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A comprehensive weighted gene co-expression network analysis uncovers potential targets in diabetic kidney disease

Shaokang Pan#, Zhengyong Li#, Yixue Wang, Lulu Liang, Fengxun Liu, Yingjin Qiao, Dongwei Liu, Zhangsuo Liu

Department of TCM-Integrated Department of Nephrology, the First Affiliated Hospital of Zhengzhou University; Research Institute of Nephrology, Zhengzhou University; Research Center for Kidney Disease, Henan Province; Key Laboratory of Precision Diagnosis and Treatment for Chronic Kidney Disease in Henan Province; Core Unit of National Clinical Medical Research Center of Kidney Disease, Zhengzhou 450052, Henan Province, China

ABSTRACT

Background and Objectives: Diabetic kidney disease (DKD) is one of the most common microvascular complications of diabetes. It has always been difficult to explore novel biomarkers and therapeutic targets of DKD. We aimed to identify new biomarkers and further explore their functions in DKD. **Methods:** The weighted gene co-expression network analysis (WGCNA) method was used to analyze the expression profile data of DKD, obtain key modules related to the clinical traits of DKD, and perform gene enrichment analysis. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to verify the mRNA expression of the hub genes in DKD. Spearman's correlation coefficients were used to determine the relationship between gene expression and clinical indicators. **Results:** Fifteen gene modules were obtained *via* WGCNA analysis, among which the green module had the most significant correlation with DKD. Gene enrichment analysis revealed that the genes in this module were mainly involved in sugar and lipid metabolism, regulation of small guanosine triphosphatase (GTPase) mediated signal transduction, G protein-coupled receptor signaling pathway, peroxisome proliferator-activated receptor (PPAR) molecular signaling pathway, Rho protein signal transduction, and oxidoreductase activity. The qRT-PCR results showed that the relative expression of nuclear pore complex-interacting protein family member A2 (*NPIPA2*) and ankyrin repeat domain 36 (*ANKRD36*) was notably increased in DKD compared to the control. *NPIPA2* was positively correlated with the urine albumin/creatinine ratio (ACR) and serum creatinine (Scr) but negatively correlated with albumin (ALB) and hemoglobin (Hb) levels. *ANKRD36* was positively correlated with the triglyceride (TG) level and white blood cell (WBC) count. **Conclusion:** *NPIPA2* expression is closely related to the disease condition of DKD, whereas *ANKRD36* may be involved in the progression of DKD through lipid metabolism and inflammation, providing an experimental basis to further explore the pathogenesis of DKD.

Key words: diabetic kidney disease, bioinformatics analysis, WGCNA, *NPIPA2*, *ANKRD36*

#These authors contributed equally to this work.

Address for Correspondence:
Prof. Zhangsuo Liu, Department of TCM-Integrated Department of Nephrology, the First Affiliated Hospital of Zhengzhou University, No. 1 Jianshe East Road, Zhengzhou 450052, Henan Province, China.
E-mail: zhangsuoliu@zzu.edu.cn

Prof. Dongwei Liu, Department of TCM-Integrated Department of Nephrology, the First Affiliated Hospital of Zhengzhou University, No. 1 Jianshe East Road, Zhengzhou 450052, Henan Province, China.
E-mail: liu-dongwei@zzu.edu.cn

Access this article online

Website: www.intern-med.com

DOI: 10.2478/jtim-2022-0053

INTRODUCTION

Diabetic kidney disease (DKD) is one of the most serious microvascular complications of diabetes and is the most common cause of end-stage renal disease.[1,2] The onset of DKD is insidious, and its early clinical symptoms are easily ignored, leading to an increase in the incidence of end-stage diabetic nephropathy.[3] Studies have shown that approximately 30% of patients with type 1 diabetes and 40% of patients with type 2 diabetes develop this microvascular complication.[4] Worse, the development of

DKD can significantly increase the risk of cardiovascular disease and mortality, causing a substantial economic and social burden.[5,6]

The pathogenesis of DKD is complex, and there is no clear clinical biomarker that reliably predicts the occurrence and development of DKD.[7,8] Most current studies have focused on screening differentially expressed genes but have ignored the intrinsic correlation between genes. Genes with similar expression patterns may be functionally significantly related,[9,10] allowing for the exploration of effective biomarkers for DKD. WGCNA is a bioinformatics method used for analyzing gene expression patterns across multiple samples, dividing the gene co-expression network of complex biological processes into highly correlated signature modules. Genes within the module change in a highly coordinated manner, allowing the identification of biomarkers or therapeutic targets by associating specific clinical phenotypes to genes with key functions.[11]

WGCNA can quickly extract gene co-expression modules related to sample characteristics from complex data and is one of the most effective methods for co-expression network analysis. To date, few studies have used WGCNA to analyze the pathogenesis and treatment of DKD. In this study, we performed WGCNA to analyze the expression profile data of DKD and used peripheral blood samples from patients to verify the selected hub genes *via* quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Correlation analysis was then conducted with the clinical indicators of patients with DKD to identify new biomarkers. Therefore, this study provides a new experimental basis for exploring the mechanisms and elucidating potential markers for the early diagnosis of DKD.

