted drugs.\(^22\) However, sepsis, the most common disease in the ICU, has no such prognostic model. Here, in this study, we develop a prognostic model based on ferroptosis-related genes and explore its prognostic effect on sepsis patients, and we also explore its association with immune infiltration.

**Material and methods**

**Sepsis datasets source and preprocessing**

The flow chart of the overall study is shown in Figure 1. We searched GEO (https://www.ncbi.nlm.nih.gov/geo/) for experiments studying sepsis in human whole blood samples and found 2 datasets (GEO accession numbers: GSE65682, GSE95233).\(^23,24\) We downloaded the data using the GEOquery package of R software (version 4.1.0, http://r-project.org/).\(^25\) GSE65682 was based on the GPL13667 [HG-U219] Affymetrix Human Genome U219 Array platform, and the dataset contained 802 blood samples, including 760 sepsis cases and 42 healthy controls. GSE95233 was based on GPL570 [HG-U133_plus_2] Affymetrix Human Genome U133 Plus 2.0 Array platform, and the data set included 51 sepsis patients and 22 control patients, a total of 73 patients. Blood samples from sepsis patients on the first day of ICU admission were included in the study. The expression profile data of sepsis samples from the above two data sets were combined into one data set, and data cleaning operations such as batch removal, standardized processing and annotation probe were carried out. The clinical information of dataset GSE65682 was extracted for clinical analysis. The next step was to obtain genes related to ferroptosis. Firstly, 304 genes related to ferroptosis were searched in the
genecards database (https://www.genecards.org/) using ferroptosis as keywords. Then a total of 288 ferroptosis genes related to Driver, Suppressor and Marker were obtained from the FerrDb database (http://www.zhounan.org/ferrdb/). Finally, a total of 470 ferroptosis genes were obtained after the combination of the two data and the removal of duplicate genes.

**Consensus clustering for ferroptosis related genes**

Consensus clustering analysis was applied to identify distinct ferroptosis patterns based on the expression of ferroptosis related genes. We used the ConsensusClusterPlus package to perform the above steps, cluster1 and cluster2 are classified according to the results. The correctness of the classification was verified by principal component analysis (PCA) of mRNA expression profile based on sepsis. Survival analysis was performed using the clinical data of GSE65682 to analyze whether the classification of ferroptosis subtypes was clinically significant. The "limma" R package was used to analyze the differentially expressed genes (DEGs) between the two clusters with a false discovery rate (FDR) < 0.05 and logarithmic fold change (LFC)>0.3. The ggplot2 R package was used to draw the volcano map and heat map of differential genes to show the expression of DEGs.

**Evaluation of Immune Cell Infiltration**

We used CIBERSORT, an analytical algorithm that deconvolutes bulk samples with a minimal representation for each immune cell type using support vector regression based on a set of reference gene expression values. CIBERSORT analyzes RNA expression data to evaluate the abundance of different immune cell subtypes in each sample, to examine immune cell types of sepsis and to estimate the proportion of infiltrating immune cells. Only samples with a CIBERSORT output of p < 0.05 were considered worthy of further analysis. The ggplot2 R package was used to visualize the expression differences of 22 immune cells between the
cluster1 and cluster2. Corrplot R package was used to draw correlation heat maps to visualize the correlation of 22 immune cell infiltration.

**Gene set variation analysis (GSVA) and Gene Set Enrichment Analysis (GSEA)**

To investigate the difference on biological process between the cluster1 and cluster2, we performed GSVA enrichment analysis using “GSVA” R packages. GSVA, in a non-parametric and unsupervised method, is commonly employed for estimating the variation in pathway and biological process activity in the samples of an expression dataset. The gene sets of “c2.all.v7.4.symbols.gmt” were downloaded for running GSVA analysis. Rank-based approaches GSEA was applied to evaluate the enrichment of ferroptosis genes. Adjusted P value less than 0.05 was considered as statistically significance.

