Identification of key genes for diabetic kidney disease using biological informatics methods

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Abstract. Diabetic kidney disease (DKD) is a common complication of diabetes, which is characterized by albuminuria, impaired glomerular filtration rate or a combination of the two. The aim of the present study was to identify the potential key genes involved in DKD progression and to subsequently investigate the underlying mechanism involved in DKD development. The array data of GSE30528 including 9 DKD and 13 control samples was downloaded from the Gene Expression Omnibus database. The differentially expressed genes (DEGs) in DKD glomerular and tubular kidney biopsy tissues were compared with normal tissues, and were analyzed using the limma package. Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed for DEGs using the GO Function software in Bioconductor. The protein-protein interaction (PPI) network was then constructed using Cytoscape software. A total of 426 genes (115 up- and 311 downregulated) were differentially expressed between the DKD and normal tissue samples. The PPI network was constructed with 184 nodes and 335 edges. Vascular endothelial growth factor A (VEGFA), α-actinin-4 (ACTN4), proto-oncogene, Src family tyrosine kinase (FYN), collagen, type 1, α2 (COL1A2) and insulin-like growth factor 1 (IGF1) were hub proteins. Major histocompatibility complex, class II, DP α1 (*HLA-DPA1*) was the common gene enriched in the rheumatoid arthritis and systemic lupus erythematosus pathways, and the immune response was a GO term enriched in module A. *VEGFA*, *ACTN4*, *FYN*, *COL1A2*, *IGF1* and *HLA-DPA1* may be potential key genes associated with the progression of DKD, and immune mechanisms may serve a part in DKD development.

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Key words: diabetic kidney disease, differentially expressed genes, Kyoto Encyclopedia of Genes and Genomes pathway, protein-protein interaction network, key genes

Introduction

Diabetic kidney disease (DKD) is a common complication of diabetes, which is characterized by albuminuria, an impaired glomerular filtration rate (GFR) or a combination of the two (1,2). DKD accounts for ~50% of all cases of end-stage renal disease (ESRD) in the United States and the US ESRD program is a large medical expense/economic burden and costs a great amount of money to run. However, a number of genes closely associated with DKD development have yet to be identified despite many years of intensive study (3). Therefore, the identification of genes associated with DKD development is urgently required, as well as the subsequent elucidation of its molecular mechanism.

Some advancements have been made in the elucidation of the pathological mechanism involved in the development of DKD. The loss of podocytes is an early feature of DKD (4). The levels of almost all podocyte-specific genes including genes for congenital nephrotic syndrome of the finish type (*NPHS1*), glomerular podocin (*NPHS2*), the Wilm's tumor gene (*WT1*) and vascular endothelial growth factor (*VEGF*) are all severely reduced in DKD (3). Some other studies have also demonstrated that *NPHS1* (5,6), *NPHS2* (7), bone morphogenetic protein 7 (8), *WT1* (4) and *VEGF* (9,10) are decreased in DKD. In addition, tubulointerstitial fibrosis is a prominent feature of progressive DKD and is likely to be one of the final common pathways leading to the development of ESRD, with patients subsequently requiring dialysis or transplantation (11,12). A previous study revealed that using angiotensin-converting-enzyme-inhibitors and angiotensin II receptor antagonists in patients with diabetes mellitus can respectively improve mortality and delay the progression of DKD (13). In addition, a human genetic study highlighted that the complement system potentially serves a role in low-grade inflammation and the development of DKD (14). Therefore, the aim of the present study was to identify the key genes associated with the development of DKD and elucidate its underlying mechanism.

In the present study, the microarray data of GSE30528 was downloaded the from Gene Expression Omnibus database (GEO; www.ncbi.nlm.nih.gov/geo/). The gene expression profiles in DKD were analyzed and functional analysis was performed for differentially expressed genes (DEGs) in DKD glomerular and tubular kidney biopsy tissues in comparison with normal tissues. In addition, the protein-protein interaction (PPI) network was also constructed. These results were used to

discover the key genes associated with DKD development and to clarify the underlying mechanism.

