Bioinformatics analysis identifies potential ferroptosis-related key genes in the pathogenesis of diabetic nephropathy

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Abstract

Objective: To identify potential ferroptosis-related key genes in the pathogenesis of diabetic nephropathy (DN) through bioinformatics analysis, thereby providing new targets for the treatment of DN.

Methods: We first downloaded the RNA expression dataset GSE30529 from the GEO database and intersected it with a ferroptosis dataset to obtain ferroptosis-related differentially expressed genes (DEGs). Venny 2.1 was used to generate Venn diagrams of the DEGs, and Heml software was used to draw heatmaps of the DEGs. DAVID 6.8, Metascape, and WebGestalt were employed for functional enrichment analysis of the DEGs. Protein-protein interactions (PPIs) were retrieved through the STRING database and visualized by Cytoscape v3.6.0 software. miRWalk 2.0 was used to predict target key miRNAs and construct related gene-miRNA interaction networks.

Results:

Our study identified 31 ferroptosis-related DEGs. Gene Set Enrichment Analysis (GSEA) revealed that the biological processes of these genes were significantly enriched in response to stress signals, starvation signals, lipids and atherosclerosis, and regulation of endogenous apoptotic signaling pathways, among others. The regulatory network of the MAPK8 molecule is the most crucial potential molecule

that may affect the occurrence of DN. The endogenous apoptotic signaling pathway is the main biological pathway involved. We screened out one key module through MCODE, which includes two downregulated genes (MAPK8 and DDIT3) and three upregulated genes (XBP1, HSPA5, and ASNS).

Conclusion:

The ferroptosis-related key genes MAPK8, has-miR-4775, HSPA5, has-miR-4712-5p/has-miR-770-5p, and XBP1 form a regulatory network, participating in the occurrence and development of DN. This provides some important references for our future basic research verification and suggests a potential target for the development of DN treatment strategies.

Keywords: Diabetic Nephropathy; Ferroptosis; MAPK8; Bioinformatics Analysis; Target

1. Introduction

Diabetic Nephropathy (DN) is one of the most common microvascular complications of diabetes [1] and a major cause of end-stage renal disease (ESRD), which is associated with inflammation and immune responses [2-3]. DN is a significant public health issue worldwide. According to statistics, there are approximately 420 million diabetics globally, with one in every four women and one in every five men having type 2 diabetes and diabetic nephropathy, a phenomenon more common in type 1 diabetes [4]. From a pathophysiological perspective, DN can be divided into glomerular lesions, tubular and interstitial atrophy and fibrosis. Its clinical features include a continuous decline in glomerular filtration rate, accompanied by persistent elevations in proteinuria and serum creatinine [5]. After the occurrence of DN, many factors are triggered, among which the reduction of renal tissues or cells that can play an active and effective role is the main factor leading to the reduction of renal function.

Previous studies have also shown that programmed cell death plays an important role in the development of DN [6-7]. Ferroptosis is a programmed cell death-like process characterized by the production and accumulation of iron-dependent lipid reactive

oxygen species (ROS) [8]. Studies have reported that ferroptosis plays an important role in the occurrence and development of many diseases, such as targeting iNOS to reduce early brain injury after experimental subarachnoid hemorrhage by promoting ferroptosis of M1 microglia and reducing neuroinflammation [9]; the loss of heart ferritin H promotes cardiomyopathy through Slc7a11-mediated ferroptosis [10]; and the loss of iron transporter proteins induces memory impairment by promoting ferroptosis in Alzheimer's disease [11]. However, the mechanism of ferroptosis in DN is still unclear, and there is also a lack of related bioinformatics research. We used data mining and data analysis techniques to screen for differentially expressed genes (DEGs) in diseased renal tissues and normal renal tissues of DN. These DEGs were then intersected with the ferroptosis dataset to obtain ferroptosis-related DEGs.

In addition, to identify key biomarkers and establish the pathogenesis of DN at the molecular level, we investigated key miRNAs that may play a major role in DN. Our research results will help to understand the state of ferroptosis after the occurrence of DN and provide new ideas for the clinical diagnosis and treatment of DN.

2. Materials and Methods

2.1 GEO Data Analysis

The RNA expression dataset GSE30529 (containing diseased renal tissues and normal renal tissues of DN) was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) using the GEOquery package. Probes corresponding to multiple molecules were removed, and when probes corresponding to the same molecule were encountered, only the probe with the largest signal value was retained.

2.2 Ferroptosis Data Analysis

Relevant ferroptosis datasets were downloaded from the ferroptosis database (http://www.zhounan.org/ferrdb), which contains 259 genes. The annotations of these genes reveal 108 driver genes, 69 inhibitory genes, and 111 gene markers [12].

