Identification of WIP1 as A Potential Ferroptosis-Related Biomarker in Sepsis and Validation in CLP Mice Model.

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Abstract

Background

Sepsis is the leading cause of death and critical illness worldwide. Ferroptosis is a new type of programmed cell death that is different from apoptosis, cell necrosis, and autophagy, which might contribute to development of sepsis. However, it lacks of a rapid, accurate diagnostic method and a therapeutic target of sepsis. In this work, we identified a novel promising biomarker related to ferroptosis in sepsis.

Methods

The expression profiles of mRNAs were downloaded from Gene Expression Omnibus (GEO) database, and ferroptosis-related genes were used to screen differential genes. Furthermore, we identified potential ferroptosis-related biomarkers through a series of comprehensive bioinformatics analysis, such as differential analysis, enrichment analysis, protein-protein interactions (PPI). Moreover, the receiver operating characteristic (ROC) was used to evaluate the diagnostic value of biomarkers and the relative expressions of biomarker were measured by quantitative real-time PCR (qRT-PCR) in cecum ligation and puncture (CLP) mice model. Finally, immune infiltration analysis was preformed to explore the relationship between ferroptosis-related biomarkers and immune regulation.

Results

Totally 52 (36 upregulated and 16 downregulated) overlapped ferroptosis-related differential expression genes differentially expressed genes (FRDEGs) from two GEO datasets were identified between sepsis and normal samples. Upregulated ferroptosis-related WD Repeat Domain, Phosphoinositide Interacting 1 (WIPI1), with high area under curve (AUC) was selected as a promising biomarker, according to expression of WIPI1, septic samples were divided into high-expression and low-expression group. There are significant differences in the infiltration of some immune cells between the two group.

Conclusion

In summary, we identified WIPI1 as a novel biomarker that may involve in immune regulation of sepsis by ferroptosis. In addition, this work contributes to the rapid, accurate and convenient diagnosis of sepsis.

Introduction

Sepsis is defined as the host inflammatory response to life-threatening infections with organ dysfunction [1]. According to conservative estimates, sepsis is the leading cause of death and critical illness worldwide[2, 3]. Although the prognosis of sepsis has improved in the past few decades, the mortality rate with shock is still higher than 25%~30%, even 40%~50%[4]. Moreover, surviving sepsis patients usually have to suffer long-term physical, psychological and cognitive impairments, it has a significant impact on healthcare and society [5]. Furthermore, sepsis is differed from other major

epidemics, the treatment of sepsis is non-specific, and there are no approved drugs, but only through the support of organ function and the administration of intravenous fluids, antibiotics and oxygen [6]. Therefore, there is an urgent need to explore new sepsis biomarkers.

Ferroptosis is an iron-dependent, non-apoptotic cell death method characterized by abnormal accumulation of lipid hydroperoxide and related lipophilic reactive oxygen species (ROS) in the cell membrane [7]. Since the term was coined in 2012, the field of research on ferroptosis has grown exponentially in the past few years [8]. Ferroptosis has been reported to be involved in various pathological processes including neurotoxicity, acute kidney failure, liver injury and heart disease, as well as myocardial ischemia reperfusion injury [9-12]. Furthermore, the development of sepsis has been reported to be related to ferroptosis [13]. However, the research on identifying ferroptosis-related biomarkers in sepsis is not sufficiently thorough.

To explore the relationships between sepsis and ferroptosis, in this study, we aim to investigate ferroptosis related genes expression profiles and their values in biomarkers in sepsis through bioinformatics analysis. Furthermore, the ferroptosis related differentially expressed genes were used for functional annotation. Moreover, to analyze the regulatory relationships, PPI network was constructed and transcription factors (TFs) were predicted. The potential ferroptosis related hub genes with high AUC were identified as biomarkers and qRT-PCR was used to validate the relative expressions of biomarkers in CLP mice model. Finally, the potential biomarker WIP1 was further used to explore immune infiltration in sepsis. This work might provide novel insight into ferroptosis in human sepsis and may suggest a promising strategy.

Methods

GEO dataset selection

The expression profile datasets of mRNAs were downloaded from the NCBI GEO (www.ncbi.nlm.nih.gov/geo) database. MRNAs from whole blood between human sepsis and healthy control were included. MRNAs expression profiling data were obtained from GSE13904 (158 sepsis sample and 18 healthy control) [34] and GSE26378 (82 sepsis sample and 21 healthy sample) [35] datasets. Validation set were GSE80496 (21 sepsis sample and 21 healthy control) [36] and GSE134347 (156 sepsis sample and 83 healthy control) [37].