Materials and methods

Affymetrix microarray data. The array data for GSE30528 was downloaded from the GEO database, which was first recorded by Woroniecka *et al* (3) and was based on the GPL571 platform (Affymetrix Human Genome U133A 2.0 Array; Affymetrix, Inc.; Thermo Fisher Scientific, Inc., Waltham, MA, USA). A total of 44 samples were used to develop the original array data, and of these 9 DKD [age, 64±13.56 years; 5 females, 4 males; body mass index (BMI), 32.74±7.9 kg/m^2] and 13 healthy, disease-free control samples (age, 51.38±12.01 years; 5 females, 8 males; BMI, 29.59±9.08 kg/m^2) were selected for analysis in the present study.

Data processing and DEG analysis. The raw expression data was preprocessed using the robust multiarray average algorithm (15) and the Affy package in Bioconductor (bioconductor.org/packages/release/bioc/html/affy.html); the expression levels of the probes were then obtained. If several probes mapped to one gene symbol, then the mean value was set as the final expression value of this gene. The DEGs in DKD glomerular and tubular kidney biopsy tissues where then compared with normal tissues using the limma package (16). |logFC| >1 and P<0.05 were considered as the cutoff criterion.

Gene Ontology (GO) and pathway enrichment analysis. GO is used for the unification of biology, collecting defined, structured and controlled vocabulary for gene annotation, which mainly includes the following 3 categories: Molecular function, biological process and cellular component (17). The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database for the classification of relevant gene sets into their respective pathways (18).

In order to analyze the DEGs on a functional level, GO annotation and KEGG pathway enrichment analyses were performed for DEGs using GO Function version 1.24.0 (19) software in Bioconductor version 3.5 (www.bioconductor.org/packages/release/bioc/html/GOFunction.html), and gene annotation information was obtained from the org. Hs. eg. db and GO. db package. P<0.05 and gene counts >2 were set as the cut off values.

PPI network analysis. The Search Tool for the Retrieval of Interacting Genes (STRING) database provides the experimental and predicted interaction information of proteins (20). Protein pair interactions in STRING were presented with a combined score. The DEGs were mapped into PPIs and a combined score of >0.7 was identified as the cutoff standard for the important protein pairs. The PPI network was constructed using Cytoscape software version 2.8.2 (www.cytoscape.org/) (21).

Module analysis. ClusterONE version 1.0 (www.paccanarolab.org/cluster-one/) in the Cytoscape software package was used to analyze the PPI network modules with a minimum size of 3 and a minimum density of 0.5. Modules with P<0.01 were set as significant clustering modules.

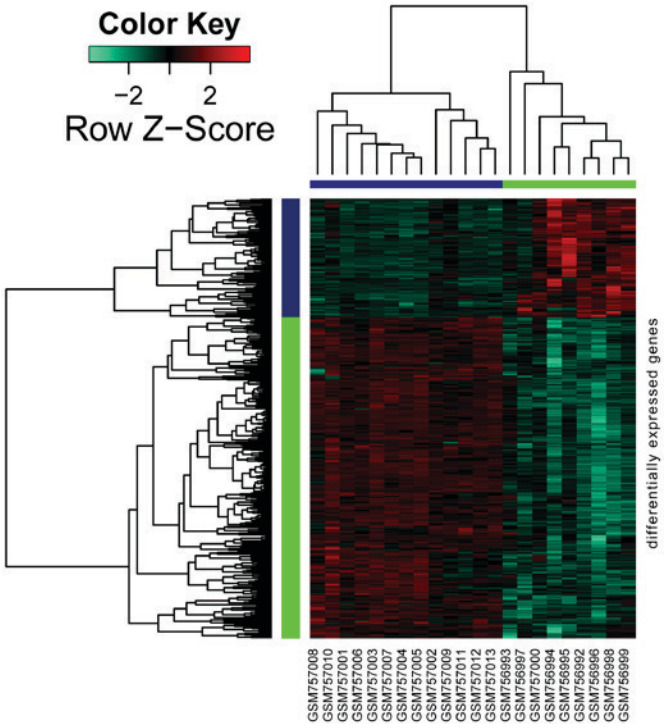

Figure 1. A heat map of the differentially expressed genes identified. Horizontal band with the tree at the top: blue, normal samples; green, diabetes samples; vertical band with the tree on the side: blue, upregulated genes; green, downregulated genes.

