Bioinformatics analysis of ferroptosis-related genes in the pathogenesis of diabetic ulcers

Li Wang  
Guangzhou University of Chinese Medicine  

Lulu Tang  
Guangzhou University of Chinese Medicine  

Jinqi Xie  
Guangzhou University of Chinese Medicine  

Haoxiang Ye  
Guangzhou University of Chinese Medicine  

Zaoyuan Kuang  
Guangzhou University of Chinese Medicine  

Aijun Liu (✉ ajunjliu@gzucm.edu.cn)  
Guangzhou University of Chinese Medicine  

Article

Keywords:

**Posted Date:** November 22nd, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-2272260/v1

**License:** ☺  This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

**Background:** Diabetic ulcers are a major complication of diabetes which causing lower extremity amputation. Nonetheless, the progression in the development of diabetic ulcers therapeutics is slow. Ferroptosis plays a key role in the pathogenesis of chronic wound in diabetic ulcers. The mechanism needs to be further clarified.

**Methods:** Ferroptosis-related differentially expressed genes (FRDEGs) in diabetic ulcers were screened from the dataset GSE92724 and FerrDb online database based in silico. Then, functional enrichment analysis and protein-protein interaction (PPI) network were implemented to recognize the potential biological pathways and mechanisms. MCODE tool was used to cluster and predict hub genes. The miRNAs corresponding to hub genes were predicted by miRWalk 2.0. Receiver operating characteristic (ROC) was applied to verify the diagnostic value of five hub genes in the dataset GSE132187 and GSE134431. The immune infiltration between diabetic ulcers samples and normal samples were analyzed by using CIBERSORTx.

**Results:** 26 FRDEGs and 5 hub genes (EGFR, SLC2A1, CD44, CA9, and PTGS2) in diabetic ulcers were identified. GO and KEGG analysis revealed that hub genes were signficantly enriched in response to oxidative stress, basolateral plasma membrane, and HIF-1 signaling pathway. ROC results suggested that hub genes have a high diagnostic accuracy for diabetic ulcers. In immune cell infiltration, T follicular helper cells and monocytes were significantly lower in diabetic ulcers.

**Conclusion:** This research firstly demonstrated that five hub genes may be potential therapeutic targets and possible diagnostic biomarkers in the pathogenesis of diabetic ulcers.

Introduction

Diabetic ulcers, including diabetic foot ulcers (DFUs), is one of the major complications of diabetes mellitus\(^1\). Most diabetic ulcers will eventually progress into chronic and incurable ulcers, often causing patients to suffer from severe pain and low-quality life. Diabetic ulcers are presently the leading cause of lower extremity amputation in the USA and consume an estimated $200 billion annually\(^2\). These ulcers cause high rates of morbidity and mortality, worse than most cancers\(^3\). The patients’ quality of life will be improved for the early diagnose and prevention of diabetic ulcers. The pathogenesis of diabetic ulcers is complex and involves a combination of oxidative stress led to cell death and hyperglycemia-induced free radicals\(^4\). Cell death patterns are key factors to elucidate the molecular mechanism of diabetic ulcers. Managing diabetic ulcers encompasses the usage of anti-inflammation agents and antibiotics. While recent progress advances in the understanding of delayed wound healing, the treatment options are still very limited. Therefore, it is essential to facilitate the study of the pathogenesis to aid in the diagnosis and treatment of diabetic ulcers.
Ferroptosis is a novel form of cell death: an iron-dependent, non-apoptotic form of programmed cell death characterized by the accumulation of intracellular reactive oxygen species and the accumulation of lipid peroxides. Ferroptosis is associated with the pathogenesis of chronic wound in diabetic ulcers. Long-term hyperglycemia elevates the expressions of lipid peroxidation products, reactive oxygen species (ROS), and ferroptosis-associated proteins. Researchers have demonstrated that the use of ferroptosis inhibitor ferrostatin-1 can attenuated the expression of oxidative stress and inflammation markers. In addition, the administration of ferrostatin-1 significantly accelerated tissue healing via activating of the PI3K/AKT signaling pathway. More interestingly, the prophylactic application of this inhibitor also prevented the formation of wounds. Increased levels of free iron in plasma also contribute to oxidative stress and ferroptosis. Increasing evidence suggests that ferroptosis and ferritinophagy are potential therapeutic targets with applications in diabetic complications. However, the exact molecular mechanism of ferroptosis in diabetic ulcers still requires further exploration.

