Crucial genes associated with diabetic nephropathy explored by microarray analysis

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Abstract

Background: This study sought to investigate crucial genes correlated with diabetic nephropathy (DN), and their potential functions, which might contribute to a better understanding of DN pathogenesis.

Methods: The microarray dataset GSE1009 was downloaded from Gene Expression Omnibus, including 3 diabetic glomeruli samples and 3 healthy glomeruli samples. The differentially expressed genes (DEGs) were identified by LIMMA package. Their potential functions were then analyzed by the GO and KEGG pathway enrichment analyses using the DAVID database. Furthermore, miRNAs and transcription factors (TFs) regulating DEGs were predicted by the GeneCoDis tool, and miRNA-DEG-TF regulatory network was visualized by Cytoscape. Additionally, the expression of DEGs was validated using another microarray dataset GSE30528.

Results: Totally, 14 up-regulated DEGs and 430 down-regulated ones were identified. Some DEGs (e.g. MTSS1, CALD1 and ACTN4) were markedly relative to cytoskeleton organization. Besides, some other ones were correlated with arrhythmogenic right ventricular cardiomyopathy (e.g. ACTN4, CTNNA1 and ITGB5), as well as complement and coagulation cascades (e.g. C1R and C1S). Furthermore, a series of miRNAs and TFs modulating DEGs were identified. The transcription factor LEF1 regulated the majority of DEGs, such as ITGB5, CALD1 and C1S. Hsa-miR-33a modulated 28 genes, such as C1S. Additionally, 143 DEGs (one upregulated gene and 142 downregulated genes) were also differentially expressed in another dataset GSE30528.

Conclusions: The genes involved in cytoskeleton organization, cardiomyopathy, as well as complement and coagulation cascades may be closely implicated in the progression of DN, via the regulation of miRNAs and TFs.

Keywords: Diabetic nephropathy, Differentially expressed gene, MicroRNA, Transcription factor, Network

Background

Diabetic nephropathy (DN) is a complication correlated with both type 1 and type 2 diabetes and is characterized by glomerulosclerosis due to accumulation of extracellular matrix [1]. Despite great attention from both clinicians and basic scientists, the morbidity of end-stage renal disease and glomerulosclerosis in diabetic patients is increasing dramatically [2].

In the past years, many molecules associated with DN have been uncovered. For example, the mammalian target of rapamycin (mTOR) complex 1 activation has a key role in podocyte dysfunction in diabetic mice [3, 4]. Rooney et al. have shown that connective tissue growth factor/CCN family protein 2 (CTGF/CCN2) can activate the Wnt signaling in mesangial cells through low density lipoprotein receptor-related protein 6 (LRP6), which may be implicated in the pathogenesis of DN [5]. In diabetic mice, Glo1 (Glyoxalase 1) overexpression completely inhibits diabetes-induced increases in methylglyoxal modification of glomerular proteins, and promotes the development of diabetic kidney disease (DKD) [6]. Furthermore, there is evidence that miR-192 enhances collagen expression by regulating the E-box repressors Zeb1/2, and locked nucleic acid–anti-miR-192 alleviates proteinuria in the diabetic mice [7]. Based on a gene expression profiling of DN, Hans et al. have shown that some genes in glomeruli from patients with DN are down-regulated, such as bone
morphogenetic protein 2, fibroblast growth factor 1, vascular endothelial growth factor, nephrin and insulin-like growth factor binding protein 2, suggesting that progression of DN might be due to diminished tissue repair ability [8]. However, the pathways which the differentially expressed genes (DEGs) participate in and regulators that target these genes remain unknown.

In this study, the microarray dataset GSE1009 deposited by Hans [8] was used to identify DEGs between diabetic glomeruli samples and healthy controls. Gene Ontology (GO) and pathway enrichment analyses were then performed for the up- and down-regulated DEGs. Furthermore, microRNAs (miRNAs) and transcription factors (TFs) regulating DEGs were predicted, and miRNA-DEG-TF regulatory network was constructed. These findings may contribute to a better understanding of the nosogenesis of DN.