Results

Data processing and DEGs analysis. As shown in Fig. 1, a total of 426 genes were differentially expressed in DKD samples when compared with normal samples, amongst which 115 genes were upregulated and 311 were downregulated.

GO and pathway enrichment analysis. GO and KEGG pathway analyses were performed for upregulated and downregulated DEGs. The top 5 GO terms are shown in Table I. The over-represented GO terms of upregulated DEGs were primarily associated with extracellular region, antigen binding, extracellular space, the defense response, the immune response and peptidase regulator activity (Table IA). The downregulated DEGs were mainly involved in cardiovascular system development, circulatory system development, actin cytoskeleton, cell junction, cytoskeletal protein binding and integrin binding (Table IB).

The upregulated DEGs were mainly enriched in 17 KEGG pathways, including primary immunodeficiency, extracellular matrix-receptor interactions, rheumatoid arthritis and systemic lupus erythematosus (Table IIA). In addition, major histocompatibility complex, class II, DP α1 (*HLA-DPA1*) was the common gene in the rheumatoid arthritis and systemic lupus erythematosus pathways. The downregulated DEGs were mainly enriched in 16 KEGG pathways, such as tight junction and adherens junction.

PPI network analysis. Based on the STRING database, a total of 335 protein pairs with a combined score of >0.7 were obtained.

Table I. Gene Ontology analysis for differentially expressed genes.

A, Upregulated

Term	Description	Counts (n)	P-value[a]
GO-BP terms			
GO:0006952	Defense response	44	<0.0005
GO:0006955	Immune response	41	$2.22x10^{-16}$
GO:0002376	Immune system process	50	$3.00x10^{-15}$
GO:0001775	Cell activation	31	$1.37x10^{-14}$
GO:0045321	Leukocyte activation	26	$1.76x10^{-13}$
GO-CC terms			
GO:0005576	Extracellular region	63	$2.06x10^{-12}$
GO:0005615	Extracellular space	32	$4.37x10^{-12}$
GO:0031982	Vesicle	53	$9.90x10^{-11}$
GO:0031988	Membrane-bounded vesicle	52	$1.13x10^{-10}$
GO:0044421	Extracellular region part	53	$3.50x10^{-10}$
GO-MF terms			
GO:0003823	Antigen binding	8	$2.97x10^{-07}$
GO:0061134	Peptidase regulator activity	10	$6.67x10^{-07}$
GO:0005539	Glycosaminoglycan binding	9	$2.84x10^{-07}$
GO:0004866	Endopeptidase inhibitor activity	8	
$8092x10^{-06}$			
GO:0061135	Endopeptidase regulator activity	8	$1.11x10^{-05}$

B, Downregulated

Term	Description	Counts (n)	P-value[a]
GO-BP terms			
GO:0072358	Cardiovascular system development	50	$3.77x10^{-15}$
GO:0072359	Circulatory system development	50	$3.77x10^{-15}$
GO:0009653	Anatomical structure morphogenesis	91	$4.66x10^{-15}$
GO:0048731	System development	121	$3.40x10^{-14}$
GO:0032502	Developmental process	147	$5.66x10^{-14}$
GO-CC terms			
GO:0015629	Actin cytoskeleton	31	$1.79x10^{-12}$
GO:0030054	Cell junction	47	$6.04x10^{-10}$
GO:0070161	Anchoring junction	28	$1.82x10^{-09}$
GO:0005912	Adherens junction	27	$3.39x10^{-09}$
GO:0044421	Extracellular region part	98	$3.00x10^{-08}$
GO-MF terms			
GO:0008092	Cytoskeletal protein binding	40	$9.40x10^{-11}$
GO:0005178	Integrin binding	12	$1.23x10^{-07}$
GO:0032403	Protein complex binding	35	$4.24x10^{-07}$
GO:0050839	Cell adhesion molecule binding	14	$8.04x10^{-07}$
GO:0003779	Actin binding	21	$1.40x10^{-06}$

[a]P<0.00001 vs. normal matched tissues. GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; Term, the identification number of GO; Description, the name of the GO term; Counts, the number of genes enriched in the GO term.