To our knowledge, the exact bioinformatics-based molecular studies of diabetic ulcers pathogenesis remain elusive. In this study, bioinformatics data mining and analysis were performed using the dataset GSE92724. The differentially expressed genes (DEGs) were screened in diabetic patients (Pat) and control individuals (Ctrl). The DEGs with ferroptosis database (FerrDb) was intersected to obtain the ferroptosis-related DEGs. Functional enrichment analysis and PPI network analysis were both implemented to detect the hub genes involved in the development of diabetic ulcers. Furthermore, the crucial microRNAs (miRNAs) associated with biomarkers that play principal roles in diabetic ulcers were recognized. The expression levels of hub genes in the dataset GSE132187 and GSE134431 were analyzed to validate the results. Together, these results have the potential to offer new thoughts on the molecular basis for diabetic ulcers.

**Methods**

**Data acquisition.** The high throughput sequencing dataset GSE92724 was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). This dataset includes tissues from four patients with types 2 diabetes mellitus patients’ tissues and six normal persons. In addition, ferroptosis-related genes (FRGs) were retrieved from the FerrDb database (https://www.zhounan.org/ferrdb).

**Differentially expressed genes recognition.** The DEGs were identified using the limma R package (version 3.52.4) with the threshold criterion of |log2FC| >0.5 and adjusted \( p \) value< 0.05. Then 259 ferroptosis-related genes with DEGs were intersected to recognize ferroptosis-related DEGs (FRDEGs). The ggplot2 package was utilized to construct the volcano plot to display the DEGs. A heatmap plot and Venn diagram were drawn to visualize the FRDEGs using R.

**Functional enrichment analysis.** GO enrichment analysis (including Biological Processes (BP), Cellular Components (CC), and Molecular Functions (MF)) and KEGG enrichment analysis were performed using the DAVID online database (https://david.ncifcrf.gov/) or clusterProfiler package (version 3.14.0). Enriched terms were defined significantly following the criteria of \( p < 0.05 \).
Protein–protein interaction network analysis and hub genes identification. STRING (http://string-db.org), an online database that determines the interaction between collections of proteins, was used to predict PPI\textsuperscript{15}. The network was set to the standard threshold (confidence score > 0.4) in the STRING database. The PPI network for DEGs was visualized by using cytoscape software. For clustering analysis of gene networks, the MCODE, a plug-in in cytoscape, was used to identify hub genes.

mRNA-miRNA regulatory network construction. The miRNAs connected to hub genes were predicted using miRWalk 2.0 online database. The predictions from MiRTarBase and miRWalk databases were intersected to ensure the reliability of the results. After the prediction, the mRNA-miRNA regulatory network was visualized by cytoscape software.

Diagnostic value of hub genes. ROC analysis was performed to validate the diagnostic accuracy of FRDEGs in the dataset GSE132187 and GSE134431. The results were visualized to display the area under the curve using the ggplot2 package.

Immune cell Infiltration analysis. CIBERSORTx (https://cibersortx.stanford.edu/), a deconvolution algorithm introduced by Stanford University, was used to analyze the immune infiltration differences between diabetic ulcers tissue and normal tissue\textsuperscript{16}. The normalized expression profile of the GSE92724 dataset were uploaded to the web using LM22 signature and 1000 permutations to access the distribution of immune cells.

Prediction of targeted transcription factor. To further investigate the targeted transcription factor (TF) related to hub genes, we next downloaded GSEA transcription factor gene set to identify transcription factor using enricher function of clusterProfiler package. Criteria of FDR < 0.05 and binding genes > 2 were used to screen reliable results.

Statistical analysis. All statistical analyses were performed by R (Version 4.1.2). Results with \( p < 0.05 \) were considered as statistically significant.

Results

Quality control of dataset. The GSE92724 dataset was downloaded from GEO. We utilized genes counts per million (cpm) > 0.05 in at least three cases for each group to filter raw count and the variance stabilizing transformation (VST) module in DESeq2 to remove the difference of two groups in this dataset (Fig. 1A). The principal component analysis (PCA) tested the consistency of the grouped data. PCA showed that the expression profile Pat samples and Ctrl samples were distinct from each other (Fig. 1B). The biological replicates of the two groups were in good agreement (Fig. 1C). These results revealed good consistency in two groups.

Identification of ferroptosis-related DEGs. Totally 1271 DEGs containing 798 upregulated and 473 downregulated DEGs were screened in the dataset (Fig. 2A). Then, DEGs were intersected with 259 ferroptosis-related genes to obtain FRDEGs. Finally, 26 FRDEGs were selected and marked in Venn
diagram and heatmap (Fig. 2B and C). These FRDEGs were further classified as ferroptosis suppressor, driver, and marker via FerrDb website (Table 1).