Differential expression analysis of mRNAs

Differential expression analysis was performed by limma R package [38], with adjusted P value < 0.05 and the log(fold-change) > 0.5 or < -0.5. Then, ferroptosis related genes were downloaded from FerrDb, the world's first manually curated database for regulators and markers of ferroptosis and ferroptosis-disease associations (<http://www.zhounan.org/ferrdb>) [39] (Table S1). Furthermore, the overlap of differential expression genes and ferroptosis-related genes were defined as ferroptosis-related differential expression genes differentially expressed genes (FRDEGs).

Gene enrichment analysis

ClusterProfiler R package was used for functional annotation of gene [40]. GO and KEGG pathway enrichment analyses were performed and visualized by ggplot2 R package [41].

Protein-Protein interaction network building

STRING database was used to construct PPI network with medium confidence (0.4), line color indicates the type of interaction evidence [42]. The hub gene were identified by Cytoscape cytohubba [43].

Development of ROC curves

To examine the diagnostic value of FRDEGs, ROC curve was applied and AUC was measured via pROC R package [44].

Transcription factors predict

NetworkAnalyst database was used to predict the TFs of FRDEGs [45]. The promoter sequences of hub genes were downloaded from NCBI, then the binding site were analyzed by JASPAR database [46], a collection of transcription factor DNA-binding preferences, modeled as matrices, with relative score >0.99.

CLP mice model

20 specific pathogens free (SPF) female C57BL/6J mice (8-10 weeks old, weighing 20~22g) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. All animal experiments were conducted in strict accordance with the Institutional Animal Care and Use Committees and Institutional Review Board (IRB) of Tongji Hospital. The all experimental procedures of the mice were specifically approved for this study by the Medical Ethics Committee of Tongji Hospital and were carried out in accordance with institutional guidelines (TJ-IRB20182677). All procedures on mice were performed under sodium pentobarbital anesthesia, all efforts were made to minimize suffering. CLP mice model is the most frequently used sepsis model and protocol of CLP mice model was referred to previous report with high-grade sepsis [47]. 12h after surgery, the blood was collected from orbital sinus.

qRT-PCR

TRIpure Reagent was used to extract total RNA from blood of CLP mice, and the PCR conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s. The relative gene expression level was calculated using the 2- $\Delta\Delta C_t$ method. To normalize the data, GAPDH was used as internal reference. The sequences of the primer are shown in Table 1.

Table 1: The sequences of qRT-PCR primer.

Gene names	Primer sequence (5'-3')
WIPI1	Forward-AGGATACTCTGAGGACGGCG
	Reverse-TCTGACTTCCACGGCACAAG
GABARAPL2	Forward-CCGTTGTTGTTGTGGTCGCT
	Reverse-TGAGAGCCCGAGACTTTTTTCC
TLR4	Forward-GTGCCAGTCAGGGTCATTCA
	Reverse-ACTCCCCAGCCCTTTATGGA
GAPDH	Forward-GCAAGTTCAACGGCACAG
	Reverse-GCCAGTAGACTCCACGACAT

Immune infiltration and GSEA

GSEA analysis were performed via GSEA/MSigDB [48] and visualized by ggplot2 R package. ImmuCellAI database was applied to estimate the difference of immune cell infiltration in two groups [49].

Results

Identification of FRDEGs

A flowchart of the study design is shown in Figure 1. 56 and 70 FRDEGs in Two sepsis public datasets, GSE13904 (n=176) and GSE26378 (n=103) were obtained from the GEO database. The limma R package was used for different gene expression analysis with thresholds of $|\text{Log}_2(\text{FC})| > 0.5$ and adjusted p-value < 0.05 , were shown in volcano plots respectively (Figure 2A). The intersection of two group of FRDEGs with consistent up-regulation and down-regulation trends were exhibited via venn diagram (Figure 2B), among them, 37 upregulated and 15 downregulated FRDEGs in sepsis samples (Table S2). Totally 52 overlapped FRDEGs were found to explore the functional annotation. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis were used (Table S3), top 10 biological process (BP), cellular component (CC), molecular function (MF) and KEGG pathways were shown in Figure 2D-E. Terms indicated that FRDEGs of sepsis were associated with 'cellular response to oxidative stress', 'response to oxidative stress' as well as 'response to starvation'. Top 3 term of CC were 'ficolin-1-rich granule', 'phagophore assembly site' and 'phagophore assembly site membrane'. In categories of MF, 'MAP kinase activity', 'coenzyme binding' and 'mitogen-activated protein kinase binding' were major terms. Top 3 of KEGG signaling pathways were 'ferroptosis', 'autophagy – animal' and 'FoxO signaling pathway'. PPI network of 52 FRDEGs was constructed by STRING database and visualized by Cytoscape as shown in Figure 3A. The hub genes were identified by Cytoscape cytohubba (Figure 3B).