**Weighted Gene Co-expression Network Analysis (WGCNA)**

based on Ferroptosis Cluster

The WGCNA R package was applied to process data. Firstly, the soft threshold value of network construction is selected, and the adjacency matrix is the continuous value between 0 and 1, so that the constructed network accords with the power-law distribution and is closer to the real biological network state. Secondly, a scale-free network was constructed by using the function of block modules, and then the co-expression modules were identified by block partitioning analysis, so that genes with similar expression patterns were grouped. These modules are defined by using a dynamic tree cutting algorithm to cut the component branches of the cluster tree and assign different colors for visualization. All modules are summarized by modular characteristic genes (ME), the most important major component of each module, which are calculated as synthetic genes representing the expression profile of all genes in a given module.
**Functional Enrichment Analysis**

We used the “clusterProfiler” R package to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses DEGs between the cluster1 and cluster2 (FDR <0.05). GO analysis is a common method for conducting large-scale functional enrichment studies, including biological processes (BP), molecular functions (MF), and cellular components (CC).

**Protein-Protein Interaction (PPI) Network Construction**

In this study, we used the STRING database (http://string-db.org, version 11.09) to predict protein-protein interaction (PPI) and selected genes with a database score greater than 0.4 to construct PPI networks. Cytoscape software (v3.7.2) was used to realize network visualization, and Cytoshubba plug-in was used to screen the top 20 hub genes according to the score.

**Constructing regulatory network**

The associations between hub genes and miRNAs were performed in this article. After identified the potential mRNAs and miRNAs through miRTarBase databases. (http://miRTarbase.mbc.nctu.edu.tw). An integrating network of regulatory connections of mRNAs and miRNAs was constructed with the R package multiMiR. Cytoscape software (v3.7.2) was used to realize network visualization.

**Construction of A Predictive Nomogram**

We used Cox regression to detect the prognosis-associated ferroptosis-related gene signature. Survival package and SurvMiner package were used to perform univariate Cox regression
analysis on GSE65682. The risk factors were selected into the multivariate Cox regression analysis and the regression model was established. A nomogram was established based on multivariate Cox model to predict the 28-day survival of sepsis patients. Finally, the correction curve is used to evaluate the accuracy and resolution of the nomogram.

Construction of animal and cell models

The healthy 8-week-old male rats used in this study were purchased from the Animal Laboratory of Nanchang University. The rat were reared in sterile cages with humidity of 45-55% and light/dark cycle of 12 h. The rat were adaptively reared for 1 week before animal experiment. All animal experiments were conducted in accordance with the Guidelines for The Use of Experimental Animal Care approved by the Ethics Committee of Nanchang University (Approval No. 81960346). Next, rat sepsis model was established by cecal ligation and puncture (CLP) and venous blood samples were obtained. The rat were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal injection), and the cecum was exposed by cutting 1.5-2 cm along the midline of the abdomen. After stripping the mesentery, except for the sham group, the caecum was ligated at half of the end of the caecum with line 4. Except for the rat in the sham operation group, the cecum was punctured at 1cm of the distal end of ligation with no. 21 sterile needle and the wound was sutured. For fluid resuscitation, rat received 1ml of preheated saline (37°C). Analgesic treatment: Rat were injected with buprenorphine (0.05 mg/kg, subcutaneously) every 6 hours for 2 consecutive days.

NR838 cell line (rat lung macrophage) and RLE-6TN cell line (rat lung epithelial type II cells) were inoculated in 6-well plates with 2ml medium per well at a density of 106. When the cells grew to 50%, the cells were stimulated with 1μg/ mL LPS, and collected 2h and 9h later, respectively, to establish the sepsis cell model.
qRT-PCR Verification

Total RNA was extracted with TRIzol reagent (Takara, Dalian, China). Prime-script RTase (Takara) was used for reverse transcription. With the help of premixed ex-Taq (Takara), gene expression levels were determined by qPCR and normalized to GAPDH expression levels. Expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Drug sensitivity analysis

According to the expression profile of GDSC (www.cancerrxgene.org) cell line and TCGA gene, pRRophetic algorithm was used to construct ridge regression model to predict drug IC50, and drugs with significant differences between groups were screened out. The top 20 drugs were selected for visualization.