2.3 Differential Expression Analysis

GEO2R is an online tool for differential expression analysis [13]. The T-test was used

to determine p-values and adjusted p-values in differential gene expression (DGE) analysis. Genes from different tissues were selected using the following criteria: |log2(fold-change)| > 1, adjusted p-value < 0.05. We also obtained a dataset that included genes from the ferroptosis database and intersected it with GSE30529 to identify ferroptosis-related DEGs. The online tool Venny 2.1 was used to generate Venn diagrams of the DEGs, and Heml software was used to draw heatmaps of the DEGs.

2.4 Functional Enrichment Analysis

DAVID 6.8, Metascape, and WebGestalt were used for functional enrichment analysis of the DEGs. These different enrichment analysis tools have different algorithms, which can serve as mutual validation. We uploaded the ferroptosis-related DEGs to WebGestalt's GSEA for further study. WebGestalt's GSEA first filters gene sets based on the number of genes they contain. KEGG analysis was obtained from WebGestalt's GSEA. Metascape is an online tool for gene function annotation analysis. The biological process annotations were performed by Metascape using genes shared between GSE30529 and the ferroptosis dataset. Additionally, the biological pathways of miRNAs were analyzed in the enrichment analysis tool Funrich. A p-value < 0.05 was considered statistically significant.

2.5 Protein-Protein Interaction Network Analysis

To predict protein-protein interactions (PPIs), we searched for interactions between these proteins through the STRING database. Furthermore, the PPI network was constructed and visualized by Cytoscape v3.6.0 software. Molecular Complex Detection (MCODE) was used for clustering analysis of the gene network to identify key PPI network modules. The function of MCODE is to select key subnetworks, i.e., modules. A PPI module refers to a PPI that serves a single function. In a module, different genes have different module scores, and key genes can be selected based on the scores. To identify key modules, a p-value < 0.05 was considered to indicate significant differences.

2.6 Gene-miRNA Interaction Network

We used miRWalk 2.0 to predict target key miRNAs and construct related gene-miRNA interaction networks. We intersected the prediction results from the MiRTarBase and miRWalk databases to ensure the accuracy of our results. We screened for miRNAs that target more than two genes.

2.7 Statistical Analysis

GraphPad Prism 7.0 software was used for graphing and statistical analysis. All data are presented as mean ± standard deviation (SD). In DGE analysis, the t-test was used to determine p-values and adjusted p-values, where p-values were adjusted by FDR. A p-value < 0.05 indicated a statistically significant difference.

3. Results

3.1 The microarray expression profile analysis dataset GSE30529 was downloaded from the GEO database, and DEGs were obtained by comparing diseased renal tissues and normal renal tissues of DN. The heatmap and Venn diagram of the DEGs are shown in Figure 1.

