European Review for Medical and Pharmacological Sciences 2012; 16: 1967-1973

Gene networks implicated in diabetic kidney disease

W. TANG[1], Y. GAO[1], Y. LI[2], G. SHI[1]

[1]Department of Endocrinology, The Affiliated Jiangyin Hospital of Southeast University Medical College, Jiangyin, Jiangsu, P.R. China
[2]Department of Endocrinology, The First Affiliated Hospital, Nanjing Medical University, Nanjing, P.R. China

Abstract. – BACKGROUND: Diabetic kidney disease (DKD) is one of the main causes of renal end-stage disease. The incidence of DKD has increased substantially over the past few years. However, our understanding to the molecular mechanism of DKD is still essential, and an effective treatment has not been developed.

AIM: We aimed to explore the molecule mechanism in the development of DKD, and provide a comparison of DKD in different compartments.

MATERIALS AND METHODS: In this study, we implemented a system biology approach and analyzed gene expression profiles in 22 microdissected human renal glomerular and 22 tubule samples from healthy patients and patients with DKD.

RESULTS: The WGCNA (Weighted Gene Co-expression Network Analysis) analysis identified 10 modules of genes with high topological overlap in tubuli and 12 modules in glomeruli. Several TFs (transcription factors) were found expressed in both compartments, such as ETS1, ETV4, JUN, LITAF, NFE2, RARG and STAT5A. These genes may be used as therapeutic targets for DKD. By comparing the modules in the two compartments, we found that dysregulation of cell proliferation may significantly contribute to the development of DKD. Furthermore, our results concluded that DKD may be an immune-mediated degenerative disease.

CONCLUSIONS: Our studies identified multiple genes that may play an important role in the pathogenesis of DKD and provided a system understanding of the potential relationships among these genes. We hope our study could aid in understanding of DKD and could provide the basis for DKD biomarker discovery.

Key Words:
Diabetic kidney disease, Gene networks, Glomeruli, Tubuli.

Introduction

Diabetic kidney disease (DKD), a major microvascular complication of both type 1 diabetes mellitus and type 2 diabetes mellitus, is an important cause of end-stage renal disease in many countries[1]. DKD develops in 35-40% of diabetic patients starting treatment for kidney failure each year, including dialysis and renal transplantation[2,3]. DKD was traditionally thought to result from intrarenal metabolic, hemodynamic and structural changes[4]. However, accumulating evidence indicates that DKD is a complex phenotype caused by the combined effects of genetic and environmental factors which are responsible for triggering a complex series of pathophysiological events[2,5]. Understanding the molecular mechanisms involved in the development and progression of DKD will enable the identification of new potential targets and facilitate the design of novel therapeutic strategies.

Genetic studies of DKD are hampered by multiple issues, including variable disease progression[6,7], frequent association with hypertension, variation in design and phenotype assessment[8], and lack of mouse models that would faithfully recapitulate changes of DKD[9]. The advent of DNA microarray technology, which facilitates the rapid investigation of genes and biological pathways that are associated with clinically defined conditions[10], has provided a powerful tool to understand the complex pathogenesis and heterogeneous diseases. The technology has been applied in the field of DKD as well[11,12]. It is demonstrated that DNA microarray is more effective when combined with bioinformatics techniques such as gene ontology (GO) databases and pathway analysis software. However, traditional microarray-based transcriptional profiling analysis usually produces large sets of genes without consideration of potential relationships among these genes. Therefore, the systems biology approach is now an arisen mean to facilitate mechanism investigation of disease. This approach allows for a higher order interpretation of

Corresponding Author: Wei Tang, MD; e-mail: drtangwei@yahoo.com.cn

gene expression relationships and identifies modules of co-expressed genes that are functionally related, and eventually characterizes causal pathways and genetic variants[13]. To date, this approach has successfully applied in many studies to identify disease-related transcriptional networks and genetic variants that contribute to the disease phenotypes[14-16].

In this study, we implemented a system biology approach and analyzed gene expression profiles in 22 microdissected human renal glomerular and 22 tubule samples from healthy patients and patients with DKD from an ethnically diverse population downloaded from Gene Expression Omnibus (GEO). We aimed to explore the molecule mechanism in the development of DKD, and provide a comparison of DKD in different compartments. We hope our study could aid in understanding of DKD and could provide the basis for DKD biomarker discovery.

