

Identification of diagnostic markers for diabetic kidney disease by weighted gene co-expression network analysis and machine learning

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Abstract. Diabetic kidney disease (DKD) represents a major complication associated with diabetes mellitus, notably contributing to patient morbidity and mortality. However, early diagnosis of DKD remains challenging due to the lack of clear diagnostic biomarkers. Therefore, in the present study, microarray and RNA-sequencing data from the Gene Expression Omnibus database were systematically analyzed. Using differential expression and weighted gene co-expression network analysis, 49 genes with marked expression changes in DKD were identified. Subsequent analyses, including functional enrichment, protein-protein interaction network construction, machine learning techniques and assessment of immune cell infiltration, led to the identification of three hub genes: Spleen-associated tyrosine kinase, apoptotic peptidase activating factor 1 and ADAM metalloproteinase domain 10, as promising diagnostic markers, which were further evaluated by receiver operating characteristic curve analysis. Expression changes of the identified hub genes were validated in both DKD mouse models and clinical patient samples. Collectively, the present study provided a novel perspective on the molecular basis of DKD, and highlighted novel candidates for potential diagnostic and therapeutic applications.

Introduction

Diabetic kidney disease (DKD) represents a notable micro-vascular complication of diabetes and has emerged as the foremost contributor to end-stage renal disease (ESRD) worldwide (1). Epidemiological investigations indicate that DKD develops in 20–40% of patients with diabetes, making it the leading cause of chronic kidney disease (CKD) worldwide (2). Moreover, DKD markedly increases the risk of cardiovascular events and early mortality in individuals with diabetes (3). According to data from the International Diabetes Federation in 2021, >537 million individuals globally are affected by diabetes, with projections estimating this number will rise to 700 million by 2045 (4). As the worldwide population of individuals with diabetes continues to increase, DKD prevalence has escalated, imposing notable financial and medical strains on both affected individuals and health-care infrastructures (5,6). From a pathological perspective, DKD is mainly typified by glomerular basement membrane thickening, tethered membrane dilatation, tubulointerstitial fibrosis and proteinuria (7–9). At present, the primary strategies for DKD management encompass lifestyle interventions, strict control of blood glucose and blood pressure, correction of lipid metabolism disorders and antioxidant therapy, all with the objective of decelerating disease progression and enhancing patient outcomes (10,11).

However, the aforementioned treatments have limited efficacy in preventing or treating diabetic nephropathy, and no specific or effective therapeutic regimen is currently available (12). Early-stage intervention remains crucial for disease reversal, highlighting the need for safe and effective treatment strategies at the early stages of DKD. Currently, commonly used diagnostic indicators such as estimated glomerular filtration rate (eGFR) and albuminuria lack sufficient specificity and sensitivity for early detection, as histopathological changes may occur even in normoalbuminuric patients, complicating accurate early diagnosis (13–15). Moreover, the pathogenesis of DKD is multifactorial, involving metabolic disturbances, inflammatory responses, oxidative stress and immune dysregulation, making it difficult for traditional research methods to fully elucidate its molecular mechanisms. Consequently, it is imperative to discover new, reliable biomarkers that can

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enhance the early diagnosis and prognostic assessment of DKD (9,16,17).

In recent years, with advances in high-throughput omics and molecular biology technologies, a series of proteins, RNAs and small-molecule metabolites have been reported as potential biomarkers for the diagnosis or prognosis of DKD (18–20). For example, urinary neutrophil gelatinase-associated lipocalin, kidney injury molecule 1, β 2-microglobulin, inflammatory cytokines (such as IL-18 and TNF- α) and specific microRNAs (miRNAs/miRs) (such as miR-21, miR-192 and miR-377) show an upward trend in early renal injury, but their specificity and clinical translational value remain controversial (21–24). Multiple studies have attempted to enhance diagnostic accuracy through biomarker panels, yet existing markers lack universally accepted standards due to sample heterogeneity, technical platform variations and insufficient clinical validation (25–27). Current major bottlenecks in the field include: The need for improved biomarker specificity and stability, the absence of large-scale, multicenter prospective cohort validation, and insufficient elucidation of underlying molecular mechanisms.

Recent advancements in high-throughput genomics and computational biology have revolutionized biomarker discovery. Weighted gene co-expression network analysis (WGCNA) is a systems biology method widely applied to identify disease-associated gene modules and hub genes in complex diseases, including renal diseases (28). WGCNA clusters genes based on similarity in expression patterns, forming multiple modules. Each module represents a set of genes exhibiting coordinated expression across different samples, providing important insights into the molecular mechanisms of DKD (29). In parallel, machine learning (ML) has become an effective approach for identifying biomarkers, supporting the creation of reliable predictive models and efficiently handling high-dimensional datasets (30–32). Integrating WGCNA with ML can enhance biomarker discovery by combining network-level insights with advanced feature selection techniques.

The aim of the present study was to discover and confirm novel diagnostic biomarkers for DKD by integrating WGCNA and ML. Using publicly available transcriptomics datasets, co-expression networks were constructed to identify disease-associated modules and pivotal genes, and the hub genes were subsequently validated using receiver operating characteristic (ROC) curve analysis and nomogram models (33). These biomarkers were further assessed in independent cohorts to evaluate their diagnostic performance. Additionally, the association between renal function and hub genes were predicted using the Nephroseq v5 database (34). Ultimately, the expression levels of hub genes were validated through *in vivo* experiments and clinical sample analysis. Collectively, the findings may provide new molecular insights into DKD, and could contribute to the development of precision medicine approaches for managing DKD.

Materials and methods

Differential gene expression analysis. In the present study, two DKD datasets from the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) (35) were screened:

GSE30529 (GPL571) containing 10 cases of DKD and 12 controls; and GSE30122 (GPL571) as a validation dataset containing 19 cases of DKD and 50 controls (Table SI) (36,37). Serial matrix files of both datasets, containing gene expression data, were downloaded for subsequent analysis. The boxplot function from the ‘graphics’ package in R (version 3.6.2; <https://rdrr.io/doc/r/packages/graphics/>) was used to visualize and evaluate the distribution of gene expression across samples. Differential genes were analyzed using the ‘limma’ R package (version 3.64.1; <https://bioinf.wehi.edu.au/limma/>) (38), with thresholds set at $\log_2$ (fold change) >0.58 or $\log_2$ (fold change) <-0.58 , and $P < 0.05$ for identifying differentially expressed genes (DEGs). Heatmaps and volcano plots were subsequently generated employing the ‘pheatmap’ (version 1.0.12; <https://cran.r-project.org/web/packages/pheatmap/>) and ‘ggplot2’ (version 3.3.5; <https://cran.r-project.org/web/packages/ggplot2/>) R packages, respectively, to display the DEGs.

WGCNA. The ‘WGCNA’ R package (version 1.73; <https://cran.r-project.org/web/packages/WGCNA/>) was employed to identify gene modules associated with DKD phenotypes in the GSE30529 dataset. A soft-thresholding power was selected to achieve a scale-free network topology ($R^2=0.85$). Subsequently, hierarchical clustering analysis grouped genes into distinct modules, with a minimum size of 30 genes per module. To identify biologically relevant modules, the ‘WGCNA’ R package was used to evaluate the associations between module eigengenes and clinical traits. Genes within these trait-associated modules were defined as key drivers if they met the criteria of module membership (MM) >0.8 and gene significance (GS) >0.2 . Venn diagram analysis was used to examine the intersection between key driver genes and previously identified DEGs to yield the final target gene set. The Venn diagram was generated using the ‘VennDiagram’ package (version 1.7.3; <https://cran.r-project.org/web/packages/VennDiagram/>).

Gene set enrichment analysis (GSEA). GSEA was applied to evaluate gene distribution patterns in DKD within the GSE30529 dataset and to investigate their related phenotypes (39). The ‘clusterProfiler’ R package (version 3.0.4; <http://www.bioconductor.org/packages/clusterProfiler/>) was used for functional enrichment analysis of the HALLMARK gene set modules with the reference gene set: c2.cp.all.v2022.1.Hs.symbols.gmt. Absolute net enrichment scores >1 , false discovery rates $q < 0.25$ and $P < 0.05$ were considered to represent significant enrichment.

Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis. The ‘clusterProfiler’ R package was also used to analyze GO and KEGG pathway enrichment (40,41). Gene-related molecular functions (MFs), cellular components (CCs), biological processes (BPs) and gene-related signaling pathways were assessed.

ML and protein-protein interaction (PPI) networks for filtering hub genes. In the present study, three ML algorithms: Random forest (RF) (42), least absolute shrinkage and selection operator (LASSO) logistic regression (43),

and support vector machine-recursive feature elimination (SVM-RFE) (44), were employed to identify key biomarkers for DKD. The RF approach was performed with the 'randomForest' R package (version 4.7-12; <https://cran.r-project.org/web/packages/randomForest/>). SVM-RFE was performed with the R package 'e1071' (version 1.7-16; <https://cran.r-project.org/web/packages/e1071/>), whereas LASSO logistic regression was conducted using the 'glmnet' R package (version 4.1-10; <https://www.rdocumentation.org/packages/glmnet/>), selecting the optimal model according to the minimum λ value. Parameter optimization was carried out using 10-fold cross-validation, with the model reaching the lowest biased likelihood deviance criterion. Genes exhibiting common traits across the three classification models were selected for further investigation. Additionally, a PPI network was built using the STRING database (<http://string-db.org/>) and was subsequently visualized with Cytoscape 3.9.1 software (<https://cytoscape.org/>). Differential genes were assessed using three algorithms: Degree, closeness and maximum clique centrality, within the cytoHubba plugin (version 0.1; <https://apps.cytoscape.org/apps/cytohubba>). The top 10 genes from each method were selected and their overlaps determined. Ultimately, genes common to these intersections were designated as hub genes. UpSet plots and petal diagrams was generated using the 'ggplot2' package.