Association between FRDEGs and transcription factors

It is known that transcription factors play a key role in the degree of gene expression. Therefore, to explore possible mechanism affecting FRDEGs expression, regulation relationships between FRDEGs and transcription factors were investigated. As shown in Figure 4A, TFs-FRDEGs regulation network was constructed by NetworkAnalyst and visualized via Cytoscape. To screen high reliability TFs, the promoter sequences of hub genes were downloaded from NCBI, then the binding site were analyzed by JASPAR database with relative score >0.99 (Table S4). Finally, forkhead box L1 (FOXL1), trans-acting T-cell-specific transcription factor GATA-3 (GATA3), homeobox protein Nkx-3.1(NKX3-1) and kruppel-like factor 5 (KLF5) were selected, as well as motif sequence diagrams of 4 TFs were shown in Figure 4B-E.

Validation of the hub genes in sepsis

To validate the hub genes in sepsis patients, GSE80496 (n=42) and GSE134347 (n=239) were downloaded for validation set. ROC analyses of hub genes were performed by pROC R package, and AUC was calculated at to estimate the predictive performance of the hub genes. As shown in Figure 5A-B, AUC of toll-like receptor 4 (TLR4), Gamma-aminobutyric acid receptor-associated protein-like 2 (GABARAPL2) and WIPI1 have high AUC (>0.75) in both two validation sets, reached 0.964, 0.975, 0.851, 0.998 in GSE80496, and 0.787, 0.964 and 0.961 in GSE134347 respectively. Moreover, the expressions of TLR4, GABARAPL2 and WIPI1 in two validation sets were exhibited in Figure 5C-G.

CLP mice model was used to simulated Sepsis, as shown in Figure5 I-K, the relative expression of TLR4, GABARAPL2 and WIPI1 were measured via qRT-PCR. Importantly, WIPI1 and GABARAPL2 showed significance difference between normal and sepsis groups, but TLR4 have no difference.

Immune infiltration and Gene set enrichment analysis (GSEA)

According to expression level of WIPI1, septic samples in GSE13904 were divided into high-expression and low-expression groups for GSEA analysis (Table S5). As shown in Figure 6A-F, GSEA showed that many of the most enriched gene sets describe KEGG signaling pathways related to immunity, inflammation, and infection. According to results of GSEA enrichment, WIPI1 high-expression group was positively correlated to B cell receptor signaling pathway, natural killer cell mediated cytotoxicity, leukocyte transendothelial migration, Fc gamma R-mediated phagocytosis, chemokine signaling pathway, Toll-like receptor signaling pathway. In consideration of the enrichment to many immune-related signaling pathways, immune infiltration analysis was performed via ImmuCellAI database. The violin plot of the immune cell infiltration shows that, the fraction naïve CD4⁺ T cell, naïve CD8⁺ T cell, cytotoxic T (Tc) cell, exhausted T (Tex) cell, natural regulatory T (nTreg) cell, induced regulatory T (iTreg) cell, follicular B helper T (Tfh) cell, central memory T (Tcm) cell, mucosal associated invariant T (MAIT) cell, dendritic cell (DC), B cell, natural killer (NK) cell, CD4⁺ T cell, CD8⁺ T cell were relatively low in high-expression group. By contrast, the fractions of macrophage, neutrophil were relatively high in high-expression group (Figure 7A). Therefore, we investigated the correlations matrix of all immune cell proportions. As shown in Figure 7B, naïve CD4⁺ T cell shows strongest positive correlation with Tcm cell (Pearson correlation = 0.84), while iTreg cell shows strongest negative correlation with macrophage (Pearson correlation = -0.67).