Methods

Affymetrix microarray data

The gene expression profile data of GSE1009 [8] were downloaded from the public database Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), which was based on the platform of [HG_U95Av2] Affymetrix Human Genome U95 Version 2 Array (GPL8300, Affymetrix Inc., Santa Clara, California, USA). This dataset contained 6 glomeruli samples, including 3 samples from 2 kidneys from patients with diabetes mellitus type 2, and 3 samples from 2 healthy kidneys.

Another gene expression dataset GSE30528 [9] in GEO, which contains a relatively big sample size and has a high data quality, was used for validation. The data in GSE30528 were produced by the platform of [HG_U133A_2] Affymetrix Human Genome U133A 2.0 Array (GPL571, Affymetrix Inc., Santa Clara, California, USA). A total of 9 diabetic glomeruli samples from patients with DKD and 13 healthy glomeruli samples were included in this dataset.

CEL files and the probe annotation files were downloaded, and the gene expression data of all samples were preprocessed via background correction, quantile normalization and probe summarization using the Robust Multi-array Average (RMA) algorithm in Affy software package of Bioconductor (available at http://www.bioconductor.org/packages/release/bioc/html/affy.html) [10].

DEGs screening

Linear Models for Microarray Data (LIMMA) package [11] of Bioconductor (available at http://www.bioconductor.org/packages/release/bioc/html/limma.html) was used to identify genes that were differentially expressed in diabetic glomeruli. Only the genes meeting \( p\)-value \(< 0.05 \) and \( \log_2 \text{FC} \) (fold change) \( \geq 1 \) were chosen as DEGs.

Enrichment analysis for DEGs

To explore the functions of DEGs in diabetic glomeruli samples, the DAVID (Database for Annotation, Visualization and Integrated Discovery) database [12] was used to perform GO and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analyses for DEGs. The \( p\)-value \(< 0.01 \) and gene count \( \geq 2 \) were set as the cut-off criteria.

Furthermore, the category of enriched GO terms and the gene number were displayed as a histogram which was constructed by WEGO (Web Gene Ontology Annotation Plot) [13] with the cut-off criterion of level = 2.

Construction of miRNA-DEG-TF regulatory network

GeneCoDis (Gene Annotation Co-occurrence Discovery, http://genecodis.cnb.csic.es/) [14], which is a grid-based tool that integrates biological information from different sources to search for biological characteristics (annotations) that frequently co-occur in a series of genes and rank them by statistical significance, was used to identify miRNAs and TFs regulating DEGs with hypergeometric algorithm, and the adjusted \( p\)-value \(< 0.01 \) was used as the cut-off criteria. The regulatory network consisting of DEGs, miRNAs and TFs were then visualized by Cytoscape (http://cytoscape.org/) [15].

Validation of the expression level of DEGs

The DEGs between DN and control samples in the dataset GSE30528 were identified using the aforementioned methods. The overlapped DEGs in both of GSE30528 and GSE1009 were then identified using the VennDiagram package. Here, if a gene was differentially expressed in both of GSE30528 and GSE1009 with the same expression pattern (up-regulated or down-regulated expression), this gene was identified as an overlapped DEG.

Results

Identification of DEGs

Based on the cut-off criteria, a set of 444 genes were identified to be differentially expressed in the diabetic glomeruli samples, including 14 up-regulated and 430 down-regulated ones, compared with the controls (Additional file 1).

GO and KEGG pathway enrichment analysis of up- and down-regulated DEGs

A total of 111 GO terms for the down-regulated DEGs were generated. Some genes (e.g. MTSS1, CALD1 and ACTN4) were related to cytoskeleton organization, such as actin filament-based process, actin cytoskeleton and cytoskeletal protein binding; some other genes were distinctly enriched in negative regulation of cell proliferation (e.g. AIF1, IGFBP7 and COLA4A3) (Table 1). Using WEGO (level = 2), 25 categories of GO terms for down-regulated DEGs were displayed. The majority of genes were enriched
Table 1: The top 5 enriched GO terms with the most low p-value for differentially expressed genes

