Uncovering the Role of Anoikis-Related Genes in Modulating Immune Infiltration and Pathogenesis of Diabetic Kidney Disease

Jiaqiong Lin, Yan Lin, Xiaoyong Li, Fei He, Qinyuan Gao, Yuanjun Wang, Zena Huang, Fu Xiong

Background: Diabetic kidney disease (DKD) is an intricate complication of diabetes with limited treatment options. Anoikis, a programmed cell death activated by loss of cell anchorage to the extracellular matrix, participated in various physiological and pathological processes. Our study aimed to elucidate the role of anoikis-related genes in DKD pathogenesis.

Methods: Differentially expressed genes (DEGs) associated with anoikis in DKD were identified. Weighted gene co-expression network analysis (WGCNA) was conducted to identify DKD-correlated modules and assess their functional implications. A diagnostic model for DKD was developed using LASSO regression and Gene set variation analysis (GSVA) was performed for enrichment analysis. Experimental validation was employed to validate the significance of selected genes in the progression of DKD.

Results: We identified 10 anoikis-related DEGs involved in key signaling pathways impacting DKD progression. WGCNA highlighted the yellow module’s significant enrichment in immune response and regulatory pathways. Correlation analysis further revealed the association between immune infiltration and anoikis-related DEGs. Our LASSO regression-based diagnostic model demonstrated a well-predictive efficacy with seven identified genes. GSVA indicated that gene function in the high-risk group was primarily associated with immune regulation. Further experimental validation using diabetic mouse models and data analysis in the single-cell dataset confirmed the significance of PYCARD and SFN in DKD progression. High glucose stimulation in RAW264.7 and TCMK-1 cells showed significantly increased expression levels of both Pycard and Sfn. Co-expression analysis demonstrated distinct functions of PYCARD and SFN, with KEGG pathway analysis showing significant enrichment in immune regulation and cell proliferation pathway.

Conclusion: In conclusion, our study provides valuable insights into the molecular mechanisms involved in DKD pathogenesis, specifically highlighting the role of anoikis-related genes in modulating immune infiltration. These findings suggest that targeting these genes may hold promise for future diagnostic and therapeutic approaches in DKD management.

Keywords: diabetic kidney disease, anoikis, immune infiltration, biomarker, PYCARD, SFN

Introduction

In the past decade, the prevalence of diabetes mellitus, a metabolic disorder marked by hyperglycemia, has been steadily increasing. Diabetes mellitus, a metabolic disorder marked by hyperglycemia, has been steadily increasing. Diabetic kidney disease (DKD) is a type of kidney disease that results from diabetes, with roughly 40% of diabetic patients developing DKD, which eventually leads to end-stage kidney disease. Pathologically, DKD is...
characterized by structural injury to the glomerulus and tubules, leading to proteinuria as a hallmark symptom. Currently, available treatment strategies primarily focus on decelerating disease progression, as there are no curative therapies for DKD. Therefore, identifying potential early diagnostic indicators and therapeutic targets for DKD is of paramount importance.

Anoikis is a distinct form of programmed cell death that differs from other types, including apoptosis, autophagy, and pyroptosis. It is initiated when cell adhesion to the extracellular matrix (ECM) is disrupted, promoting mismatched cellular death to maintain tissue homeostasis. Anoikis activation consists of two major pathways: endogenous and exogenous. The endogenous pathway, known as the mitochondrial death pathway, is triggered by stimuli such as high glucose and oxidative stress, which cause a decrease in mitochondrial membrane potential and release of oxygen radicals. This event triggers the activation of internal mitochondrial protein kinases, which eventually activate downstream execution molecules of anoikis, such as caspase 9 and caspase 3. The exogenous pathway, called the Fas/FasL pathway, mediates the onset of anoikis through activating downstream execution molecules via receptor and ligand binding.