MATERIALS AND METHODS

Gene chip data and patient sample collection

Diabetic nephropathy and diabetic kidney disease were used as keywords to search the appropriate data sets in the Gene Expression Database (GEO) (https://www.ncbi.nlm.nih.gov/geo/). The inclusion criteria were: the number of samples from patients was more than 30, the expression profile was obtained from kidney tissue rather than serum or cell lines, the species studied was human, and data processing was performed using common sequencing platforms. Thirty-four human peripheral blood samples were collected from the First Affiliated Hospital of Zhengzhou University between December 2019 and December 2020, including 12 patients in the DKD group, 11 patients in the diabetes (DM) group, and 11 patients in the healthy control (CON) group. Patients in the DKD group had diabetic glomerulopathy, as confirmed through renal biopsies.[12] The patients in the DM group had at least one of the following classic symptoms: random plasma glucose ≥ 200 mg/dL (11.1 mmol/L), fasting plasma glucose (FPG) ≥ 126 mg/dL (7.0 mmol/L), 2-h plasma glucose (2-h PG) ≥ 200 mg/dL (11.1 mmol/L), or A1C ≥ 6.5% (48 mmol/L).[13] The study was approved by the institutional review board (Ethical Lot Number:2019-KY-015), and all participants gave informed consent.

Data acquisition and preprocessing

Raw data from the GSE96804 dataset, which met the screening criteria, were downloaded from the GEO database. The dataset included kidney biopsy specimens from 41 cases of DKD and 20 normal cases. The R package (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.) was used to convert the probe IDs to Ensembl serial numbers, and mismatched Ensembl serial numbers were deleted. The HUGO Gene Naming Committee (https://www.genenames.org/) was used to convert the Ensembl serial number to a common Entrez ID, and the Ensembl sequence number was removed without pairing the Entrez ID. For multiple identical Entrez IDs, the average value of multiple probes was considered the final expression value of the Entrez ID.

Differentially expressed gene analysis

The "limma" package in R was used to analyze the differential gene expression of the experimental groups (DKD tissues *vs*. normal tissues). The standard for interception was $|\log_2 FC| > 1.0$, with an adjusted *P*-value < 0.01. The "ggplot2" R package was used to visualize the results as a volcano map.

WGCNA analysis

The WGCNA package in R was used for this analysis. The data were normalized to screen out genes in the top 75% of the median absolute deviation (MAD) and MAD greater than 0.01. The "goodSamplesGenes" function was utilized to detect and process the missing values. Outliers were removed using sample cluster analysis to reduce errors caused by sample factors. The "pickSoftThreshold" function was used to filter the optimal soft threshold β value. We then constructed a co-expression network based on the β value, divided the resulting modules using the "blockwiseModules" function dynamic clipping method, indicated the name of each module with a separate color, and visualized the network using a hierarchical clustering tree. Taking the occurrence of DKD as the main external biological parameter, module membership (MM) was obtained by calculating the gene significance (GS) and module significance (MS). According to the MS, modules related to the selected clinical phenotype could be selected, and genes with high GS and MM were selected as the key genes. The "clusterprofiler" package was used for gene ontology (GO) enrichment and Kyoto Encyclopedia of

Genes and Genome (KEGG) pathway enrichment analysis to explore the main biological functions and pathways of the genes related to DKD. The GO and KEGG enrichment results were displayed as bar and bubble graphs, respectively. The module connectivity of the key module genes was calculated and visualized using Cytoscape software. Finally, the top 50 genes were selected as the key genes for preliminary screening and were intersected with the differentially expressed genes. The intersection genes obtained through these two methods were considered the hub genes related to DKD.

Total RNA extraction and qRT-PCR analysis

The primary role of the hub genes in DKD was verified using qRT-PCR. Briefly, total RNA from blood samples was extracted using TRIzol reagent. The purity and concentration of the RNA samples were measured using a UV spectrophotometer, and the samples were used to synthesize cDNA. Subsequently, gene expression levels were determined using qRT-PCR with the following reaction conditions: 95°C for 10 min; then 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The sequence of the forward primer used to amplify the human *NPIPA2* gene was 5'-CATCTCCCTCTGCACAAGTTAC-3' and the reverse primer sequence was 5'-TTCCACCTGTCTCCA TAAAGTAAA-3'. The sequence of the forward primer used to amplify the human *ANKRD36* gene was 5'-TCCTTTAT CCACGCTTACTCTG-3' and the reverse primer sequence was 5'-ATTGGTCCCTCCTTTATTTCTG-3'. Human *GAPDH* was used as the internal reference gene (forward primer: 5'-CAGGAGGCATTGCTGATGAT-3'; reverse primer: 5'-GAAGGCTGGGGCTCATTT-3'.) Finally, the relative expression levels of *NPIPA2* and *ANKRD36* were calculated using the $2^{-\Delta\Delta CT}$ method and are displayed as fold changes. All experiments were repeated thrice.

Clinical data collection, processing, and correlation analysis

Upon admission, the initial test results of the patients were collected, including the urine albumin/creatinine ratio (ACR), hemoglobin (Hb), serum creatinine (Scr), albumin (ALB), triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), estimated glomerular filtration rate (eGFR), and glycated hemoglobin (HbA1c). Spearman's correlation coefficient was used to analyze the correlation between clinical data and relative gene expression and identify clinical indicators related to the target genes.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software (IBM Corporation, Armonk, NY, USA). Measurement data are expressed as the mean ± standard deviation. An independent sample *t*-test or analysis of variance was used for comparisons between groups. Spearman's correlation coefficient was used for the correlation analysis between clinical data and gene expression. Statistical significance was set at $P < 0.05$.

