Potential Biomarkers of Diabetic Kidney Disease Based on Weighted Gene Co-expression Network Analysis

Yang Chen

Wuhan Xiehe Hospital: Wuhan Union Hospital <https://orcid.org/0000-0002-6108-6173>

Kangli Xiao

Department of Endocrinology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, 430022, China. 2Hubei provincial Clinical Research Center for Diabetes and Metabolic Disorders, Wuhan, China;

Ningjie Shi

Department of Endocrinology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, 430022, China. 2Hubei provincial Clinical Research Center for Diabetes and Metabolic Disorders, Wuhan, China;*Corresponding A

Fengying Yang

Department of Endocrinology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, 430022, China. 2Hubei provincial Clinical Research Center for Diabetes and Metabolic Disorders, Wuhan, China;*Corresponding A

Zhenhai Cui

Department of Endocrinology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, 430022, China. 2Hubei provincial Clinical Research Center for Diabetes and Metabolic Disorders, Wuhan, China;*Corresponding A

Jiaoyue Zhang

Department of Endocrinology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, 430022, China. 2Hubei provincial Clinical Research Center for Diabetes and Metabolic Disorders, Wuhan, China;*Corresponding A

Huiqing Li (✉ lhqing5@126.com)

Department of Endocrinology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, 430022, China. 2Hubei provincial Clinical Research Center for Diabetes and Metabolic Disorders, Wuhan, China;*Corresponding A

Research

Keywords: Diabetic kidney disease, WGCNA, Functional modules, Key genes

Posted Date: October 22nd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-94431/v1>

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Abstract

Background: Diabetic kidney disease (DKD) is one of the serious complications of diabetes. However, there is no unified and clear understanding of the pathogenesis of DKD at present. The evidence suggests that genetic predispositions play a significant role in an individual's susceptibility to DKD. WGCNA analysis may provide a new way to search for genes closely related to DKD.

Methods: In this study we analysed key genes and pathways involved in DKD from RNA-seq count results using 23 peripheral blood samples from patients with DKD. Weighted gene co-expression network analysis (WGCNA) was performed with the WGCNA package.

Results: We found turquoise and purple module were characterized as having the highest correlation with large proteinuria stage in DKD. Furthermore, the key genes, including DCN, F2R, LTBP2, B2M, PSMB8, and NLRC5, were evaluated through a protein-protein interaction (PPI) network combined with a co-expression network, which were expected to be potential diagnostic and therapeutic targets of DKD.

Conclusions: These signalling pathways and hub genes will provide effective targets and ideas for further study of the pathogenesis of DKD and are expected to have roles in the prevention of, early screening for, and the treatment of DKD.

1. Background

Diabetic kidney disease (DKD) is one of the most common serious complications of diabetes. The prevalence of DKD in diabetic patients is approximately 20–60%¹. Those cases that do not receive a timely intervention in the early stage gradually develop into chronic renal insufficiency or even end-stage renal disease (ESRD). At present, DKD has become the leading cause of ESRD in developed countries. It seriously reduces the quality of life of patients and threatens their lives².

DKD is the result of kidney damage caused by chronic exposure to a high blood glucose level. Its main clinical manifestations are a blood glucose metabolism disorder, haemodynamic changes, and albuminuria³. The main pathological features of DKD are glomerular hypertrophy, mesangial hyperplasia, thickening of the basement membrane, loss of podocytes and fusion of podocytes⁴. The pathogenesis of DKD is complex and multifactorial. The evidence suggests that genetic predispositions play a significant role in an individual's susceptibility to hyperglycaemia-induced kidney disease. Studies by Khodaeian M showed that single nucleotide polymorphisms of a variety of genes are involved in the occurrence and development of DKD⁵. Rizvi S indicated that the risk of DKD was increased by several times in people who inherited high-risk alleles in the susceptibility sites of ACE, IL, TNF- α , COL4A1, GLUT and other genes⁶. Many other genes have also been linked to the occurrence and development of DKD^{7–8}. However, these candidate genes are still not sufficient to explain the susceptibility to DKD.