<table>
<thead>
<tr>
<th>Suppressor</th>
<th>Driver</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP63, MT1G, CD44, ENPP2, CA9, ARNTL</td>
<td>SLC1A5, DUOX1, EGFR, TFRC, FBXW7, CHAC1, DPP4, CDO1</td>
<td>TFAP2C, SLC2A1, PSAT1, JDP2, SLC7A5, NCF2, SLC1A4, CHAC1, RRM2, NNMT, PTGS2, SLC2A6, HBA1</td>
</tr>
</tbody>
</table>

Table 1 The FRDEGs were divided into ferroptosis suppressor, driver, and marker.

GO and KEGG enrichment analysis of FRDEGs. GO and KEGG pathway enrichment analysis were performed to explore the pathophysiological activities of FRDEGs based on DAVID database and clusterProfiler R package. The results of GO enrichment analysis were divided into three categories, namely BP, CC, and MF (Fig. 3A). Regarding BP, FRDEGs were significantly enriched in response to oxidative stress (GO:0006979), basolateral plasma membrane (GO:0016323), and cellular response to chemical stress (GO:0062197). For the CC, the enriched terms included apical plasma membrane (GO:0016324), apical part of cell (GO:0045177), and melanosome (GO:0042470). MF analysis showed that FRDEGs were mainly involved in organic anion transmembrane transporter activity (GO:0008514), anion transmembrane transporter activity (GO:0008509), and virus receptor activity (GO:0001618) (Fig. 3A). Subsequently, the KEGG pathway analysis was associated with central carbon metabolism in cancer (hsa05230), HIF-1 signaling pathway (hsa04066), cysteine and methionine metabolism (hsa00270), miRNAs in cancer (hsa05206), and glutathione metabolism (hsa00480) (Fig. 3B).

Protein-protein interaction network analysis. A PPI network was constructed to visualize the 26 FRDEGs with thresholds for interaction scores \( \geq 0.4 \) (Fig. 4). The results indicated that there were 26 nodes and 46 edges in the network. Using the MCODE plugin of cytoscape, five hub genes were determined as hub genes including EGFR, SLC2A1, CD44, CA9, and PTGS2 (Table 2). Then, KEGG functional analysis was implemented to illustrate the pathway of hub genes. KEGG results indicated that hub genes were significantly enriched in miRNAs in cancer, central carbon metabolism in cancer, and HIF-1 signaling pathway. The top eight KEGG pathways are illustrated in a bubble chart and a chord diagram, respectively (Fig. 5 A and B).
### Table 2 Details of five hub genes.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Degree</th>
<th>Full names</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>11</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>SLC2A1</td>
<td>9</td>
<td>Solute carrier family 2 member 1</td>
</tr>
<tr>
<td>CD44</td>
<td>7</td>
<td>CD44 molecule</td>
</tr>
<tr>
<td>CA9</td>
<td>6</td>
<td>Carbonic anhydrase 9</td>
</tr>
<tr>
<td>PTGS2</td>
<td>6</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
</tr>
</tbody>
</table>

**mRNA–miRNA regulatory network construction.** mRNA-miRNA regulatory analysis was investigated using miRWalk 2.0 online database. The crosslinked miRNAs were obtained from miRWalk and miRTarBase online databases with \( p < 0.05 \) and the 3'UTR as the gene-binding region to ensure the accuracy and reliability of the result. Totally 176 miRNAs pairing three genes (CD44, DGFR, and SLC2A1) were screened to interpret the underlying mutual mechanism (Fig. 6; Supplementary Table S1).

**Validation of key genes in diabetic ulcers.** ROC analysis was performed in two datasets GSE132187 and GSE134431 to evaluate the predictive value of five FRDEGs in diabetic ulcers (Fig. 7; Supplementary Table S2). In the dataset GSE132187, CA9 and PTGS2 had higher accuracy than other hub genes in the diagnosis of diabetic ulcers (AUC = 1.000). In the dataset GSE134431, SLC2A1 had higher accuracy than other hub genes (AUC = 1.000). These results indicated that all five hub genes have good accuracy in the diagnosis of diabetic ulcers.

**Differences in immune infiltration.** Immune cell infiltration data of 22 types of immune cells were analyzes by using CIBERSORTx online database. The fractions of T follicular helper cells and monocytes were lower in diabetic ulcers than in normal tissues (Fig. 8; Supplementary Table S3).

**Targeted transcription factor.** We searched for significant TFs as shown in Table 3. The four TFs are TGASTMAGC_NFE2_01, HIF1_Q5, P53_02, and NF-κB_C.