Statistical analysis

All of the analyses were performed using R with a two-sided significance threshold of $P< 0.05$. For the comparison of the two groups of continuous variables, the statistical significance of the normally distributed variables was estimated using the independent Student t test, and the differences between the non-normally distributed variables were analyzed using the Mann-Whitney U test (i.e. Wilcoxon rank-sum test). The Chi-square test or Fisher’s exact test was used to compare and analyze the statistical significance between the two groups of categorical variables.
Results

**Subtype construction of sepsis based on ferroptosis**

Firstly, ConsensusClusterPlus software was used to construct subtypes based on ferroptosis gene expression profiles to explore the biological differences among different ferroptosis subtypes of sepsis. The classification is reliable and stable when \( k = 2 \) (Figure 2A-C). So we divided the samples into cluster1 and cluster2. Further, we conducted survival analysis based on clinical data from GSE65682 dataset, and found that there was a significant difference in the survival rate between cluster1 and cluster2 groups (\( P<0.05 \)), indicating that this grouping has clinical significance (Figure 2D). Therefore, it is of practical significance for us to further explore the biological characteristics of ferroptosis.

PCA was used to further verify the correctness of classification of ferroptosis subtypes. It can be clearly seen that cluster1 group and cluster2 group are clearly distinguished from (Figure 3A). A differential expression analysis using R package limma identified 106 differentially DEGs between the two groups, with a p. adjusted (FDR) value < 0.05 and \(|\log_2\text{FC}| > 0.3\) as the cut-offs. Among these genes, 95 were downregulated and 11 were upregulated. (Figure 3B-C)

**Gene Set Variation Analysis and Gene Set Enrichment Analysis**

“c2.all.v7.4.symbols.gmt” was selected as the reference gene set for GSVA analysis. There were significant differences in the following 10 gene sets based on ferroptosis subtype grouping. Such as VALK_AML_CLUSTER_7, VALK_AML_CLUSTER_8, XIE_ST_HSC_S1PR3_OE_DN, WANG_LMO4_TARGETS_DN, CHYLA_CBFA2T3_TARGETS_DN, KYNG_RESPONSE_TO_H2O2_VIA_ERCC6, REACTOME_METABOLISM_OF_PORPHYRINS, BIOCARTA_AHSP_PATHWAY, NICK_RESPONSE_TO_PROC_TREATMENT_DN and NIKOLSKY_BRDEAST_CANCER_16P13_AMPLICON. Most of these different gene sets were
related to iron metabolism, inflammation, oxidative stress, immunity, blood diseases and other pathways (Figure 3D, Table 1).

In order to explore the functional enrichment of sepsis, GSEA analysis was performed on genes in sepsis expression profile, with reference gene set h.all.v7.4.entrez.gmt. Results showed that differential genes were significantly enriched in data sets HALLMARK_HEME_METABOLISM, HALLMARK_TNFA_SIGNALING_VIA_NFKB, HALLMARK_MYOGENESIS, HALLMARK_APICAL_JUNCTION, HALLMARK_XENOBIOTIC_METABOLISM, HALLMARK_COAGULATION, HALLMARK_PROTEIN_SECRETION, HALLMARK_INTERFERON_GAMMA_RESPONSE, HALLMARK_ANDROGEN_RESPONSE, HALLMARK_INFLAMMATORY_RESPONSE (Figure 4A-I, Table 2). It has obvious correlation with inflammation, immunity, iron metabolism, coagulation, etc.

**Evaluation of immune cell infiltration**

Both GSEA and GEVA enrichment analysis found that sepsis was highly correlated with immunity. In order to further explore immune-related cell changes in sepsis patients, CIBERSORT was used to analyze the proportion of sepsis immune cells and construct 22 immune cell maps in sepsis samples (Figure 5A). In addition, We analyzed the correlation between immune cells (Figure 5B). Immune cell correlation matrix shows that the infiltration level of activated memory CD8+ T cells is highly correlated with neutrophils, indicating that these two types of immune cells play an important role in the course of sepsis (Figure 5B). In addition, we compared the differences of immune cells infiltration between cluster1 and cluster2, and the results showed that B cells naïve, B cells memory, Plasma cells, T cells CD8, T cells gamma delta, Monocytes, Dendritic cells resting, Mast cells resting, Mast cells activated and Neutrophils were significantly different between the two clusters (Figure 5C).
WGCNA analysis based on ferroptosis subtypes

The gene coexpression networks of the dataset were established via the WGCNA package (Figure 6A). To establish scale-free networks, the soft thresholding power was set $\beta=5$ based on scale independence and mean connectivity (Figure 6B). The dynamic tree cut package was used to generate a gene cluster dendrogram containing 20 co-expression models (Figure 6C). The modules with the highest correlation with immune characteristics are brown ($r = 0.76, P = 7e^{-149}$) (Figure 6D). We intersected 106 DEGs grouped based on ferroptosis characteristics with 1251 genes in the brown module to obtain 94 intersection gene (Figure 6E).