Discussion

In spite of advances made in comprehending of septic pathophysiology, the definition and management of sepsis has been a significant evolved in over the last three decades [14]. It is benefited for understanding clinical syndrome, advancements in hemodynamic monitoring tools, and resuscitation measures, but sepsis remains one of the major causes of morbidity and mortality in critically ill patients [15]. Moreover, it lacks of a rapid, accurate diagnostic method and a therapeutic target of sepsis. Recent studies have shown that ferroptosis is volved in regulations of the occurrence and development of many diseases, including sepsis, indicating that ferroptosis has broad implications for health. The protein expression level of Glutathione peroxidase 4 (GPX4), a ferroptosis mainly relies on regulator, was reported to be decreased in septic ventricular tissues [16]. In addition, the protein expression level of a ferroptosis driver heme oxygenase 1(HMOX1) was increased, thereby regulated ferroptosis in sepsis [17]. Fang et al. found that nuclear receptor coactivator 4 (NCOA4) mediated ferritinophagy is leads to ferroptosis in sepsis-induced cardiac injury, and inhibition of ferroptosis in cardiomyocytes improves the cardiac function and survival rate of mice [18]. However, the mechanism and effect of ferroptosis in sepsis is unclear, and need to be explored.

In this work, a comprehensive bioinformatics analysis was performed based on expression profile of sepsis from GEO database. Furthermore, expression levels of ferroptosis-related genes were analyzed in septic data sets. Combining GO/KEGG enrichment analysis, construction of PPI network, ROC analysis and calculation of AUC, upregulated FRDEGs WIPI1, GABARAPL2 and TLR4 showed high sensitivity and specificity in sepsis diagnosis. In order to validate the results, blood from CLP mice model was collected and analyzed via qRT-PCR. WIPI1 and GABARAPL2 both showed significant difference between normal and sepsis groups. Considering of WIPI1 with high AUC and mRNA fold change, WIPI1 was identified as a potential biomarker of sepsis.

WIPI1 was first discovered because of its role in the formation of nascent autophagosomes, and then related to classical and nonclassical autophagy pathways [19]. Subsequently, RNAi screening analysis demonstrated that WIPI1 is a potential positive regulator of ferroptosis, WIPI1 promoted accumulation of lipid-based ROS to result in ferroptosis [20].

Sepsis is a complex interaction that triggers the host's pro-inflammatory and anti-inflammatory processes. There are several studies showed that sepsis induces numerous overlapping mechanisms of immunosuppression involving both innate and adaptive immunity [21, 22]. The race to death in these immune mechanisms determines the fate of the septic patients. Therefore, in order to explore the underlying immune mechanisms, GSEA and immune infiltration analysis were performed. According to results of GSEA enrichment analysis, high-expression of WIPI1 group was significantly associated immunity, inflammation, and infection, demonstrated that ferroptosis related WIPI1 was relevant to immune regulations. Moreover, immune infiltration analysis showed that, WIPI1 was correlated with infiltration of various immune cells. The results of immune infiltration showed that fraction of B cell, DC, CD4+ T cells and CD8+ T cell were relatively low in high-expression group, because sepsis leads to

profound depletion of these critical immune effector cells [23-27]. The number of NK cells are significantly decreased in septic patients, and it lasts for several weeks as well as it is associated with increased mortality [28-30]. However, the fractions of neutrophil, macrophage were relatively high in high-expression group. Neutrophils are the most abundant cells in innate immunity, which are important for early controlling of invade pathogens. In contrast to lymphocytes which accelerated apoptosis, neutrophil death was delayed during sepsis. In the early stage of sepsis, macrophages exacerbate the inflammatory response by secreting a large amount of pro-inflammatory factors and chemokines, and the excessive apoptosis of macrophages in the late stage of sepsis leads to immune dysfunction and organ damage [31]. In sepsis, Tfh cell showed high positive correlated with CD4+ naïve T cell owing to CD4+ naïve T cell differentiated into Tfh cells, but fraction of CD4+ naïve T cell was relatively low in high-expression; therefore, it may limit differentiation of CD4+ naïve T cell into Tfh cell [32]. On the contrary, macrophage showed high negative correlated with nTreg cell, might be due to Treg cell can limit suppress innate immune cells [33]. These results demonstrated that ferroptosis-related gene WIPI1 can affect immune cell infiltration characteristics. We inferred that WIPI1 might promote ferroptosis of B cell, DC, CD4+ T cells, CD8+ T cell and NK cells, which aggravates sepsis.

In this work, expression of WIPI1 was proven to be a promising biomarker for sepsis by bioinformatics analysis. Interestingly, it contributes to the rapid, accurate and convenient diagnosis of sepsis, because it only needs peripheral blood collected from patinaed. This biomarker also can serve as a potential therapeutic target. Moreover, WIPI1 was inferred to facilitate sepsis by promoting ferroptosis of immune cells, and it benefits in understanding the pathogenesis and mechanism of sepsis. Nevertheless, the lack of large quantity of clinical verify results is a limitation of our work. In addition, the mechanism of WIPI1 promote ferroptosis of immune cells need to be identified in vitro and in vivo via multi-omics analyses.