Validation and clinical relevance of hub genes. Hub gene expression was validated using the GSE30122 dataset, followed by the use of the Nephroseq v5 tool (<https://www.nephroseq.org/>), which was employed to analyze the correlations between serum creatinine (Scr), eGFR and identified hub genes (45). Scatter plots illustrating these correlations were re-plotted using the 'ggplot2' R package. A nomogram model for diagnosing DKD was developed using the rms package (version 8.1-0; <https://hbiostat.org/R/rms/>) based on hub genes. Decision curve analysis (DCA) (version 3.8-3; <https://github.com/therneau/survival>) demonstrated the clinical utility of the nomogram model.

Analysis of immune cell infiltration and associated correlation studies. The CIBERSORTx algorithm (<https://cibersortx.stanford.edu/>) (46) was employed to quantify the infiltration levels of various immune cell subtypes within the DKD gene expression profiles. Subsequently, based on the relative abundance of 22 types of infiltrating immune cells, pairwise Pearson correlation coefficients were calculated. The 'ggplot2' R package was utilized to visualize the correlation results. Principal component analysis (PCA) was subsequently applied to compare immune cell infiltration patterns between the DKD and control groups, with visualization performed using the 'ggplot2' R package. Furthermore, the interrelationships among the 22 immune cell subsets were illustrated in a heatmap.

Animal model and sampling. A total of 12 healthy male C57BL/6J mice (age, 8 weeks; weight, 20-22 g), six male C57BLKS/J db/db mice and six male C57BLKS/J db/m mice (age, 8 weeks; weight, 20-22 g) were purchased from Nanjing Junke Biological Engineering Co., Ltd. Due to their leptin receptor deficiency, db/db mice are commonly used as a classic

animal model for spontaneous type 2 diabetes, with their littermate heterozygous db/m mice serving as a control group (47). The mice were housed and experimented on in the animal laboratory at Shanghai Rat & Mouse Biotech., Ltd. (Shanghai, China). The mice were maintained a temperature of 24°C and relative humidity of 50-60%, under a 12-h light/dark cycle. The mice had free access to standard laboratory chow and clean drinking water, and underwent a 1-week acclimation period. C57BL/6J mice were randomly allocated to two groups: The sham group (n=6) was maintained on a standard diet, whereas the DKD model group (n=6) received a high-sugar, high-fat diet (67% maintenance chow, 10% lard, 20% sucrose, 2.5% cholesterol and 0.5% sodium bile acid). Under anesthesia, induced with 2% isoflurane and maintained with 1.5% isoflurane, left uninephrectomy (UNx) was performed.

After 1 week of postoperative recovery, the mice were fasted for 12 h and received a single intraperitoneal injection of streptozotocin (STZ; 40 mg/kg body weight; Sigma-Aldrich; Merck KGaA) dissolved in sodium citrate buffer (pH 4.5). Control animals received an equivalent volume of sodium citrate buffer alone. After 4 consecutive days of injection, plasma glucose levels were assessed from 10- μ l tail vein blood samples using an AccuChek glucometer (Roche Diagnostics) over 3 consecutive days. A diabetic mouse model was confirmed when plasma glucose concentrations reached >16.7 mmol/l. The early DKD mouse model was established by further defining diabetic models based on the urine albumin-to-creatinine ratio (UACR). 24-h urine samples collected via metabolic cages for analysis. When the UACR increases and meets the criteria for early DKD, the model is considered successfully established. All animal procedures were approved by the Institutional Animal Care and Use Committee of Shanghai Rat & Mouse Biotech., Ltd. (approval no. LL-2024-060).

The health and body weight of the mice were monitored regularly. Mice were weighed every Wednesday, and fasting blood glucose levels were measured after an 8-12-h fast with free access to water. Urine samples were collected at the conclusion of the 16-week experimental period. At the end of the experimental period, the mice were euthanized via intravenous injection of an overdose of pentobarbital (100 mg/kg). Subsequently, 0.8-1.0 ml blood was collected from the retro-orbital sinus and kidney tissue was obtained for subsequent analysis. To reduce variability arising from environmental or individual factors, each sample was collected in triplicate.

Cell culture and in vitro studies. HK-2 cells (an immortalized proximal tubule epithelial cell line from a normal adult human kidney) were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The cells were cultured in DMEM/F12 (cat. no. 11320033) supplemented with 10% FBS (cat. no. A5670701) and 1% penicillin/streptomycin (cat. no. 15140148) (all from Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured in a 37°C, 5% CO₂ incubator and subjected to a 24-h low glucose (LG) environment at 5.5 mmol/l glucose. Cells were then divided into the following groups: i) LG group: Treated with 5.5 mmol/l glucose plus 24.5 mmol/l mannitol for 24 h to serve as an osmotic control; ii) high-glucose (HG) group: stimulated with 30 mmol/l glucose for 24 h. Mannitol (cat. no. ST2362;

Beyotime Biotechnology) was used as an osmotic control to prevent hyperosmolarity.

Transfection of small interfering RNA (siRNA) into HK-2 cells. Cells were transfected with siRNAs targeting apoptotic peptidase activating factor 1 (APAF1; cat. no. HY-RS00801), ADAM metallopeptidase domain 10 (ADAM10; cat. no. HY-RS00265) and spleen-associated tyrosine kinase (SYK; cat. no. HY-RS14075) (all from MedChemExpress). Cells were cultured in 6-well plates for transfection. When cells reached 60% confluence, they were transfected using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly cells were transfected with siRNAs (final concentration: 50 nM) in a 5% CO₂ incubator at 37°C. After incubation of the cells with the siRNAs for 6 h, the medium was replaced with conventional complete medium containing 10% FBS, and the cells were further cultured at 37°C under 5% CO₂. Subsequent functional assays and relevant parameter measurements were conducted 48 h post-transfection. The sense and antisense sequences of the siRNAs (including the non-targeting negative control; MedChemExpress) are shown in Table I.

Renal function testing and serum cytokine detection. For renal function testing and cytokine detection, 0.8–1.0 ml blood drawn from the retro-orbital sinus of euthanized mice was collected into an anticoagulant-free centrifuge tube, which was allowed to stand at room temperature for 30 min, before being centrifuged at 1,500 × g for 10–15 min at 4°C. The supernatant was thus separated to obtain the serum for subsequent analyses. Blood urea nitrogen (BUN) was quantified using a urea assay based on the urease method (BUN; cat. no. C013-2-1; Nanjing Jiancheng Bioengineering Institute), whereas Scr was assessed via a creatinine oxidase assay (Scr; cat. no. C011-2-1; Nanjing Jiancheng Bioengineering Institute). At the end of the 16-week study, mice were housed in metabolic cages for 24-h urine collection. The urine samples were thoroughly mixed and centrifuged at 2,000 × g for 10 min at 4°C. The resulting supernatant was carefully collected, and urinary protein levels were determined using UACR assay kit (microalbumin; cat. no. C035-2-1; Nanjing Jiancheng Bioengineering Institute, Inc.) via the turbidimetric method. All kits were used in accordance with the manufacturer's protocol.

Histological analysis. Renal tissues from the mice were collected and fixed in 4% paraformaldehyde solution at room temperature (20–25°C) for 24-h, followed by paraffin embedding. After preparing 3-μm renal tissue sections, multiple staining techniques including hematoxylin and eosin (H&E), and Masson's trichrome staining were performed. For H&E staining, samples were stained with hematoxylin for 5–10 min, followed by eosin counterstaining of the cytoplasm for 1–3 min. The degree of tubular damage was recorded using the Paller tubular injury scoring system (48), wherein 10 randomly selected tubules per high-power field were observed: Marked tubular dilatation, flattened or swollen cells scored 1 point; brush border damage or loss scored 1 or 2 points; cast formation scored 2 points; and necrotic cells shed into the tubular lumen (without forming casts or fragments) scored 1 point. The

median score was calculated for each histopathological section by selecting 10 fields of view (representing 100 tubules).

For Masson's trichrome staining, first, the dewaxed tissues were incubated overnight in Bouin's fixative (solution A) at room temperature, then processed at 65°C for 30 min. Subsequently, the nuclei were stained with a mixture of Wiegner's stain A (solution B) and Wiegner's stain B (solution C) for 1 min, followed by differentiation with 1% hydrochloric acid in ethanol. The processed tissue was then treated with phosphomolybdic acid (solution E) for 1 min. Subsequently, muscle fibers were stained red with Ponceau red (solution D) for 8 min, while collagen fibers were stained blue with aniline blue (solution F) for 8–30 sec. Differentiation with 1% glacial acetic acid enhanced contrast between the two colors. The Masson's trichrome staining kit (cat. no. G1340) was obtained from Beijing Solarbio Science & Technology Co., Ltd. Pathological images were captured using a BX51 brightfield microscope (Olympus Corporation) and were semi-quantitatively analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Expression levels of the three hub genes identified through the analyses were validated in both blood samples from patients with DKD, as well as in kidney tissue samples from DKD and sham mice (n=6/group). Renal tissues were flash-frozen in liquid nitrogen upon collection to preserve RNA integrity and maintained at -80°C prior to processing. For the detection of gene expression in cells, the cells were collected 48 h after siRNA transfection. Total RNA was isolated using TRIzol reagent (cat. no. CW0580S; Jiangsu CoWin Biotech Co., Ltd.) according to the manufacturer's protocol. RT was performed using Evo M-MLV RT Premix (cat. no. AG11706; Hunan Accurate Bio-Medical Technology Co., Ltd.), with all procedures strictly adhering to the manufacturer's instructions. qPCR was performed using an ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the SYBR Green Premix Pro Taq HS qPCR kit (cat. no. AG11701; Accurate Biology). The following thermal cycling conditions were adhered to: 95°C pre-denaturation for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Curve analysis was performed after amplification. Gene expression was quantified using the 2^{-ΔΔC_q} method with GAPDH as the reference gene (49). The primer sequences of the hub genes used in RT-qPCR are shown in Table II.

