

Identifying hub genes associated with clinical characteristics in IgA nephropathy by WGCNA

Chengyu Yang

the Affiliated Hospital of Qingdao University

Chenyu Li

the Affiliated Hospital of Qingdao University

Long Zhao

the Affiliated Hospital of Qingdao University

Bin Zhou

the Affiliated Hospital of Qingdao University

Congjuan Luo

the Affiliated Hospital of Qingdao University

Wei Jiang

the Affiliated Hospital of Qingdao University

Hong Luan

the Affiliated Hospital of Qingdao University

Yan Xu (✉ xuyanqfy@126.com)

The Affiliated Hospital of Qingdao University <https://orcid.org/0000-0003-2049-3976>

Research

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Abstract

Background Clinically, IgA nephropathy (IgAN) has a variety of symptoms including paroxysmal gross hematuria, nephritic syndrome and nephrotic syndrome. However, the currently research of IgAN research is not thoroughly enough. This study was aimed at investigating hub genes and genes modulars related to IgAN clinical characteristics. Results We collected microarray data from 66 human samples to construct the gene co-expression network by weighted gene co-expression network analysis and identify the hub genes associated with clinical characteristics. The GO-enriched analysis, pathway analysis and protein-protein interaction network were used to study the specific clinically relevant hub genes. The results showed there were 1470 differentially expressed genes (DEGs) in IgAN glomeruli, of which 48 hub genes associated with blood pressure (Bp) and enriched in ERK1 and ERK2 cascade and Rap1 signaling pathway, 223 hub genes associated with body Mass Index (BMI) and related to organic acid catabolic process and fatty acid degradation pathway, 136 hub genes associated glomerular filtration rate (GFR) enriched in immune response and PI3K-Akt signaling pathway, 82 hub genes associated with proteinuria enriched in extracellular matrix organization and PI3K-Akt signaling pathway. Moreover, there were 480 DEGs IgAN in tubulointerstitium. Among 480 DEGs, 35 hub genes associated with Bp which enriched in positive regulation of apoptotic process, 87 hub genes associated with GFR which related in negative regulation of macromolecule metabolic process and RNA transport, 33 hub genes associated with proteinuria and enriched in regulation of apoptotic process and FoxO signaling pathway. Conclusions We made a preliminary investigation of the transcriptome of IgAN and identified hub genes and pathways closely related to BMI, GFR and proteinuria in IgAN.

Introduction

Immunoglobulin A nephropathy (IgAN), also known as Berger's disease, initially described in 1968 by Berger and now was considered as the most common chronic glomerular disease in the world¹. This condition is defined by the predominant deposition of IgA in glomerular mesangium by immunofluorescence. Main clinical symptoms of IgAN include slight histological changes with glomerular mesangial proliferation, microscopic hematuria, gross hematuria and latent onset. About 30% of patients with IgAN reached end-stage renal disease (ESRD) after 10 ~ 20 years of initial diagnosis². Epidemiologically, IgAN may occurs at all age groups, with the peak incidence at 20 ~ 30 years old. Males are more susceptible to IgAN than females with a males to females ratio of 2 ~ 5: 1, however, the effect of age or sex for prognosis of IgAN is still uncertain. Therefore, it is of great significance to investigate the molecular mechanisms of IgAN and its relationship with the clinical characteristics, in order to understand the pathogenesis of IgAN. This is crucial for the evaluation of IgAN prognosis and guiding diagnosis and treatment of IgAN.

Weighted Gene Co-expression Network Analysis (WGCNA)³ is an advanced and comprehensive algorithm for co-expression analysis based on R programming language. Currently, gene co-expression networks are increasingly being used to look for functionally similar genes, using a very straightforward concept: the nodes in the network represent the genes, and the significantly co-expressed genes will be clustered

together by selecting the appropriate tissue samples. In fact, it is difficult to define whether the two nodes can be connected in a network. Traditionally, the binary method was used to construct genes co-expression network by defining 1 as connected and 0 as unconnected, but it cannot interpret biological significance between the “hard” threshold 1 or 0. To solve this problem, WGCNA uses a soft threshold to define a weight value to determine the probability of interaction among a set of genes. Using this concept, a weighted co-expression network is formed. In order to use soft threshold, the co-expression network is transformed into a weight connection network, and parameters of soft threshold were set by scale-free topology criterion based on biological and statistical significance³.

In our study, we used 66 human samples from the European Renal cDNA Bank to construct the gene co-expression of differential expression genes by WGCNA, and identified the hub genes associated with clinical characteristics in renal interstitium and glomeruli respectively.

Results

Differentially expressed genes between IgAN and healthy control

Series GSE37463, including 25 samples of tubulointerstitium and 27 samples of glomeruli from IgAN patients, 6 samples of tubulointerstitium and 5 samples of glomeruli from Healthy Living Donors (LD), were downloaded from GEO with clinical parameters including age, sex, blood pressure (BP), Body Mass Index (BMI), glomerular filtration rate (GFR), serum creatinine (Scr), and proteinuria (Fig. 1ab). For glomeruli, there were 1802 probes (fold change > 1.2, false discovery rate (FDR) < 0.05) between IgAN and LD, containing 1470 unique genes annotated by official annotations, among which consists of 935 up-regulated differentially expressed genes (DEGs) and 535 down-regulated DEGs; for tubulointerstitium, there were 586 probes (fold change > 1.2, FDR < 0.05) containing 480 unique genes, among which there are 225 up-regulated DEGs and 255 down-regulated DEGs.