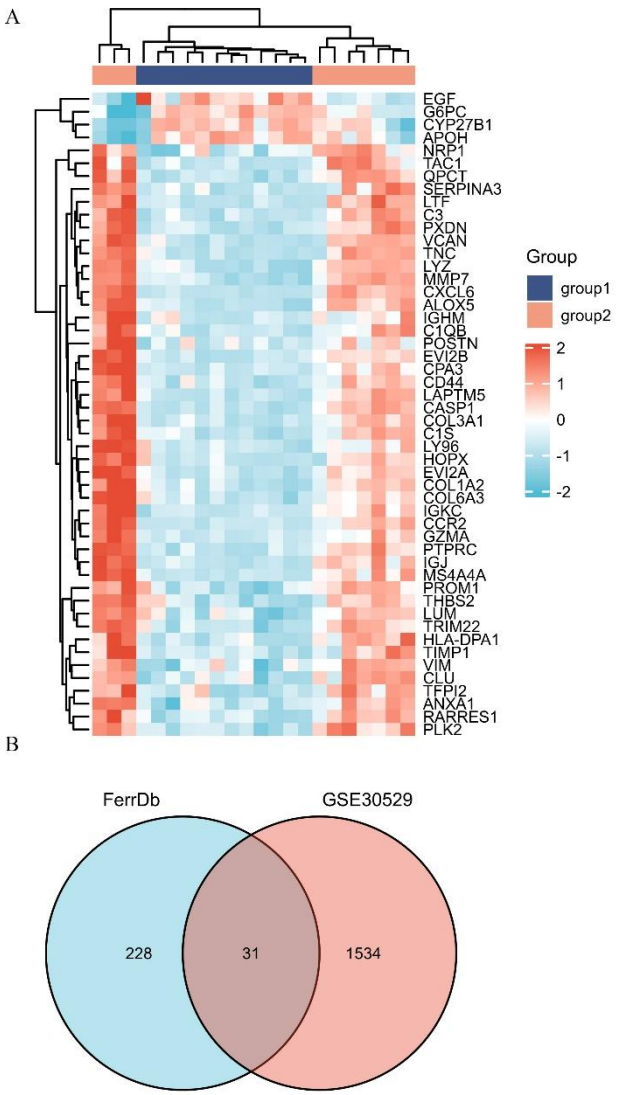


Figure 1. (A) There are 1565 differentially expressed genes (DEGs) in diseased renal tissues and normal renal tissues of DN. The top 50 DEGs are shown in the heatmap, with red representing genes significantly upregulated in the samples and blue representing significantly downregulated genes; (B) Venn diagram of ferroptosis-related DEGs. We intersected the ferroptosis dataset with GSE30529 to identify 31 ferroptosis-related DEGs.

3.2 Enrichment Pathways and Analysis of Ferroptosis-related DEGs

The enrichment analysis of DEGs was conducted using online tools such as Metascape, WebGestalt, and DAVID. Firstly, we uploaded the relevant information of DEGs from both DN-affected and normal kidney tissues containing DN to the

WebGestalt software. The results of the enriched gene dataset analysis indicated that the significantly enriched genes were predominantly involved in metabolic pathways, Epstein-Barr virus infection, proteoglycans in cancer, MAPK signaling pathway, etc. (Figure 2). DAVID was used to analyze the biological pathways and processes of these 31 DEGs in the samples. The KEGG functional analysis revealed that the gene set was significantly activated in areas such as prion diseases, fluid shear stress and atherosclerosis, spinocerebellar ataxia, pancreatic cancer, and protein processing in endoplasmic reticulum (Figure 3). Secondly, the 31 DEGs were uploaded to Metascape, and the results showed that the biological processes were significantly enriched in cellular responses to stress signals, responses to starvation signals, lipids and atherosclerosis, regulation of endogenous apoptotic signaling pathways, etc. Moreover, the biological processes were significantly activated in response to stress (Figure 4). Notably, the endogenous apoptotic signaling pathway was the primary biological pathway involved.