<table>
<thead>
<tr>
<th>TF</th>
<th>BgRatio</th>
<th>FDR</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGASTMAGC_NFE2_01</td>
<td>195/27137</td>
<td>0.0130</td>
<td>CD44/CA9</td>
</tr>
<tr>
<td>HIF1_Q5</td>
<td>249/27137</td>
<td>0.0131</td>
<td>SLC2A1/CA9</td>
</tr>
<tr>
<td>P53_02</td>
<td>255/27137</td>
<td>0.0131</td>
<td>EGFR/CA9</td>
</tr>
<tr>
<td>NF-κB_C</td>
<td>268/27137</td>
<td>0.0131</td>
<td>CA9/PTGS2</td>
</tr>
</tbody>
</table>

**Table 3.** Regulatory relations of TFs with hub genes.

**Discussion**
Diabetic ulcers, as one of the serious complications of diabetes, mostly appearing in the legs and feet of patients, usually result in amputation and death\textsuperscript{17}. Ferroptosis is a novel form of programmed cell death linking metabolism, redox biology, and diseases\textsuperscript{18}. Recent studies suggest that ferroptosis is a potential mechanism of diabetic ulcers\textsuperscript{10}. Understanding the correlation between diabetic ulcers and ferroptosis may provide a new therapeutic target for wound healing.

In the present study, there were 798 upregulated and 473 downregulated DEGs in the dataset. 26 ferroptosis-related DEGs were identified by analyzing diabetes mellitus cases compared to healthy donors. GO analysis revealed that the response to oxidative stress seems to be a key biological process modulating ferroptosis in diabetic ulcers. The result was consistent with previous studies\textsuperscript{10}. It was noted that FRDEGs were mainly enriched in cysteine and methionine metabolism and HIF-1 signaling pathway in KEGG analysis. Oxidation of cysteine residues and methionine in hemoglobin is associated with the pathogenesis of diabetes complications\textsuperscript{19}. Hypoxia-inducible factors (HIFs) are critical regulators of oxygen balance responding to hypoxia. Emerging evidence indicates that skin or wound tissue in diabetes is hypoxic, indicating that hypoxia plays a core role in diabetic ulcers. However, HIF-1-mediated adaptive responses to hypoxia are impaired in diabetes, resulting in cellular dysfunction\textsuperscript{20}. These results suggest a potential role in the activation of oxidative stress response in diabetic ulcers.

MCODE tool plug-in in the Cytoscape was used to visualize five hub genes in the PPI network of FRDEGs. The five hub genes including EGFR, SLC2A1, CD44, CA9, and PTGS2 might be related to the occurrence of diabetic ulcers. Further, the KEGG analysis on the five hub genes was conducted. The KEGG pathway analysis suggested that hub genes mostly enriched in miRNAs in cancer, Central carbon metabolism in cancer, and HIF-1 signaling pathway. Two recently published papers proved that three miRNAs could inhibit the expression of vascular endothelial growth factor A (VEGFA) negatively regulated angiogenesis and wound healing in patients with diabetic foot ulcers\textsuperscript{21, 22}. Two high throughput sequencing datasets GSE132187 and GSE134431 were selected to validate diagnostic value of five hub genes. These five hub genes have a good performance in the prediction and diagnosis of diabetic ulcers. A previous study found that EGRF knockdown decreased the proliferative capacity of cells and delayed the healing of full-thickness of back wounds \textit{in vivo}\textsuperscript{23}. Solute carrier family 2 member 1 (SLC2A1) has been related to diabetic microvascular complications in multiple projects\textsuperscript{24, 25}. CD44 is associated with the regulation of tissue repair process. Jia-Hong Gong et al. found the low expression of CD44 in adipose stem cells (ASCs) from diabetic ulcers rats\textsuperscript{26}. A published research paper revealed that CD44 expression inhibited ferroptosis\textsuperscript{27}. CA9 is an expressed gene induced by hypoxia in several tumors\textsuperscript{28}. CA9 can counteract ferroptosis in malignant mesothelioma\textsuperscript{29}. However, little study on CA9 and diabetic ulcers has been reported. PTGS2 encoding cyclooxygenase-2(COX-2) was associated with the development of diabetic ulcers. Based on powerful evidence, it is worthwhile to conduct in-depth research.

miRNAs are a category of small, endogenously expressed noncoding RNA molecules of 18–25 nucleotides in length. miRNAs have been reported to play essential roles in the pathogenesis, severity, and prognosis of diabetic foot ulcers\textsuperscript{30}. The genes-miRNAs network was established and the results showed
that only three hub genes linked to target miRNAs. The rigorous filtering criteria in this study may be the possible reason for the result. Recent studies revealed that miR-155, miR-211, and the let-7 family are involved in glucometabolic disorder in diabetes mellitus, which are consistent with results in this study\textsuperscript{31}. Downregulation of endothelial miR-200b suppressed the expression and GATA2 and VEGFR2 to accelerate delayed wound healing\textsuperscript{32}. Moreover, miR-130 delayed epithelization in diabetic wounds models\textsuperscript{33}. These researches confirmed the predicted results in this study. Despite the regulatory network has been shown to be changed in diabetic ulcers, further studies should be carried out to clarify the ferroptosis-related chronic nonhealing wounds.