Western blot analysis. Kidney tissue and HK-2 cell proteins were extracted using RIPA lysis buffer (cat. no. P0013B; Beyotime Biotechnology) and quantified using the BCA protein assay. Protein samples were denatured according to the 5X protein loading buffer protocol. The denatured proteins (30 μg/lane) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% resolving gel and 5% concentrating gel. The voltage was set at 80 V for 20 min, then increased to 100 V for 40 min. After electrophoresis, the proteins were transferred to PVDF membranes, which were subsequently blocked with 5% non-fat milk for 1 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies against SYK (1:1,000; cat. no. ab40781), APAF1 (1:1,000; cat. no. ab234436), ADAM10 (1:1,000; cat. no. ab242389), fibronectin (1:5,000;

Table I. siRNA sequences.

Gene	Sense	Antisense
siSYK	5'-UGUCUUUGAUCCUAUCAGGAC-3'	5'-CCUGAUAGGAUCAAAAGACAAA-3'
siADAM10	5'-UAAUCCUUAUAAUGUCUGAU-3'	5'-CAGACAUUAUGAAGGAUUAUC-3'
siAPAF1	5'-AAUUUCGAGCUUUUGCAUCCA-3'	5'-GAUGCAAAAGCUCGAAAUUGU-3'
Negative control	5'-UUCAGUUGUGUGUUGUCAAUU-3	5'-GUCAACACACAACAGUUAUU-3

ADAM10, ADAM metalloproteinase domain 10; APAF1, apoptotic peptidase activating factor 1; si, small interfering; SYK, spleen-associated tyrosine kinase.

Table II. Sequences of the primers utilized for reverse transcription-quantitative PCR.

Gene	Forwards primer sequence	Reverse primer sequence	Organism
SYK	5'-TGAAAGGGAGCGAAGTGACC-3'	5'-ATTGCGAAGCCTCAGTTCCA-3'	Mouse
ADAM10	5'-GGGCTGGGAGGTCAGTATGG-3'	5'-CACGCTGGTGTGTTTGGTGTA-3'	Mouse
APAF1	5'-TCCCTGGGCTGCTTTCTTTC-3'	5'-CCGGATGTGTAACCAAGCCT-3'	Mouse
GAPDH	5'-AGGTCGGTGTGTGAACGGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'	Mouse
SYK	5'-CGTATGAGCCAGAACTTG-CACC-3'	5'-CTTTCGGTCCAGGTAAA-CCTCC-3'	Human
ADAM10	5'-CACGAGAAGCTGTGATTGCC-3'	5'-TCCGGAGAAGTCTGTGGTCT-3'	Human
APAF1	5'-ATGAAGCCATGTCTATAAGTGTT-3'	5'-GCACCTTAACGTCCTTCTG-3'	Human
GAPDH	5'-CACGGCAAATTCAACGGC-3'	5'-GGTGGTGAAGACACCAGTA-3'	Human

ADAM10, ADAM metalloproteinase domain 10; APAF1, apoptotic peptidase activating factor 1; SYK, spleen-associated tyrosine kinase.

cat. no. ab45688), vimentin (1:2,500; cat. no. ab137321), Snail (1:1,000; cat. no. ab180714) and GAPDH (1:10,000; cat. no. ab181602) (all from Abcam), and β -actin (1:10,000; cat. no. 6009-1-1; Proteintech Group, Inc.). This was followed by incubation with anti-rabbit secondary antibodies (1:5,000; cat. no. 7074) and anti-mouse secondary antibodies (1:5,000; cat. no. 7076) (both from Cell Signaling Technology, Inc.) for 1 h at room temperature. Protein bands were visualized using an ultrasensitive ECL chemiluminescence kit (cat. no. P0018S; Beyotime Biotechnology) and documented with a gel imaging system. The gray values of the strips were analyzed semi-quantitatively using Image-Pro Plus 6.0 software.

Sample collection. The present study was approved by the Ethics Committee of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine (approval no. 2024-7th-HIRB-097) and adhered to ethical guidelines consistent with the principles of The Declaration of Helsinki. All participants provided written informed consent. Patients with DKD and healthy controls were consecutively recruited from the outpatient clinic and inpatient ward of the Department of Nephrology, Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine. Between July 2025 and August 2025, 15 patients with DKD and 15 healthy controls were enrolled in the study. Peripheral venous blood samples were collected from all participants for total RNA extraction and RT-qPCR was used to detect the expression of three hub genes. The inclusion criteria were established based on the KDIGO 2022 Clinical Practice Guideline for the

Management of Chronic Kidney Disease in Diabetes (50), as follows; i) UACR ≥ 30 mg/g; ii) eGFR < 60 ml/min/1.73 m² for ≥ 3 months; and iii) renal biopsy demonstrating pathological changes consistent with DKD. The exclusion criteria were as follows: i) Individuals < 18 years of age or > 65 years of age; ii) women who were pregnant or breastfeeding; iii) individuals with a history of diabetic ketoacidosis or urinary tract infection within the past month; and iv) patients diagnosed with primary diseases affecting other systems (including cardiac, hepatic, renal and hematopoietic systems).

Statistical analysis. The R software 'ggpubr' package (version 0.6.2; <https://cran.r-project.org/web/packages/ggpubr/>) was used to perform statistical analyses. Statistical comparisons between groups were performed using either the parametric Student's t-test or the nonparametric Mann-Whitney U test, depending on the data distribution. Multi-group comparisons were performed using one-way analysis of variance; when significant differences were detected, Tukey's or Bonferroni-corrected multiple comparisons were conducted. The correlation between hub gene expression and eGFR was assessed using Spearman's or Pearson's rank correlation coefficient analysis (Nephroseq v5 platform). $P < 0.05$ was considered to indicate a statistically significance difference. To evaluate the diagnostic potential of candidate genes, ROC analysis was performed utilizing the 'pROC' package (version 1.19.0.1; <https://www.rdocumentation.org/packages/pROC/>) in R. Diagnostic performance was quantified by calculating the area under the ROC curve (AUC), with genes

exhibiting AUC values >0.85 considered to have significant diagnostic capability. Visualization of data distributions and ROC curves was accomplished using the 'ggplot2' R package.

Results

Identification of DEGs. For the present investigation, transcriptomics data from the GSE30529 dataset were obtained from the GEO repository. The quality of data normalization was visualized using boxplot analysis (Fig. 1A). Analysis demonstrated that sample median values exhibited consistent horizontal alignment, validating the high quality of the microarray data and ensuring comparability between the experimental groups. Differential expression analysis was performed using the 'limma' R package, which identified 1,281 DEGs. Among these, 817 genes showed increased expression, whereas 464 demonstrated reduced expression. The distribution of DEGs was illustrated using a volcano plot (Fig. 1B) and hierarchical clustering heatmap (Fig. 1C).

Gene co-expression network analysis was implemented using WGCNA methodology to elucidate patterns of gene coordination. For network construction, a scale-free topology criterion with $R^2=0.85$ as the soft-thresholding parameter was employed, which optimized biological network fidelity (Fig. 1D). Module detection was performed through a combination of average linkage hierarchical clustering and dynamic branch cutting algorithms, yielding 22 discrete modules designated by distinct colors (Fig. 1E). The relationships between identified modules across control and DKD specimens were visualized using a correlation heatmap (Fig. 1F). Subsequently, MM and GS metrics were calculated to prioritize functionally relevant genes, resulting in 420 candidate hub genes. The overlap between these hub genes and the 1,281 DEGs identified by differential expression analysis was determined through Venn diagram analysis, which identified 49 genes of interest (Fig. 1G).

GSEA, and GO and KEGG functional enrichment analysis. To identify relevant biological pathways associated with the disease phenotype, GSEA was performed comparing transcriptomics profiles between renal tissue from patients with DKD and healthy control individuals. The HALLMARK analysis in the GSE30529 dataset revealed the top 10 significantly enriched biological signaling pathways associated with DKD (Fig. 2A). These pathways include 'Integrin cell surface interactions', 'Antigen processing cross presentation', 'Neutrophil degranulation', 'Interferon signaling', 'Extracellular matrix organization', 'Core matrisome', 'Signaling by interleukins', 'Leishmania infection', 'TYROBP causal network in microglia' and 'Allograft rejection', suggesting their potential regulatory roles in the pathogenesis of diabetic nephropathy (Fig. 2B-K).