Functional Enrichment Analysis

In order to understand the biological functions and mechanisms between intersection genes and ferroptosis, we used GO enrichment analysis and KEGG pathway analysis on the intersection genes in this study. Erythrocyte development, Myeloid cell development, Erythrocyte differentiation, Cortical cytoskeleton, Hemoglobin complex, Cell cortex part, Drug transmembrane transporter activity, 2 Iron 2 Sulfur cluster binding, Neutral amino acid transmembrane transporter activity and other biological function or characteristic were significantly enriched. However, when it comes to KEGG pathway enrichment analysis, only Malaria pathway was significantly enriched (Fig 7A). In these inflammation, autophagy, apoptosis, iron metabolism and other related pathways, logFC is decreased in these pathways, and the genes in them are also down-regulated genes (Fig 7B). The correlation between CISD2, SNCA, SESN3, FLCN, STK11, OPTN, PIP4K2A, SLC4A1, DMTN, EPB42, BPGM, NPRL3 and GO terms such as Erythrocyte development, TORC1 signaling, Negative regulation of TOR signaling, regulation of autophagy, Positive regulation of autophagy were observed (Fig 7C).

PPI and miRNA-mRNA network construction

In order to explore the functions of hubgenes transcribed and translated proteins, we used Cytoscape software to build a PPI network based on STRING database. 62 proteins had close
interactions with each other (Figure 7D). CytoHubba plug-in was used to screen the key genes, and the top 20 genes in the cytoHubba were selected as hub genes (Figure 7E). The top 20 genes were SLC4A1, EPB42, AHSP, DMTN, SNCA, FECH, KLF1, GYPB, GYPA, CA1, HBD, OSBP2, ANK1, TNS1, BPGM, SELENBP1, TMOD1, SPTB, FKBP8, and GMPR.

In order to further explore the upstream regulatory relationship, based on miRTarBase V.8 database, the results of "Functional MTI" evidence were selected to predict the miRNA interacting with hub genes. The mRNA transcribed from the hub genes interacted with 330 miRNA (Figure 7F).

**Construction of A Predictive Nomogram**

The clinical characteristic data and the expression data of 20 hub genes were extracted from GSE65682 datasets for univariate Cox regression analysis. The results showed that AHSP, DMTN, KLF1, GYPB, GYPA, CA1, HBD, BPGM, SPTB and GMPR had statistical significance (P<0.05). We further conducted multivariate Cox regression analysis on these genes and constructed multivariate Cox regression model, which was visualized by forest map (Figure 8A, Table 4). Through the forest plot of cox regression, we can see that genes such as AHSP, GYPA, CA1, and BPGM may have important values for the prognosis of sepsis(Figure 8A). In addition, a nomogram was constructed using this model to predict 7-day, 14-day, and 28-day survival for patients with sepsis (Figure 8B). We further evaluate the predictive power of the line chart. The c-index of the line graph is 0.68, indicating that it has high confidence. The calibration curves of days 7, 14 and 28 were consistent (Figure 8C-E), indicating that the nomogram has a good accuracy and resolution.

**Experimental Validation**

AHSP, DMTN, KLF1, GYPB, GYPA, CA1, HBD, BPGM, SPTB and GMPR were included in the previous multivariate Cox regression model construction. We further verified the expression of
these genes by constructing septic animal and cell models. We verified in CPL animal models and cell models. We found that the expression of BPGM in the sepsis model was significantly higher than that in the control group, and the results were statistically different (Figure 9A). In addition, consistent results were obtained in the two cell models MR8383 and RLE cell lines (Figure 9A). This proves that the expression of BPGM will increase significantly during the course of sepsis. Subsequently, we further verified several other genes included in the model, such as GYPA, AHSP, CA1, GMPR, SPTB, KLF1, HBG, and DMTN, and obtained consistent results. Since GYPB was not expressed in rat, other genes were used for subsequent experimental verification. The results showed that the expression level in the blood of sepsis animals was significantly higher than that in the control group, and similar results were obtained in cells experiments (Figure 9A-I).