In recent times, the significance of anoikis has emerged in various pathological processes associated with disease like cancers, diabetes, and cardiovascular disorders. The intricate interactions between cells and the ECM are predominantly maintained through the binding of collagen and integrins. These interactions play a pivotal role in governing essential cellular functions such as adhesion, migration, and differentiation. However, in diabetic vascular disease, changes in the modification of collagen can trigger endothelial cell anoikis, resulting from the detachment from the ECM. Excessive activation of anoikis can lead to the exacerbation of diabetic retinopathy. In diabetic retinopathy, increased expression of Cyr61 and CTGF has been shown to mediate anoikis through MMP2 activation. Moreover, recent research has identified the engagement of molecular pathways, including MAPK, NF-κB, and AKT signaling pathways, in the activation of anoikis. Importantly, these same pathways have also been implicated in the progression of DKD, emphasizing the potential role of anoikis in disease advancement.

The involvement of anoikis-related genes in DKD has not been extensively studied, leading to an insufficient understanding of their comprehensive role. Addressing this gap, our study aimed to comprehensively investigate the relationship between anoikis-related genes and DKD. To accomplish this, we identified differentially expressed genes (DEGs) associated with anoikis in DKD, followed by pathway enrichment analysis to gain insight into the underlying biological mechanisms. A predictive signature based on seven anoikis-related DEGs were developed using LASSO regression analysis. The expression level of genes in the predictive signature was validated in a mouse model of DKD, leading to the identification of PYCARD and SFN as potentially important candidate molecules. The potential molecular mechanisms underlying these two genes were further elucidated using single-cell datasets and correlation analysis. This investigation not only deepens our comprehension on the pathogenesis of DKD, but also identifies potential early diagnostic indicators and therapeutic targets for DKD.

**Method**

**Animals**

Animal experiments conducted in this study were carried out in accordance with guidelines for the treatment and welfare of Animals, including adherence to the Public Health Service Policy on Humane Care and Use of Laboratory Animals, as well as the NIH Guide for the Care and Use of Laboratory Animals. The ethical approval was granted by the animal ethical committee of Guangzhou Medical University. As for the experimental subjects used in our study, we utilized male mice of the C57BL/6 strain at six weeks of age, and diabetic mice were modeled using established research protocols. Initially, the mice were randomly assigned to either a normal diet group (consisting of 10% fat calories, 20% protein calories, and 70% carbohydrate) or a high-fat diet group (consisting of 45% fat calories, 20% protein calories, and 35% carbohydrate). Following 35, 36, and 37 days of high-fat feeding, STZ injections were administered at a dose of 40 mg/kg to induce diabetes. Throughout the study, regular recordings of body weight and blood glucose levels were conducted on a weekly basis. Once successful modeling was achieved, the mice were humanely euthanized under anesthesia. The success of the DKD model was confirmed by histopathological analysis.
Cell Lines and Culture
RAW 264.7 and TCMK-1 cells (Procell Life Science&Technology Co., Ltd) were cultured in a DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37°C under controlled conditions of 37°C and 5% CO2. To examine the effect of glucose on these cell lines, they were classified into two groups: a normal glucose group and a high glucose group. The normal glucose group was maintained in DMEM medium containing 5.5 mM glucose, while the high glucose group was cultured in DMEM medium supplemented with 40 mM glucose.

RNA Extraction and RT-PCR
The total RNA from kidney tissue was extracted using Trizol reagent (Invitrogen, California, USA) and subjected to reverse transcription utilizing HiScript III RT SuperMix (Vazyme, Nanjing, China). The ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) was used for quantitative polymerase chain reaction (qPCR). GAPDH was employed as the reference gene. Table 1 lists the primer sequences utilized in this experiment.

Data Preparation
To obtain gene expression profiles related to DKD, we downloaded the relevant datasets from the Gene Expression Omnibus (GEO) dataset (https://www.ncbi.nlm.nih.gov). The datasets GSE30528, GSE96804, GSE142025 and GSE131882 were derived from kidney biopsy samples, while GSE142153 consisted of peripheral blood samples collected from normal control (NC) and DKD patients. Specifically, we utilized the GSE30528 dataset for identifying DEGs, conducting weighted gene co-expression network analysis (WGCNA), and establishing training set. The external validation sets of GSE96804, GSE142025, and GSE142153 were used to validate the findings. Furthermore, the gene expression pattern in DKD was investigated using the GSE131882 dataset.