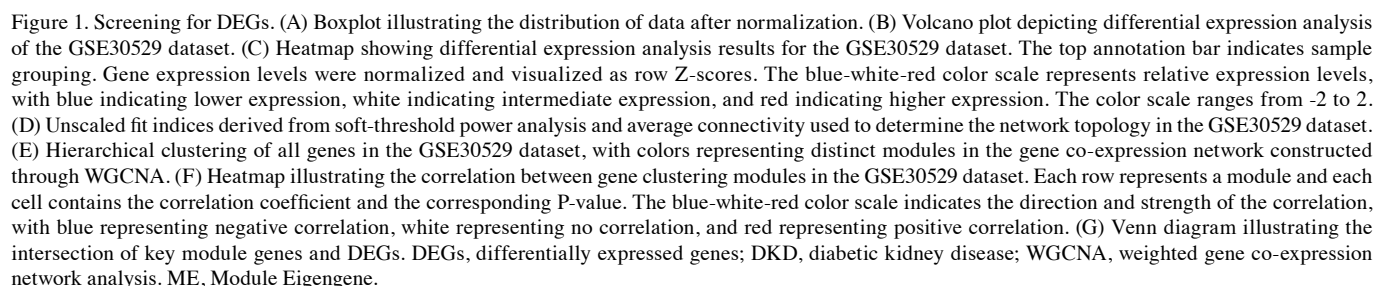
To elucidate the biological significance of the identified key genes, comprehensive functional characterization was performed. GO enrichment analysis revealed 457 significantly enriched terms, categorized into 351 BP, 50 CC and 56 MF terms. The most statistically significant terms in each category were selected for detailed examination (Fig. 2M-O). Within the BP category, prominent enriched pathways included 'neutrophil degranulation', 'neutrophil activation involved in immune

responses', 'response to nutrient levels', 'T cell activation', 'response to nutrient', 'antigen processing and presentation', 'antigen processing and presentation of peptide antigen', 'regulation of innate immune response', 'positive regulation of defense response' and 'response to drug'. CC terms were mainly related to 'endocytic vesicle', 'phagocytic vesicle', 'specific granule', 'tertiary granule', 'recycling endosome', 'secretory granule membrane', 'glutamatergic synapse', 'focal adhesion', 'cell-substrate junction' and 'specific granule membrane'. MF terms included 'GTPase activity', 'lipase activity', 'magnesium ion binding', 'G protein-coupled receptor binding', 'Wnt-activated receptor activity', 'H4 histone acetyltransferase activity', 'apolipoprotein binding', 'mannose binding', 'triglyceride lipase activity' and 'Wnt-protein binding'.

Pathway enrichment analysis using the KEGG database identified 118 significantly associated signaling cascades for the candidate genes. After applying a statistical threshold of $P<0.05$, 11 pathways emerged as particularly relevant. These pathways encompassed neurodegenerative disorders ('Alzheimer disease'), infectious disease responses ('Tuberculosis', 'Epstein-Barr virus infection', 'Human cytomegalovirus infection' and 'Epithelial cell signaling in *Helicobacter pylori* infection'), immune functions ('Phagosome', 'Antigen processing and presentation' and 'IL-17 signaling pathway'), metabolic processes ('Glycerolipid metabolism' and 'Lipid and atherosclerosis') and neurological signaling ('GABAergic synapse') (Fig. 2L).

Hub gene screening. For examination of molecular interactions, the 49 identified DEGs were submitted to the STRING database to generate a PPI network. Network visualization was accomplished using Cytoscape software (Fig. 3A). To prioritize genes based on topological properties, three distinct algorithmic approaches were used (Fig. 3B-D). Further refinement of critical gene identification was performed through the application of three independent ML methodologies to extract feature genes of biological relevance. Feature selection was performed using the LASSO regression technique, which identified 13 genes with significant predictive capacity from the pool of variables demonstrating statistical significance in univariate analysis (Fig. 3E and F). The SVM-RFE recursive method was employed for core gene screening, identifying 16 predictive genes (Fig. 3G). Additionally, RF with feature selection, a robust and interpretable method, was used to adjust the number of classification trees and the importance of the screened genes, resulting in the selection of the top 19 hub genes in descending order (Fig. 3H). Integration of these six algorithms highlighted SYK, ADAM10 and APAF1 as hub genes associated with DKD (Fig. 3I and J).

A nomogram model was subsequently developed for diagnosing DKD based on the marker genes SYK, ADAM10 and APAF1, using the rms software package (Fig. 3K). DCA indicated that the nomogram model provided substantial clinical benefits (Fig. 3L). ROC curves demonstrated that the AUC values for SYK, ADAM10 and APAF1 were 0.950, 0.942 and 0.950, respectively, demonstrating their promise for biomarker development (Fig. 3M) and highlighting their high predictive accuracy. External validation using the GSE30122 dataset (quality of data normalization shown in Fig. S1) revealed significantly elevated transcript abundance of SYK, ADAM10



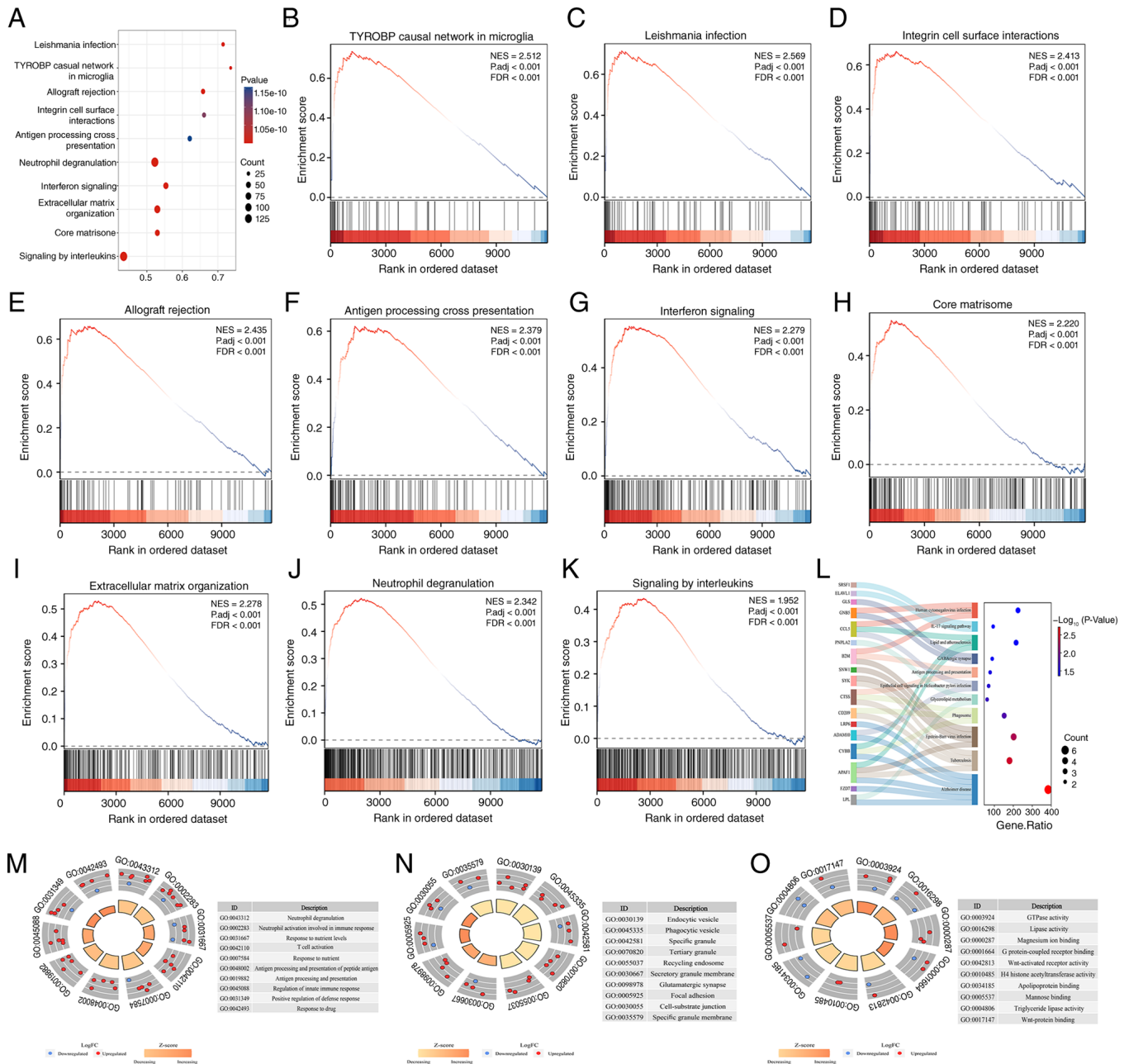


Figure 2. GSEA, GO and KEGG functional enrichment analysis. (A) Bubble plot illustrating biological signaling pathways associated with diabetic kidney disease. (B) 'TYROBP causal network in microglia'; (C) 'Leishmania infection'; (D) 'Integrin cell surface interactions'; (E) 'Allograft rejection'; (F) 'Antigen processing cross presentation'; (G) 'Interferon signaling'; (H) 'Core matrisome'; (I) 'Extracellular matrix organization'; (J) 'Neutrophil degranulation'; and (K) 'Signaling by interleukins'. (L) Sankey diagram depicting KEGG enrichment analysis results. Circle diagrams representing functional enrichment in (M) biological process categories, (N) cellular component categories and (O) molecular function categories. FDR, false discovery rate; GO, Gene Ontology; GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, normalized enrichment score.

and APAF1 in the renal tissues of patients with DKD compared with that in healthy control samples (Fig. 3N-P). To assess the role of these hub genes in the context of DKD, hub gene analysis was performed using the Nephroseq v5 bioinformatics platform; the results showed that the expression levels of SYK, ADAM10 and APAF1 were positively correlated with Scr (Fig. 3Q-S) and negatively correlated with GFR (Fig. 3T-V).

Immunological infiltration analysis. Immunological profile analysis revealed marked distinctions in cellular composition between DKD specimens and normal renal tissue. Dimensionality reduction through PCA demonstrated clear

separation between pathological and healthy samples, emphasizing the importance of immune system dysregulation in DKD progression (Fig. 4A). Computational deconvolution of the GSE30529 transcriptomic dataset using CIBERSORTx algorithms identified significant alterations in specific lymphocyte populations, particularly activated memory CD4⁺ T cells and T follicular helper cells (P<0.05; Fig. 4C and D). Additionally, correlation network analysis among 22 distinct immune cell subpopulations within glomerular compartments revealed intricate interconnections, indicating a sophisticated and highly coordinated immunoregulatory landscape in DKD pathophysiology (Fig. 4B).