CIBERSORT\textsuperscript{x} is a machine learning tool for the inference cell-type-specific gene expression profile from bulk tissue transcriptomes. Immune cells are critical in the occurrence of diabetic foot ulcers\textsuperscript{34}. So, the immune cells infiltration between diabetic ulcers and normal samples were assessed. T follicular helper cells and monocytes have lower proportions in diabetic ulcers than normal tissues. The results in this study indicated that immune cells may be involved in the pathogenesis of diabetic ulcers. J Moura et al. have confirmed the acceleration of immune aging delayed wound healing in diabetic chronic inflammation complications.

Transcription factors (TFs) are proteins that finely tune transcriptional regulation and directly shape organism phenotypes by maintaining cell fate\textsuperscript{35};\textsuperscript{36}. In the present study, we recognized four TFs TGASTMAGC\textsubscript{NFE2}\textsubscript{01}\textsuperscript{37};\textsuperscript{38}, HIF1\textsubscript{Q5}\textsuperscript{39};\textsuperscript{40}, P53\textsubscript{02}\textsuperscript{41};\textsuperscript{42}, and NF-κB\textsubscript{C}\textsuperscript{43};\textsuperscript{44} binding to two hub genes in cellular system. So far, no significant data reveal the role of TFs with two hub genes in diabetic ulcers by commonational approaches. More studies could focus on the function of TFs regulators affect downstream gene expression.

There are several limitations in this study. First, this study was lacked of clinical or animal experiments to further validate the above findings. Second, this study only analyzed the datasets including four diabetic ulcers patients and six normal donors, which may induce bias. Finally, due to the drawback of bulk RNA sequencing, it was unable to distinguish cell population heterogeneity. The single-cell RNA sequencing analysis will be focused on to explore cell-specific expression profiles in diabetic ulcers.

**Conclusion**

This study identified five hub ferroptosis-related DEGs, which were related to the pathogenesis of diabetic ulcers. GO and KEGG analysis were implemented to reveal the potential biological pathway and mechanism. There were differences in immune cell infiltration between diabetic ulcers and normal samples. This research offers a new sight into the pathogenesis and diagnosis of diabetic ulcers.

**Declarations**

**Data availability**
The datasets analyzed in this study are available in the GEO database.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant number 82174368) and Traditional Chinese Medicine Bureau of Guangdong Province (grant number 20231128).

Author contributions

L.W. and A.L. conceptualized the study design. L.T. and J.X. analyzed the data. J.X. and H.Y. revised the images. L.W. and L.T. wrote the manuscript. A.L. and Z.K. helped to accurately process the data, and reviewed and revised the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Correspondence and requests for materials should be addressed to Z.K. or A.L.

References


**Figures**

(A) Boxplot of GSE92794 dataset. (B) PCA plot. (C) The correlation of different samples.
Figure 2

(A) A volcano plot showing DEGs in GSE92724 dataset. (B) Venn diagram of 26 FRDEGs in diabetic ulcers. (C) Heatmap of the 26 FRDEGs.
Figure 3

GO and KEGG analysis of FRDEGs. (A) GO enrichment analysis of FRDEGs. (B) The top 10 significant KEGG pathways.
Figure 4

PPI analysis of FRDEGs. Red nodes represent hub genes.

Figure 5
KEGG pathway analysis of hub genes. (A) A bubble chart of the top eight KEGG pathways. (B) A chord diagram of the top eight pathways.

**Figure 6**

Interaction of hub genes with targeted miRNAs. Red octagons and blue triangles represent hub genes and miRNAs.
Figure 7

ROC analysis of five hub genes. (A) On the dataset GSE132187. (B) On the dataset GSE134431.
Figure 8

Distribution of immune cell clusters. (A) Proportions of each cell cluster in bar-plot. (B) The fraction of immune cells between normal and diabetic ulcers samples in box-plot (Wilcoxon test, \( *p < 0.05 \)).

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

- SupplementaryTableS1.tif
- SupplementaryTableS2.tif
- SupplementaryTableS3.tif