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ORIGINAL RESEARCH

The Mitochondrial Metabolism Gene ECH1 Was Identified as a Novel Biomarker for Diabetic Nephropathy: Using Bioinformatics Analysis and Experimental Confirmation

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Background: Diabetic nephropathy (DN) is a major cause of kidney failure, and its incidence is increasing worldwide. Existing studies have shown that mitochondrial dysfunction is potentially related to the pathogenesis of DN. This study aims to explore novel biomarkers related to mitochondrial metabolism that may affect the diagnosis and treatment of DN.

Methods: The Gene Expression Omnibus (GEO) database and MitoCarta3.0 database were used to download the DN datasets and mitochondrial metabolism-related genes (MRGs), respectively. Differentially expressed genes (DEGs) were identified using the "limma" R package, and their functional analysis was performed through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Important gene modules were identified by weighted gene Coexpression network analysis (WGCNA) clustering. Next, we obtained key genes by intersecting DEGs, important gene modules and MRGs. The ROC curve was employed to assess the sensitivity and specificity of the diagnostic indicators for DN. Finally, the expression of key genes was assessed in the in vitro DN model and the mechanisms of key gene were investigated.

Results: A total of 343 DEGs were identified, with functional analysis revealing a primary focus on metabolic biological processes. A sum of 752 important module genes was ascertained. PDK4, ECH1, and ETFB were selected as key genes. Then, the expression level and specificity of key genes were verified by the GSE104954 dataset, which confirmed the high diagnostic value of PDK4 and ECH1 (AUC>0.9). Finally, the q-PCR, flow cytometry, and Western blot results indicated that key genes were significantly decreased in high glucose induced HK-2 cells. ECH1 could promote fatty acid oxidation and inhibit cell apoptosis, oxidative stress, and inflammation.

Conclusion: This study identified biomarkers related to mitochondrial metabolism in DN, providing new insights and directions for the diagnosis and treatment of DN.

Keywords: ECH1, diabetic nephropathy, DN, mitochondrial metabolism

Introduction

Diabetic nephropathy (DN) is characterized by proteinuria, hypertension, and a gradual loss of kidney function, making it a leading cause of end-stage renal disease and imposing significant social and economic burdens.[1–3] Statistics indicate that as global diabetes cases rise from 537 million to an expected 783 million in the next 20 years, the number of patients with DN is also increasing.[4] Currently, the primary treatments for DN include renin-angiotensin system blockade, blood pressure management, and glycemic control, but these therapeutic approaches can not fully eliminate the residual risks of DN.[5] The molecular mechanism of DN is extremely complex, with the characteristics of multifaceted, multistage, and multigenic. Numerous studies have demonstrated that changes in the renal microenvironment, genetics, and epigenetic factors are involved in the pathogenesis of DN.[6] Therefore, new biomarkers for DN are urgently required.

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Increasing evidence indicates that mitochondrial dysfunction significantly contributes to the onset and progression of DN. As the kidney is a highly metabolic organ rich in mitochondria, it is particularly sensitive to mitochondrial dysfunction.[7] Notably, mitochondrial dysfunction can accelerate the progression of chronic kidney disease.[8] High blood sugar levels directly damage renal tubular cells, resulting in extensive metabolic and cellular dysfunction.[9] Although renal tubular cells contain the highest levels of mitochondria, podocytes, mesangial cells, and glomerular endothelial cells may also be impacted by diabetes-induced mitochondrial damage.[10–12] Additionally, excessive production of reactive oxygen species (ROS), activation of apoptosis pathways, and defects in mitochondrial autophagy are key mechanisms involved in the progression of DN.[13] In summary, understanding the pathological biology of mitochondrial dysfunction in DN will facilitate the development of novel therapeutic strategies.

In the current investigation, to investigate the potential of mitochondrial metabolism-related genes (MRGs) as diagnostic markers and therapeutic targets in DN patients, we obtained DN-related microarray datasets from the GEO database and identified DEGs. Functional annotations of the DEGs were performed using GO and KEGG pathway enrichment analyses. Subsequently, WGCNA was employed to identify gene modules closely associated with DN. By intersecting DEGs, module genes, and MRGs, the key genes linked to mitochondrial metabolism in DN were screened. ROC curves were plotted to assess the sensitivity and specificity of these key genes for disease diagnosis. Validation was conducted using additional GEO datasets and in vitro experiments. In conclusion, our study highlights potential biomarkers related to mitochondrial metabolism in DN, offering new insights for its diagnosis and treatment.

Materials and Methods

Data Acquisition

DN microarray datasets were collected and downloaded from the Gene Expression Omnibus (GEO) database. GSE96804 based on the GPL17586 platform was used as a training set which contained 41 DN patients and 20 control samples,[14] and GSE104954 based on the GPL22945 platform served as validation set, which contained 7 DN patients and 18 control samples.[15] Human mitochondrial metabolism-related genes (MRGs) were downloaded from the MitoCarta3.0 database,[16] a professional mammalian mitochondrial protein database.

Acquisition of DEGs and the Construction of Co-Expression Networks

The R package "limma"[17] was employed to discern differentially expressed genes (DEGs) between the DN and control group, with the cutoff as $p<0.05$ and $|logFC|>1$. The volcano map of DEGs was plotted using the "ggplot2" R package.

Weighted Gene Go-expression Network Analysis (WGCNA) was used to identify gene modules that are highly associated with the disease and determine candidate biomarkers for the disease. The "WGCNA" R package[18] was utilized to identify the genes most associated with DN.