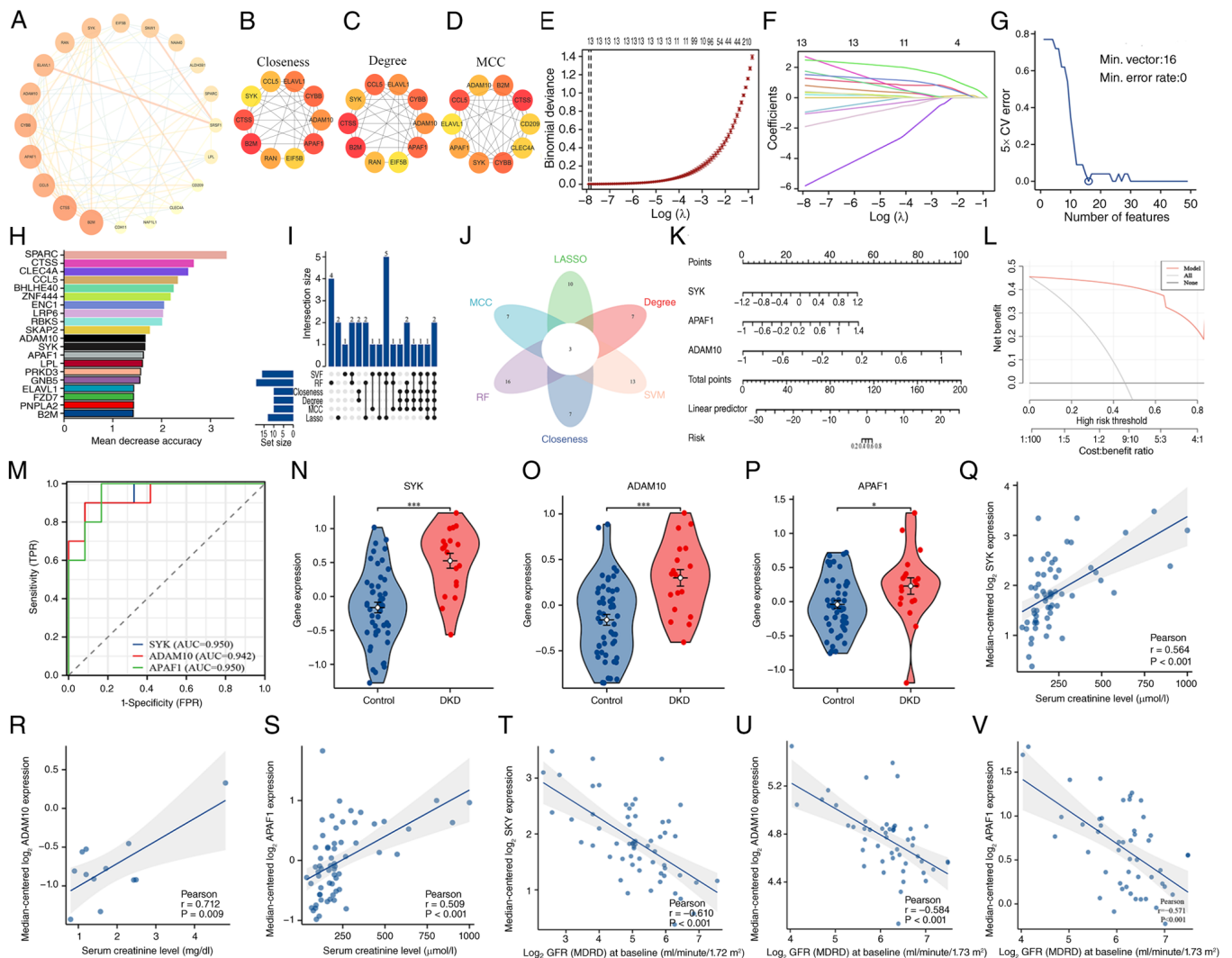


Figure 3. Hub genes for DKD diagnosis. (A) Protein-protein interaction network of intersecting genes. Diagnostic markers identified by the (B) closeness, (C) degree and (D) MCC algorithms via the cytoHubba plugin. (E and F) Screening of diagnostic markers using the LASSO logistic regression algorithm. (G) Biomarker screening using the SVM-RFE method. (H) Biomarker screening based on the RF algorithm. (I and J) UpSet plots and petal diagrams showing intersecting diagnostic markers identified by all six algorithms. (K) Nomogram for predicting DKD. (L) Decision curve analysis curve for validation of diagnostic efficacy. (M) ROC curve for validation of diagnostic efficacy of key genes. Violin plots comparing (N) SYK expression levels, (O) ADAM10 expression levels and (P) APAF1 expression levels in DKD vs. control samples within the GSE30122 validation cohort. * $P < 0.05$, *** $P < 0.001$. Analysis of the relationships between (Q) SYK and serum creatinine, (R) ADAM10 and serum creatinine, (S) APAF1 and serum creatinine, (T) SYK and GFR, (U) ADAM10 and GFR, and (V) APAF1 and GFR. ADAM10, ADAM metalloproteinase domain 10; APAF1, apoptotic peptidase activating factor 1; AUC, area under the ROC curve; DKD, diabetic kidney disease; FPR, false positive rate; GFR, glomerular filtration rate; LASSO, least absolute shrinkage and selection operator; MCC, maximum clique centrality; ROC, receiver operating characteristic; RF, random forest; SVM-RFE, support vector machine-recursive feature elimination; SYK, spleen-associated tyrosine kinase; TPR, true positive rate.

To further elucidate the immunomodulatory mechanisms, associations between the expression profiles of hub genes (SYK, ADAM10 and APAF1) and signature transcripts of various immune cell subsets were assessed. Comprehensive correlation matrices (Fig. 4E-G) revealed that SYK expression exhibited significant positive associations with naïve B lymphocytes, T follicular helper cells, plasma cells and quiescent dendritic cells, while showing inverse relationships with activated memory CD4⁺ T cells, monocytes and memory B lymphocytes (all $P < 0.05$). The ADAM10 transcript abundance demonstrated significant positive correlation with activated mast cells and resting natural killer cells, whereas significant negative associations were observed with activated natural killer cells, activated memory CD4⁺ T cells, M0 macrophages, monocytes, CD8⁺ T lymphocytes and resting

mast cells (all $P < 0.05$). For APAF1, expression levels positively correlated with resting dendritic cells, but negatively correlated with M0 macrophages, activated memory CD4⁺ T cells, γ - δ T cells, monocytes and memory B lymphocytes (all $P < 0.05$). These relationship patterns suggest that these three hub genes may function as critical modulators of immune cell dynamics in the pathogenesis of DKD.

Experimental verification of hub regulatory genes in a DKD model. To elucidate the expression profiles of hub candidate genes during the progression of DKD, fasting blood glucose levels were monitored ensuring they remained >16.7 mmol/l, and two distinct mouse models of DKD were successfully established (Fig. 5A). Subsequently, key clinical parameters in both DKD and control groups were evaluated, including