In recent years, the rapid development of genomics, transcriptomics, high-throughput technology has provided new ideas and directions for gene analysis, which not only contributes to a better understanding

of the pathogenesis of DKD but also contributes to finding potential biomarkers for DKD diagnosis and therapy. However, most of the current research focused on screening for differentially expressed genes (DEGs) and ignored any possible correlations among the genes. Genes do not lead an independent existence. To obtain a better understanding of the relationships among genes, more candidate genes related to DKD and gene regulatory network models should be identified.

Weighted gene co-expression network analysis (WGCNA) is one of the most representative methods of developing gene co-expression networks. It can simultaneously analyse multiple complex sample gene expression patterns. Compared with the analysis of differentially expressed genes, WGCNA does not need to carry out multiple hypothesis testing of the relationship between the phenotype and the gene, but instead it directly mines the genes with similar expression patterns and clusters them into gene modules. Genes in the same module may be tightly functionally related or members of the same signalling pathway. Then, a significant correlation analysis between the gene modules and clinical traits is carried out to determine the relationship between the modules of interest and the related traits.

Currently, WGCNA has been used in the genetic analysis of multiple species, including humans, mice and plants, and has obtained meaningful results⁹⁻¹². However, most of the current research on WGCNA uses genome-wide expression dataset downloaded from the database, and few studies have been performed using this method to identify the significant modules and highly connected key genes in DKD. In this study, RNA sequencing was performed on the collected clinical samples, and the RNA-seq results of the samples were analysed by weighted gene co-expression network analysis, aiming to find the functional modules and key genes that were significantly correlated with DKD, with the ultimate goal of providing more ideas for the early prevention, diagnosis and treatment of DKD.

2. Materials And Methods

2.1 Study Objectives

This study recruited 23 patients with diabetic kidney disease admitted to the Affiliated Union Hospital of Tongji Medical College of Huazhong University of Science and Technology between Jan 2018 and Jan 2019.

The inclusion criteria were as follows: the diagnostic criteria and classification for DM, given by the World Health Organization (WHO) in 1999, was adopted to define a diabetic patient. DKD patients were defined by the standard 24 h urine albumin excretion rate (UAER) and glomerular filtration rate (GFR). All patients were divided into Group A (normal proteinuria stage, $\text{UAER} < 30 \text{ mg}/24 \text{ h}$), Group B (microproteinuria stage, $30 \text{ mg}/24 \text{ h} \leq \text{UAER} < 300 \text{ mg}/24 \text{ h}$) and Group C (large proteinuria stage, $\text{UAER} \geq 300 \text{ mg}/24 \text{ h}$) according to the UAER.

The exclusion criteria were as follows: we excluded patients with anaemia, neoplasms, severe cardiovascular, cerebrovascular and liver diseases, chronic glomerulonephritis, known kidney diseases

other than DKD, infection, autoimmune diseases, and acute diabetic complications such as ketoacidosis. Moreover, patients with poorly controlled hypertension, fever, vigorous physical activity, urinary tract infections, pregnant women, and those on their menstrual period were excluded to avoid non-specific albuminuria.

Written informed consent was obtained from all patients analysed in this study. The study protocol was reviewed and approved by the Medical Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology (Ethics Approval Number: S1039).

2.2 Data Collection

Demographic and clinical measurements, including age, sex, height, body weight, body mass index (BMI), diabetic duration, and blood pressure, were collected via interview and confirmed by checking the patients' records. Medication history was carefully recorded. All blood samples were drawn from the patients after 12 h overnight fasting. Routine tests included fasting blood glucose(FBG), serum creatinine, serum uric acid(SUA), and lipid profiles [total cholesterol(TC), triglyceride(TG), high-density lipoprotein(HDL), low-density lipoprotein(LDL)] were conducted using a AU5800 automatic biochemical analyser. Haemoglobin A1c (HbA1c) was measured using the HLC-723G8 HbA1c analyser. To determine the level of 24 h UAER, we collected urine (24 h urine collection for two consecutive days), and the mean value was adopted. All specimens were tested in the Department of Clinical Laboratory at Huazhong University of Science and Technology Tongji Medical College Affiliated Hospital.