| Category | Terms | Description | Count | p-value | Genes |
|----------|-------|-------------|-------|---------|-------|
| BP       | GO:00300029 | actin filament-based process | 23 | 5.38E-07 | MTSS1, ROCK1, ACTN4, AIF1, CALD1, ARF6, MYH9, CAPZB, TNNT2, ACTG1… |
|          | GO:00300036 | actin cytoskeleton organization | 22 | 7.32E-07 | MTSS1, ROCK1, ACTN4, AIF1, CALD1, ARPCS, MYH9, CAPZB, TNNT2, ACTG1… |
|          | GO:0008285 | negative regulation of cell proliferation | 28 | 1.48E-06 | AIF1, IGFBP7, PTH1R, IN1G1, COL4A3, BMP2, CTBP1, GAS1, CD164, TGFBR3… |
|          | GO:0007010 | cytoskeleton organization | 31 | 2.17E-06 | MTSS1, AIF1, CALD1, ARF6, ACTG1, PAK1, PLS3, ACTN4, ROCK1, MYH9… |
|          | GO:0030832 | regulation of actin filament length | 11 | 6.01E-06 | ACTR3, PFN2, CAPZA1, TMSB4X, RDX, ARF6, ARPC5, CAPZB, SPTAN1, DSTN… |
| CC       | GO:0015629 | actin cytoskeleton | 31 | 2.60E-11 | MTSS1, AIF1, CALD1, MYL9, ACTR3, ACTG1, ACTN4, MYO1B, MYH9, CTNNA1… |
|          | GO:0005938 | cell cortex | 20 | 1.02E-08 | SEPT2, ACTN4, SEPT1, CALD1, ARF6, MYH9, SEPT10, ACTG1, FNBPI, CLCS… |
|          | GO:0005856 | cytoskeleton | 70 | 1.28E-07 | MTSS1, AIF1, CALD1, MYL9, ACTR3, ACTG1, ACTN4, MYO1B, MYH9, CTNNA1… |
|          | GO:0044448 | cell cortex part | 13 | 1.78E-06 | SEPT2, ACTN4, SEPT1, CALD1, PRKCI, MYH9, CAPZB, SEPT10, DSTN, ACTG1… |
|          | GO:0043232 | intracellular non-membrane-bounded organelle | 104 | 5.54E-06 | SEPT2, ACTN4, SEPT1, CALD1, PRKCI, MYH9, CAPZB, SEPT10, DSTN, ACTG1… |
| MF       | GO:0008092 | cytoskeletal protein binding | 40 | 7.17E-10 | MTSS1, AIF1, TNNC1, CALD1, EZR, TARDBP, ACTN4, MYO1B, SUN2, MYH9… |
|          | GO:0003779 | actin binding | 30 | 5.79E-09 | MTSS1, AIF1, TNNC1, CALD1, ACTN4, MYH9, PALD, NEBL, TNNT2, PARVA… |
|          | GO:0005520 | insulin-like growth factor binding | 6 | 3.80E-04 | CTGF, HTRA1, IGFBP7, IGFBP2, CRIM1, CYR61 |
|          | GO:0005200 | structural constituent of cytoskeleton | 9 | 6.07E-04 | TNNT2, ACTG1, DMD, VM, AGRN, ARPC5, CD2AP, ADD3, SPTAN1 |
|          | GO:0050839 | cell adhesion molecule binding | 6 | 9.18E-04 | EZR, PVR12, NPTN, CTNNA1, CD2AP, CTNBB1 |

GO: Gene Ontology, BP: biological process, MF: molecular function, CC: cellular component

in biological processes (BP), such as anatomical structure formation, biological regulation, death and response to stimulus. Moreover, several GO terms in cellular component (CC) were obtained, such as cell part and organelle. Furthermore, three GO terms in molecular function (MF) were enriched by the down-regulated genes, including binding, structural molecular and transporter (Fig. 1). In addition, three up-regulated DEGs (CD69, LGALS2 and FBPI) were significantly enriched in sugar binding.

According to KEGG enrichment analysis, the down-regulated DEGs were significantly enriched in 6 pathways, such as arrhythmogenic right ventricular cardiomyopathy (ARVC) (e.g. ACTN4, CTNNA1 and ITGB5), regulation of actin cytoskeleton (e.g. ACTN4, MYL9 and ITGB5) and complement and coagulation cascades (e.g. CIR and CIS). There were no significant pathways for the up-regulated DEGs (Table 2).