Functional Enrichment Analysis

KEGG pathway enrichment analysis is a popular approach to identify pathways or sets of genes which are significantly enriched in the context of DEGs.[19] GO is an internationally standardized classification system for gene function, which helps explain the pathogenesis of diseases.[20,21] In this study, the R package "clusterProfiler"[22] was utilized for functional analysis of DEGs.

Assessment of Key Genes Diagnostic Value

The diagnostic performance of candidate genes was evaluated using the Area Under the Curve (AUC) from ROC curve analysis in GSE96804 and GSE104954, employing the "pROC" R package to generate the curves.[23]

Cell Culture and Treatment

HK-2 cells (ATCC®, USA) were cultured in RPMI-1640 medium (Procell, China) supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin, at 37°C in a 10% CO_2 atmosphere.[24] Cells in the logarithmic

phase were used for subsequent experiments. Cells were treated with either 5.5 mm glucose as the control or 25.5 mm glucose for the high glucose (HG) treatment.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from treated HK-2 cells using TRIzol reagent (Invitrogen, USA), and cDNA was synthesized by PrimeScript™ RT Master Mix (Takara, Japan.) and quantitative real-time PCR was conducted with SYBR Premix Ex TaqII (Takara).[25] The thermocycling conditions of PCR amplification consisted of an initial denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 30 seconds and extension at 70°C for 30 seconds. The results were calculated using the $2^{-\Delta\Delta Ct}$ method. The following primers were used: PDK4: F: AATGAGCAGCGGAGTCCAAACTCTT; R: CAAGCTGGGCTTAAGATTAGCCTCTT. ECH1: F: ATAGTGGCTTCTCGCAGACTC; R: CAGTGAGGCGAAGGCTAATAC. ETFB: F: GGTCACGGATGGTGTGAAG; R: GCGGTACGAATCGTCTCCTG.

Plasmid Construction

After being amplified by PCR, ECH1 gene was inserted into the pcDNA3.1 vector. Then the ECH1 overexpression plasmid was constructed and was termed as OE-ECH1. The empty plasmid was termed as OE-NC. OE-ECH1 and OE-NC were transfected into HK-2 cells using Effectene transfection reagent (QIAGEN) following the manufacturer's instructions.

Cell Viability Assay

HK-2 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and treated with normal glucose (5.5 mm) and high glucose (25.5 mm). Following incubation, cell viability was assessed using the Cell Counting Kit 8 (CCK8, Yeasen, China). Ten microliters of CCK8 solution were added to each well and incubated at 37°C for 3 hours. The absorbance was measured at 450 nm using an enzymatic reader.

Western Blot (WB) Analysis

Cells were lysed with RIPA lysis buffer (Betotime) and sonication, and total protein concentration was measured using an enhanced BCA assay kit (Betotime). Protein samples (30 μg/lane) were separated using 8–15% sodium dodecyl sulfate-polyacrylamide gels and transferred to PVDF membranes. After the blots were blocked in 5% milk for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C and incubated with HRP-conjugated anti-rabbit secondary antibody for 2 h at room temperature. The protein bands were visualized using ECL and analyzed with Image software. The following antibodies (Abclonal, China) were used: anti-PDK4 (A13337), anti-ECH1 (A12944), anti-ETFB (A16043), anti-BAX (A0207), anti-Bcl-2 (AP0314), and anti-Caspase-3 (A2156).

Measurement of ROS Generation

5.6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, USA) and Rosamine-based MitoTracker probe (MitoTracker Red CM-H_2XROS, USA) were used to assess intracellular and mitochondrial ROS levels.[26] After PBS washing, cells were incubated with 10 μM CM-H_2DCFH-DA or 0.2 μM CM-H_2XROS at 37°C for 30 min. Images were visualized and analyzed using a laser scanning microscope (LSM 510, Carl Zeiss) and Image software, with magnifications of x200 or x400.

Determination of Mitochondrial Membrane Potential (MMP)

MMP was measured using the JC-1 kit (Biyuntian, China) by incubating cells with the JC-1 working solution at 37 °C for 20 min in the dark.[27] After two washes with cold JC-1 staining buffer, the images were observed under a fluorescence microscope. Normal cells displayed red J-aggregate emission (Ex/Em=525/590nm), while a decrease in membrane potential led to the production of JC-1 monomers (Ex/Em=490/530nm), resulting in green emission.

Assessment of Fatty Acid Oxidation (FAO)

FAO activity was measured using the fatty acid oxidation detection reagent FAOBlue (Funakoshi, Japan). FAOBlue was dissolved in fresh HEPES buffer (HBS) to the final concentration. After removing the medium, cells were washed twice with HBS, and then cultured in HBS containing FAOBlue at 37°C for 1 h. The fluorescence was observed via a microscope (Ex. 405 nm/Em. 450 nm).

Detection of Malondialdehyde (MDA) and Superoxide Dismutase (SOD) Level

The SOD and MDA levels in HK-2 cells were measured using a lipid peroxidation MDA assay kit and a SOD assay kit (Beyotime Biotechnology, China),[28] following the manufacturer's instructions. The absorbance was measured with a microplate reader (Benchmark; Bio-Rad Laboratories, Inc).

ELISA Detection

Cells were plated in 6-well plates (8×10^4 cells/mL) and incubated for 24h. After incubation, the cell supernatant was collected. Then IL1β, TNF-α and TGF-β were measured by ELISA kits according to the manufacturer's instructions.[29] The absorbance at 450 nm and 570 nm were detected using a microplate reader.