2.3 RNA Extraction and Analysis

We collect 3–5 ml of cubital venous blood using a DKDA/RNA Shield blood collection tube containing 6 ml of Shield protective solution (ZYMO RESEARCH). Then, the samples were transported to Beijing Huajie Gene Medical Technology Co. Ltd. for further analysis. Genomic RNA was extracted from blood leukocytes using the DKDA/RNA Blood tube kit. TruSeq RNA Library Prep (48 Samples) and TruSeq RNA CD Index Plate (96 Indexes, 96 Samples) were used to construct a transcriptomic library. RNA sequencing was performed using the Illumina Nextseq500 sequencing platform and the Illumina/MiniSeq Mid Output Kit (300 cycles).

2.4 Weighted Gene Correlation Network Analysis (WGCNA)

Co-expression networks were built according to the protocols of WGCNA in an R environment. Briefly, we used a measure of similarity between the gene expression profiles that was based on a matrix of pairwise Pearson's correlation coefficients. This similarity is a measure of the level of concordance between gene expression profiles across the samples. Then, the similarity matrix was transformed into an adjacency matrix using a power adjacency function, which encodes the connection strengths between pairs of nodes. The power $\beta = 7$ was chosen based on the scale-free topology criterion, resulting in a scale-free topology index (R2) of 0.95 for the cohort. To detect the modules, we used average linkage hierarchical clustering with a dissimilarity measure (the Topological Overlap Measure-TOM). This measure represents the overlap observed between shared neighbours, which is a robust measure of network

interconnectedness. Modules were detected as branches of the dendrogram, which were cut using the Dynamic Tree-Cut algorithm. To represent and summarize each module, the module eigengene was calculated. This measure is defined as the first principal component of a given module. It can be considered to be representative of gene expression profiles and to capture the maximal amount of variation in the module. To quantify module trait associations, given that we had a summary profile (eigengene) for each module, we correlated the eigengene with external traits and looked for the most significant associations. This calculation was referred to as the Module-Trait Relationships (MTRs). For intramodular analysis, we evaluate the Gene Significance (GS) and Module Membership (MM), the latter of which is also known as eigengene-based connectivity (kME). GS is the absolute value of the correlation between a specific gene and a trait. The MM is the correlation between the module eigengene and the gene expression profile. Using the GS and MM, we can identify genes that have a high significance for a clinical trait and important module membership.

2.5 Functional enrichment analysis of co-expression modules

Functional enrichment analysis was performed on the genes in the modules of interest. The corresponding genes' information was mapped to the DAVID (Database for Annotation, Visualization, and Integrated Discovery) dataset. GO (Gene Ontology) analysis results and KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis results were extracted out. P value ≤ 0.05 after correction was used as the threshold.

2.6 Module visualization and hub genes

The modules of interest were visualized by Cytoscape software, and the maximum intramodular connectivity of a gene was informally referred to as an intramodular hub gene.

3. Results

3.1 Construction of co-expression modules of DKD

The expression values of 5000 genes in 23 cases of DKD were used to construct the co-expression module with the WGCNA package tool. The flashClust tools package was used to perform the cluster analysis on these samples. One sample was not in the cluster and did not pass the cuts. One of the most critical parameters was the power value, which mainly affected the independence and the average connectivity degree of co-expression modules. First, the power value was screened out (Fig. 1). When the power value was equal to seven, the independence degree was up to 0.95, and the average connectivity degree was higher. Therefore, the power value was used to construct co-expression modules, and 12 distinct gene co-expression modules were identified in DKD. These co-expression modules were constructed and are shown in different colours (Fig. 2). These modules ranged from large to small by the number of genes they included. The number of genes in the 12 modules are shown in Table 1. Interactions of the 12 co-expression modules were analysed (Fig. 3).

Table 1
The number of genes in
the 12 modules

Module Colors	Freq
black	233
blue	843
brown	799
green	363
greenyellow	65
grey	501
magenta	131
pink	204
purple	126
red	323
turquoise	846
yellow	566

3.2 Construct module-trait relationships of DKD.

Interaction analysis of co-expression modules with common expression patterns that were associated with particular clinic traits was conducted based on the correlation between the module eigengene and the clinic traits, including gender,, height, weight, HbA1c, FBG, Group A, Group B, and Group C (Fig. 4). ME of the purple moudle had a positive correlation with gender($r = 0.58$, $p = 5 \times 10^{-3}$), ME of the greenyellow module had a negative correlation with weight ($r = -0.67$, $p = 7 \times 10^{-4}$), ME of the greenyellow module had a positive correlation with HbA1c ($r = 0.52$, $p = 1 \times 10^{-2}$),