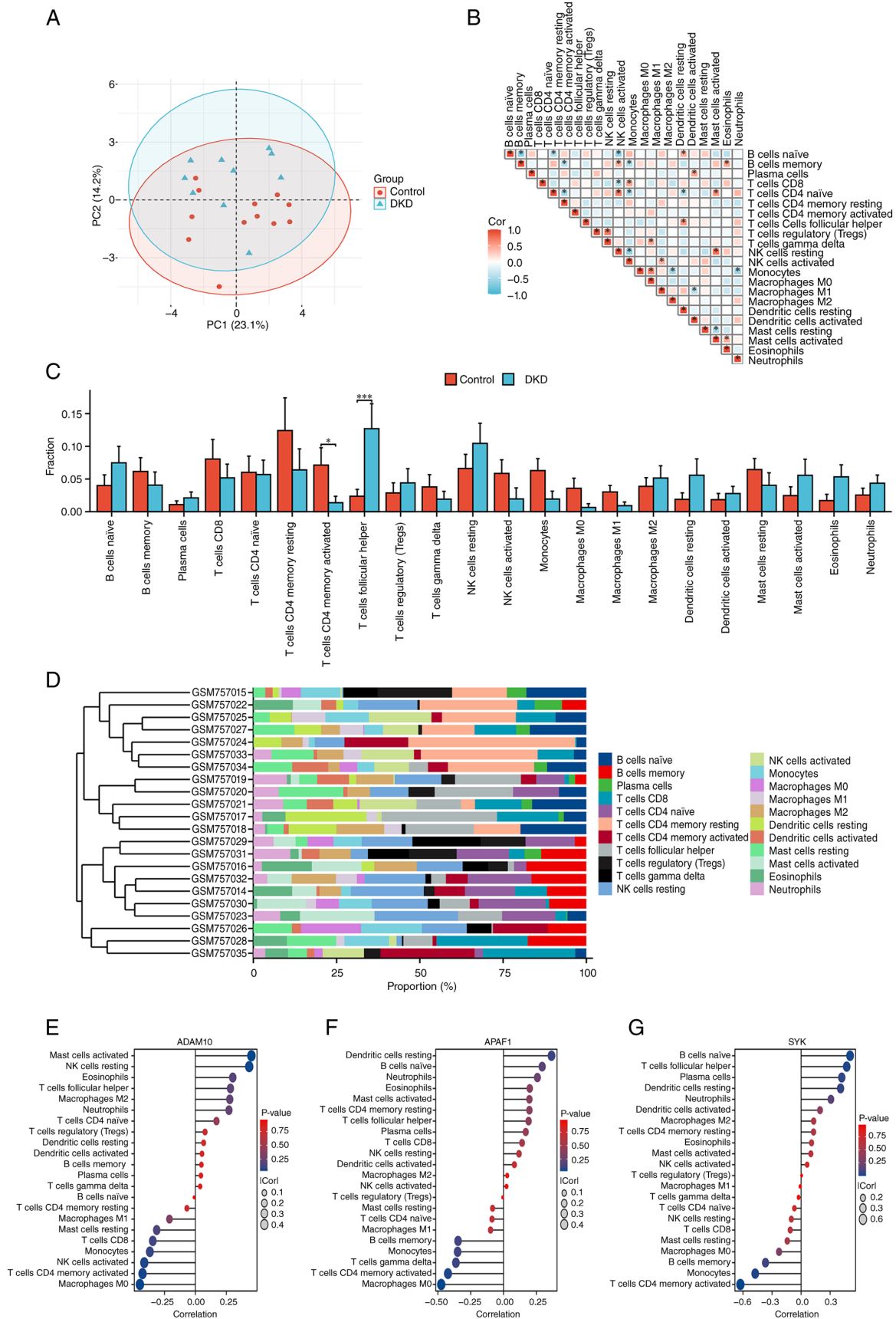


Figure 4. Analysis of the immune microenvironment. (A) Principal component analysis clustering plot illustrating immune cell infiltration patterns. The diagram presents differences in immune profiles between the DKD group and controls. (B) A heatmap displaying immune cell type correlations, where red signifies positive and blue negative associations, and the color saturation corresponds to the strength of the correlation. (C) Analysis illustrating disparities in immune cell infiltration between DKD and controls kidney tissue samples. *P<0.05, ***P<0.001. (D) Relative proportions of 22 immune cell subpopulations. Correlations between (E) ADAM10 and immune cells, (F) APAF1 and immune cells, and (G) SYK and immune cells. Point size corresponds to correlation strength between the hub gene and different immune cells; stronger correlations are shown by larger points, while weaker ones are shown by smaller points. ADAM10, ADAM metallopeptidase domain 10; APAF1, apoptotic peptidase activating factor 1; DKD, diabetic kidney disease; SYK, spleen-associated tyrosine kinase.

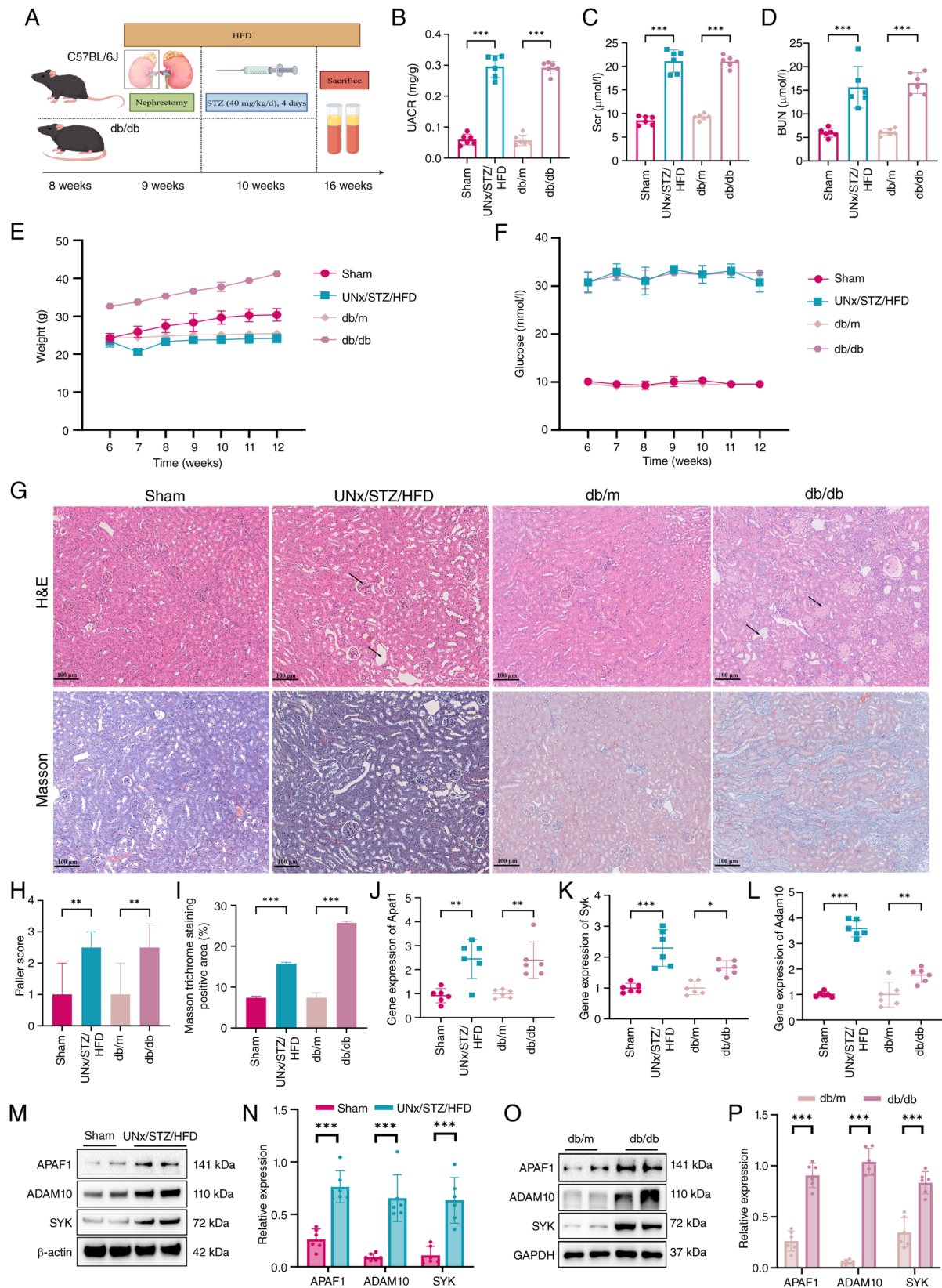


Figure 5. Validation of pivotal gene expression in the mouse DKD model. (A) Schematic illustration of the mouse DKD model. (B) Quantification of UACR in mice. (C) Scr and (D) BUN levels in the two experimental groups. (E) Body weight changes in mice over time. (F) Blood glucose alterations in mice. (G) H&E staining of mouse kidney tissue sections, highlighting the extent of renal injury. Renal fibrosis was assessed using Masson's trichrome staining (magnification, x200; scale bar, 100 μm). The arrows point to dilated renal tubules and atrophic glomeruli. Semi-quantitative analysis of (H) H&E and (I) Masson staining results, with error bars in (H) representing the 95% confidence interval for the median. Reverse transcription-quantitative PCR detection of (J) APAF1, (K) SYK and (L) ADAM10 mRNA expression. (M) Western blot analysis showing the expression levels of SYK, ADAM10 and APAF1 in the UNx/STZ/HFD model, and (N) semi-quantitative analysis. (O) Western blot analysis showing the expression levels of SYK, ADAM10 and APAF1 in the db/db model, and (P) semi-quantitative analysis. Data are presented as the mean ± SD or median (n=6). *P<0.05, **P<0.01, ***P<0.001. ADAM10, ADAM metalloproteinase domain 10; APAF1, apoptotic peptidase activating factor 1; BUN, blood urea nitrogen; DKD, diabetic kidney disease; H&E, hematoxylin and eosin; HFD, high-fat diet; Scr, serum creatinine; STZ, streptozotocin; SYK, spleen-associated tyrosine kinase; UACR, urine albumin-to-creatinine ratio; UNx, uninephrectomy.

the UACR, blood glucose, body weight, BUN and Scr. The results revealed that the UNx/STZ/HFD-induced DKD group exhibited higher UACR, blood glucose, BUN and Scr levels compared with the sham counterparts, coupled with substantial weight loss (Fig. 5B-F). Likewise, the db/db mice showed elevated UACR, body weight, blood glucose, BUN and Scr levels compared with the db/m control group (Fig. 5B-F).

Histopathological analysis was performed on renal tissue sections, employing H&E staining and Masson's trichrome staining. H&E staining identified hallmark pathological features of DKD, including glomerular hyperplasia, vacuolization of tubular epithelial cells and thickening of the glomerular basement membrane, when compared with controls. Masson's trichrome staining further revealed pronounced interstitial fibrosis in both DKD groups (Fig. 5G). Quantitative analysis results of H&E and Masson staining are shown in Fig. 5H and I.

To corroborate these histological observations, western blotting and RT-qPCR analyses were performed on renal tissues from both DKD mouse models. RT-qPCR results demonstrated a significant upregulation in the mRNA expression levels of SYK, ADAM10 and APAF1 in both DKD models (Fig. 5J-L). Western blot analysis further validated these findings, showing significantly increased protein expression of SYK, ADAM10 and APAF1 in the UNx/STZ/HFD model (Fig. 5M and N), with a similar pattern observed in the db/db mice (Fig. 5O and P). These results align with the bioinformatics predictions, providing robust evidence for the involvement of these genes in DKD progression.

siRNA-mediated silencing of hub genes ameliorates HG-induced fibrosis-associated protein expression in HK-2 cells. To further investigate the role of hub genes in renal fibrosis, an *in vitro* model was established using HG-induced HK-2 cells. Western blot analysis revealed that, compared with in the LG group, three hub genes-SYK, ADAM10 and APAF1, alongside increased expression of fibrosis-related markers, including fibronectin, vimentin, and Snail, in HG-treated HK-2 cells (Fig. 6B, C, E, F, H and I). These findings suggested that the aberrant upregulation of hub genes may contribute to the progression of renal fibrosis.