Statistical Analysis

Statistical analysis in the bioinformatics analyses was conducted using R software (version 4.4.1), and statistical analyses in cell experiments were performed with GraphPad Prism 9.3 (GraphPad Software, San Diego, CA, USA). The differences among multiple groups were compared via one-way ANOVA. The P-value < 0.05 indicated statistical significance.

Results

Characterization of DEGs and Functional Annotation

A total of 343 DEGs were screened between the DN patients and 20 control samples. Among these, 121 genes exhibited significant upregulation, while 222 genes displayed significant downregulation (Figure 1A).

We conducted KEGG and GO functional annotation on the DEGs. KEGG analysis demonstrated that DEGs were significantly enriched in protein digestion and absorption, drug metabolism, amino acid metabolism, fatty acid degradation, AGE-RAGE signaling and other pathways (Figure 1B). The GO analysis indicated that the DEGs primarily

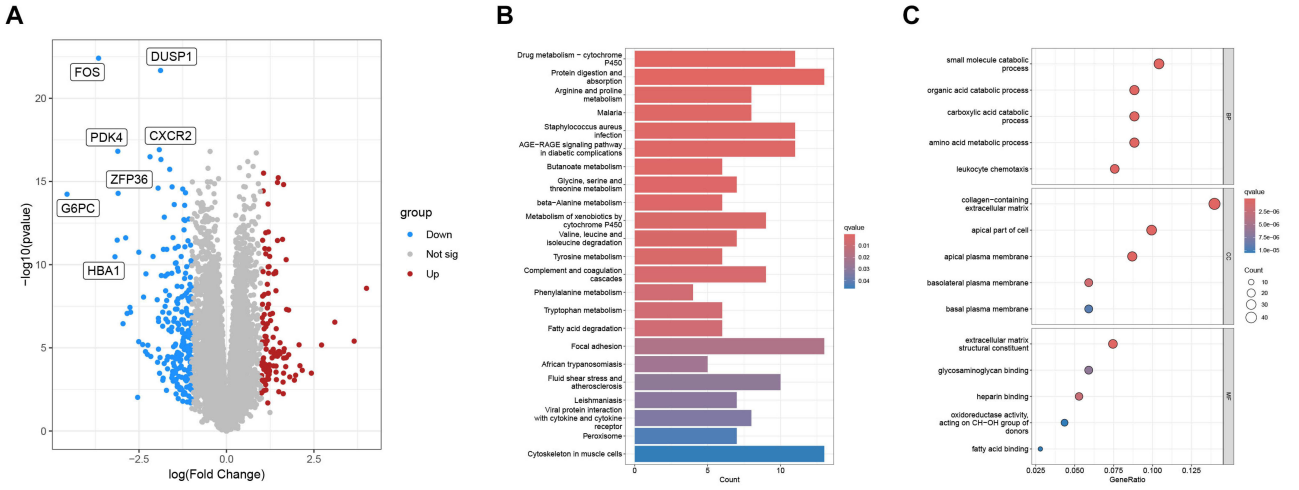

Figure 1 Identification of DEGs and functional enrichment analysis of DEGs. (**A**) Volcano map of DEGs in GSE96804. (**B**) Results of KEGG enrichment analysis based on DEGs. (**C**) Results of GO analysis based on DEGs.

involved small molecule metabolic processes, including fatty acid, organic acids, and amino acid metabolism (Figure 1C). Overall, these findings suggest that DEGs were mainly linked to metabolic biological processes.

Identification of Module Genes Based on WGCNA

A co-expression network was constructed between the DN and control group using an optimal soft threshold of β=10 (Figure 2A). Dynamic hybrid cutting revealed 11 distinct modules (Figure 2B). Pearson correlation coefficients and significance levels for each module against clinical traits were calculated and displayed in a heatmap (Figure 2C). Modules with an absolute correlation value over 0.8 were selected for further analysis. The green, yellow, and pink modules showed a strong correlation with DN (Figure 2D-F), leading to their classification as DN-related modules, with 732 genes identified as DN-related genes.

Screening of Key Genes in DN and Identification of Diagnostic Value

Three key genes (PDK4, ECH1, and ETFB) were identified through intersections with DEGs, DN-related genes, and MRGs (Figure 3A). Subsequently, we analyzed the expression patterns of these MRGs in DN and control samples in the training and validation sets, revealing a consistent downward trend for PDK4, ECH1, and ETFB in DN (Figure 3B and C). To assess their sensitivity and specificity for DN diagnosis, ROC curves and AUC values were utilized. The AUC values for PDK4, ECH1, and ETFB were 0.982, 0.927, and 0.933 in the training set, and 1, 0.905, 0.595 in the validation set (Figure 3D and E). These results indicated that PDK4 and ECH1 possessed strong diagnostic potential for DN, while the diagnostic ability of ETFB for DN required more data verification.

Expression of Key Genes and Effects of OE-ECH1 on Key Genes

To replicate the renal tubular cell environment in DN patients, high glucose was used to induce HK-2 cells. CCK-8 assays indicated that HK-2 cells activity decreased in HG group (Figure 4A). Subsequently we employed q-PCR and WB to assess the expression of key genes in control and HG group (Figure 4B-D, 4H-K). The results indicated down-regulation of PDK4, ECH1, and ETFB in HG, aligning with previous data analyses.