ME of the turquoise module had a negative correlation with Group C ($r = -0.69$, $p = 4 \times 10^{-4}$), ME of the purple module had a positive correlation with Group C ($r = 0.65$, $p = 1 \times 10^{-3}$), Finally, we made a scatterplot of Gene Significance vs. Module Membership for the above modules, and the correlation between GS for Group_C and MM in turquoise module is particularly significant($cor = 0.7$, $p = 1.5 \times 10^{-125}$) (Fig. 5).

3.3 Functional enrichment analysis of co-expression modules.

The "clusterProfiler" package, "ggplot2" package and "Hmisc" package in R language were used to annotate the GO function and analyse the KEGG pathways of the turquoise module genes (Fig. 6).

The GO analysis results for the turquoise module genes showed that most of these genes had MF such as extracellular matrix structural constituent, glycosaminoglycan binding, integrin binding, collagen binding, heparin binding, sulfur compound binding, Wnt – protein binding. These genes were mainly concentrated in proteinaceous extracellular matrix, extracellular matrix component, basement membrane, complex of collagen trimers, focal adhesion, as revealed by the GO cellular component analysis. GO biological process analysis showed these genes were mainly involved in extracellular matrix organization, extracellular structure organization, cell – substrate adhesion, collagen metabolic process, glycosaminoglycan catabolic process, mucopolysaccharide metabolic process. KEGG pathway analysis indicated that multiple pathways were involved. According to P value, the top ten were included focal adhesion, ECM-receptor interaction, protein digestion and absorption, AGE-RAGE signaling pathway in diabetic complications, arrhythmogenic right ventricular cardiomyopathy (ARVC), amoebiasis, hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), PI3K-Akt signaling pathway, Small cell lung cancer.

The GO analysis results for the purple module genes showed that these genes were mainly concentrated in MHC class I protein complex, phagocytic vesicle membrane, early endosome membrane, recycling endosome membrane, COPII-coated ER to Golgi transport vesicle, as revealed by the GO cellular component analysis. GO biological process analysis showed these genes were mainly involved in type I interferon signaling pathway, cellular response to type I interferon, response to type I interferon. KEGG pathway analysis indicated that multiple pathways were involved. According to P value, the top ten were included antigen processing and presentation, viral myocarditis, allograft rejection, graft-versus-host disease, type I diabetes mellitus, autoimmune thyroid disease, cellular senescence, human cytomegalovirus infection, human papillomavirus infection, Epstein-Barr virus infection.

3.4 Module visualization and hub genes.

The turquoise and purple modules were constructed using Cytoscape software (shown in Fig. 7), and we calculated the intramodular connectivity. The hub genes in the two modules are in bold with yellow in Fig. 7.

4. Discussion

There is an appreciable inter-individual variation in the rate of progression of kidney disease in diabetic patients. A simple biological model may not be sufficient to observe both the clinical and theoretical modalities to understand the mechanisms of gene-environment interactions that result in diabetic kidney disease. In this study, WGCNA was used to identify the gene module of co-expressed genes in patients with diabetic kidney diseases and to explore the key genes in the regulatory gene network. The results of this could be used to identify the population at risk of developing kidney disease among diabetic patients

in order to implement early therapeutic strategies to prevent untoward sequelae, which has important clinical implications.

In this study, we first clustered the top 5000 genes of the RNA-seq count data of DKD samples based on gene expression patterns. Then, we divided these genes into 12 modules based on co-expression patterns. Finally, we combined these modules with eight clinical features, included sex, height, weight, fasting glucose, glycosylated haemoglobin, normoalbuminuria stage (Group_A), microalbuminuria stage (Group_B) and macroalbuminuria stage (Group_C).