Identification of Anoikis-Related DEGs
An initial list of genes related to anoikis was obtained from the Harmonizome web portal (https://maayanlab.cloud/Harmonizome/) and the GeneCards database (https://www.genecards.org/). Next, R package “Limma” was utilized to detect DEGs in healthy individuals and patients with DKD after normalization. DEGs were visualized by a volcano plot with “ggplot2” R package. The criteria for DEGs were established as: |log2 fold change| (>1 and an adjusted P-value<0.05. Lastly, we identified anoikis-related DEGs by intersecting between anoikis-related genes and the DEGs by employing the “ggvenn” R package.

Pathway Enrichment Analysis
To gain deeper insights into the biological functions of genes, pathway enrichment analysis was conducted using Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Gene set enrichment analysis (GSEA) was performed utilizing

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
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<tr>
<td>Itga3-F</td>
<td>CCTCTTCGGCTACTCGGTC</td>
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<tr>
<td>Itga-R</td>
<td>CCGTTGGTATAGTCATCACCC</td>
</tr>
<tr>
<td>Vegfa-F</td>
<td>CTGCCGTCCGATTGAGACC</td>
</tr>
<tr>
<td>Vegfa-R</td>
<td>CCCCTCCTTGATCACCCTGC</td>
</tr>
<tr>
<td>Nqo1-F</td>
<td>AGGATGGGAGGTACTCAATC</td>
</tr>
<tr>
<td>Nqo1-R</td>
<td>AGGCCGTCCTCTTATATGCTA</td>
</tr>
<tr>
<td>Igf1-F</td>
<td>CTGCCGTCGGACTGCTTC</td>
</tr>
<tr>
<td>Igf1-R</td>
<td>GAAGGGGCTTCTGAGTCTT</td>
</tr>
<tr>
<td>Hmox1-F</td>
<td>AAGCCCGAATGCTGAGTCTC</td>
</tr>
<tr>
<td>Hmox1-R</td>
<td>GCCCGTGATAGATGCAAAGGA</td>
</tr>
<tr>
<td>Pycard-F</td>
<td>CTTGTACAGGGGATGAACTCA</td>
</tr>
<tr>
<td>Pycard-R</td>
<td>GCCATACGACTGATCACATAG</td>
</tr>
<tr>
<td>Sfn-F</td>
<td>GTGTGTACACCCGAGTACT</td>
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<td>Sfn-R</td>
<td>CCTGGTAGTGAGCCGTAG</td>
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KEGG gene sets from the GSEA C2 dataset to identify pathways that were significantly enriched for DEGs. Furthermore, the R package “clusterProfiler” was utilized to conduct KEGG enrichment analyses, enabling further exploration of molecular pathways associated with DEGs related to anoikis. In addition, pathway activation scores were determined by employing Gene-set variation analysis (GSVA) based on Hallmarks and KEGG pathway. And differences in pathway activation scores between different groups were compared using “Limma” R package.

**Weighted Co-Expression Network Analysis**

Weighted co-expression network analysis (WGCNA) was carried out using “WGCNA” R package. Initially, cluster analysis was conducted to identify and remove outlier samples in order to ensure integrity and reliability of the subsequent analyses. A soft-thresholding power of eight was selected to construct a scale-free network, and gene co-expression modules were generated by computing the adjacency matrix. Subsequently, the correlation between each module and phenotype was calculated, and the results were presented as a heatmap. In addition, these modules were annotated with KEGG analysis to identify the involved biological processes.

**Construction and Validation of a Predictive Signature**

We employed the Lasso regression Methodology to develop a predictive signature comprising seven genes, utilizing anoikis-associated DEGs. The risk score for each sample was calculated using the formula: \( \text{Risk score} = \sum_{j=1}^{n} \text{Exp}_j \times \text{Coe}_j \), where \( \text{Exp}_j \) and \( \text{Coe}_j \) signify gene expression and coefficient, respectively. Following the Youden index, samples were stratified into low-risk or high-risk groups. Subsequently, receiver operating characteristic (ROC) curve analysis was conducted to evaluate the predictive signature’s diagnostic potential in both training and test datasets.