As presented in Fig. 2, the PPI network was constructed with 335 edges and 184 nodes. The nodes of VEGFA (degree score, 19), α-actinin-4 (ACTN4; degree score, 17), proto-oncogene, Src family tyrosine kinase (FYN; degree score, 17), collagen, type

Table II. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of the top 10 DEGs.

A, Upregulated

Term	Description	Counts (n)	P-value
5150	*Staphylococcus aureus* infection	5	$1.89x10^{-04c}$
5020	Prion diseases	4	$3.58x10^{-04c}$
5340	Primary immunodeficiency	4	$3.58x10^{-04c}$
4512	Extracellular matrix-receptor interaction	5	$1.42x10^{-03b}$
5323	Rheumatoid arthritis	5	$1.92x10^{-03b}$
5142	Chagas disease (American trypanosomiasis)	5	$3.45x10^{-03b}$
4610	Complement and coagulation cascades	4	$4.61x10^{-03b}$
4974	Protein digestion and absorption	4	$8.12x10^{-03b}$
5322	Systemic lupus erythematosus	5	$1.06x10^{-02a}$
4640	Hematopoietic cell lineage	4	$1.08x10^{-02a}$

B, Downregulated

Term	Description	Counts (n)	P-value
4520	Adherens junction	8	$3.85x10^{-05d}$
4510	Focal adhesion	13	$4.51x10^{-05d}$
4810	Regulation of actin cytoskeleton	13	$8.65x10^{-05d}$
4530	Tight junction	9	$5.06x10^{-04c}$
5410	Hypertrophic cardiomyopathy	7	$6.17x10^{-04c}$
5414	Dilated cardiomyopathy	7	$1.00x10^{-03b}$
5412	Arrhythmogenic right ventricular cardiomyopathy	6	$1.87x10^{-03b}$
4610	Complement and coagulation cascades	5	$7.26x10^{-03b}$
4360	Axon guidance	7	$7.65x10^{-03b}$
5200	Pathways in cancer	12	$1.21x10^{-02a}$

[a]P<0.05, [b]P<0.01, [c]P<0.001 and [d]P<0.0001 vs. normal matched tissues. KEGG, Kyoto Encyclopedia of Genes and Genomes; Term, the identification number of the KEGG pathway; Description, the name of the KEGG pathway; Counts, the number of genes enriched in the KEGG pathway.

1, α2 (COL1A2; degree score, 15) and insulin-like growth factor 1 (IGF1; degree score, 15) were hub proteins in the network.

Modules analysis. Two significant clustering modules were obtained using ClusterONE software (Fig. 3). A total of 8 and 6 nodes were enriched in modules A and B, respectively. As shown in Table III, nodes in module A (density, 1.0; quality, 0.800; $P=1.606x10^{-4}$) were mainly enriched in GO: G-protein coupled receptor protein signaling pathway, cell surface receptor linked signal transduction, the immune response, the chemokine signaling pathway and the cytokine-cytokine receptor interaction pathway. Nodes in module B (density, 1.0; quality, 0.789; P=0.001) were mainly enriched in GO: regulation of ATPase activity, regulation of system process and regulation of hydrolase activity.

Discussion

In the present study, using the gene expression patterns downloaded from the GEO database, 426 DEGs in DKD glomerular and tubular kidney biopsy tissues were obtained and compared with matched normal tissues, identifying 115 upregulated genes and 311 downregulated DEGs. The results demonstrated that *HLA-DPA1* was the common gene enriched in the rheumatoid arthritis and systemic lupus erythematosus pathways, and the immune response was a significant GO term enriched in module A. In addition, *VEGFA*, *ACTN4*, *FYN*, *COL1A2* and *IGF1* had higher degrees and were established as hub nodes in the PPI network; they may therefore contribute to the progression of DKD.