Data and Methods

Affymetrix Microarray Data

The transcription profile of GSE30122 was downloaded from GEO (http://www.ncbi.nlm.nih.gov/geo/), a public functional genomics data repository, which are based on the Affymetrix GPL571 platform data (Affymetrix Human Genome U133A 2.0 Array). We selected 44 samples for further analysis, including 22 microdissected human renal glomerular and 22 tubule samples from healthy patients and patients with DKD from an ethnically diverse population. The 22 renal glomerular samples consisted of 9 glomeruli samples from patients with diabetic human kidney and 13 control human glomeruli. The 22 tubule samples consisted of 10 tubuli samples from patients with diabetic human kidney and 13 control human tubuli. We downloaded the raw and analyzed data files.

Regulation Data

Transcriptional Regulatory Element Database (TRED) has been built to increase the needs of an integrated repository for both cis- and trans-regulatory elements in mammals[17]. TRED did the curation for transcriptional regulation information, including transcription factor binding motifs and experimental evidence. TRANSFAC (Transcription Factor) database contains data on transcription factors, and their experimentally-proven binding sites and regulated genes[18].

A total of 774 pairs of regulatory relationship between 219 transcription factors (TFs) and 265 target genes were collected from TRANSFAC (http://www.gene-regulation.com/pub/databases.html). A total of 5722 pairs of regulatory relationship between 102 Transcription Factors (TFs) and 2920 target genes were collected from TRED (http://rulai.cshl.edu/TRED/).

By combining the two regulation datasets, total 7234 regulatory relationships between 376 TFs and 2653 target genes were collected.

Differentially Expressed Genes (DEGs) Analysis

For the GSE30122 dataset, the two sample *t*-test method was used to identify DEGs. We used Affy package in R[19] to preprocess the data of profile GSE30122. The raw expression datasets from all conditions were processed into expression estimates using the robust multiarray averaging (RMA) method with the default settings implemented in Bioconductor, and then the linear model was constructed. To circumvent the multi-test problem which might induce too much false positive results, the BH (Benjamini and Hochberg) method[20] was used to adjust the raw P-values into false discovery rate (FDR). The DEGs only with FDR less than 0.01 were selected.

Co-expression Network Construction

To explore the relationships among the DEGs, we applied a systems biology approach using a Weighted Gene Co-expression Network Analysis (WGCNA)[21]. WGCNA can be used for finding modules of highly correlated genes, for summarizing such modules using the module eigengene or an intramodular hub gene, for relating modules to one another and to external sample traits, and for calculating module membership measures[22]. We selected the DEGs and the top 1000 undifferentially expressed genes, and then inputted expression profiles of these selected genes to construct Weighted Gene Co-expression networks using the WGCNA R package[23]. We defined modules using static method by hierarchically clustering the genes using 1-TOM as the distance measure with a height cutoff = 0.95 and a minimum size (gene number) cut-off = 40 for the resulting dendrogram.

Co-expression Module Similarity Analysis

For the modules of tubuli and modules of glomerli, we performed similarity analysis. We calculated the number of overlapping genes of each module in tubuli and glomeruli, then ran-

dom 10^4 times of the same size two modules in module distribution of genes. If the number of overlapping genes between two modules larger than actual overlapping number and the *p*-value less than 0.01 in random conditions, then the two modules are considered as significant overlapping, that is, they are similar.

Gene Ontology Analysis

DAVID (The Database for Annotation, Visualization and Integrated Discovery) consists of an integrated biological knowledgebase and analytic tools aimed at systematically extracting biological meaning from large gene/protein lists[24]. To explore whether genes in each target group share a common biological function, DAVID was used to identify over-represented GO categories based on the hypergeometric distribution. *p* values < 0.05 were considered statistically significant for GO enrichment analysis.

Results

Gene Ontology Analysis for Differentially Expressed Genes in Tubuli and Glomeruli Samples

For dataset GSE 30122, FDR less than 0.01 was chosen as cutoff criterion. We got 783 DEGs in tubuli samples: 325 out of the 783 genes were high-expressed and the remaining 458 genes were low-expressed. We got 839 DEGs in glomeruli samples: 239 out of the 839 genes were high-expressed and the remaining 566 genes were low-expressed. The number of overlapping DEGs in both compartments is shown in Table I.

The percentage of overlapping genes of the two compartments is 7.64%, and the common direction rate is 74.19%. Both of the two parameters are not high, which suggests that the gene detection is variable in different compartments, although they are both diabetic kidney disease.