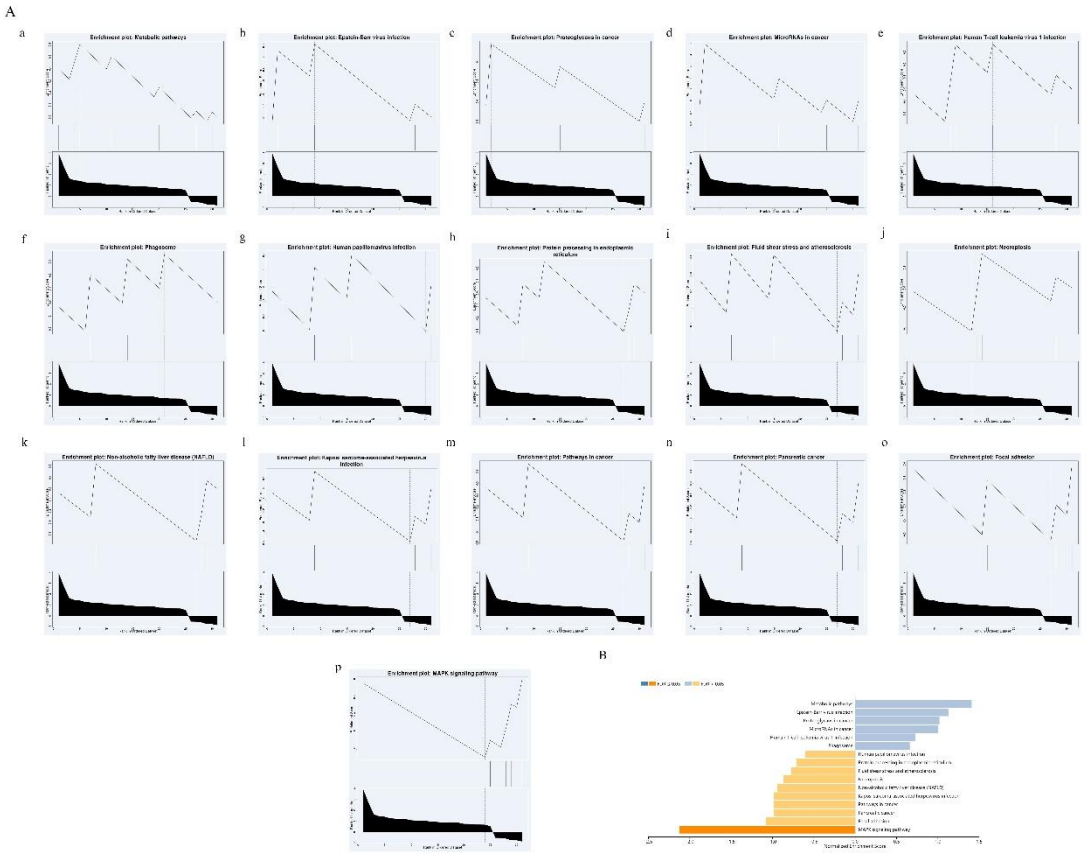


Figure 2. The results of the enriched gene dataset analysis showed that the

significantly enriched genes were mainly involved in the MAPK signaling pathway. The gene set enrichment analysis of WebGestalt first filtered the gene sets based on the number of genes contained, with a default minimum of 7 genes and a maximum of 2000 genes.

ONTOLOGY	Description	GeneRatio	pvalue	qvalue
KEGG	Prion disease	6/27	2.30e-04	0.025
KEGG	Fluid shear stress and atherosclerosis	4/27	0.001	0.044
KEGG	Spinocerebellar ataxia	4/27	0.001	0.044
KEGG	Pancreatic cancer	3/27	0.002	0.051
KEGG	Protein processing in endoplasmic reticulum	4/27	0.002	0.051

Figure 3. The pathway directions that the gene sets may participate in were selected and displayed based on enrichment scores.

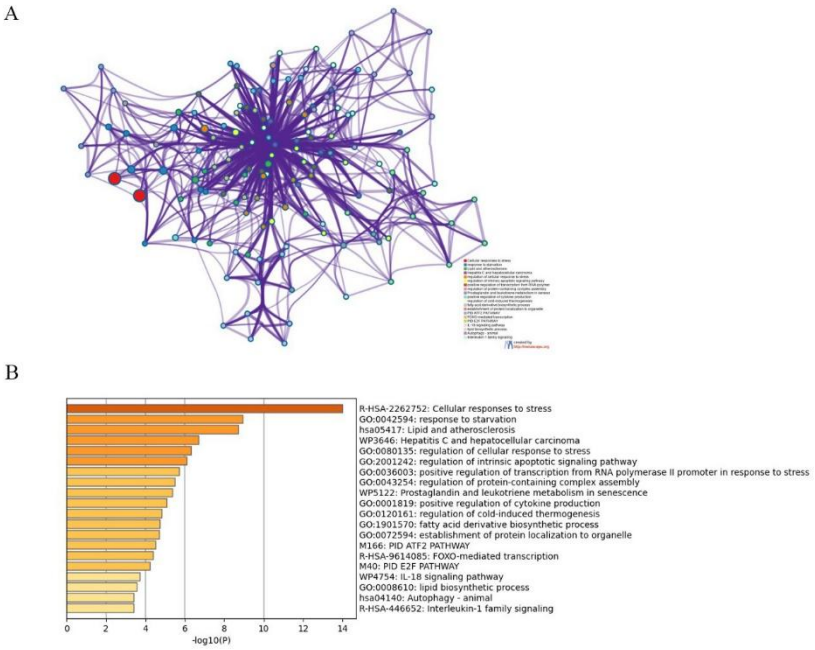


Figure 4. (A) Enrichment network. (B) Metascape plotted a bar chart of 20 biological pathways based on P-values and gene percentages, where biological pathways with P-values < 0.01 were statistically significant.

3.3 Protein-Protein Interaction Network Analysis of Ferroptosis-related DEGs

We obtained a network containing 26 nodes. The network was set to the default cutoff value (interaction score > 0.4) in the STRING online database. Genes are represented by nodes, and interactions between genes are represented by edges. Up-regulated genes are marked in red, while down-regulated genes are marked in blue. MCODE, an application of Cytoscape, was used for cluster analysis of the gene network to identify key modules. One key module was established, consisting of 2 down-regulated genes (MAPK8 and DDIT3) and 3 up-regulated genes (XBP1, HSPA5, and ASNS). These 5 genes are the key genes screened by MCODE. Additionally, functional analysis using Metascape indicated that these 5 genes are mainly involved in protein folding responses in photodynamic therapy-induced starvation responses, endoplasmic reticulum stress responses in coronavirus infections, and cellular responses to starvation (Figure 6).