**Drug sensitivity analysis**

Based on IC50 of different cell lines for different small molecule drugs in the GDSC database, we analyzed changes in sensitivity of different ferroptosis classifications to these drugs. The results suggest that NU7441, AMG706, SL01011, GDC0941, Sunnib, Metformin and other small molecule drugs have higher IC50 in cluster2 than cluster1, and the difference is statistically significant (P<0.001) (Figure 10A-T). The results showed that different classification of ferroptosis may lead to changes in the sensitivity of sepsis to 54 different small molecule drugs. The results showed that 20 small molecule drugs were statistically significant (Figure 10A-T).

**Discussion**

Sepsis is a life-threatening medical emergency caused by a malregulated host response to inflammation in which microbial biological processes play an important role. Iron is an important element in microbial biological processes, and many studies have revealed the relationship between changes in iron metabolism and sepsis. Most microorganisms depend on iron for their
pathogenicity, and some bacteria such as Escherichia coli and Klebsiella pneumoniae have evolved the ability to remove iron from host iron-binding proteins. Previous studies have shown that iron imbalance is associated with lower survival rates in patients with sepsis.

A number of studies have proposed that ferroptosis is a novel regulation of cell death closely related to iron overload. Ferroptosis plays an important role in immune infiltration, but the specific mechanism remains unclear. In this study, 10 ferroptosis-related genes were used to construct a sepsis prognostic model. Nine of these genes were validated in animal and cell models. Some of these genes have been shown to play different roles in immune infiltration. For example, the KLF1 gene is associated with immune activation. Other genes may play an important role in ferroptosis. Sepsis is a highly heterogeneous disease. In the past, many studies have explored the biomarkers and prognostic models of sepsis, but these studies are limited to a single hematological index and do not comprehensively consider the level of gene expression. In addition, these predictive models are only valuable in one type of population, but have limited accuracy in another type of patient. Our prediction model is based on the expression of iron death-related genes, which is more accurate. The present prognostic model provides significantly better performance than previous prognostic models. In addition, our nomogram can provide personalized prediction of mortality risk, which is of great significance to clinicians in clinical application.

In this study, we constructed subtypes based on the expression profile of ferroptosis genes to explore the biological differences among different types of ferroptosis subtypes of sepsis. Furthermore, 106 DEGs were studied and genes related to OS were screened out. Functional enrichment analysis showed that the DEGs were significantly correlated with inflammation, immunity and iron metabolism. The mechanism of inflammation susceptibility to ferroptosis has been a hot topic of research in the past decades, but the complex relationship between immunity and ferroptosis is still unknown. Studies have shown that inflammation can easily induce ferroptosis, and the activation of ferritin related pathways under inflammation may provide a new
therapeutic target for the treatment of sepsis.\textsuperscript{12, 47} We further explored the relationship between ferroptosis and immune cell infiltration. Notably, we found that ferroptosis significantly affected both B lymphocyte and CD8+ T cell function in the analysis of infiltrating immune cells. The correlation matrix of immune cells showed that the infiltration level of activated memory CD8+ T cells was highly correlated with Neutrophils cells. However, we found that these results are somewhat similar to studies of features associated with ferroptosis in other diseases.

Through the GDSC database,\textsuperscript{48} based on the differential genes associated with ferroptosis, several small molecule drugs targeting the characteristic components associated with ferroptosis were identified. The results showed that 20 small molecule drugs were statistically significant. These small molecule drugs may provide a new therapeutic target for the treatment of sepsis.

There are some limitations to the study. Due to limited knowledge of ferroptosis, most of the characteristic components of our study involve not only ferroptosis-related pathways, but other pathways as well. In addition, our research lacks validation of animal models. Our follow-up studies will further conduct IHC and FISH experiments on animal models of sepsis in order to provide pathological evidence. What's more, because of our small sample size, it is difficult for our risk score to fully accurately assess the role of ferroptosis in sepsis. Therefore, our predictive model should be further validated in a cohort of sepsis from multicenter studies.