**Correlation Analysis**

To evaluate the levels and function of immune cell infiltration, single-sample gene set enrichment analysis (ssGSEA) was utilized to generate an enrichment score. In order to determine any difference in immune infiltration within the high-risk and low-risk groups, the Wilcoxon test was employed. Spearman correlation analysis was carried out to investigate the association regarding LASSO genes and ssGSEA enrichment scores, which were subsequently represented visually by corrplots. Scatter plots were utilized to demonstrate the strongest correlations. In addition, gene co-expression analyses of PYCARD and SFN were performed using Spearman correlation analysis.

**Single-Cell RNA Data Analysis**

To further investigate the expression patterns of PYCARD and SFN in DKD, we downloaded the raw count matrix of GSE131882 from the GEO dataset, which included samples from three healthy individuals and three DKD patients. Data analysis was conducted utilizing the “Seurat” R package. Cells with transcript counts below 200 or above 2500, as well as those with mitochondrial content greater than 10%, were removed through filtering. Batch effects were eliminated by applying the “Harmony” R package. Ultimately, 12 distinct cell types were identified through clustering.

**Statistical Analysis**

The results of the analysis were presented as mean ± SEM values. Two independent samples \( t \)-tests were conducted to compare gene expression levels between NC and DKD mice.

**Results**

**Identification of Anoikis-Related DEGs and Functional Enrichment Analysis**

A total of 322 DEGs (|log2FC|>1, adjusted \( P<0.05 \)) between healthy individuals and DKD patients of GSE30528 were identified, including 234 downregulated and 88 upregulated DEGs (Figure 1A). GSEA was employed to measure the enrichment of KEGG pathways. The Results revealed that immune regulation related pathways, including cytokine and cytokine receptor interaction, as well as the T cell receptor signaling pathway were highly enriched in DKD (Figure 1B). Subsequently, 10 anoikis-related DEGs were screened via overlapping DEGs and anoikis-related genes (Figure 1C). The expression level of 10 anoikis-related DEGs was...
Figure 1 Expression landscape and functional analysis of anoikis-related genes in diabetic kidney disease (DKD). (A) Volcano plot of differentially expressed genes (DEGs) in GSE30528. (B) GSEA analysis of DEGs. (C) Venn diagram of DEGs and anoikis-related genes. (D) The expression level of anoikis-related DEGs between normal control (NC) and DKD. (E) KEGG enrichment analysis of anoikis-related DEGs. (F) Correlation analysis of anoikis-related DEGs. *p<0.05 vs NC group. **p<0.01 vs NC group. ***p<0.001 vs NC group.

Abbreviations: NC, normal control; DKD, diabetic kidney disease.
displayed in Figure 1D. KEGG enrichment analysis of these 10 genes indicated their involvement in chemical carcinogenesis, reactive oxygen species, HIF-1, PI3K-Akt and p53 signaling pathways (Figure 1E). Strong correlations were found among these genes, indicating they may act together in DKD progression (Figure 1F).

Annotation of Cluster Modules Using WGCNA
To investigate key modules correlated with DKD, WGCNA was carried out. A soft-power value of 8 was chosen to derive mean connectivity and scale-free network (Figure 2A and B). Totally, 13 modules were identified (Figure 2C). The heatmap was utilized to depict the relationship between modules and clinical phenotypes, with the turquoise, red and yellow module exhibiting the highest correlation with DKD (Figure 2D). Subsequently, KEGG enrichment analysis was conducted to uncover the potential mechanisms underlying DKD (Figure 2E). The yellow module, displaying the strongest positive correlation with DKD, exhibited a noteworthy enrichment in immune response and regulation pathways.

Correlation Between Immune Infiltration and Anoikis-Related DEGs
Above results indicated a crucial role of immune deregulation in the pathogenesis of DKD. To investigate this further, we analyzed immune infiltration levels between NC and DKD group. Our results revealed higher levels of immune infiltration, including B cell, T cell, dendritic cell and kill cell, in the DKD group than those observed in the NC group (Figure 3A). Subsequently, we carried out correlation analysis to explore the association between immune infiltration and anoikis-related DEGs. The results demonstrated a closed association between immune infiltration and anoikis-related DEGs. Specifically, we found a distinctly negative correlation between different immune cell types and HTRA1, while PYCARD exhibited an obvious positive correlation with these cell types, indicating their contribution to immune infiltration regulation (Figure 3B). Furthermore, we explored the association between immune function and anoikis-related DEGs. In line with our previous findings, we noted a negative and positive correlation between HTRA1 and PYCARD, respectively, with immune function. Additionally, we identified LTF as the gene that is most positively correlated with APC co-inhibition, while NQO1 exhibits the most significant negative correlation (Figure 3C). These results suggest that deregulation of anoikis-related DEGs potentially participated in the progression of DKD through modulating immune infiltration, highlighting their potential therapeutic implications and warranting further investigation.