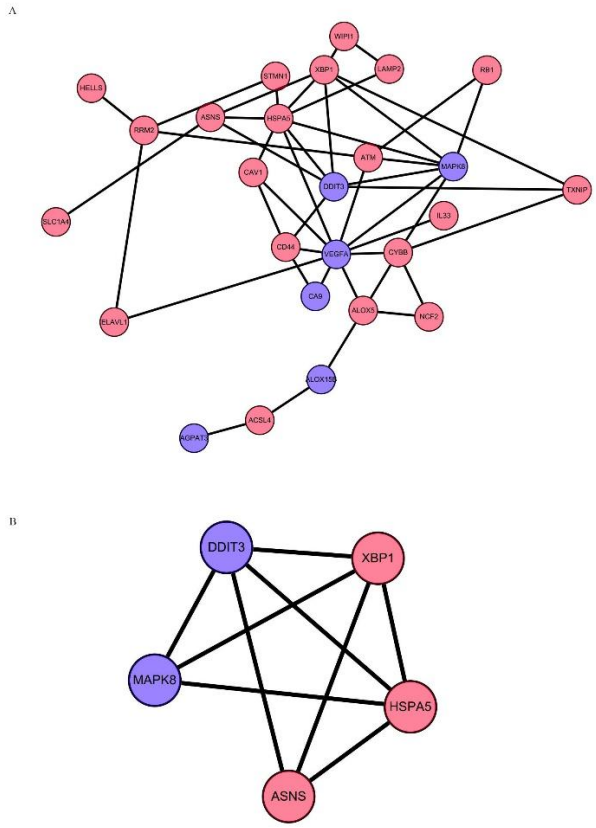


Figure 5. (A) Cytoscape network visualization of 26 nodes obtained from the STRING online database, with an interaction score > 0.4. Nodes represent genes, and edges represent connections between genes. Red represents up-regulated genes, and

blue represents down-regulated genes. (B) MCODE identified one key module for identifying gene clusters in the network.

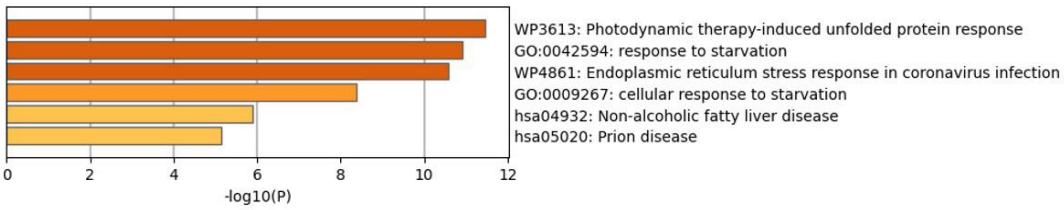
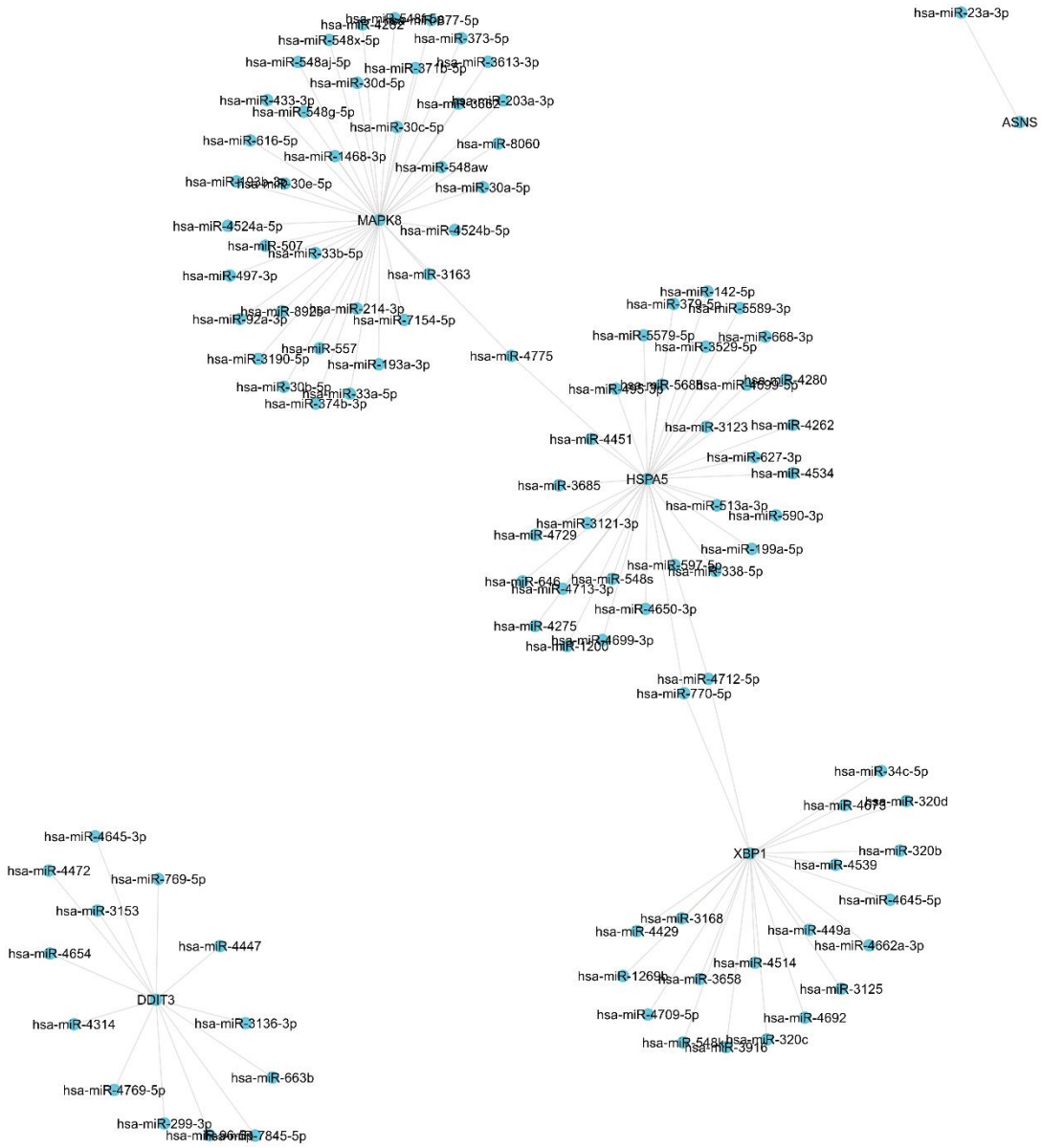