RESULTS

Differentially expressed genes between DKD and normal specimens

According to the screening threshold, 418 significantly differentially expressed genes were obtained from the GSE96804 dataset. Among these, 123 genes were upregulated and 295 were downregulated in the DKD group compared to the control group (Figure 1).

Weighted Gene Co-expression Network Analysis (WGCNA)

Sample quality inspection

Hierarchical clustering was performed on the 61 samples from the GSE96804 dataset. No obvious outlier samples were found in the test, indicating that the quality of the original data was good and that a co-expression network could be constructed (Figure 2).

Determination of the soft threshold and construction of a co-expression network

According to the soft threshold β screening criteria, the correlation coefficient R2 was set as 0.85, and the "pickSoftThreshold" function was used to calculate the optimal soft threshold β as 8. In this case, the scale-free topology network construction and connectivity were good (Figure 3).

WGCNA with an optimal soft threshold of β = 8 was then constructed, and the minimum number of genes in the module was set to 30. The dynamic shearing method was used to identify gene modules, and 15 gene modules were obtained (Figure 4).

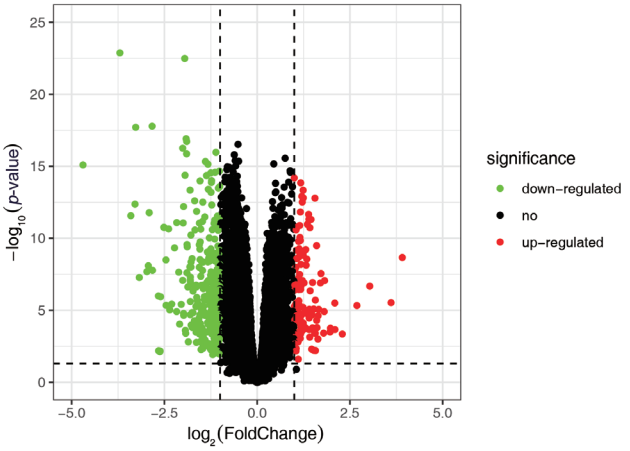

Figure 1: The differentially expressed genes are shown as a volcano map. Red dots represent upregulated genes, green dots represent downregulated genes, and black dots represent genes with no significant difference in expression.

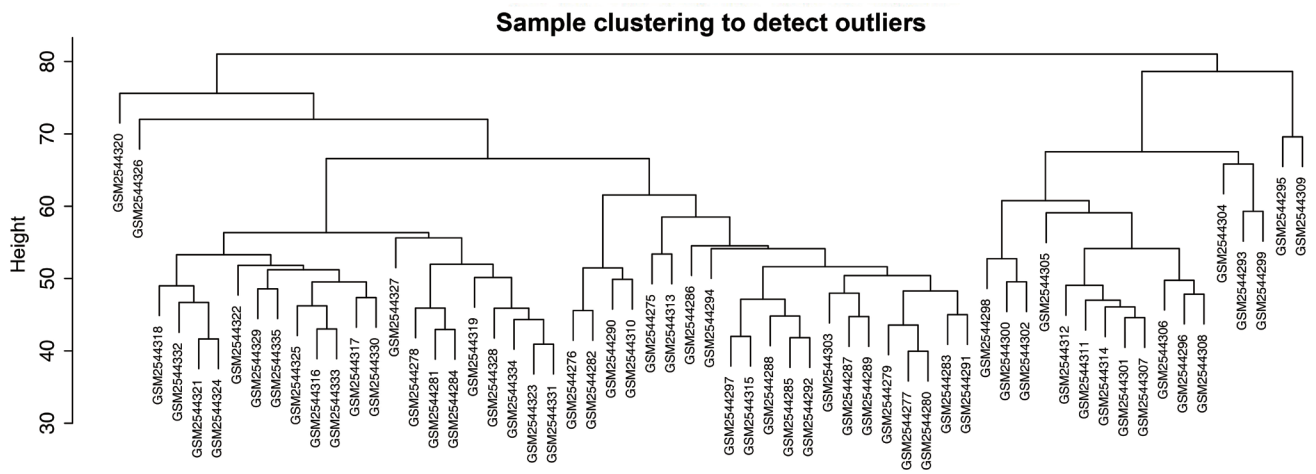

Figure 2: Sample hierarchical clustering diagram.

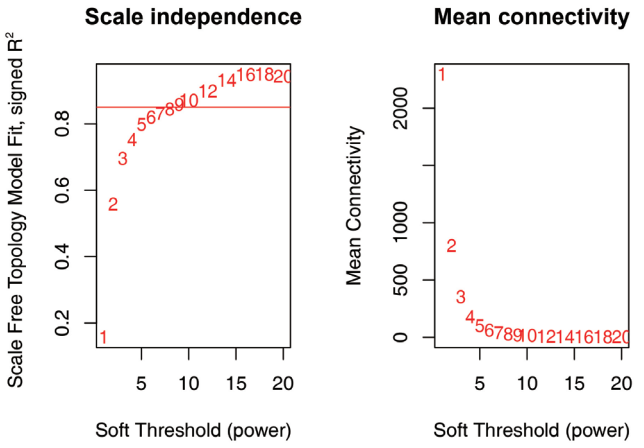

Figure 3: Screening of soft thresholds. Analysis of the scale-free fit index (left) and the mean connectivity (right) for various soft-thresholding powers.