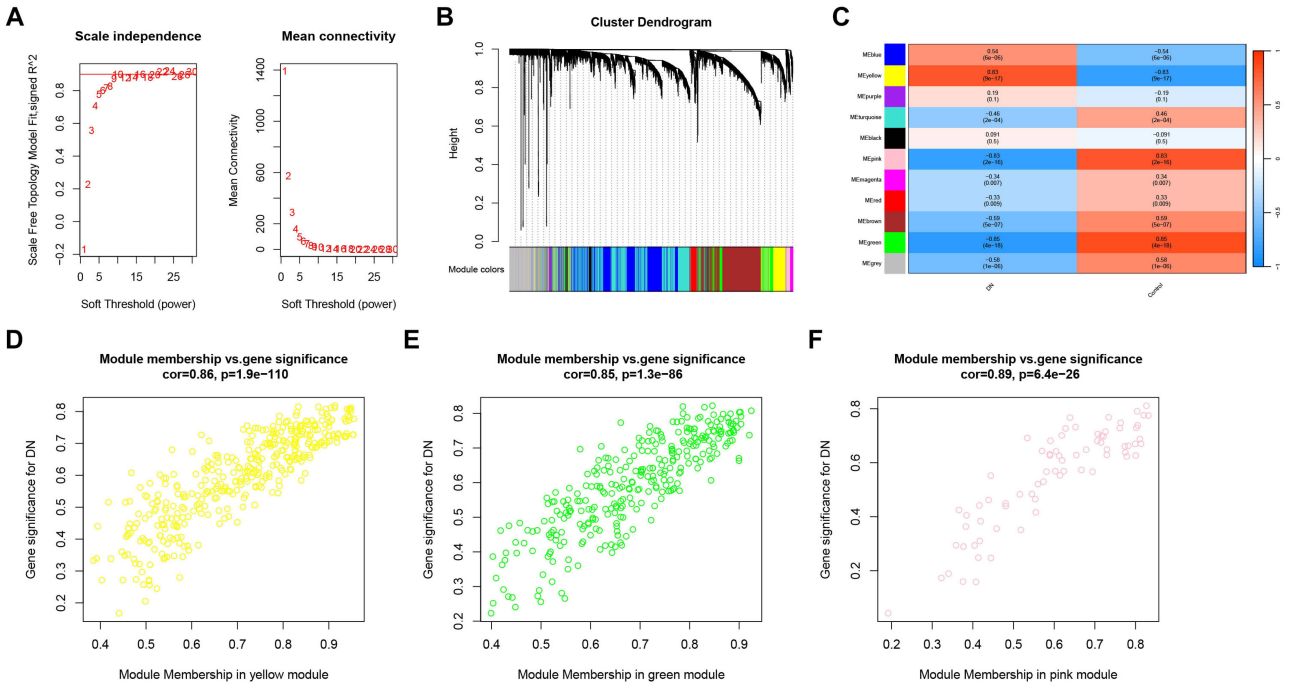

Figure 2 Construction of coexpression gene module based on WGCNA algorithm. (**A**) Determining the soft threshold of GSE96804. (**B**) Clustering tree of origin and merge gene module in GSE96804. (**C**)Correlation heatmap of DN occurrence and modular characteristic genes. (**D-F**) Dotplot of module membership in yellow, green and pink modules.

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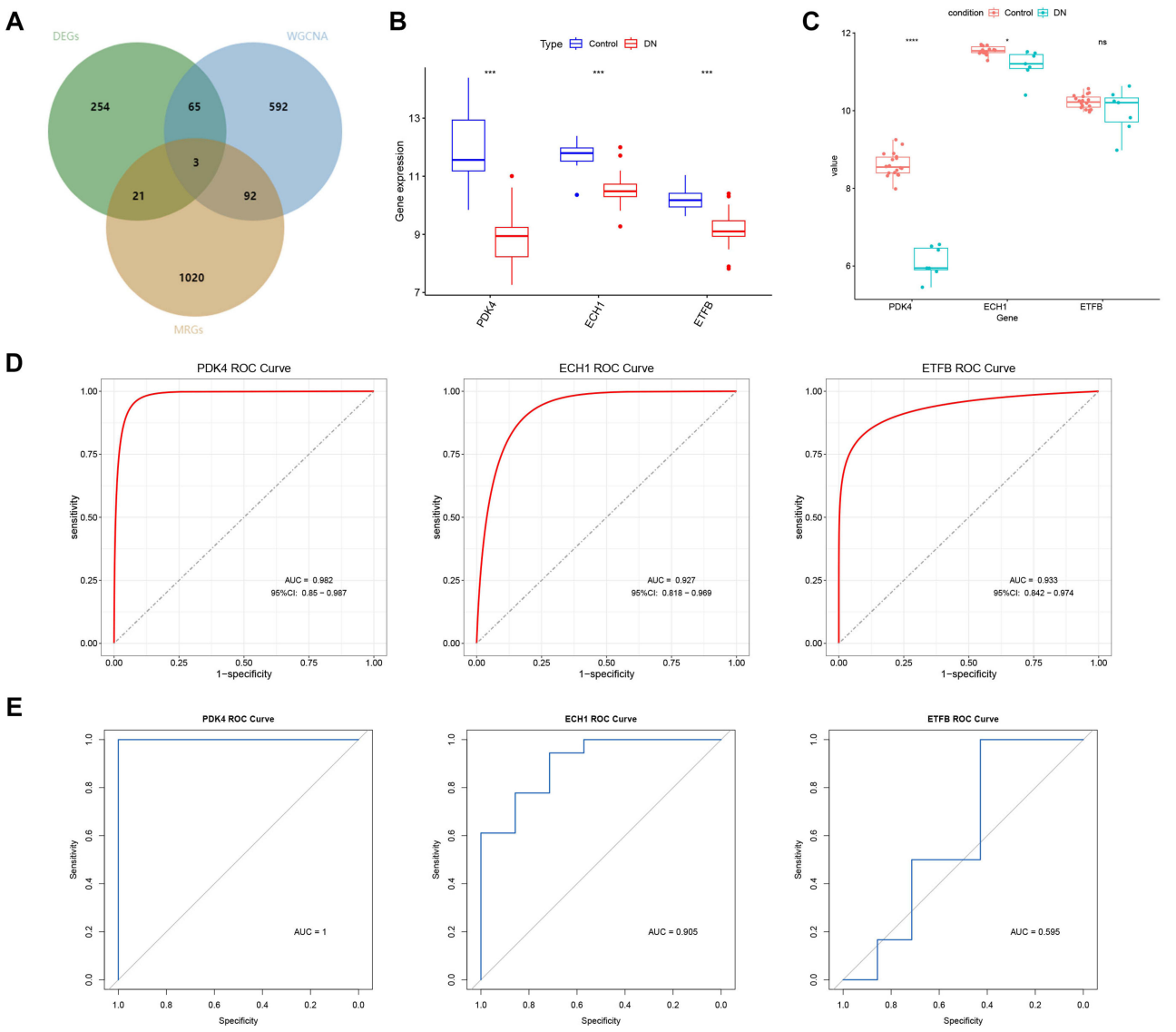