We found that the turquoise and purple modules had significant correlations with the occurrence and development of the macroalbuminuria stage of DKD, with the turquoise module having a negative correlation and the purple module having a positive one. These findings suggest that the genes whose expression levels change in these modules have a significant impact on the occurrence and development of DKD. Further analysis of the genes in these modules revealed that all of these modules had a high degree of internal connectivity. GO function enrichment analysis and KEGG pathway analysis were carried out on the genes in these modules. The results of KEGG showed that the genes in these modules participated in the development of diabetes complications, including the AGEs-RAGE signalling pathway, the PI3K-Akt signalling pathway, the ECM-receptor interaction pathway, etc. Finally, PPI analysis was carried out on these module genes, and the top three genes were determined by their degree algorithm using the CytoHubba plug-in in Cytoscape. The hub genes of the turquoise module were DCN, F2R and LTBP2; and the hub genes of the purple module were B2M, PSMB8, and NLRC5.

These findings are consistent with another study. Our previous research showed that differentially downregulated genes were mainly distributed in the cytoplasmic membrane and extracellular matrix; had the functions of protein binding, integrin binding and other molecular functions; and participated in extracellular matrix tissues and other biological processes¹³. The analysis of the KEGG pathways suggested that the differentially downregulated genes were mainly involved in the ecm-receptor interaction signalling pathway, the PI3K-Akt signalling pathway, and the signalling pathways of dilated cardiomyopathy, hypertrophic cardiomyopathy and arrhythmia-induced right ventricular cardiomyopathy. As mentioned above, the change of gene expression in the turquoise module had a significant negative influence on the occurrence and development of the large albuminuria stage of DKD, which is consistent with the results of downregulating differentially expressed genes in our previous study. It also confirmed that the gene modules divided by the WGCNA method have biological and clinical significance in the study of DKD.

For the AGEs-RAGE signalling pathway, the advanced glycosylation end products (AGEs) and the receptor of AGEs (RAGE) play important roles in the occurrence and development of DKD¹⁴. AGEs are irreversible products generated by a series of complex processes such as dehydration, oxidation and chemical reordering of amino, aldehyde or ketone groups of macromolecular substances. The rate of generation of AGEs is significantly accelerated in a state of hyperglycaemia. Studies have shown that inhibiting the glycation process can effectively delay the occurrence and development of DKD¹⁵.

RAGE, as an immunoglobulin superfamily receptor, is a receptor for AGEs that has been studied thoroughly. It is poorly expressed in healthy tissues but is significantly upregulated in tumours in response to inflammation and elevated blood sugar¹⁶. The mechanisms downstream of the AGEs-RAGE signalling pathway are complex, and recent studies have shown that the interaction of this pathway with NF- κ B, VEGF, TGF- β 1, MCP-1 and other genes may play an important role in the development and progression of DKD¹⁷⁻¹⁹. However, most of the current studies on this pathway only involve in vitro experiments, and additional experiments are necessary to elucidate the role of this pathway in DKD.

The PI3K-Akt signalling pathway, namely, the phosphatidylinositol 3 kinase-protein kinase B signalling pathway, plays an important role in the regulation of cell differentiation and apoptosis. At present, a large number of studies have shown that over-activation of this signalling pathway is closely related to podocyte injury and the fibrosis process of DKD²⁰⁻²¹. Pfeiffer et al. showed that an arrhythmogenic right ventricular cardiomyopathy signalling pathway, a hypertrophic cardiomyopathy signalling pathway and a dilated cardiomyopathy signalling pathway might be involved in the occurrence and development of diabetic cardiovascular complications and chronic kidney disease²².

Many of these hub genes have been reported to be involved in the pathogenesis of DKD. Decorin (DCN) is one of the components of the extracellular matrix. Wei Lanji et al. found that increased DCN expression in a high-glucose environment may be one of the important mechanisms leading to vascular calcification²³. Vascular calcification is common in patients with DKD. Therefore, the high level of DCN expression in the presence of a high blood glucose level may be one of the important mechanisms of the development of DKD.

Coagulation factor 2 receptor (F2R), also well-known as a protease-activated receptor 1 (PAR1), is the first known thrombin receptor and plays a critical role in transmitting thrombin-mediated activation of intracellular signalling in many types of cells. Recent studies have suggested that F2R may be critically involved not only in mediating bacteria-induced detrimental coagulation but also in innate immune and inflammatory responses²⁴. Other studies have suggested that F2R might be used as an adipogenic marker to provide a potential target for understanding metabolic syndromes such as obesity, type-2 diabetes, and atherosclerosis²⁵.