To functionally classify these differentially expressed genes, we used the biological classification tool DAVID and observed significant enrichment of these genes in multiple GO categories. The most significant enrichment of DEGs in tubuli was the GO category of regulation of transferase activity with *p*-value = 2.6E-4. The other significant GO categories included regulation of protein kinase activity (*p* = 3.1E-04), ncRNA metabolic process (*p* = 4.2E-4), regulation of kinase activity (*p* = 5.7E-4), regulation of mitogen-activated-protein (MAP) kinase activity (*p* = 8.0E-4) and steroid biosynthetic process (FDR = 2.10 × 10-13). There was only one significant GO category in glomeruli, innate immune response with *p*-value = 1.8E-3.

Table I. The overlapping DEGs in tubuli samples and glomeruli samples.

	H_Tubuli	L_Tubuli	Total
H_Glomeruli	37	0	37
L_Glomeruli	16	9	25
Total	53	9	62

Gene co-Expression Network Analysis

Because co-expressed genes are biologically related, grouping these highly connected genes by network analysis may shed light on underlying functional processes in a manner complementary to standard differential expression analyses. We selected the DEGs and the top 1000 undifferentially expressed genes to construct co-expression module using the WGCNA R package. We defined modules using static method by hierarchically clustering the genes using 1-TOM as the distance measure with a height cutoff = 0.95 and a minimum size (gene number) cut-off = 40 for the resulting dendrogram. Finally, we got 10 modules of genes with high topological overlap in diabetic tubuli samples (Figure 1) and 12 modules in DKD glomeruli samples (Figure 2). The modules were defined as a cluster of highly connected genes (nodes). Each major branch in the figure represented a color-coded module containing a group of highly correlated genes. The 10 modules of diabetic tubuli included 562, 248, 231, 198, 174, 77, 71, 70, 47 and 46 genes, respectively. There were 59 genes that were not included in any modules. The 12 modules of DKD glomeruli included 539, 169, 159, 153, 143, 125, 119, 89, 73, 66, 64 and 55 genes, respectively. There were 85 genes that were not included in any modules.

Co-expression Module Similarity Analysis

We chose *p*-value less than 0.01 as the threshold, and got 13 pairs of similar modules when random module distribution of genes in tubuli samples, and 14 pairs of similar modules when random module distribution of genes in glomeruli samples. There are 11 pairs of overlapping modules between tubuli samples and glomeruli samples. Therefore, we think that these 11 pairs of overlapping modules are the common modules of tubuli and glomeruli (shown in Table II).

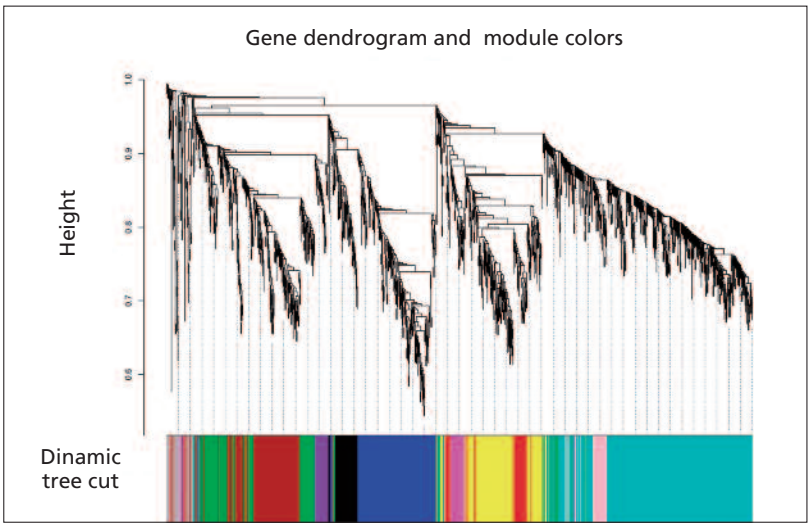

Figure 1. Gene co-expression network analysis in diabetic tubuli samples. Gene modules of highly correlated genes by average linkage hierarchical clustering of genes. The colored bars directly corresponded to the module (color) designation for the clusters of genes. Grey denoted genes that were not part of any module. The remaining colors were used for the 10 modules.

Because the gene expression is regulated by transcription factors, we analysed if there are same transcription factors in similar modules. We chose *p*-value less than 0.01 as the threshold. The target genes of TFs NFE2 [nuclear factor (erythroid-derived 2)], RARG (retinoic acid receptor gamma) were significantly enriched in both M2 of tubuli and M6 of glomeruli. The target genes of TFs ETS1 (protein C ets-1), ETV4 (ETS translocation variant 4), JUN, and LITAF (lipopolysaccharide-induced tumor necrosis alfa factor) were enriched in M7 of tubuli and M6 of glomeruli. The target genes of TF STAT5A (signal transducers and activators of transcription 5A) were enriched in M5 of tubuli and M8 of glomeruli. We also identified specific modules of each compartment. In tubuli samples, M8, M9, M10 are specific modules and M1, M5, M9 are specific modules of glomeruli.