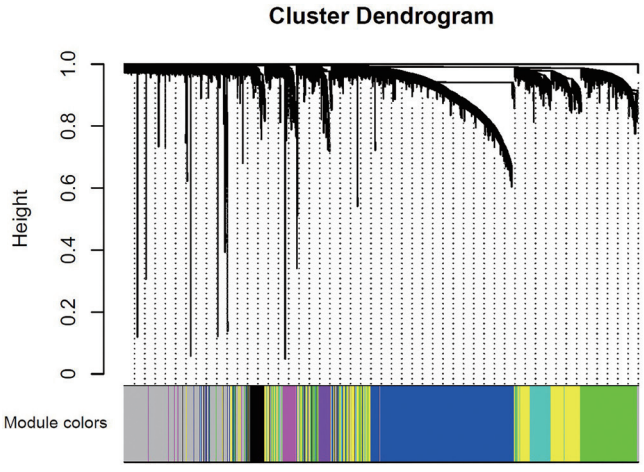

Figure 4: Gene cluster tree. The upper part of the figure is a diagram of the cluster tree, and the lower part is a cluster of gene modules with similar expression patterns. Different colors represent different modules.

Correlation analysis between gene modules and clinical information

The correlation between the samples, clinical information, and gene modules is shown in Figure 5A. Each cell therein contains a corresponding correlation coefficient and *P*-value. The darker the color, the smaller the *P*-value, and the higher the correlation. As shown in the figure, there was a significant correlation between the green module and DKD. Parallel phenotypic data of vector genes adjacent to the heatmap also showed similar results (Figure 5B).

Next, we constructed a scatter plot for the genes in the green module based on their gene significance (GS) and module membership (MM). The results showed a significant positive correlation; the module genes were highly correlated with the corresponding modules and the corresponding clinical phenotype (Figure 5C).

Gene enrichment analysis of the green module

Gene enrichment analysis of GO includes three main items: biological pathway (BP), cellular component (CC), and molecular function (MF). As shown in Figure 6A, the enrichment results based on GO_BP showed that the genes related to DKD were mainly involved in small-molecule catabolic processes, fatty acid metabolic processes, regulation of small GTPase-mediated signal transduction, adenylate cyclase-activating G-protein-coupled receptor signaling pathway, acyl-CoA metabolic process, cAMP-mediated signaling, lipid transport, regulation of GTPase activity, and Rho protein signal transduction. The enrichment results based on GO_CC showed that the genes related to DKD were mainly concentrated in the AP-type membrane coat adaptor complex, membrane coat, coated membrane, clathrin adaptor complex, peroxisomes, microbodies, and mitochondrial matrix (data not shown). Further, GO_MF analysis revealed

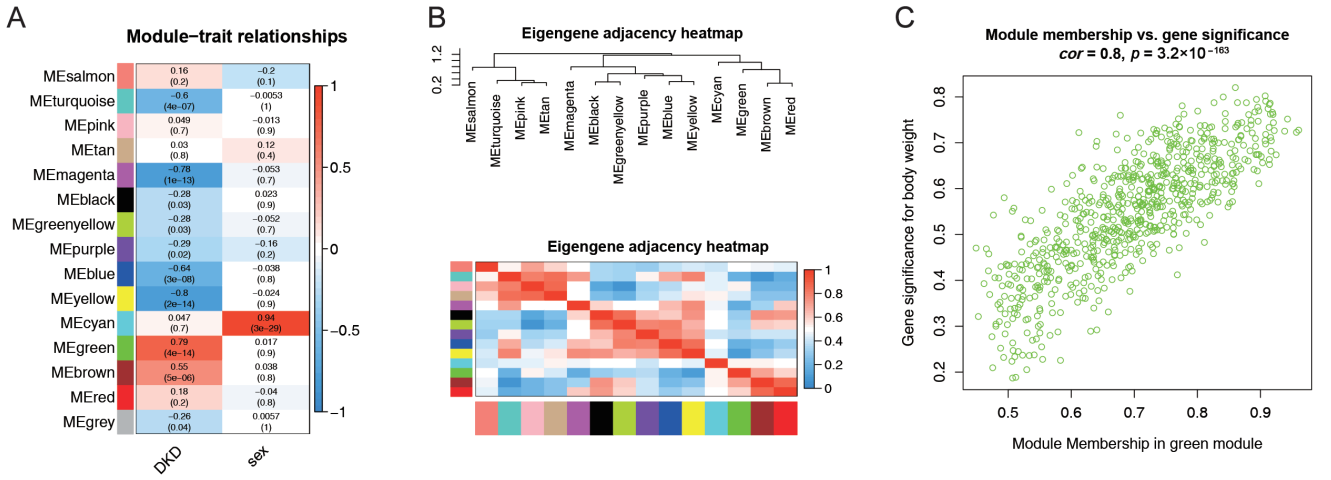

Figure 5: Correlation analysis between gene modules and clinical information. (A) Module-phenotype correlation diagram. The horizontal axis was for DKD and sex, and the vertical axis contained the gene co-expression modules. (B) Adjacent heat map of the featured vector genes. The upper part of the figure is a cluster tree composed of the modules and clinical phenotypes of DKD. The lower part of the figure is the corresponding heat map of the cluster tree. (C) Scatter plot showing the correlation between the green module genes and DKD. The horizontal axis represents the degree of module membership, and the vertical axis represents gene significance. DKD: diabetic kidney disease.