Figure 6. Functional enrichment analysis.

3.4 Further miRNA Interaction and Exploration

We screened the 5 genes in the previous key module and conducted gene-miRNA analysis using miRWalk 2.0 software. The cross-linked miRNAs were selected from the miRWalk and miRTarBase databases to ensure the accuracy and reliability of our results. The following criteria were used to filter the results: $p < 0.05$, seed sequence length > 7, and 3′UTR as the target gene binding region. Therefore, we speculate that the ferroptosis-related key genes MAPK8, has-miR-4775, HSPA5, has-miR-4712-5p/has-miR-770-5p, and XBP1 form a regulatory network, thereby participating in the occurrence and development of diabetic nephropathy (Figure 7). The enrichment analysis results showed that their molecular functions were significantly enriched in the regulation of nucleobase, nucleotide, and nucleic acid metabolism, and cellular signal transduction. The enriched biological pathways include proteoglycan synthesis-mediated signaling events, ErbB receptor signaling network, and IFN-γ signaling pathway (Figure 8).



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210 Figure 7. Interaction network between genes in the key module and their targeted

211 miRNAs.

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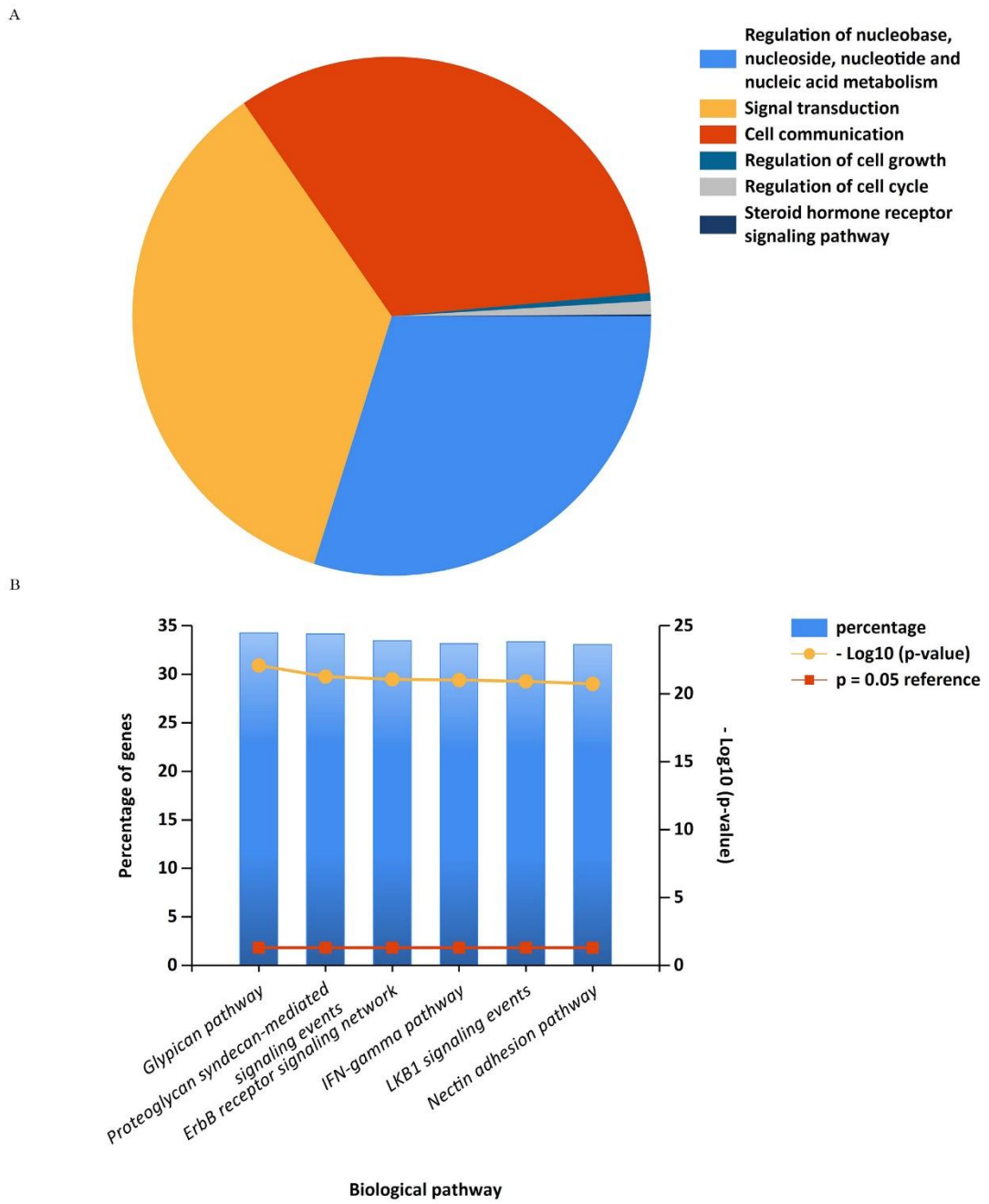