Figure 3 Screening of key genes and analysis and validation of their diagnostic value. (**A**)Venn map of DEGs, the WGCNA module genes and MRGs. (**B**) The expression of key genes in DN and control group in GSE96804. (**C**) The expression of key genes in DN and control group in GSE104954. (**D**) ROC curves of key genes for predicting DN in GSE96804. (**E**)ROC curves of key genes for predicting DN in GSE104954. *p<0.05, ***p<0.001, ****p<0.0001, ns represented p>0.05.

Prior studies have established that deficient PDK4 leads to abnormal glucose and lipid metabolism, contributing to DN,[30–32] while ETFB deficiency is also known to induce kidney damage.[33] However, ECH1's specific role in DN has yet to be reported. Consequently, we concentrated on analyzing ECH1. The q-PCR and WB analyses demonstrated strong transfection efficacy with the OE-ECH1 (Figure 4E-G). Additionally, OE-ECH1 significantly upregulated ECH1 expression only in the HG group and did not affect PDK4 or ETFB expression (Figure 4H-K).

ECH1 Enhanced FAO and Supported Mitochondrial Activity

ECH1 is a crucial enzyme in the β-oxidation of unsaturated fatty acids.[34] These fluorescence experiments confirmed that ECH1 enhanced fatty acid oxidation in the HG group (Figure 5A). The findings indicated a significant increase in FAO activity in HG after introducing OE-ECH1, whereas HG and HG+OE-NC exhibited notably lower activity. Given that mitochondria were the primary site of fatty acid oxidation, we employed the JC-1 reagent to assess mitochondrial activity under various conditions (Figure 5B). The results revealed that most mitochondria in HG and HG+OE-NC had lost