**Conclusion**

In conclusion, we identified two molecular subgroups by analyzing the expression of matrix based on genes associated with ferroptosis in this study. The two molecular subgroups showed significantly different survival rates and immune status. Moreover, a sepsis prognostic model based on ferroptosis-related genes was developed and its predictive efficiency was well demonstrated. In addition, gene function enrichment analysis indicated that ferroptosis-related genes could affect the immune cell infiltration and then affect the occurrence, development and
prognosis of sepsis. Our works indicated that the important role of ferroptosis and immune interaction in the occurrence and development of sepsis, and provides a new idea for exploring the molecular mechanism and treatment of sepsis.

Data Availability Statement:
The expression profile data and related clinical information were obtained from GEO databases (GSE65682, GSE95233). All relevant data and materials are available from the corresponding author upon reasonable request.

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Disclosure
All authors claim that there are no potential conflicts of interest in the study.

Author Contributions:
WD and TY conceived the idea. WD designed the study. DL, QX, FL, QS and NZ collected the data and performed the experiments. TY drafted the manuscript. WD and TY reviewed and corrected the manuscript. KQ supervision, project administration, funding acquisition and paper finalization. All authors contributed to the article and approved the submitted version.
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Table 1  GSVA enrichment analysis results

| Pathway name                          | logFC  | AveExpr | t      | P.Value   | B       |
|---------------------------------------|--------|---------|--------|-----------|---------|
| VALK_AML_CLUSTER_7                    | 0.7660 | -0.0268 | 31.0918| 1.37E-140 | 310.5310|
| VALK_AML_CLUSTER_8                    | 0.7967 | -0.0305 | 30.0983| 1.94E-134 | 296.4196|
| XIE_ST_HSC_S1PR3_OE_DN                | 0.6401 | -0.0164 | 28.8664| 8.53E-127 | 278.8851|
| WANG_LMO4_TARGETS_DN                  | -0.3373| 0.0109  | -28.8027| 2.12E-126 | 277.9779|
| CHYLA_CBFA2T3_TARGETS_DN              | 0.3751 | -0.0067 | 28.3197| 8.53E-127 | 271.0997|
| KYNG_RESPONSE_TO_H2O2_VIA_ERCC6      | 0.5893 | -0.0084 | 28.2910| 3.19E-123 | 270.6908|
| REACTOME_METABOLISM_OF_PORPHYRINS     | 0.5606 | -0.0137 | 28.0679| 7.74E-122 | 267.5122|
| BIOCARTA_AHSP_PATHWAY                 | 0.7278 | -0.0298 | 26.8280| 3.81E-114 | 249.8658|
| NICK_RESPONSE_TO_PROC_TREATMENT_DN    | -0.4814| 0.0131  | -26.0573| 2.25E-109 | 238.9187|
| NIKOLSKY_BREAST_CANCER_16_P13_AMPLICON| 0.4482 | -0.0128 | 25.8906| 2.41E-108 | 236.5542|

Table 2  GSEA enrichment analysis results

| Description                        | SetSize | EnrichmentScore | NES    | P-value  | Leading_edge          |
|------------------------------------|---------|-----------------|--------|----------|------------------------|
| HALLMARKHEME_METABOLISM            | 176     | -0.8075         | -3.6367| 1.00E-10 | tags=58%, list=7%, signal=55% |
| HALLMARKTNFA_SIGNALLING_VIA_NFKB   | 150     | 0.4199          | 1.9435 | 1.56E-06 | tags=46%, list=28%, signal=34% |
| HALLMARKMYOGENESIS                 | 115     | -0.4649         | -1.9668| 9.42E-06 | tags=50%, list=25%, signal=38% |
| HALLMARKAPICAL_JUNCTION            | 127     | -0.4325         | -1.8592| 5.73E-05 | tags=33%, list=15%, signal=28% |
| Hallmark | Count | log2FoldChange | p-value | Tags | List | Signal |
|----------|-------|---------------|---------|------|------|--------|
| Xenoergic Metabolism | 132 | -0.4069 | 1.78E-04 | 30% | 15% | 26% |
| Coagulation | 83 | -0.4376 | 6.77E-04 | 45% | 26% | 33% |
| Protein Secretion | 86 | 0.3686 | 5.27E-03 | 38% | 20% | 31% |
| Interferon Gamma Response | 175 | 0.2967 | 7.36E-03 | 26% | 18% | 21% |
| Androgen Response | 76 | 0.3756 | 7.81E-03 | 34% | 18% | 28% |
| Inflammatory Response | 149 | 0.3022 | 9.95E-03 | 48% | 33% | 33% |