A previous study suggested that cells in the immune system may be involved in the progression of DKD (22). Immune cells take part in vascular injury under DKD-associated conditions (23). Other previous studies have also indicated that the immune system is associated with DKD development (24-26). Primary immunodeficiency (27), rheumatoid arthritis (28) and systemic lupus erythematosus (29) are associated with the immune system. In the present study, primary immunodeficiency, rheumatoid arthritis and systemic lupus erythematosus were 3 significantly enriched pathways, and the immune response was a GO term enriched in module A. Thus, the results of the present study are in agreement with previous findings,

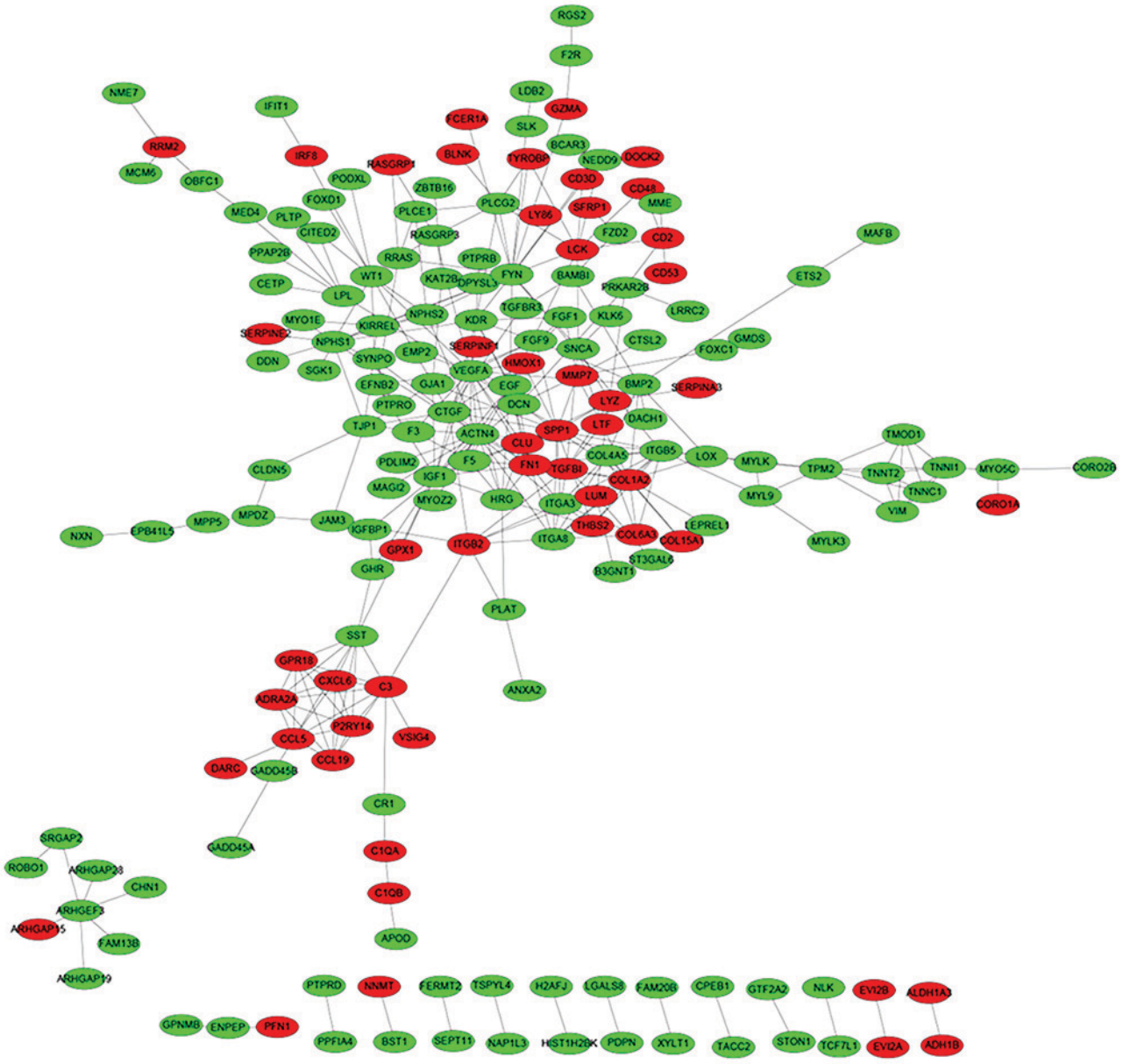

Figure 2. Protein-protein interaction networks of differentially expressed genes. The red nodes represent the upregulated genes and the green nodes represent the downregulated genes.

and therefore indicate that immune mechanisms may serve a role in DKD development.

The work of Woroniecka *et al* (3) suggested that HLA-DPA1 was a differentially expressed transcript in the tubulointerstitium of patients with DKD when compared with normal samples. Previous studies revealed that *HLA-DPA1*, which is the closest centromeric gene expressed to HLA-DOα, may contribute to the differences in the associated risks of diabetes (30,31), including DKD, which is a complication of diabetes. In the present study, *HLA-DPA1* was the common gene enriched in the rheumatoid arthritis and systemic lupus erythematosus pathways. Therefore, these results are in line with previous findings and suggest that *HLA-DPA1* may contribute to DKD development.

In addition, VEGFA, ACTN4, FYN, COL1A2 and IGF1 were identified as hub proteins in the PPI network. VEGFA is an important angiogenic growth factor that regulates endothelial cells' permeability and vasculogenesis (32). It is also important for the differentiation, proliferation, survival and migration of endothelial cells within the glomerulus (33). Previous studies have suggested that VEGFA may serve a significant role in