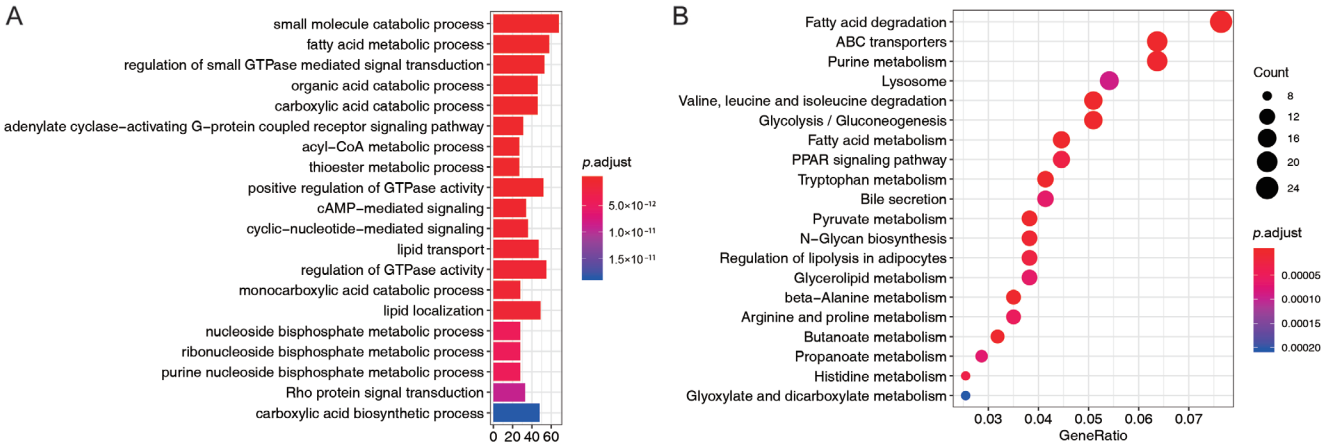

Figure 6: Gene enrichment analysis of the green module. Gene ontology enrichment (A) and Kyoto Encyclopedia of Genes and Genome pathway enrichment analysis (B). PPAR: peroxisome proliferator-activated receptor.

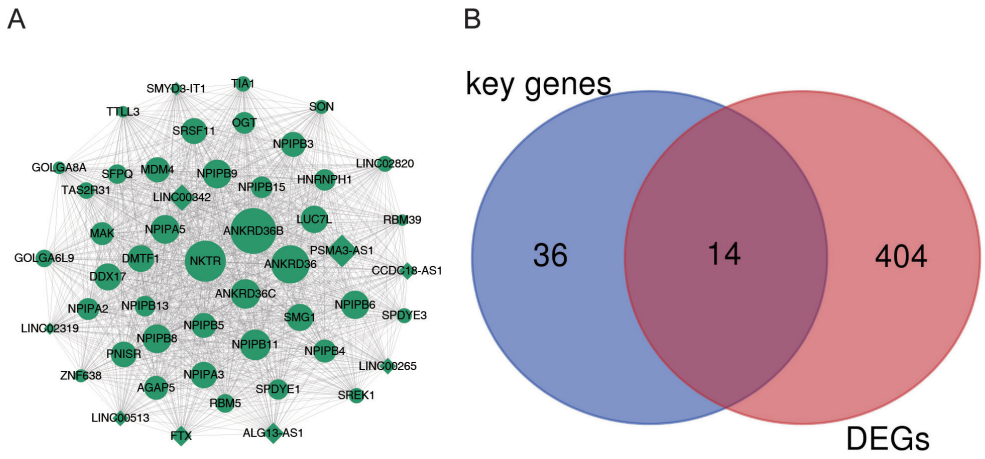

Figure 7: Selection of hub genes. (A) Visualization of the gene co-expression network created using Cytoscape software. The node size represents the connectivity between the modules. (B) The intersection between differentially expressed genes (DEGs) and key genes is shown as a Venn diagram. DEGs: differentially expressed genes.