To explore the functional relevance of these hub genes, siRNAs were utilized to knock down their expression. Compared with in the HG group, knockdown of SYK using SYK-siRNA resulted in a significant reduction in fibrosis marker expression (Fig. 6A-C). Similarly, silencing ADAM10 with ADAM10-siRNA led to a significant decrease in fibrosis markers (Fig. 6D-F). Furthermore, silencing APAF1 with APAF1-siRNA reversed the upregulation of fibrosis markers (Fig. 6G-I). These results provide strong evidence that hub genes serve a pivotal role in the regulation of renal fibrosis, and their targeted suppression can effectively attenuate fibrosis associated with DKD. This highlights the therapeutic potential of hub gene inhibition in mitigating the progression of renal fibrosis.

Clinical validation of hub genes as diagnostic biomarkers in DKD. To further assess the diagnostic potential of hub genes in human DKD, the expression levels of three hub genes, SYK, ADAM10 and APAF1, were evaluated in peripheral blood samples from both patients with DKD and non-DKD

(healthy) individuals using RT-qPCR. The results revealed significantly elevated expression levels of SYK, ADAM10 and APAF1 in the DKD cohort compared with those in the non-DKD group ($P < 0.05$; Fig. 7A-C). These findings are consistent with the results of the bioinformatics analysis and provide robust evidence supporting the involvement of these genes in DKD progression. Furthermore, these results underscore the potential of these hub genes as valuable diagnostic biomarkers, providing new options for future clinical research and therapeutic exploration.

Discussion

DKD is a form of CKD resulting from diabetes mellitus and remains among the most prevalent and serious chronic complications in diabetic populations (51). In developed countries, individuals with ESRD due to DKD account for 30-50% of all patients on dialysis or receiving kidney transplant, resulting in a considerable burden on healthcare systems and socioeconomic structures (52,53). Epidemiological studies have indicated that the incidence of DKD among individuals with type 2 diabetes mellitus is increasing annually, primarily due to the insidious progression of the disease. The incidence of DKD is rising notably, particularly in emerging economies such as those in Asia and Africa (6,54). Beyond hyperglycemia, other factors such as hypertension, obesity, genetic predisposition and lifestyle choices markedly increase the risk of DKD (55-57). Notably, early-stage DKD often presents with minimal or no symptoms, usually manifesting only as microalbuminuria (58). As the disease progresses, it can lead to massive proteinuria, gradual renal function decline and eventually end-stage renal failure. Thus, early screening, accurate diagnosis and risk stratification of DKD are crucial for improving clinical outcomes (59). Despite the implementation of various preventive and therapeutic strategies in recent years, the incidence and disability rates of DKD remain high (60). Therefore, investigating specific molecular markers underlying DKD is essential for enhancing patient prognosis and reducing the worldwide impact of kidney disease (61). The present investigation utilized DKD tissue transcriptomics profiles obtained from the GEO repository to identify hub genes involved in DKD. Immune cell infiltration patterns using DEG analysis, PPI networks, WGCNA and ML approaches were also investigated. Moreover, hub gene expression was validated in DKD tissues using mouse models and clinical samples.

In the present study, 1,281 DEGs significantly associated with DKD were identified. To further clarify their involvement in disease pathogenesis, functional enrichment analyses were performed. GO analysis demonstrated that these DEGs were predominantly enriched in BPs associated with regulation of the innate immune system and neutrophil activation in immune response. KEGG analysis highlighted associations with glycerolipid metabolism, antigen processing and presentation, the IL-17 signaling pathway, as well as lipid metabolism and atherosclerosis. These findings align with the work of Zhong *et al.* (62); this previous study demonstrated that hub DKD-associated genes are mainly involved in pathways linked to humoral immune regulation, activation of the immune response and leukocyte-mediated immunity.

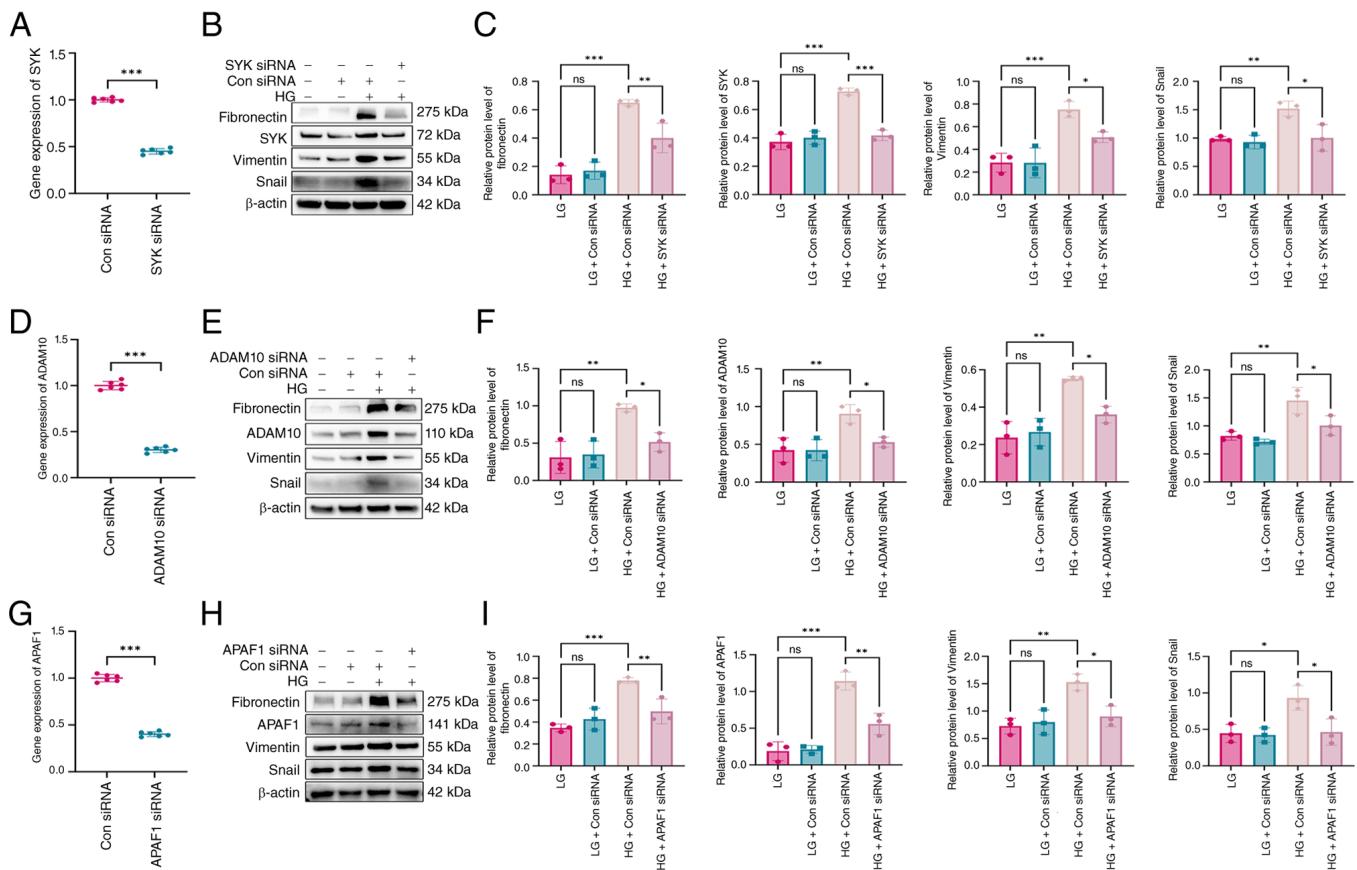


Figure 6. siRNA-mediated silencing of pivotal genes can alleviate fibrosis. (A) SYK siRNA reduced SYK mRNA levels. (B) Western blot analysis showing the expression levels of SYK, fibronectin, vimentin and Snail, and (C) semi-quantitative analysis. (D) ADAM10 siRNA reduced ADAM10 mRNA levels. (E) Western blot analysis showing the expression levels of ADAM10, fibronectin, vimentin and Snail, and (F) semi-quantitative analysis. (G) APAF1 siRNA reduced APAF1 mRNA levels. (H) Western blot analysis showing the expression levels of APAF1, fibronectin, vimentin and Snail, and (I) semi-quantitative analysis. Data are presented as the mean $\pm$ SD (n=6). *P<0.05, **P<0.01, ***P<0.001. ADAM10, ADAM metalloproteinase domain 10; APAF1, apoptotic peptidase activating factor 1; Con, control; HG, high glucose; LG, low glucose; ns, not significant; siRNA, small interfering RNA; SYK, spleen-associated tyrosine kinase.