Analysis of miRNA-DEG-TF regulatory network
Totally, 896 regulatory relationships between miRNAs, TFs and DEGs were identified to construct the miRNA-DEG-TF regulatory network, including 24 miRNAs, 44 TFs and 275 down-regulated genes (Fig. 2). The transcription factor LEF1 regulated most of genes (degree = 79), such as ITGB5, CALD1, RTN4 and PAK1. Hsa-miR-33a modulated 28 genes, such as CIS and RTN4.

Besides, no miRNAs were predicted to regulate the up-regulated genes, whereas, two TFs (AML1 and NFKB) were identified to modulate several up-regulated genes. Among them, AML1 regulated CD96 and CYP17A1.

Screening of overlapped DEGs in the two datasets
To validate the expression of the identified DEGs in the dataset GSE1009, another dataset GSE30528 was used. A total of 635 DEGs were identified in GSE30528. Among them, 143 DEGs, including one upregulated gene (TRIM16) and 142 downregulated genes (e.g. MTSS1, ACTN4 and ITGB5), were also differentially expressed in the dataset GSE1009 (Fig. 3), indicating that the expression of the 143 DEGs in GSE1009 identified above were validated by the dataset GSE30528.

Discussion
In the present study, a set of 444 DEGs in the dataset GSE1009 were identified from diabetic glomeruli samples, including 14 up-regulated ones and 430 down-regulated...
ones, compared with healthy glomeruli samples. Among them, the expression of 143 DEGs (one upregulated gene and 142 downregulated genes) were validated by another dataset GSE30528. According to the GO functional enrichment analysis, a set of DEGs were related to the function of cytoskeleton organization, such as \( MTSS1 \), \( ACTN4 \) and \( CALD1 \).

\( MTSS1 \) encodes metastasis suppressor 1, which is also known as MIM (missing in metastasis). It is an actin and membrane binding protein that was originally identified as a potential tumor metastasis suppressor [16]. \( MTSS1 \) can induce actin-rich protrusions at the plasmalemma and promote disintegration of actin stress fibers [17], indicating that it may be crucial in regulating cytoskeletal dynamics. Renal tubular cell and podocyte apoptosis is an inevitable event in the progression of glomerulosclerosis [18, 19], and major modifications of the cytoskeleton are involved in the apoptosis process, including dynamic membrane blebbing, nuclear disintegration, chromatin condensation and cell fragmentation [20]. Therefore, \( MTSS1 \) may play a pivotal role in DN, via participation in the regulation of cytoskeleton organization. \( ACTN4 \) encodes an actinin, which participates in cytoskeleton action. Previous studies have been reported that mutations in \( ACTN4 \) cause focal segmental glomerulosclerosis [21–23]. There is evidence that two single-nucleotide polymorphisms in \( ACTN4 \) are associated with DN in women [24]. Moreover, the up-regulation of \( ACTN4 \) was observed during the proteinuria stage [25]. Thereby, \( ACTN4 \) may be also important in the progression of DN. \( CALD1 \) encodes a caldesmon that plays a key role in the regulation of smooth muscle and nonmuscle contraction [26]. A previous study has shown that \( CALD1 \) is over-expressed in fibroblasts from the diabetic patients with nephropathy, compared with those from the controls [27]. Conway et al. have