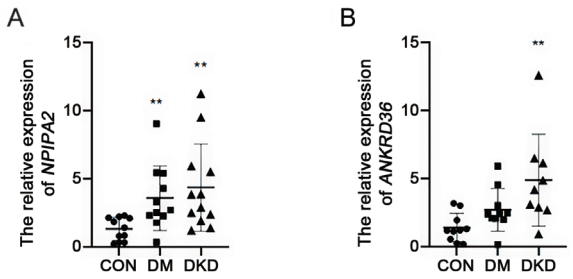

Figure 8: *NPIPA2* and *ANKRD36* were highly expressed in DKD. The relative expression levels of *NPIPA2*(A) and *ANKRD36*(B) in each group were detected *via* qRT-PCR. CON: healthy control group; DM: diabetes; DKD: diabetic kidney disease. **$P < 0.01$, as compared to CON.

that these genes might participate as GTPase activator, oxidoreductase, aldehyde dehydrogenase (NAD), and in nucleoside-triphosphatase regulator activities (data not shown). The results of KEGG analysis suggested that the genes related to DKD were primarily involved in fatty acid degradation and metabolism, amino acid metabolism, glycolysis/gluconeogenesis, regulation of lipolysis in adipocytes, glycerolipid metabolism, ABC transporters, lysosome, and the PPAR signaling pathway (Figure 6B).

Network visualization and selection of hub genes

The differential expression data and interaction relationships of the green module genes were imported into Cytoscape software for visualization. The top 50 genes with the highest connectivity are shown in Figure 7A. The first 50 genes were preliminarily screened as key genes, and 14 pivotal genes related to DKD were obtained through cross-comparison with differentially expressed genes, including (*ANKRD36, ANKRD36B, ANKRD36C*), (*NPIPA2, NPIPB3, NPIPB4, NPIPB5, NPIPB11, NPIPB13*), (*SPDYE1, SPDYE3*), *TAS2R31, GOLGA8A, and LINC00342* (Figure 7B).

NPIPA2 and ANKRD36 were highly expressed in DKD

NPIPA2 and *ANKRD36*, which accounted for the largest proportion of the two gene clusters, were selected for expression verification. Blood samples were collected from the three patient groups and used for qRT-PCR analysis. The results showed that the expression level of *NPIPA2* was significantly higher in the DKD group and DM group than in the healthy control (CON) group (Figure 8A). Similarly, *ANKRD36* expression was dramatically increased in the DKD group compared to the CON group (Figure 8B). Compared to the other two groups, both *NPIPA2* and *ANKRD36* showed the highest expression levels in the DKD group.

Correlation analysis between the expression level of NPIPA2 or ANKRD36 and clinical indicators in patients with DKD

Considering that the expression levels of *NPIPA2* and *ANKRD36* were significantly increased in the DKD group, we investigated whether these two genes were correlated with the clinical parameters of the patients. Correlation analysis showed that the relative expression of *NPIPA2* in patients with DKD was significantly positively correlated with the urine albumin/creatinine ratio ($r = 0.7343$, $P < 0.01$) and serum creatinine ($r = 0.5874$, $P < 0.05$), and significantly negatively correlated with albumin ($r = –0.6503$, $P < 0.05$) and hemoglobin ($r = –0.6014$, $P < 0.05$) (Figure 9). The relative expression of *ANKRD36* in patients with DKD was also positively correlated with the urine albumin/creatinine ratio and creatinine levels but was not statistically significant. We observed a significant positive correlation between *ANKRD36* expression and triglycerides ($r = 0.7950$, $P < 0.05$) and white blood cell count ($r = 0.9667$, $P < 0.001$) (Figure 10).

DISCUSSION

The incidence of diabetes is rapidly increasing, affecting more than 415 million people worldwide.[14] By 2040, the prevalence of diabetes is expected to increase to 642 million people, of whom 30%–40% will develop DKD.[15] Diabetic kidney disease (DKD) is the leading cause of chronic kidney disease, resulting in enormous economic and social burdens. However, owing to the complex pathogenesis of DKD and the lack of specific and effective interventions, therapies targeting this disease are not ideal, and the incidence of end-stage DKD is still increasing.[16] In this study, a co-expression network related to DKD was constructed through WGCNA analysis. Potential therapeutic targets were screened, providing a new paradigm for an in-depth investigation of the pathogenesis and treatment of DKD.

WGCNA can be used to effectively analyze gene expression profile data, with full consideration of the associated gene interactions. It has been widely used in studying the molecular pathogenesis of various diseases, such as cancer, stroke, immune diseases, and genetic diseases.[17–20] To better understand the pathogenesis of DKD, we first conducted a WGCNA analysis and obtained a total of 15 gene modules. We then selected the genes in the green modules closely related to DKD for further study. The enrichment analysis results showed that the key genes screened were mainly involved in sugar and lipid metabolism, regulation of small GTPase-mediated signal transduction, the G protein-coupled receptor signaling pathway, PPAR molecular signaling pathway, Rho protein signal transduction, and oxidoreductase activity, which is consistent with the results of previous studies. Diabetes-related metabolic changes can lead to glomerular hypertrophy, sclerosis, tubulointerstitial inflammation, and fibrosis.[3,21] In addition, several studies have confirmed that the pathogenesis of DKD is accompanied by serious lipid metabolism disorders, suggesting that abnormal lipid metabolism is closely related