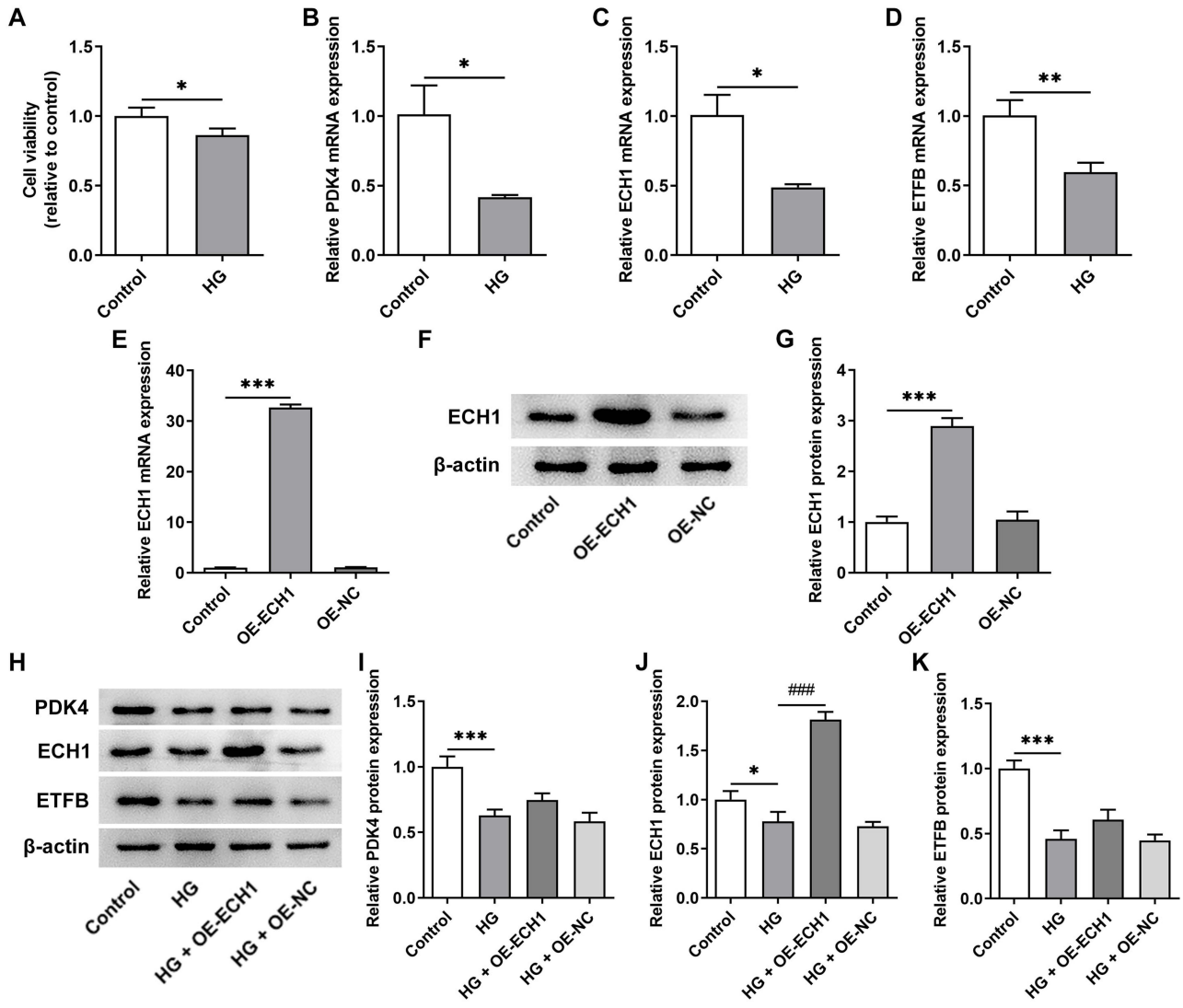

Figure 4 Experimental verification of key gene expression and analysis of the influence of OE-ECH1 on key genes. (**A**)Cell viability was assessed using the CCK-8 assay. (**B-D**) The gene expression of PDK4, ECH1 and ETFB. (**E-G**) Effects of OE-ECH1 on ECH1 gene expression and protein levels. (**H-K**) Effects of OE-ECH1 on protein levels of key genes. *p<0.05, **p<0.001, ***p<0.0001 versus control. ###p<0.0001 versus HG.

activity, while only a few in HG+OE-ECH1 did. These results demonstrated that ECH1 promoted FAO in high glucose HK-2 cells and maintained mitochondrial homeostasis.

ECH1 Reduced Oxidative Stress, Apoptosis, and Inflammation in HG Group

Increased oxidative stress in diabetic patients can lead to endothelial cell apoptosis, inflammation, autophagy, and fibrosis, resulting in abnormal renal tissue and function, ultimately causing renal damage.[35] To investigate the role of ECH1 in DN, we examined the relationship between OE-ECH1 and oxidative stress, apoptosis, and inflammation in HK-2 cells. The results showed that oxidative stress was increased in HG group, while OE-ECH1 reduced levels of ROS and malondialdehyde (MDA), but did not affect superoxide dismutase (SOD)(Figure 6H-J). Additionally, the pro-apoptotic protein Bax and the apoptosis execution protein Casp3 were significantly elevated, whereas the anti-apoptotic protein Bcl-2 was decreased in HG group, while OE-ECH1 promoted Bcl-2 and inhibited Bax and Casp3 (Figure 6A-D). Furthermore, levels of inflammatory factors (IL-1β, TNF-α, and TGF-β) were significantly increased in HG group, and OE-ECH1 suppressed their expression (Figure 6E-G). These findings suggested that ECH1 could mitigate oxidative stress, apoptosis, and inflammatory responses in high glucose HK-2 cells. Notably, the activation of oxidative stress,

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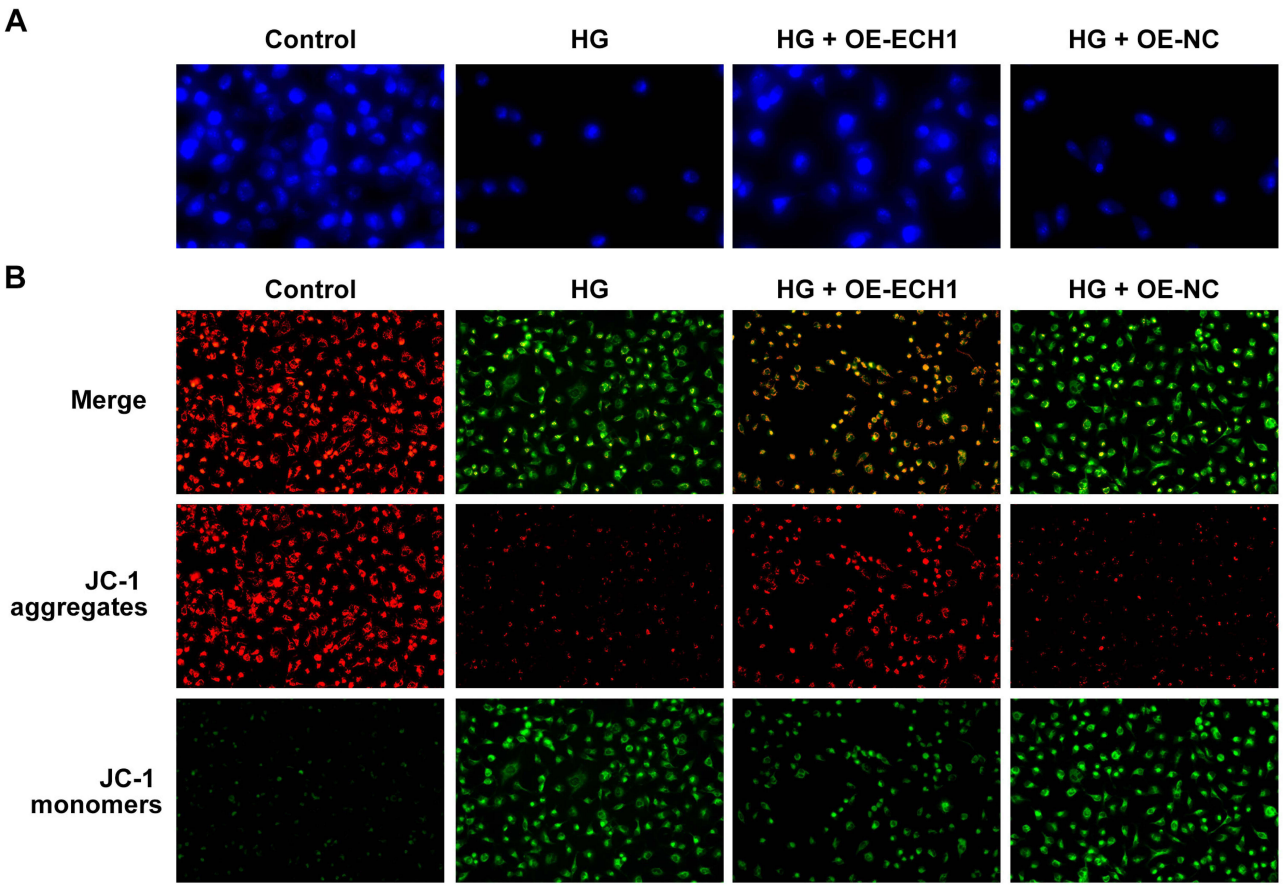