**Table 3** GO, KEGG enrichment analysis results

| Ontology ID | Description | Count | p-value |
|-------------|-------------|-------|---------|
| GO:0048821 | erythrocyte development | 5 | 4.19E-07 |
| GO:0061515 | myeloid cell development | 6 | 8.88E-07 |
| GO:0030218 | erythrocyte differentiation | 7 | 1.03E-06 |
| GO:0038202 | TORC1 signaling | 4 | 4.72E-05 |
| GO:0032007 | negative regulation of TOR signaling | 4 | 9.30E-05 |
| GO:0010506 | regulation of autophagy | 8 | 1.43E-04 |
| GO:0010508 | positive regulation of autophagy | 5 | 2.18E-04 |
| GO:1903432 | regulation of TORC1 signaling | 3 | 6.77E-04 |
| GO:2001240 | negative regulation of extrinsic apoptotic signaling pathway in absence of ligand | 3 | 4.87E-04 |
| GO:2001239 | regulation of extrinsic apoptotic signaling pathway in absence of ligand | 3 | 1.09E-03 |
| GO:0030863 | cortical cytoskeleton | 5 | 2.11E-04 |
| Variables | Univariate analysis | Multivariate analysis |
|-----------|---------------------|-----------------------|
|           | HR | 95%CI of HR | pvalue | HR | 95%CI of HR | pvalue |
| Gender    | 1.04 | 0.71-1.51 | 0.832 |     |     |     |
| Age       | 1.22 | 0.84-1.76 | 0.284 |     |     |     |
| SLC4A1    | 1.08 | 0.94-1.22 | 0.271 |     |     |     |
| EPB42     | 1.11 | 0.97-1.24 | 0.109 |     |     |     |
| AHSP      | 1.15 | 1.01-1.29 | 0.029 | 0.67 | 0.44-0.99 | 0.047 |
| DMTN      | 1.18 | 1.00-1.39 | 0.046 | 1.08 | 0.67-1.72 | 0.741 |
| SNCA      | 1.01 | 0.89-1.12 | 0.923 |     |     |     |
| FECH      | 1.06 | 0.95-1.18 | 0.261 |     |     |     |
| KLF1      | 1.22 | 1.08-1.37 | 0.001 | 1.06 | 0.78-1.44 | 0.697 |
| GYPB      | 1.18 | 1.05-1.31 | 0.003 | 1.23 | 0.89-1.7 | 0.201 |
| GYP A     | 1.26 | 1.13-1.39 | 0.000 | 1.37 | 1.09-1.71 | 0.007 |
| CA1       | 1.24 | 1.11-1.37 | 0.000 | 1.94 | 1.45-2.58 | 0.000 |
| HBD       | 1.18 | 1.05-1.31 | 0.004 | 1.09 | 0.71-1.66 | 0.701 |
| OSBP2     | 1.09 | 0.94-1.24 | 0.224 |     |     |     |
| Gene     | Fold Change | 95% CI | p-value |
|----------|-------------|--------|---------|
| ANK1     | 1.11        | 0.98-1.24 | 0.075   |
| TNS1     | 1.12        | 0.97-1.27 | 0.106   |
| BPGM     | 1.12        | 1.01-1.23 | 0.018   | 0.62    | 0.47-0.81 | 0.001 |
| SELENBP1 | 1.06        | 0.94-1.18 | 0.348   |
| TMOD1    | 1.07        | 0.93-1.22 | 0.303   |
| SPTB     | 1.22        | 1.02-1.46 | 0.029   | 0.85    | 0.58-1.24 | 0.412 |
| FKBP8    | 1.17        | 0.98-1.39 | 0.080   |
| GMPR     | 1.16        | 1.00-1.32 | 0.037   | 0.76    | 0.47-1.2  | 0.239 |

**Figure 1** Flow chart of the overall analysis of the biological characteristics of sepsis.

**Figure 2** Characteristics of sepsis subtypes. (A) The consensus matrix when k = 2. Both rows and columns of the matrix represent samples. (B) Consensus CDF; (C) Delta area. (D) Grouped by ferroptosis subtype, the difference in survival rate between the two groups p=0.043.