Figure 8. (A) The molecular functions of miRNAs targeting genes in the key module were significantly enriched in the regulation of nucleobase, nucleotide, and nucleic acid metabolism, and cellular signal transduction. (B) Biological pathways were enriched in proteoglycan synthesis-mediated signaling events, ErbB receptor signaling network, and IFN-γ signaling pathway.

4 Discussion

This study identified key genes involved in ferroptosis and further explored the possible mechanisms related to ferroptosis in DN. Our study obtained 31 DEGs from the intersection of dataset GSE30529 and ferroptosis-related DEGs. Then, we used online tools such as Metascape, GSEA, and DAVID to conduct GSEA enrichment analysis of DEGs. The results showed that the biological processes of these genes were significantly enriched in cellular responses to stress signals, responses to starvation signals, lipids and atherosclerosis (free radicals, etc.), and regulation of endogenous apoptotic signaling pathways. Moreover, these biological processes were significantly activated in response to stress. Additionally, the regulatory network of the MAPK8 molecule may affect signaling pathway changes in DN. The study also identified several genes that have not been mentioned in the field of DN and ferroptosis. This study can provide an effective reference for the pathological mechanism of DN from the perspective of bioinformatics analysis.

Ferroptosis is characterized by the intracellular accumulation of lipid ROS, which are closely related and ultimately lead to lipid oxidation, causing cell membrane damage and cell death. Ferroptosis is associated with oxidative stress generated by excessive accumulation of ROS during aerobic metabolism [14]. After oxidative stress, some signaling pathways are activated, such as the MAPK pathway. According to previous studies, the MAPK pathway may induce the production of free radicals after DN, which can induce ferroptosis and apoptosis [15-16]. In this context, antioxidant therapy may reduce apoptosis after the occurrence of DN. A previous study showed that the MAPK pathway is activated after iron accumulation, and inhibiting MAPK activation can improve functional outcomes and reduce cell death [17]. Therefore, along with the induction of ferroptosis, the MAPK signaling pathway may be activated to promote the production of ROS, exacerbate cell damage, and lead to a vicious cycle.

The endogenous apoptotic signaling pathway is the primary biological pathway involved. Glomerular cell apoptosis is directly related to hemoglobin A1c and systolic blood pressure, while tubular cell apoptosis is related to the duration of diabetes and

low-density lipoprotein cholesterol. Enhanced expression of Fas, Fas ligand, and p38 mitogen-activated protein kinase in glomeruli and tubules suggests that apoptosis may be a clinically relevant mechanism for the loss of glomerular and tubular cells in type 2 diabetic patients [18]. Diabetic nephropathy is associated with glomerulosclerosis and impaired renal perfusion. Increasing capillary formation and improving perfusion may help halt or reverse damage. Transplanting anti-apoptotic p53-silenced endothelial progenitor cells (p53sh-EPCs) may help improve angiogenesis and renal perfusion and may be more beneficial than another type of stem cell, such as mouse mesenchymal stromal cells (mMSCs) [19]. Therefore, the apoptotic signaling pathway may play a crucial role in ferroptosis after DN.