B2M mRNA expression in cells in the urinary sediment is higher in T1D patients with DKD, maybe reflecting a tubulointerstitial injury promoted by albumin. Researchers have suggested that this protein contributes to diabetic (and possibly, to non-diabetic) tubulopathy²⁶. Another study showed that higher serum B2M was an independent risk factor for subclinical atherosclerosis and diabetic nephropathy in patients with T2D without renal impairment²⁷.

Many studies have shown that proteasome subunit beta type 8 gene (PSMB8) participates in the PI3K-Akt signalling pathway, and is involved in various diseases such as glioma, LAML, DKD, etc.²⁸⁻²⁹.

NOD-like receptor family caspase recruitment domain family domain containing 5 (NLRC5) has important roles in inflammation and innate immunity. NLRC5 is highly expressed in the kidney in streptozotocin-induced diabetic mice, db/db mice and patients with diabetes. NLRC5 promotes inflammation and fibrosis during DN progression, partly through its effects on the NF- κ B and TGF- β /Smad pathways. NLRC5 may be a promising therapeutic target for DN treatment³⁰. There are not many studies on RAB42 and NTM in the pathogenesis of DKD.

5. Conclusion

In summary, the gene modules significantly correlated with macroalbuminuria of DKD were divided by WGCNA. The multiple signalling pathways involved in these modules were all involved in the development of DKD. Then, we selected the top ten hub genes, and these screened genes were involved in the process of macroalbuminuria and fibrosis of DKD. These signalling pathways and hub genes will provide effective targets and ideas for further study of the pathogenesis of DKD and are expected to have roles in the prevention of, early screening for, and the treatment of DKD.

Declarations

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Medical Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology (Ethics Approval Number: S1039).

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its Additional files.

Competing interests

The authors confirm that there are no conflicts of interest.

Funding

There is no funding for this research.

Authors' contributions

JY.Z. and HQ.L. conceived and designed the study. Y.C. and NJ.S. performed the analysis procedures. FY.Y. and ZH.C. analyzed the results. Y.C. KL.X. and HQ.L contributed to the writing of the manuscript. All authors reviewed the manuscript.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No.81974111), the Health Research Foundation of HuBei Province(Grant No.WJ2017M104), the Diabetes Research Foundation of Bethune-Merck.

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Figures

Figure 1

Selection and determination of soft threshold power of co-expressed network. A, The left panel shows the scale-free fit index (y-axis) as a function of the soft-thresholding power (x-axis). The right panel displays the mean connectivity (degree, y-axis) as a function of the soft-thresholding power (x-axis). B, The left panel shows histogram of connectivity distribution when $\beta = 7$. The right panel checks the scale-free topology when $\beta = 7$.

Figure 2

Clustering dendrograms of genes, with dissimilarity based on topological overlap, together with assigned module colors. As a result, 12 co-expression modules were constructed and was shown in different color. These modules were ranged from large to small by the number of genes they included. The number of genes in the 12 modules were listed in Table 1.

Figure 3

Visualizing the gene network using a heatmap plot. The heatmap depicts the Topological Overlap Matrix (TOM) among all genes in the analysis. Light color represents low overlap and progressively darker red color represents higher overlap. Blocks of darker colors along the diagonal are the modules. The gene dendrogram and module assignment are also shown along the left side and the top.

Figure 4

Visualizing the gene network using a heatmap plot. The heatmap depicts the Topological Overlap Matrix (TOM) among all genes in the analysis. Light color represents low overlap and progressively darker red color represents higher overlap. Blocks of darker colors along the diagonal are the modules. The gene dendrogram and module assignment are also shown along the left side and the top.

Figure 5

A, Scatterplots of Gene Significance (GS) for Group_C vs.Module Membership (MM) in the turquoise module. B, Scatterplots of Gene Significance (GS) for Group_C vs.Module Membership (MM) in the purple module.

Figure 6

A, Scatterplots of Gene Significance (GS) for Group_C vs.Module Membership (MM) in the turquoise module. B, Scatterplots of Gene Significance (GS) for Group_C vs.Module Membership (MM) in the purple module.

Figure 7

The visualization of turquoise and purple modules