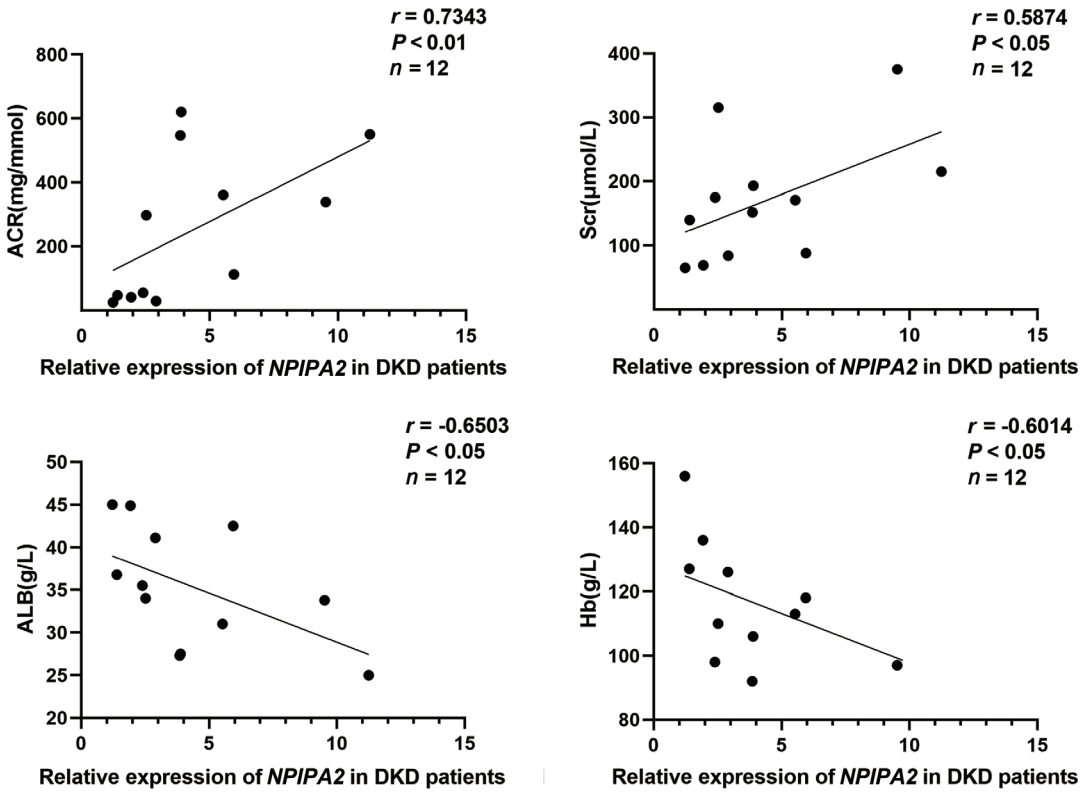

Figure 9: Correlation analysis between the relative expression level of *NPIPA2* and clinical indicators in patients with DKD. DKD: diabetic kidney disease; ACR: urine albumin/creatinine ratio; Scr: serum creatinine; ALB: albumin; Hb: hemoglobin.

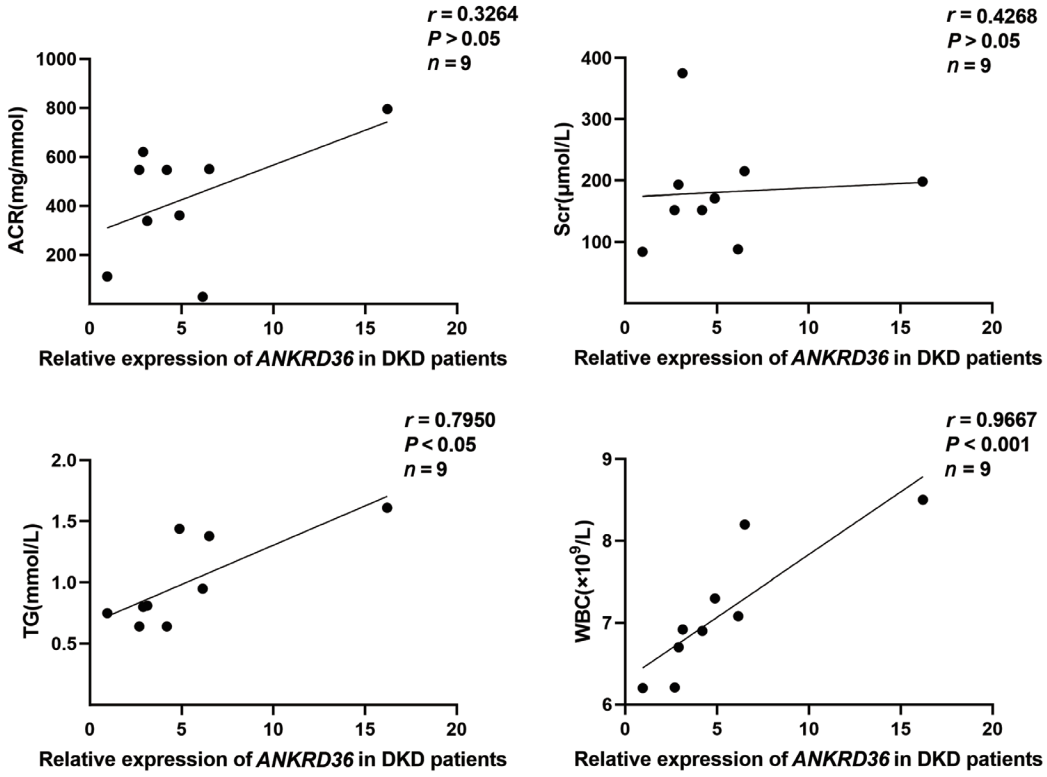

Figure 10: Correlation analysis between the relative expression level of *ANKRD36* and clinical indicators in patients with DKD. DKD: diabetic kidney disease; ACR: urine albumin/creatinine ratio; Scr: serum creatinine; TG: triglycerides; WBC: white blood cells.