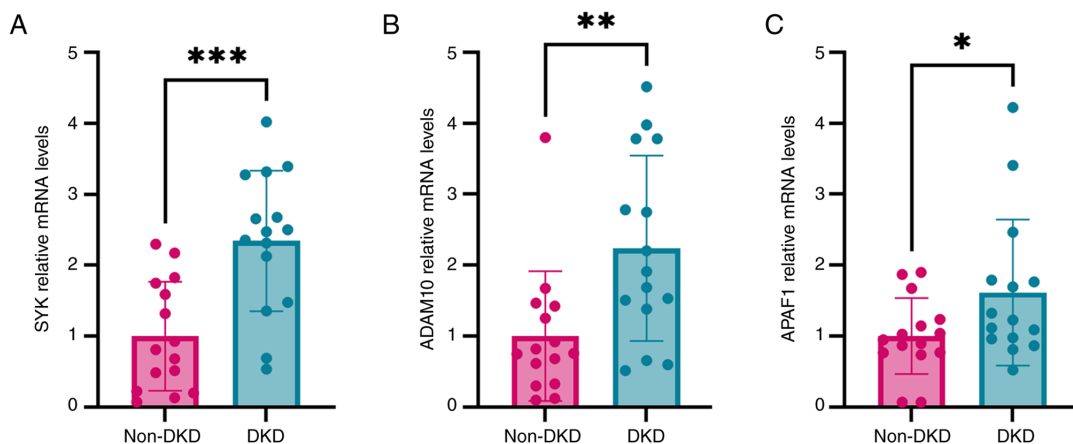


Figure 7. Clinical validation of hub genes. Relative mRNA levels of (A) SYK, (B) ADAM10 and (C) APAF1 in non-DKD patients and patients with DKD. Data are presented as the mean $\pm$ SD (n=15). *P<0.05, **P<0.01, ***P<0.001. ADAM metalloproteinase domain 10; APAF1, apoptotic peptidase activating factor 1; DKD, diabetic kidney disease; SYK, spleen-associated tyrosine kinase.

Using the identified DEGs, WGCNA, PPI networks and three ML algorithms (RF, LASSO and SVM-RFE) were integrated for the identification of potential diagnostic biomarkers for DKD. Ultimately, three hub genes, SYK, ADAM10 and APAF1, were identified. ROC curve analysis revealed that the AUC value for each of these genes was >0.85, underscoring

their significant diagnostic potential. Furthermore, analysis using the Nephroseq v5 online tool demonstrated that the expression of these genes was inversely associated with eGFR and positively associated with Scr levels. Finally, qPCR and western blot analysis revealed that, in addition to increased expression of hub genes in DKD mice, these changes were

accompanied by significant renal fibrosis and elevated levels of fibrosis markers, such as fibronectin, vimentin and Snail. The concurrent upregulation of these genes and fibrosis-related markers suggests that they may be involved in the progression of renal fibrosis. To further elucidate the direct association between these three hub genes and the DKD pathological process, siRNA-mediated targeted suppression of the hub genes was performed and was shown to improve the progression of fibrosis associated with DKD. Thus, the integrated approach adopted in the present study effectively identified potential biomarkers with practical relevance for DKD.

Numerous studies have underscored the marked role of the three hub genes identified in the current study in the progression of DKD. SYK, in particular, has garnered increasing attention for its involvement in the pathogenesis of various renal disorders, including proliferative glomerulonephritis, acute kidney transplant rejection, diabetic nephropathy and kidney fibrosis (63). Evidence indicates that SYK contributes to the activation of NF- κ B in human glomerular endothelial cells and proximal tubular cells under HG conditions, thus serving a pivotal role in regulating immune and inflammatory responses (64,65). Recent studies have detected elevated SYK expression in diabetic nephropathy, which can influence macrophage activity and exacerbate fibrosis (66,67). By contrast, inhibition of SYK has been shown to mitigate oxidative stress and apoptosis associated with DKD by down-regulating proteins involved in the PKC β /P66shc signaling pathway (68). Recent findings have also suggested that hyperglycemia enhances ADAM10 expression, promoting the shedding of its extracellular domain and proteolytic activity, while simultaneously augmenting NADPH oxidase function, reactive oxygen species (ROS) generation and permeability to albumin (69). Moreover, α Klotho deficiency has been associated with elevated ADAM10 activity, increased NADPH oxidase function and augmented ROS levels, all of which contribute to enhanced albumin permeability (69). ADAM10 protein expression and activity have also been observed in both glomerular and urine samples from diabetic rats (70). According to Sui *et al* (71), hyperglycemic conditions induce increased ADAM10 expression in podocytes, whereas silencing ADAM10 in these cells attenuates inflammation, apoptosis and pyroptosis, and suppresses activation of the MAPK signaling pathway. Although APAF1 has not been extensively studied in the context of DKD (72), it remains a promising therapeutic target, warranting further investigation.

In the present study, the CIBERSORTx algorithm was utilized to analyze patterns of immune cell infiltration throughout DKD development, providing insights into the impact of immune cells on the disease process. Growing evidence has underscored the critical involvement of immune regulation in DKD progression (73,74). Alterations in immune cell infiltration appear to serve a notable role in both the initiation and advancement of DKD, with lymphocytes actively participating in its underlying pathological mechanisms. T and B lymphocytes contribute to kidney injury in DKD by releasing pro-inflammatory cytokines, with T cells, particularly the CD4 and CD8 subsets, being central to disease onset and progression. Abnormal T-cell infiltration and activation in the renal interstitium may represent a key immunopathological mechanism in DKD, as activated T cells can cause renal

injury directly through cytotoxicity or indirectly by promoting macrophage recruitment and activation. These insights may inform the development of novel therapeutic strategies for DKD (75).

Research has revealed substantial accumulation of CD4⁺ and CD8⁺ T cells within the renal interstitium of diabetic rats (76). Similarly, individuals with type 2 diabetes exhibit elevated numbers of these T-cell subsets in the renal interstitium, and an increased CD4⁺ T-cell count has been shown to positively associate with the severity of proteinuria (76). T follicular helper cells, a specialized subset of CD4⁺ T cells, serve a key role in humoral immunity and are predominantly located in secondary lymphoid tissues, such as the tonsils, lymph nodes and spleen (77). These cells promote the maturation of germinal center B cells into antibody-producing plasma cells and memory B cells, thereby enhancing immune responses (78,79). The development of T helper (Th) cell subsets is influenced by both CD4⁺ T-cell differentiation and the specific cytokine environment. Established differentiation pathways for Th1, Th2, Th17 and regulatory T (Treg) cells have been described, with most studies suggesting that Th1 and Th17 cells are pathogenic in DKD. Zhang *et al* (80) demonstrated a positive association between UACR and the number of Th1 and Th17 cells in patients with type 2 diabetic nephropathy. It has also been shown that Th1 and Th17 cells, along with pro-inflammatory cytokines, collectively contribute to renal tissue damage in DKD (81). Moreover, Anand *et al* (82) proposed that Th2 cytokines may ameliorate insulin resistance, suggesting that the onset of DKD could be associated with suppressed Th2 responses. This study evaluated Th2 cytokine levels in healthy individuals, diabetic patients and patients with DKD, revealing a suppression of Th2 cytokines in the DKD group, particularly characterized by a decrease in the expression of IL-13 and IL-33, which are key regulators of Th2 differentiation. Furthermore, IL-33 levels were inversely correlated with urinary microalbumin levels. These observations correspond to those of Zamauskaite *et al* (83); this previous study reported that following peritoneal dialysis, the percentage of Th2 cells in patients with DKD are markedly increased, with IL-4 levels produced by Th2 cells positively associated with dialysis duration.

In contrast to Th cells, Treg cells are essential for preserving immune tolerance and safeguarding against immune-related kidney injury in diabetes (84). Evidence from animal experiments and certain clinical studies have indicated that diminished Treg cell function may be associated with the advancement of DKD (85,86). Eller *et al* (87) demonstrated that removal of Treg cells in db/db mice may intensify the features of DKD, such as increased proteinuria, glomerular hyperfiltration, greater insulin resistance, and a heightened pro-inflammatory state in both visceral adipose tissue and the kidneys. Conversely, introducing CD4⁺/Foxp3⁺ Treg cells led to a notable decrease in inflammatory cell populations, and markedly improved both insulin resistance and DKD symptoms. These results highlight the beneficial impact of Treg cells in DKD, as they help suppress inflammation.

The present study identified SYK, ADAM10 and APAF1 as potential diagnostic biomarkers for DKD. Additionally, the findings indicated that immune cells could be crucial contributors to both the onset and development of diabetic nephropathy.

Notably, the three hub genes showed significant associations with multiple immune cell types, indicating that these immune cells may exert a substantial influence on the development of DKD. A thorough investigation of these immune cells could aid in identifying novel immunotherapeutic targets and optimizing immunomodulatory therapies for patients with diabetic nephropathy. Additionally, advanced methodologies were applied, including SVM-RFE, LASSO logistic regression and RF algorithms, to identify diagnostic indicators for diabetic nephropathy, demonstrating high diagnostic efficacy. Nonetheless, several limitations warrant consideration. First, the present study does not encompass the entire clinical course of diabetic nephropathy, and the diagnostic markers identified may not be universally applicable. Moreover, the present study did not perform comparative analyses of the expression characteristics of these genes in other common kidney diseases, such as chronic glomerulonephritis, IgA nephropathy and membranous nephropathy. Consequently, further long-term follow-up and validation in a large, multicenter clinical cohort are needed before these biomarkers can be reliably integrated into clinical decision-making.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

QX contributed to the conceptualization and design of the study. CX provided administrative support and study conception. ZL contributed to study design and data interpretation, in addition to administrative support. JL provided study materials or patients, and collected and analyzed clinical data. JH and QX were responsible for conducting the experiments. Data collection and assembly were carried out by LL. Data analysis and interpretation were performed by QX and CX. LL and JH confirm the authenticity of all the raw data. The

manuscript was collaboratively written by all authors, who read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval for the animal experiments was obtained from the Institutional Animal Care and Use Committee of Shanghai Rat & Mouse Biotech., Ltd. (approval no. LL-2024-060). All procedures performed involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki, and its later amendments or comparable ethical standards. All study procedures were approved by the Ethics Committee of Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine (approval no. 2024-7th-HIRB-097). Patients included in the present study have provided written informed consent for participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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