| Table 2 | The results of pathway enrichment analysis for the down-regulated genes |
|----------------|-----------------|-----------------|-----------------|-----------------|
| Category               | Pathway Name                                      | Count | p-value | Genes                                      |
|-----------------------|---------------------------------------------------|-------|---------|--------------------------------------------|
| KEGG_PATHWAY          | Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 11    | 1.41E-04| ACTG1, ATP2A2, ACTN4, DMD, DAG1, CACNB2, ITGB5, ITGA3, CTNNA1, CTNNB1… |
| KEGG_PATHWAY          | Regulation of actin cytoskeleton                  | 19    | 1.65E-04| ACTN4, ROCK1, ITGB5, RDX, ITGA3, ARPC5, MYL12A, MYH9, MYL9, ACTG1… |
| KEGG_PATHWAY          | Hypertrophic cardiomyopathy (HCM)                 | 9     | 0.00575 | TNNT2, ACTG1, ATP2A2, TNNC1, DMD, DAG1, CACNB2, ITGB5, ITGA3 |
| KEGG_PATHWAY          | Complement and coagulation cascades               | 8     | 0.00641 | CD55, F3, CD46, CD59, C1R, SERPING1, C1S, F2R |
| KEGG_PATHWAY          | Dilated cardiomyopathy                            | 9     | 0.00920 | TNNT2, ACTG1, ATP2A2, TNNC1, DMD, DAG1, CACNB2, ITGB5, ITGA3 |
| KEGG_PATHWAY          | Pathogenic Escherichia coli infection             | 7     | 0.00956 | ACTG1, EZR, ROCK1, TUBB2A, VVHAQ, ARPC5, CTNNB1 |

*KEGG, Kyoto Encyclopedia of Genes and Genomes*
demonstrated that caldesmon is a candidate susceptibility gene for DN [28]. In this study, CALD1 was found to be regulated by several TFs, such as LEF1. LEF1 (lymphoid enhancer-binding factor 1) is a nuclear transcription factor modulated by Wnt, and it expedites epithelial to mesenchymal transition (EMT) when its activity is activated by β-catenin [29]. Rooney et al. have reported that overexpression of CCN2 during the progression of DN likely results in the activation of Wnt signaling and subsequent initiation of TCF/LEF transcription [5]. Collectively, CALD1 may play an essential role in the development of DN likely through the regulation of LEF1.

Furthermore, a series of downregulated DEGs were discovered to be enriched in the pathways of arrhythmogenic right ventricular cardiomyopathy and hypertrophic cardiomyopathy in our study, such as ITGB5. Diabetic cardiomyopathy is a frequent event in diabetic patients due to hyperglycemia [30]. ITGB5 encodes integrin beta 5 [31], and it has been found to be differentially expressed in DKD glomeruli and tubuli [9]. Furthermore, integrins are the primary receptors for intercellular adhesion molecule 1 (ICAM1), which has been reported to have a close relationship with DN [32]. Additionally, in our study, ITGB5 was also regulated by LEF1. These results suggest that ITGB5 may have a significant function in the development of DN, via participating in cardiomyopathy pathways.

Additionally, a set of genes were markedly enriched in complement and coagulation cascades, such as C1S and C1R. These two genes both encode members of the human complement subcomponent C1, which is involved in immune response [33]. Migration of immune cells into the renal is a feature of early DN, and it is implicated in the development of this complication [34–37]. Previous studies also have found C1S and C1R to be differentially expressed in DN [38, 39]. In our study, C1S and C1R were regulated by LEF1 and hsa-miR-33a. MiR-33a has been reported to be implicated in diabetes due to its regulation of insulin signaling and fatty acid metabolism [40, 41]. Therefore, C1S and C1R may be crucial in the development of DN.

However, this study has some limitations. For example, these predictions were not validated by experiments.
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Additional file

Additional file 1: The list of differentially expressed genes. (XLSX 30 kb)

Abbreviations

ARVC: Arrhythmogenic right ventricular cardiomyopathy; BP: Biological processes; CC: Cellular component; DEGs: Differentially expressed genes; DKD: Diabetic kidney disease; DN: Diabetic nephropathy; EMT: Epithelial to mesenchymal transition; GO: Gene Ontology; ICAM1: Intercellular adhesion molecule 1; LIMMA: Linear Models for Microarray Data; MF: Molecular function; mTOR: Mammalian target of rapamycin; RMA: Robust Multi-array Average; TFs: Transcription factors; WEGO: Web Gene Ontology Annotation Plot

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Availability of data and materials

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Authors’ contributions

ZKW and ZXW participated in the design of this study, and they both performed the statistical analysis. ZZ carried out the study, collected important background information, and drafted the manuscript. YR conceived of this study, and participated in the design and helped to draft the manuscript. All authors read and approved the final manuscript.

Authors’ information

None.

Competing interests

The Authors declare that they have no competing interest.

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