Gene Ontology Analysis for the Common Module and Specific Module

From the above results, we conclude that M2, M3, M4, M5, M6, M7 of tubuli and M2, M4, M6, M7, M8, M10, M11 of glomeruli are the common co-expression modules. Genes in these common modules play important roles both in tubuli and glomeruli disease. M8, M9, M10 are specific modules for tubuli and M1, M5, M9 are specific modules for glomeruli. To biologically characterize these modules, we applied the DAVID tool to classify these genes in each module and observed various levels of GO category enrichment in all modules (Table III). The common modules of tubuli and glomeruli were enriched in regulation of muscle contraction (p = 1.3E-3), regulation of protein kinase activity (p = 4.4E-3), regulation of kinase activity (p = 5.4E-3)

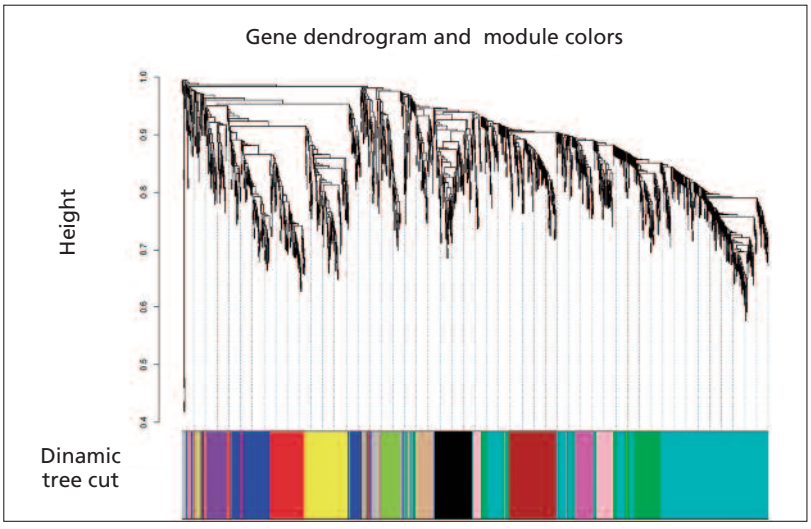

Figure 2. Gene co-expression network analysis in DKD glomeruli samples. Gene modules of highly correlated genes by average linkage hierarchical clustering of genes. The colored bars directly corresponded to the module (color) designation for the clusters of genes. Grey denoted genes that were not part of any module. The remaining colors were used for the 10 modules.

Table II. The similar module pairs (random 104, p-value < 0.01).

Tubuli module	Glomeruli module	Number of overlapping genes	p-value (random tubuli)	p-value (random glomeruli)
M6	M2	20	< 0.0001	< 0.0001
M7	M2	18	< 0.0001	< 0.0001
M2	M4	26	< 0.0001	0.0023
M2	M6	86	< 0.0001	< 0.0001
M7	M6	16	< 0.0001	< 0.0001
M3	M7	49	< 0.0001	< 0.0001
M3	M8	31	< 0.0001	< 0.0001
M5	M8	17	< 0.0001	< 0.0001
M4	M10	18	< 0.0001	< 0.0001
M7	M10	7	0.0013	0.0014
M4	M11	13	0.0006	< 0.0001

and so on. The tubuli specific modules were enriched in protein transport (p = 9.8E-3), and the glomeruli specific modules were enriched in positive regulation of immune system process (p = 2.2E-2).

Discussion

Diabetic kidney disease is the number-one cause of end-stage renal disease in many developed countries. The incidence of DKD in these countries has increased substantially over the past few years because the incidence of diabetes is rising. However, our understanding to the molecular mechanism of DKD is still essential, and an effective treatment has not been developed. Thus, there is an urgent need to investigate the molecular mechanisms underlying this disease, in order to develop better detection, diagnostic and prognostic markers as well as therapeutic targets that could aid in improving clinical management and therapeutic outcome for the patients. DKD is a main glomerular disease, and tubular gene-expression changes may not be relevant to changes that occur in glomeruli[25]. Therefore, in this study, we applied a systems biology approach to study the molecular mechanism of DKD by comparing the gene expression in glomerular and tubular.