Predictive Power Evaluation of LASSO Genes for DKD
LASSO regression was employed to establish a diagnostic model for DKD. Upon analysis, we identified seven genes as indicators for the model (Figure 4A and B). The risk score was calculated as followed: expression of ITGA3 \( \times (-8.281) \) + expression of VEGFA \( \times (-0.609) \) + expression of NQO1 \( \times (-0.151) \) + expression of IGF1 \( \times (-2.997) \) + expression of HMOX1 \( \times (1.694) \) + expression of PYCARD \( \times (0.605) \) + expression of SFN \( \times (1.284) \). To evaluate the predictive performance of the model, we performed ROC analysis on both training set and validation sets. Our results revealed a well predictive efficacy with a diagnostic efficacy of 1 in the training set, while achieving diagnostic efficacies of 0.873, 1, and 0.939 in validation sets, indicating its potential clinical usefulness (Figure 4C–F).

Biological Characteristics of the Diagnostic Model
According to the LASSO regression model, risk scores were calculated to evaluate the risk of developing DKD. Our results showed a significant increase of risk scores in the DKD group (Figure 5A). In addition, setting the optimal cutoff value at 6.54 resulted in a Youden index of 1, signifying that this risk score threshold achieved the maximum diagnostic accuracy for identifying patients with DKD within this model, which can accurately distinguish all cases of DKD (Figure 5B). GSVA-KEGG pathway analysis of high and low-risk groups revealed that gene function in the high-risk group was primarily associated with immune regulation, including primary immunodeficiency, intestinal immune network for IgA production and metabolic pathways (Figure 5C). Subsequently, Hallmark pathway activity was assessed in each sample using GSVA, and correlation analysis between LASSO genes and pathway activity enabled us to investigate the potential functions of individual genes (Figure 5D). The results demonstrated that PYCARD was primarily involved in regulating a significant number of pathways such as allograft rejection, IL2-STAT5, IL-6-JAK-STAT3, and inflammatory responses pathways. Furthermore, HMOX was predominantly associated with metabolic pathways such as adipogenesis, glycolysis, and bile acid metabolism. Notably, multiple anoikis-related genes were associated with crucial signaling pathways, including inflammatory responses, KRAS, NOTCH, and TGF-β.
Figure 2  WGCNA analysis of GSE30528. (A) Identification of the soft threshold power. (B) Analysis of the mean connectivity. (C) Cluster dendrogram. (D) Correlation between cluster modules and clinical phenotypes. (E) KEGG analysis of genes in different cluster modules.
Figure 3 Characteristics of immune cell infiltration. (A) Immune infiltration levels between NC and DKD group. (B) The correlation between immune cell infiltration and anoikis-related DEGs. The most positively or negative correlated results were presented by scatterplots. (C) The correlation between immune function and anoikis-related DEGs. The most positively or negative correlated results were presented by scatterplots. *p<0.05 vs NC group. **p<0.01 vs NC group. ***p<0.001 vs NC group.

Abbreviations: NC, normal control; DKD, diabetic kidney disease.
signaling pathways, suggesting a potential mechanism through which these genes may participate in the modulation of immune responses.