to the progression of this disease.[22–25] Lipid toxicity and deposition can cause podocyte dysfunction and apoptosis, while enhanced fatty acid synthesis and oxidation inhibition can lead to diabetic lipid kidney damage.[26,27] In addition to metabolic pathways, Rho kinase, an effector of the small GTPase binding protein Rho, is also considered an important factor in DKD pathogenesis.[28] An imbalance in NADPH oxidase activity is another reason for DKD caused by oxidative stress.[1]

Originating from human chromosome 16, the NPIP gene family is primate-specific and has undergone strong adaptive evolution.[29,30] At present, there are few studies on *NPIPA2*, and some studies have reported its role in tumorigenesis.[31,32] Subsequently, to better study the relationship between biological traits and gene expression, we performed an integrated analysis of DEGs and WGCNA. We selected *NPIPA2* and *ANKRD36*, the two gene clusters with the largest proportions, to verify their expression in DKD and their correlation with clinical indicators. The results showed that *NPIPA2* and *ANKRD36* were significantly overexpressed in DKD, which was consistent with the results of the WGCNA. Thus, our study is the first to show that *NPIPA2* may be closely related to the progression of DKD. Proteinuria, a common clinical manifestation and the main basis for diagnosing DKD, is the result of multiple factors.[33,34] The expression level of *NPIPA2* was significantly increased in DKD specimens, and the correlation analysis results with clinical indicators showed that *NPIPA2* had the most significant correlation with ACR, suggesting that it may play an important role in proteinuria and may be a potential biomarker for screening in patients with DKD.

Ankyrin repeat domain proteins (ANKRD) are among the most common protein-protein interaction domains and exist in 270 human proteins with diverse functions.[35,36] *ANKRD36*, located on chromosome 2, has 36 exons and contains six repeating units consisting of two antiparallel helices and a hairpin structure repeatedly stacked on the superhelix. To date, there have been few reports on *ANKRD36*, most of which are correlation studies rather than functional studies, so its function in human diseases is largely unknown. In renal cell carcinoma (RCC), a high expression level of *ANKRD36* is significantly associated with poor patient prognosis.[37] In hypertension, *ANKRD36* is involved in blood pressure regulation by interacting with YY1.[38] Variations in the ANKRD gene family, particularly *ANKRD36*C and its paralogs, are more prevalent in immune-mediated thrombotic thrombocytopenic purpura (iTTP) than in healthy controls.[39] Our study showed that the expression of *ANKRD36* was significantly higher in DKD samples than in normal samples. Considering that DKD is a risk factor for hypertension,[40] we would like to explore whether *ANKRD36* regulates YY1 in future studies.

Previous studies have also shown that *ANKRD36* is associated with inflammatory responses in patients with pneumonia and myocarditis, plays a pro-inflammatory role by regulating intracellular NF-κB inflammation-related signaling pathways, and alleviates LPS-induced cell damage by silencing *ANKRD36* expression.[41–43] Clinically, *ANKRD36* expression is significantly positively correlated with triglyceride (TG) and white blood cell (WBC) levels, suggesting that it may participate in the development of DKD through lipid metabolism and inflammation. Therefore, inhibiting *ANKRD36* expression to reduce inflammatory damage may provide a new strategy for treating DKD-related inflammation. Hayward *et al.* found that the cell metabolic state can markedly alter the interaction between ANKRD protein and its targets and change the functional outcomes caused by such interactions.[44] Changes in glucolipid metabolism may also be another factor leading to the abnormal expression of *ANKRD36* in DKD; however, the specific mechanism needs to be further explored.

CONCLUSION

In this study, WGCNA was used to comprehensively analyze the gene regulatory network related to DKD. The high expression of *NPIPA2* and *ANKRD36* in DKD was elucidated for the first time through clinical specimen verification. Moreover, clinical correlation analysis revealed that *NPIPA2* was closely related to DKD and that *ANKRD36* may be involved in the progression of DKD through lipid metabolism and inflammation. Our findings provide novel insights into understanding the pathogenesis and treatment of DKD and a reference for future research on the function and mechanism of *NPIPA2* and *ANKRD36* in DKD.

Source of Funding

This work was supported by a grant from the National Natural Science Foundation of China (No. 81970633). This study was also supported by the Natural Science Foundation of Henan Province (No. 202300410363).

Ethics Approval and Consent to Participate

The study was approved by the institutional review board (Ethical Lot Number:2019-KY-015), and all participants gave informed consent.

Conflict of Interest

Dongwei Liu is an Editorial Board Member of the journal.

The article was subject to the journal's standard procedures, and peer review was handled independently of this editor and his research groups.

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How to cite this article: Pan S, Li Z, Wang Y, Liang L, Liu F, Qiao Y, *et al*. A comprehensive weighted gene co-expression network analysis uncovers potential targets in diabetic kidney disease. J Transl Intern Med 2022; 10: 359-368.