Glomerular disorders are responsible for the majority of end-stage renal disease cases, thus, identification of glomerular-specific genes are important to analyse DKD mechanism. Here, we identified the DEGs in glomeruli samples and tubuli samples. We got 783 DEGs in diabetic tubuli samples and 839 DEGs in DKD glomeruli samples. By analyzing the percentage of overlapping genes (POG) of the two compartments and the common direction rate, we conclude that the gene detection is variable in different compartments, although they are both diabetic kidney disease. To functionally classify these differentially expressed genes, we used the biological classification tool DAVID and observed significant enrichment of these genes in multiple GO categories. The most significant enrichment of DEGs in diabetic tubuli was the GO category of

Table III. GO enrichment analysis of modules.

Class	Term	Gene count	p-value
Common	Regulation of muscle contraction	17	1.3E-3
Common	Regulation of protein kinase activity	48	4.4E-3
Common	Regulation of kinase activity	49	5.4E-3
Common	Regulation of transferase activity	50	6.1E-3
Common	Regulation of system process	45	6.7E-3
Common	Digestion	17	6.8E-3
Common	Purine nucleotide biosynthetic process	24	7.5E-3
Common	Neuron recognition	7	8.4E-3
Common	Cation transport	61	8.6E-3
Tubuli specific	Protein transport	17	9.8E-3
Glomeruli specific	Positive regulation of immune system process	22	2.2E-2

regulation of enzyme activity, while DEGs in DKD glomeruli only enriched in one GO category of innate immune response. This result is interesting because DKD is traditionally considered a nonimmune-mediated degenerative disease of the glomerulus. However, complement and immunoglobulins sometimes can be detected in diseased glomeruli[26]. In our findings, genes related to innate immune response expressed differentially in diseased glomeruli, suggesting that DKD may be an immune-mediated degenerative disease. However, further experimental analysis is still needed to verify our results.

Intense researches in DKD were focused on glomeruli; however, the tubulointerstitial injury is also a major feature of DKD. By examining expression profiles of glomeruli and tubuli, respectively, we observed 10 co-expression modules in tubuli and 12 modules in glomeruli. By comparing the similarity of these modules, we found several TFs which play roles in both compartments. The target genes of TFs NFE2, RARG were significantly enriched in both M2 of tubuli and M6 of glomeruli. The target genes of TFs ETS1, ETV4, JUN, LITAF were enriched in M7 of tubuli and M6 of glomeruli. The target genes of TF STAT5A were enriched in M5 of tubuli and M8 of glomeruli. Specific modules of each compartment were also identified, such as M8, M9, M10 in tubuli samples and M1, M5, M9 in glomeruli samples. Further data mining revealed significant involvement of these common TFs in cell proliferation and development. For example, the TF ETS1 regulates numerous genes and is involved in stem cell development, cell senescence and death, and tumorigenesis. The multifunctional cytokine transforming growth factor-β (TGF-β) has been implicated as a principal mediator of diabetic kidney disease[27,28]. TGF-β also induces ets-1 expression[29]. ETS-1 also is essential for the development of kidneys, integrity of glomeruli, and expression of matrix metalloproteinase[30]. The TF c-JUN regulates the expression of genes involved in proliferation and inflammation in many cell types. Previous study has demonstrated that c-JUN is activated in glomerular and tubular cells in human renal disease. Activation of c-JUN may be involved in the regulation of inflammation and/or fibrosis in human renal disease[31]. The TF STAT5A is a member of STAT (signal transducers and activators of transcription) family. STAT5A involved in the JAK/STAT (Janus associated kinase/signal transducer and activator of transcription) pathway which is an important link between cell surface receptors and nuclear transcriptional events leading to cell growth. Therefore, our results suggest that the same TFs in the similar modules of the two compartments can regulate cell proliferation. Our finding is in line with previous study which demonstrated that the key features of DKD include glomerular and tubuloepithelial hypertrophy, followed by thickening of glomerular and tubular basement membranes and progressive accumulation of extracellular matrix proteins in the mesangium and the interstitium[32].

From the result of GO enrichment analysis of modules, we can find that the specific modules of glomeruli were enriched in positive regulation of immune system process, which once again suggest that DKD may be an immune-mediated degenerative disease.

Conclusions

Overall, this study used a systems biology approach to identify genes that are potentially involved in the development of DKD. This approach moves beyond single gene investigation to provide a systems level perspective on the potential relationships between members of a network. Our results suggest that dysregulation of cell proliferation may significantly contribute to the development of DKD. Furthermore, our result concluded that DKD may be an immune-mediated degenerative disease. However, further experimental studies are still needed to confirm our conclusions.

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