**Elevated Expression of PYCARD and SFN Observed in Mouse Models and DKD Patients**

To further validate the involvement of LASSO genes in DKD, we established a DKD mouse model and assessed the expression levels of these genes in kidney tissue using qPCR. Our findings demonstrated that Itga3 and Hmox1 were downregulated, whereas Pycard and Sfn expression levels exhibited significantly increased expression levels in mice with DKD (Figure 6A). However, no significant differences in the expression levels of other genes were observed between the two groups. Immunohistochemical staining of Pycard and Sfn displayed consistent results, demonstrating increased expression in DKD mouse (Figure 6B). For further validation, we analyzed the expression levels of PYCARD and SFN in single-cell dataset derived from healthy individuals and patients suffering from DKD (Figure 6C). Consistent with our results obtained from mice, both PYCARD and SFN expression levels exhibited a significant increase in DKD patients compared to normal subjects. Furthermore, glomerular filtration rate (GFR) is an important index for evaluating the progression of DKD. Hence, we conducted an analysis of Nephroseq database to determine whether PYCARD and SFN contributed to DKD progression. The results revealed a significant negative correlation between PYCARD/SFN and GFR, highlighting their crucial roles in DKD (Figure 6D).
Figure 5 Biological Characteristics of the diagnostic model. (A) Risk scores between NC and DKD group. (B) Youden index and cut-off value for the diagnostic model. (C) GSVA analysis between high and low-risk groups. The Orange column represents activated pathways in high-risk group, while the green column means activated pathways in low-risk group. (D) Correlation analysis between LASSO genes and pathway activity. *p<0.05. **p<0.01. ***p<0.001.

Abbreviations: NC, normal control; DKD, diabetic kidney disease.
Figure 6 Validation of LASSO genes expression. (A) mRNA expression levels of LASSO genes in kidney tissue of NC and DKD mouse. (B) Immunohistochemistry of Pycard and Sfn in kidney tissue of NC and DKD mouse. (C) Expression level of PYCARD and SFN in single-cell sequencing dataset (GSE131882). (D) The correlation between GFR and PYCARD or SFN. *p<0.05 vs NC group. **p<0.01 vs NC group.

Abbreviations: NC, normal control; DKD, diabetic kidney disease.
Deciphering the Potential Mechanisms of PYCARD and SFN in DKD Pathogenesis

To investigate the potential mechanisms underlying the roles of PYCARD and SFN in DKD progression, we analyzed their expression levels across various cell types using the GSE131882 dataset. Our findings revealed that PYCARD exhibited the highest expression level in leukocytes, while SFN expression was highest in CD-PC cells (Figure 7A). Based on these findings, we established high glucose cell models in RAW264.7 and TCMK-1 cells, respectively. Following exposure to high glucose stimulation, a significant upregulation in both Pycard and Sfn expression levels was detected, as determined by qPCR analysis (Figure 7B and C). Furthermore, we conducted co-expression analyses of

**Figure 7** Exploration of potential mechanisms of PYCARD and SFN in DKD. (A) Visualization of PYCARD and SFN expression in different cell types in single-cell sequencing dataset (GSE131882). (B) mRNA expression levels of Pycard in RAW264.7 cells. (C) mRNA expression levels of Sfn in TCMK-1 cells. (D) Identification of co-expression genes with PYCARD. (E) KEGG enrichment analysis of top 200 PYCARD-correlated genes. (F) Identification of co-expression genes with SFN. (G) KEGG enrichment analysis of top 200 SFN-correlated genes. n=3. **p<0.01 vs NC group.

**Abbreviations:** NC, normal control; DKD, diabetic kidney disease.
PYCARD and SFN using the GSE30528 dataset and presented the top 20 co-expressed genes with the strongest correlations for each gene (Figure 7D and F). KEGG pathway analysis of the 200 co-expressed genes most highly correlated with PYCARD showed significant enrichment in pathways related to phagosome, leukocyte transendothelial migration, and chemokine signaling (Figure 7E). In addition, the top 200 genes most highly correlated with SFN were significantly enriched in hippo, Wnt, NOD-like, and TGF-β signaling pathways, indicating distinct functions for PYCARD and SFN in DKD progression (Figure 7G).