Conclusions

In summary, this study revealed a novel biomarker based on ferroptosis and immune in sepsis was identified and validated. Moreover, increased expression of WIPI1 involved in immune infiltrating cells such as B cells, DC, CD4+ T cells, CD8+ T cells, NK cells and neutrophils, which might contribute to development of sepsis. The results proved that WIPI1 could be a promising biomarker with precise diagnostic accuracy related to immune infiltration in sepsis. Certainly, more ingenious experimental design and validation is required to conclude the roles of WIPI1 in sepsis.

Abbreviations

GEO: Gene Expression Omnibus

PPI: protein-protein interactions

ROC: receiver operating characteristic

qRT-PCR: quantitative real-time PCR

CLP: cecum ligation and puncture

FRDEGs: ferroptosis-related differential expression genes differentially expressed genes

WIPI1: WD Repeat Domain, Phosphoinositide Interacting 1

AUC: area under curve

ROS: reactive oxygen species

TFs: transcription factors

BP: biological process

CC: cellular component

MF: molecular function

GO: Gene ontology

KEGG: Kyoto encyclopedia of genes and genomes

FOXL1: forkhead box L1

GATA3: trans-acting T-cell-specific transcription factor GATA-3

NKX3-1: homeobox protein Nkx-3.1

KLF5: kruppel-like factor 5

TLR4: toll-like receptor 4

GABARAPL2: Gamma-aminobutyric acid receptor-associated protein-like 2

GSEA: Gene set enrichment analysis

Tc: cytotoxic T cell

Tex: exhausted T cell

nTreg: natural regulatory T cell

iTreg: induced regulatory T cell

Tfh: follicular B helper T cell

Tcm: central memory T cell

MAIT: mucosal associated invariant T cell

DC: dendritic cell

NK: natural killer cell

GPX4: Glutathione peroxidase 4

HMOX1: heme oxygenase 1

NCOA4: nuclear receptor coactivator 4

SPF: specific pathogens free

IRB: Institutional Review Board

Declarations

Data Availability Statement

The data used to support the findings of this study are available from supplementary materials and the corresponding author upon request

Ethics declarations

Ethics approval and consent to participate

The all experimental procedures of the mice were specifically approved for this study by the Medical Ethics Committee of Tongji Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author Contributions

SZ and CY conceived, devised the study and performed the bioinformatics analysis. SZ performed validation experiments. YH found related data and analysis tool. SZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Figures

Figure 1

Flowchart of overall study design.

Figure 2

Volcano plots of differentially genes in (A) GSE13904 and (B) GSE26378 with P value < 0.05, $|\log(\text{fold-change})| > 0.5$; (C) A venn diagram indicating that FRDEGs were identified in GSE13904 and GSE26378 cohorts; Bubble charts of GO and KEGG enrichments (D) BP, (E) CC, (F) MF, (G) KEGG pathway.

Figure 3

(A) PPI network constructed via Cytoscape, blue rectangles represent downregulated and red rectangles represent upregulated genes; (B) hub genes identified by Cytohubba.

Figure 4

(A) TFs-FRDEGs network constructed via Cytoscape, green triangles represent TFs, red ellipses represent FRDEGs; Sequence motifs of (B) FOXL1 (C) GATA3 (D) NKX3-1 and (E) KLF5.

Figure 5

ROC curves and AUC of hub genes in (A) GSE13904 and (B) GSE26378; WIPI1 GABARAPL2 and TLR4 expression in (C-E) GSE13904, (F-H) GSE26378 and (I-J) CLP mice model.

Figure 6

GSEA between high-expression and low-expression groups of WIPI1 in GSE13904 (A) leukocyte transendothelial migration; (B) natural killer cell mediated cytotoxicity; (C) B cell receptor signaling pathway; (D) Fc gamma R-mediated phagocytosis; (E) chemokine signaling pathway; (F) Toll-like receptor signaling pathway.

Figure 7

(A) Immune infiltration of the high-expression group (red), and the low-expression group (blue) of WIP11;
 (B) Correlation matrix of all immune cell proportions.

Supplementary Files

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

- [TableS1.FerroptosisrelatedgenesfromFerrDB.xlsx](#)
- [TableS2.Overlapgenes.xlsx](#)
- [TableS3.GOKEGGenrichment.xlsx](#)
- [TableS4.TranscriptionfactorsfromJASPAR.xlsx](#)
- [TableS5.GSEAreport.xlsx](#)