retaining glomerular endothelial cell function as a reduction in VEGFA levels induced abnormal remodeling of glomerular capillaries (34,35). VEGF may also serve a role in the pathogenesis of DKD (36) and the dysregulation of VEGFA may serve a pathogenic role in inducing glomerular injury (37). In DKD, VEGFA has reduced mRNA expression and may be a potential factor that can lead to the development of DKD by inducing microvascular rarefaction and tubular atrophy (9). In addition, neoangiogenesis, which is caused by overexpression of VEGFA, may stimulate the development of DKD and therefore blocking VEGFA or its signaling may ameliorate DKD (38). These findings indicate that VEGFA may serve a role in DKD progression.

ACTNs are actin-binding proteins that are critical in cell adhesion and in the organization of the cytoskeleton (39).

Table III. Functional enrichment analysis of protein-protein interaction network clustering modules.

Term	Description	Counts (n)	P-value
Module A			
GO_BP:0007186	G-protein coupled receptor protein signaling pathway	8	$2.67x10^{-08a}$
GO_BP:0007166	Cell surface receptor linked signal transduction	8	$9.06x10^{-07a}$
GO_BP:0006955	Immune response	5	$2.08x10^{-04b}$
KEGG_ hsa04062	Chemokine signaling pathway	3	$1.25x10^{-02c}$
KEGG_ hsa04060	Cytokine-cytokine receptor interaction	3	$2.38x10^{-02c}$
Module B			
GO_BP:0043462	Regulation of ATPase activity	3	$1.31x10^{-05d}$
GO_BP:0044057	Regulation of system process	3	$4.97x10^{-03e}$
GO_BP:0051336	Regulation of hydrolase activity	3	$5.89x10^{-03f}$
KEGG_ hsa04260	Cardiac muscle contraction	2	$2.32x10^{-04b}$
KEGG_ hsa05410	Hypertrophic cardiomyopathy	3	$2.76x10^{-04b}$
KEGG_ hsa05414	Dilated cardiomyopathy	2	$3.24x10^{-04b}$

[a]P<0.00001, [b]P<0.0005, [c]P<0.05, [d]P<0.0001, [e]P<0.005 and [f]P<0.01vs. normal matched tissues. GO, Gene Ontology; KEGG, KEGG, Kyoto Encyclopedia of Genes and Genomes; Term, the identification number of GO-Biological Process or KEGG pathway; Description, the name of the GO- Biological Process or KEGG pathway; Counts, the number of genes enriched in GO- Biological Process or KEGG pathway.