Figure 5 Effects of OE-ECH1 on fatty acid oxidation and mitochondrial activity. (**A**) The stronger the blue fluorescence, the stronger the oxidation activity of fatty acids. (**B**) Red fluorescence indicated polymer formation by JC-1 in mitochondria, while green fluorescence signified JC-1 monomers in mitochondria.

apoptotic protein expression, and inflammatory factor levels in the HG+OE-ECH1 group differed significantly from those in the control group, indicating the involvement of other genes or factors in regulating these processes in high glucose HK-2 cells.

Discussion

DN is a kidney disease that affects more than 100 million people worldwide and has become the leading cause of renal failure. Although the pathogenesis of DN is currently understood to a certain extent, the complex molecular mechanisms of its occurrence and progression have not been fully explained, resulting in limited treatment options for DN.[10,36,37] Previous studies have shown that damage to mitochondrial structure and function plays a key role in the onset and development of DN.[38,39] This discovery offers a theoretical basis for researchers to use bioinformatics technology to explore new DN diagnostic and therapeutic targets.

In this study, we obtained renal tissue microarray data of DN patients and healthy samples from the GEO database and identified DEGs. The DEGs were subjected to GO and KEGG enrichment analysis. DN-related module genes were screened using WGCNA, and three key MRGs (PDK4, ECH1, and ETFB) for DN were obtained by taking the intersection of DEGs, DN-related module genes, and MRGs. We further validated the key genes in HK-2 cells in vitro, and the results showed that the expression of the three key genes was significantly reduced in the HG group, which was consistent with the transcriptional results. However, among the key MRGs, only PDK4 and ECH1 showed good diagnostic efficacy for DN in both the training set and the validation set (AUC>0.9). Since some studies have shown that PDK4 is a key gene for DN,[40] but there is a lack of research on the relationship between ECH1 and DN, this