Discussion

DKD is a prevalent vascular complication associated with diabetes that often presents with insidious early renal injury and nonspecific symptoms, which hinders prompt diagnosis and treatment. Apoptosis has been identified as a critical contributor to DKD pathogenesis. Specifically, anoikis, a form of apoptosis induced by cell detachment, has emerged as a critical player in the DKD progression. It has been demonstrated that podocyte loss is the main underlying cause of basement membrane damage and proteinuria in DKD. 10 Both in DKD patients and animal models, high glucose-induced suppression of podocyte integrin expression led to podocyte detachment from the basement membrane, ultimately triggering anoikis. 11 Additionally, DKD can lead to epithelial-to-mesenchymal transition (EMT) in podocytes, which could contribute to anoikis and disrupt the proper function of the glomerular filtration barrier. 12 Similarly, previous research has shown that FSP1 may promote anoikis through mediating EMT of podocytes in DKD. 13 Furthermore, overactive mTORC1 has been associated with podocyte loss by inducing endoplasmic reticulum stress and an EMT-like phenotype, which may initiate anoikis. 14 These findings emphasize the critical role of anoikis in DKD pathology.

In the present study, we identified anoikis-related DEGs in DKD and conducted KEGG enrichment analysis to determine the biological processes and pathways they were involved in. Our results revealed significant enrichments in focal adhesion, PI3K-AKT, P53, and HIF-1 signaling pathway. The focal adhesion signaling pathway played a pivotal role in regulating the process of anoikis activation. 15,16 Interestingly, this pathway has also been implicated in immune cell migration, 17 suggesting that anoikis-related genes may modulate the immune system in DKD by influencing immune cell adhesion and recruitment within the kidney tissue. On the other hand, PI3K-AKT and P53 were implicated in regulating cell proliferation, which could potentially contribute to DKD progression. 16,18 In addition, numerous studies have identified HIF-1 signaling pathway as pivotal pathway in DKD. 19,20 These findings suggest that these identified genes may impact cellular processes involve in anoikis induction and cell proliferation regulation, thereby contributing to DKD progression. Notably, these pathways were closely related to immune cell activation and infiltration. 21-23 Previous research has indicated the significant contribution of immune infiltration in DKD progression. Macrophage contributed to aggravate DKD damage via generating a negative feedback loop with tubular cells, while the administration of IL233, an immunomodulatory cytokine, alleviated inflammation and protected against DKD. 24,25 Further investigation in DKD mice revealed that inflammation and immune regulation were key factors in determining DKD susceptibility. 26 Our results supported these findings, as we found a significant positive correlation between the gene module and DKD, which was significantly enriched in immune response and regulation pathways. Moreover, high levels of immune cell infiltration were observed in DKD patients. These results highlighted the potential roles of immune deregulation and inflammatory responses in DKD pathogenesis. Importantly, our results also identified a close correlation between anoikis-related DEGs and immune infiltration, highlighting the interaction between anoikis and immune regulation in DKD pathogenesis. Importantly, our study revealed a significant correlation between multiple anoikis-related genes and key signaling pathways, including inflammatory responses, KRAS, NOTCH, and TGF-β. These pathways have been widely implicated in immune regulation. 27-30 This suggest that these identified pathways may serve as crucial mediators through which anoikis-related genes modulate immune responses in DKD.

The absence of reliable early diagnostic biomarkers for DKD presents a significant challenge in clinical practice. To resolve this issue, we established a diagnostic model using anoikis-related DEGs. Our study demonstrated that this model exhibited an effective predictive capacity, indicating its potential usefulness as a screening tool for DKD. Diabetic Hmox1 deficiency mice were more sensitive to microvascular injury, resulting in more severe albuminuria and serum urea nitrogen level. 31 ITGA3 encodes an integrin alpha chain which holds important significance in maintaining podocyte functionality and glomerular basement membrane integrity. Mutations in ITGA3 have been related to the development of
nephrotic syndrome. Studies in mice have shown that Itga3 deficiency leads to serious podocytopathy and GBM disorganization, ultimately resulting in significant proteinuria. Also, Itga3 was suspected as target gene of miR-124 mediating podocytic adhesive capacity injury. Both in vivo and in vitro, NQO1 overexpression has been found to significantly inhibit ROS generation and cell apoptosis, while renal fibrosis was exacerbated in Nqo1 knockout diabetic mice compared to wild-type ones. VEGFA, belonging to the family of growth factors that facilitate the proliferation and mobility of vascular endothelial cells, has been closely associated with DKD. In a diabetic mouse model, excessive expression of Vegfa led to the development of severe glomerulosclerosis. The glomerular endothelial protective effect of empagliflozin in diabetic mice was associated with the modulation of Vegfa paracrine pathway. However, the administration of anti-VEGFA clinically was under controversial, as worsened kidney injury observed in some DKD patients. These indicated that investigating other molecular mechanisms of VEGFA was essential, and that exploring the role of anoikis may be a promising direction. The role of IGF1 in DKD is complex and has yielded conflicting results in different studies. Yang et al found a significantly decreased IGF1 expression in diabetic mice. However, another study demonstrated that repression of IGF1 contributed to the protective effect of ramipril in DKD rats. Thus, it was necessary to experimentally confirmed its role in DKD.