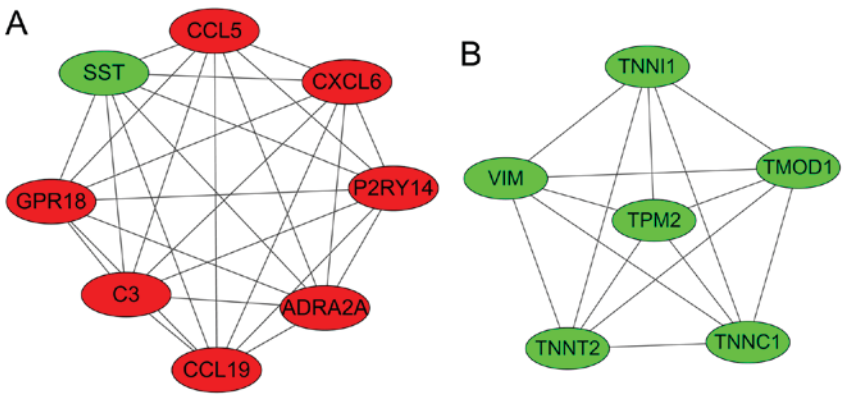

Figure 3. Two significant clustering modules (modules A and B) that were identified in the PPI networks. The red nodes represent the upregulated genes and the green nodes represent the downregulated genes. SST, somatostatin precursor; CCL5, chemokine C-C motif ligand 5; CXCL6, chemokine C-X-C motif ligand 6; P2RY14, P2Y purinoceptor 14; ADRA2A, adrenoceptor α2A; C3, complement component 3; GPR18, G-protein-coupled receptor 18; VIM, vimentin; TNNI1, troponin I1; TMOD1, tropomodulin 1; TNNC1, troponin C1; TNNT2, troponin T2; TPM2, tropomyosin 2β.

Increasing evidence has revealed that in diabetes, there are cytoskeletal changes in podocytes. For instance, advanced glycosylation end products and high glucose can decrease the expression of ACTN4 (40), and a reduced expression of ACTN4 may lead to proteinuria (a symptom of DKD) (41). In addition, FYN is a tyrosine-specific phospho-transferase that belongs to the Src family of tyrosine protein kinases (42). FYN phosphorylation is transiently stimulated by high glucose levels (43). Src/FYN kinase inhibitors disrupt signaling molecules in the VEGF signal transduction pathway (44), and as mentioned above, VEGF may be associated with DKD; thus, FYN may in turn be involved in DKD. In addition, the accumulation of extracellular matrix proteins such as COL1A2 is a key feature of DKD (45). A previous report demonstrated that some key microRNAs (miR) act as effectors of transforming growth factor (TGF)-β and the actions of high glucose in DKD (46). In mesangial cells and the kidney, experimental diabetes was associated with the increased expression of COL1A2, and miR-192 was increased by TGF-β treatment (45). IGF1 as a growth factor receptor has been associated with type 1 DKD (47). In addition, IGF-1 has the capacity to mediate the histological changes characteristic of DKD (48). These previous studies all indicate that these proteins are associated with DKD. Therefore, the results of the present study are in agreement with these findings and provide further evidence that VEGFA, ACTN4, FYN, COL1A2 and IGF1 may serve important roles in DKD development directly or indirectly.

In conclusion, the results of the present study indicated that in addition to *VEGFA*, *ACTN4*, *FYN*, *COL1A2*, *IGF1* and *HLA-DPA1,* immune mechanisms may also serve an important role in the development of DKD. These genes may serve as target genes for the treatment of DKD in future clinical practice. However, this conclusion has no experimental verification; therefore, further evaluation of the potential applications in clinical practice is required.

Acknowledgements

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