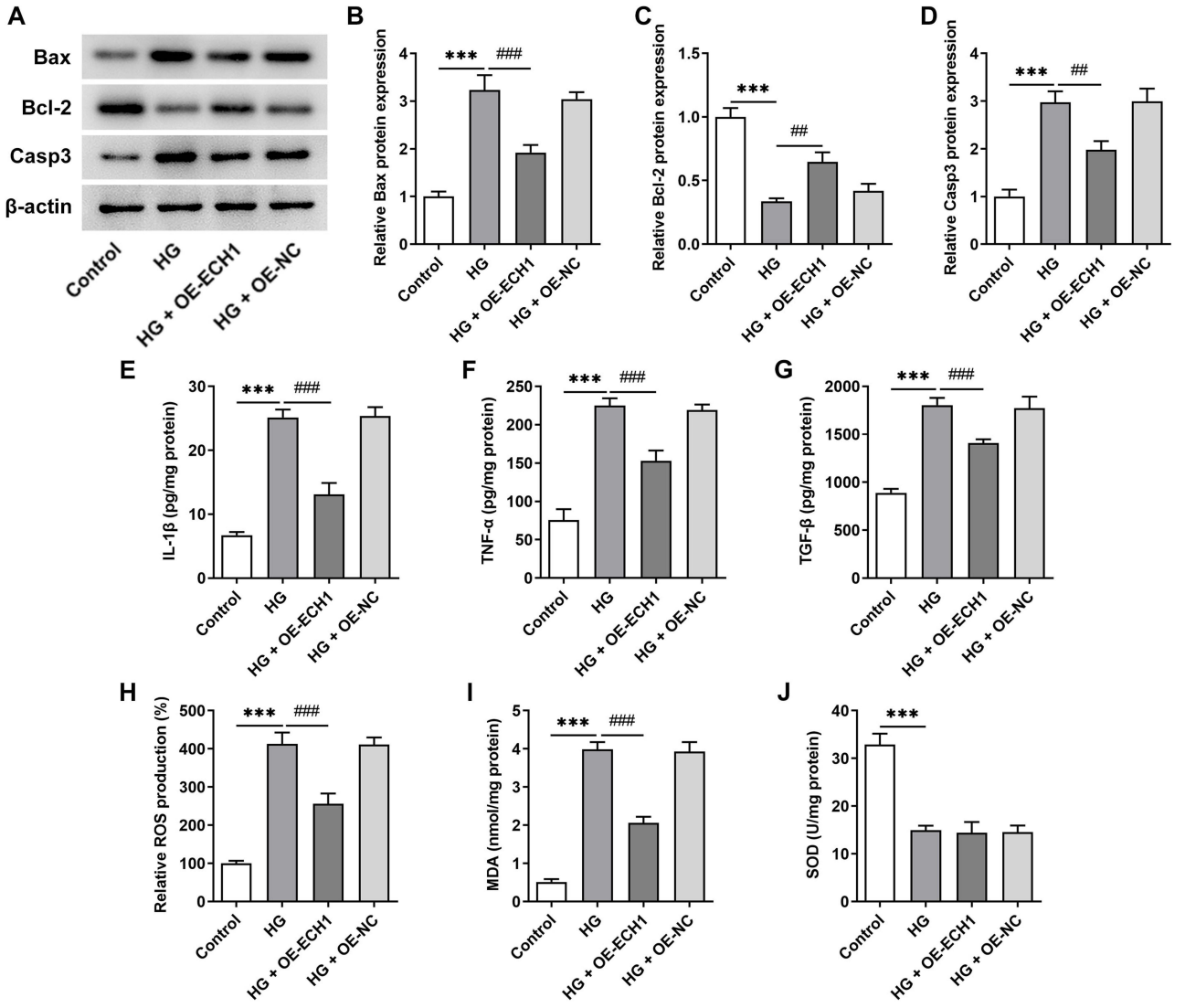

Figure 6 Effects of OE-ECH1 on oxidative stress, apoptosis and inflammation in HK-2 cells. (**A-D**) Expression levels of apoptosis proteins (Bax, BCl-1, and Casp3). (**E-G**) Levels of inflammatory cytokines (IL-1β, TNF-α, and TGF-β). (**H-J**) Changes in oxidative stress levels. ***p<0.0001 versus control. ##p<0.001, ###p<0.0001 versus HG.

study selected ECH1 for further examination. We analyzed the effects of ECH1 on ROS and mitochondrial activity, and also explored the effects of ECH1 on inflammatory factors, oxidative stress factors, and apoptotic proteins.

ECH1 is an enzyme involved in mitochondrial fatty acid β-oxidation. It has been reported that ECH1 can participate in the pathological process of obesity by regulating the browning of white adipose tissue,[41] and ECH1 can inhibit liver steatosis and insulin signaling in response to a high-fat diet.[42] ECH1 may be a potential diagnostic and therapeutic target for obese cardiovascular disease.[43] Some studies have indicated that obesity is not only a risk factor for diabetes, but also a risk factor for DN.[44–46] This study showed that ECH1 had the potential to be a diagnostic marker for DN (AUC>0.9) through ROC curve analysis. ECH1 may have a crucial role in DN, but its mechanism of action remains poorly understood.

Oxidative stress is considered as a core pathogenic mechanism of DN, because the enhanced oxidative stress in diabetic patients can activate pathways such as PI3K/Akt, TGF-β1/p38-MAPK, and NF-κB, induce endothelial cell apoptosis, inflammation, autophagy, and fibrosis, leading to abnormal renal tissue and function, and ultimately renal damage.[35] High glucose in diabetic patients can increase the production of ROS through polyol, PKC, AGE/RAGE, and hexosamine pathways, thereby enhancing the oxidative stress response.[35,47] At the same time, inflammation and apoptosis can induce tubular damage and promote the progression of DN.[48–50] An early sign of apoptosis is

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mitochondrial damage, and mitochondrial damage is also an important component of DN.[51,52] This is consistent with our research results, and the functional enrichment based on DEGs also revealed that the functions of AGE-RAGE signaling pathway and other functions were abnormal in DN patients. At the same time, In vitro experiments similarly revealed that mitochondria were inactivated in HG group, and the levels of oxidative stress factors, inflammatory factors and pro-apoptotic proteins increased significantly. Vitro experiments on ECH1 further explained its mechanism of action in high glucose HK-2 cells, indicating that ECH1 could promote FAO and maintain mitochondrial activity, while inhibiting cell apoptosis, oxidative stress and cell inflammation. These evidence showed that ECH1 had the potential to be a target for DN treatment.

Although we discovered for the first time that ECH1 was an important regulatory gene of DN and had the potential to be used as a diagnostic marker and therapeutic target, and further identified the mechanism of action of ECH1 in DN, this study still has some shortcomings. Firstly, the sample size in the microarray data set was limited, while some results have been experimentally confirmed, avoiding false positives in microarray analysis remains challenging. Second, the in vitro DN model cannot totally simulate the pathogenesis of human DN, therefore further cross-validation of transgenic mouse models may be needed.

Conclusion

ECH1 was one of the key genes in DN, which could regulate fatty acid oxidation, apoptosis, oxidative stress and inflammatory response, indicating that ECH1 could be a diagnostic marker and therapeutic target for DN. In addition, future experimental studies on the key gene ECH1 are needed to investigate its exact role in the pathophysiology of DN, providing a new perspective for exploring the diagnosis and treatment of DN.

Data Sharing Statement

The study datasets GSE96804 and GSE104954 are available for download in the GEO database (https://www.ncbi.nlm.nih.gov/geo/).

Ethics Approval and Informed Consent

Ethical approval to conduct studies using publicly available databases is exempt under the following legislation: Item 1 and 2 of Article 32 of "the Measures for Ethical Review of Life Science and Medical Research Involving Human Subjects", which was reviewed by the National Science and Technology Ethics Committee, approved by the State Council of China, and jointly promulgated by the National Health Commission, the Ministry of Education, the Ministry of Science and Technology and the State Administration of Traditional Chinese Medicine on Feb. 18, 2023.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that the study has no potential conflicts of interest.

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