Notably, significantly elevated expression levels of Pycard were observed in DKD mice, as evidenced by qPCR analysis and immunohistochemical staining. PYCARD, also called ASC, is an essential component of the NLRP3 inflammasome, assuming a vital function in activating inflammasome. Previous study has reported that inhibiting inflammasome activation mitigated the progression of DKD mice. Numerous studies have demonstrated that targeting the NLRP3 inflammasome could provide renoprotective effects against DKD-induced kidney injury. Our correlation analysis unveiled a positive association between PYCARD expression and immune cell infiltration, which was further supported by the analysis of single-cell sequencing datasets, demonstrating heightened PYCARD expression in immune cells. Co-expression analysis indicated the potential involvement of PYCARD in immune response mechanisms, thereby contributing to immune activation in DKD. Importantly, our findings were corroborated by the significant increase in Pycard expression observed under high glucose stimulation in RAW264.7 cells. Furthermore, our study found SFN to be another gene that significantly increased in DKD samples. SFN has been demonstrated to contribute to renal dysfunction. For instance, in acute kidney injury, silencing SFN resulted in a remarkably repression of inflammatory response and cell injury. Another study found that an expanded cell subset expressing stratifin was closely related to unfavorable outcomes in crescentic glomerulonephritis. Our study showed that SFN was relatively high expressed in tubular cell and high glucose stimulation in TCMK-1 cells induced an upregulation of Sfn expression, indicating its crucial role in the progression of DKD. Our investigation of SFN’s potential mechanisms underlying DKD progression revealed its association with signaling pathways related to cell proliferation, the cell cycle, and inflammation. It is worth noting that data extracted from the Nephroseq database highlighted the critical involvement of PYCARD and SFN in DKD, as evidenced by their significantly negative correlation with GFR. These findings shed light on several promising candidates contributing to DKD pathogenesis, thereby facilitating the identification of potential therapeutic targets. While our research provides fundamental theoretical foundations for further investigation, it has certain limitations. Although we have validated candidate genes through animal-based and cell-based experiments, comprehensively in vitro and in vivo investigations are imperative to elucidate their molecular mechanism in DKD pathogenesis. Moreover, while we have validated the predictive accuracy of our model using different external data, its effectiveness needs be tested in a larger and more diverse population to establish its robustness and clinical utility fully.

**Conclusion**

In Conclusion, our study comprehensively analyzes anoikis-related genes and identifies key genes significantly associated with DKD. We also develop a diagnostic model based on these genes and investigate potential molecular mechanisms underlying DKD progression, providing novel insights into DKD pathogenesis. Our study provides valuable insights into the genetic factors contributing to DKD pathogenesis, which will aid in the development of new diagnostic tools and treatments.
Abbreviations
DKD, Diabetic kidney disease; DEGs, differentially expressed genes; WGCNA, weighted gene co-expression network analysis; GSVA, Gene set variation analysis; ECM, extracellular matrix; GEO, Gene Expression Omnibus; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROC, receiver operating characteristic; ssGSEA, single-sample gene set enrichment analysis; EMT, epithelial-to-mesenchymal transition.

Ethical Approval and Consent to Participate
Ethical approval and consent to participate were obtained.

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Author Contributions
The reported work has been significantly contributed to by all the authors. Their contributions encompass a range of areas, such as conceptualization, study design, data acquisition, execution, analysis, and interpretation. Moreover, drafting, revising, and critically reviewing the article involved the participation of all authors. They have provided their final approval for the version intended for publication and have reached a consensus on the target journal. Additionally, all authors acknowledge their responsibility for every aspect of the work.

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