In our study, we identified a crucial module containing two downregulated genes (MAPK8 and DDIT3) and three upregulated genes (XBP1, HSPA5, and ASNS) using MCODE. Furthermore, we conducted gene-miRNA analysis on these genes using miRWalk 2.0 software. Based on our findings, we hypothesize that the ferroptosis-related key genes MAPK8, has-miR-4775, HSPA5, has-miR-4712-5p/has-miR-770-5p, and XBP1 interact to form a regulatory network, thereby participating in the initiation and progression of diabetic nephropathy (DN). Previous studies have shown that myriocin, an active component of Cordyceps sinensis, may exert effects on DN through various targets such as MAPK8 and TP53, which could facilitate the development of innovative therapeutic drugs for DN [20]. Immunohistochemical analysis of renal biopsy samples from DN patients confirmed the upregulation of HSPA5 protein in renal tubular epithelial cells. These cells undergo endoplasmic reticulum (ER) stress, and HSPA5 may induce an adaptive and protective unfolded protein response. While this can protect cells from ER stress, the persistent presence of hyperglycemia and proteinuria may ultimately lead to apoptosis [21]. Wang et al. [22] also demonstrated that XBP1 inhibits glomerular mesangial cell apoptosis in response to oxidative stress through the PTEN/AKT pathway in DN. Madhusudhan [23] proposed that signaling through the insulin receptor, p85, and XBP1 maintains podocyte homeostasis, and disruption of this pathway impairs podocyte function in

DN. miRNAs are endogenous non-coding RNA molecules that target the 3′UTR region of genes, regulating gene expression by degrading or inhibiting the translation of target genes [24]. In our study, we ultimately identified miR-4775, miR-4712-5p, and miR-770-5p as potentially relevant miRNAs. Therefore, the hypothesis derived from our data analysis, suggesting that the ferroptosis-related key genes MAPK8, has-miR-4775, HSPA5, has-miR-4712-5p/has-miR-770-5p, and XBP1 form a regulatory network involved in the initiation and progression of DN, holds a certain degree of reliability. This provides important insights for our future fundamental research and validation efforts, and also suggests a potential target for the development of therapeutic strategies for DN.

The current research has identified several genes closely associated with ferroptosis in the pathogenesis of DN, some of which have not been previously mentioned in the context of DN. These novel genes offer new perspectives on ferroptosis in DN and may provide new therapeutic targets for this process. The identification of these genes suggests that the regulatory network of MAPK8 molecules may be involved in the pathogenesis of DN-related ferroptosis.

Conclusion

In summary, this study employed bioinformatics analysis to identify potential ferroptosis-related key genes in the pathogenesis of diabetic nephropathy, thereby providing new targets for the treatment of DN. The ferroptosis-related key genes MAPK8, has-miR-4775, HSPA5, has-miR-4712-5p/has-miR-770-5p, and XBP1 interact to form a regulatory network that participates in the initiation and progression of DN. This provides important references for our future fundamental research validation efforts and suggests a potential target for the development of therapeutic strategies for DN.

Declaration

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets generated and/or analyzed during the current study are available in the [GSE30529] repository, [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30529].

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contribution

All authors contributed to this present work: [SQZ] and [FZ] designed the study. [SQZ] and [FZ] collected and analyzed data. [SQZ] and [FZ] drafted the manuscript. [SQZ] and [FZ] reviewed and revised the manuscript. The manuscript has been approved by all authors for publication.

Acknowledgement

None

Abbreviation

DN, Diabetic Nephropathy

ROS, Reactive Oxygen Species

iNOS, inducible nitric oxide synthase

Slc7a11,Solute Carrier Family 7 Member 11

MAPK, Mitogen-Activated Protein Kinase

DEGs, Differentially Expressed Genes

DGE, Differential Gene Expression

GSEA, Gene Set Enrichment Analysis

KEGG, Kyoto Encyclopedia of Genes and Genomes

PPIs, Protein-Protein Interactions

MCODE, Molecular Complex Detection

miRNAs, microRNAs

ERK, Extracellular signal-Regulated Kinase

p38 MAPK, p38 Mitogen-Activated Protein Kinase

PTEN, Phosphatase and Tensin Homolog

AKT, AKT Serine/Threonine Kinase

ER, Endoplasmic Reticulum

GSE, Gene Expression Omnibus Series

FDR, False Discovery Rate

DAVID, Database for Annotation, Visualization and Integrated Discovery

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