**Figure 3** Difference analysis of ferroptosis subgroups and GSVA analysis. (A) PCA analysis of ferroptosis subgroup, light blue is cluster1, light red is cluster2; (B) The heat map of the different analyses of ferroptosis subgroups, light blue is cluster1, purple is cluster2, small blue squares represent low expression, and small red squares represent high expression; (C) The volcano map of the different analyses of ferroptosis subgroups. the light blue point represents low expression, and the light red point represents high expression; (D) According to GSVA analysis, light blue is cluster1, purple is cluster2, small blue squares represent low enrichment, and small red squares represent high enrichment.

**Figure 4** GSEA enrichment analysis. (A-I) GSEA analysis of the most significant gene set.
Figure 5 Immune cell infiltration and immune correlation analysis. (A) Barplot of the ratio of 22 kinds of immune cells in sepsis samples; (B) Heat map of 22 kinds of immune cell infiltration; blue means positive correlation, red means negative correlation, the darker the color, the stronger the correlation; (C) Immune cells in the subtypes related to ferroptosis.

Figure 6 Construction of weighted co-expression network analysis. (A) Clustering dendrogram based on Euclidean distance; (B) Network topology analysis under various soft threshold powers. Left: The x-axis represents the soft threshold power. The y-axis represents the fit index of the scale-free topology model. Right: The x-axis represents the soft threshold power. The y-axis reflects the average connectivity (degrees); (C) Cluster dendrograms of topologically overlapping genes with different similarities and assigned module colors; (D) Module-t trait association. Each row corresponds to a module, and each column corresponds to a feature. Each cell contains the corresponding correlation and P-value. This table is color-coded according to the relevance of the color legend; (E) Venn diagram of the intersection of WGCNA's most relevant modular genes and differential genes.

Figure 7 GO, KEGG enrichment analysis, and PPI network analysis. (A) GO, KEGG analysis dot chart; (B) GO, KEGG analysis circle chart, the center histogram represents the z-score score, dark green represents a decline, light green represents an increase, the middle dot represents genes, and blue represents down-regulated genes; (C) Chord diagram of the selected 5 GO paths; (D) The PPI network of 62 common genes constructed by STRING database, the greater the degree value of the gene, the darker the color and the larger the diameter; (E) For the first 20 HUB genes calculated by cytoHubba, the higher the enrichment score, the darker the color; (F) The miRNA-mRNA interaction network, the light blue circle represents miRNA, and the brown triangle represents mRNA.
Figure 8 Correlation analysis of clinical features. (A) Multi-factor Cox regression analysis forest plot; (B) Multi-factor Cox regression analysis nomogram plot; (C-E) 7 days, 14 days, 28 days calibration curve.

Figure 9 Expression of the genes in sepsis prognostic models increased in vivo and in vitro. The expression of BPGM (A), GYPA (B), AHSP(C), CA1 (D), GMPR (E), SPTB (F), KLF1 (G), HBD (H) and DMTN (I) in the previous multivariate Cox regression model construction.

Figure 10 Sensitivity analysis of small molecule drugs. (A-T) The sensitivity of different ferroptosis classifications to these small molecule drugs changes. NU.7441 (A), AMG.706 (B), SL.0101.1 (C), WO2009093972 (D), GSK269962A (E), EHT.1864 (F), GDC0941 (G), PD.173074 (H), Sunitinib (I), NVP.TAE684 (J), Metformin (K), PHA.665752 (L), GNF.2 (M), ABT.888 (N), CGP.082996 (O), LFM.A13 (P), Parthenolide (Q), PF.02341066 (R), BMS.536924 (S), Bexarotene(T), etc. all had higher sensitivity in cluster2 group.
The Role of Ferroptosis-Related Gene in the Immune Activity and Prognosis of Sepsis

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