Weighted Gene Co-expression Network Analysis

WGCNA³ is a systematic and robust gene co-expression network algorithm based on R programming language to describe the correlation of gene expression matrix, detecting highly correlated gene modules as well as evaluating the correlation between gene modules and clinical traits. According to the official protocol, WGCNA can be briefly divided into the following steps: 1. Construct co-expression network specified by its adjacent matrix which is calculated by soft threshold power; 2. Transform adjacency into topological overlap matrix, using hierarchical clustering and dynamic tree-cutting method to screen module; 3. Select module and gene related to external information. The significance of a gene module was measured by combining correlation coefficient and Gene Significance (GS)³ across a module. GS was provided by WGCNA, which was used to incorporate clinical characteristics into the gene co-expression network. The higher the absolute GS value, the more biologically significant is the gene. On

this basis, $q.Weight < 0.01$ (local FDR)¹⁸, a corrected weighted p-value of the association with a clinical characteristic was considered as the threshold to select hub genes.

Gene Ontology And Kegg Pathways Analysis Of Hub Genes

Firstly, we performed GO enrichment analysis. For glomeruli, hub genes associated with Bp were enriched in ERK1 and ERK2 cascade and cellular response to organic substance; hub genes associated with BMI were enriched in small molecule catabolic process and organic acid catabolic process; hub genes associated with GFR were enriched in immune response and regulation of immune response; and hub genes associated with proteinuria were enriched in extracellular matrix and structure organization (Table 1). For tubulointerstitium, hub genes associated with Bp were enriched in positive regulation of apoptotic process and positive regulation of programmed cell death; hub genes associated with GFR were enriched in negative regulation of macromolecule metabolic process and negative regulation of metabolic process; genes significantly associated with proteinuria were enriched in regulation of apoptotic process and regulation of programmed cell death (Table 2).

Table 1
GO and KEGG pathways analysis of hub genes in glomeruli of IgAN

Relevant Clinical Characteristics	Category	Term	Count	P-Value
BMI	GOTERM_BP_FAT	small molecule catabolic process	22	8.5E-10
	GOTERM_BP_FAT	organic acid catabolic process	17	1E-08
	GOTERM_BP_FAT	carboxylic acid catabolic process	14	6.1E-07
	GOTERM_BP_FAT	single-organism catabolic process	28	2.5E-06
	GOTERM_BP_FAT	monocarboxylic acid metabolic process	22	3.6E-06
	GOTERM_BP_FAT	cellular response to chemical stimulus	58	3.6E-06
	KEGG_PATHWAY	Type I diabetes mellitus	6	5.90E-04
	KEGG_PATHWAY	Fatty acid degradation	6	7.30E-04
	KEGG_PATHWAY	Antigen processing and presentation	7	1.50E-03
GFR	GOTERM_BP_FAT	immune response	38	7.9E-11
	GOTERM_BP_FAT	regulation of immune response	28	5.3E-10
	GOTERM_BP_FAT	innate immune response	26	2.1E-09
	GOTERM_BP_FAT	cellular response to chemical stimulus	48	2.3E-09
	GOTERM_BP_FAT	defense response	35	3E-09
	GOTERM_BP_FAT	regulation of immune system process	33	4.2E-09
	KEGG_PATHWAY	PI3K-Akt signaling pathway	11	4.70E-03

Relevant Clinical Characteristics	Category	Term	Count	P-Value
	KEGG_PATHWAY	Endocytosis	10	2.10E-03
	KEGG_PATHWAY	Tuberculosis	9	7.40E-04
proteinuria	GOTERM_BP_FAT	extracellular matrix organization	13	2.10E-08
	GOTERM_BP_FAT	extracellular structure organization	13	2.20E-08
	GOTERM_BP_FAT	movement of cell or subcellular component	25	3.20E-07
	GOTERM_BP_FAT	leukocyte migration	12	6.80E-07
	GOTERM_BP_FAT	regulation of actin filament-based process	11	1.90E-06
	GOTERM_BP_FAT	cell migration	19	2.40E-06
	KEGG_PATHWAY	Amoebiasis	6	4.70E-04
	KEGG_PATHWAY	PI3K-Akt signaling pathway	6	6.10E-02
	KEGG_PATHWAY	ECM-receptor interaction	5	2.00E-03
Bp	GOTERM_BP_FAT	ERK1 and ERK2 cascade	6	2.80E-04
	GOTERM_BP_FAT	regulation of ERK1 and ERK2 cascade	6	3.10E-04
	GOTERM_BP_FAT	cellular response to organic substance	15	3.60E-04
	GOTERM_BP_FAT	cellular response to chemical stimulus	16	7.70E-04

Relevant Clinical Characteristics	Category	Term	Count	P-Value
	GOTERM_BP_FAT	hair cycle process	4	9.00E-04
	GOTERM_BP_FAT	hair follicle development	4	9.00E-04
	KEGG_PATHWAY	Central carbon metabolism in cancer	4	7.70E-04
	KEGG_PATHWAY	Epstein-Barr virus infection	4	1.70E-02
	KEGG_PATHWAY	Rap1 signaling pathway	4	2.20E-02

Table 2
GO and KEGG pathways analysis of hub genes in tubulointerstitium of IgAN

Relevant Clinical Characteristics	Category	Term	Count	P-Value
GFR	GOTERM_BP_FAT	negative regulation of macromolecule metabolic process	25	5.20E-05
	GOTERM_BP_FAT	negative regulation of metabolic process	26	6.60E-05
	GOTERM_BP_FAT	regulation of protein metabolic process	24	4.80E-04
	GOTERM_BP_FAT	regulation of multicellular organismal development	19	5.00E-04
	GOTERM_BP_FAT	regulation of response to stress	16	5.30E-04
	GOTERM_BP_FAT	regulation of protein phosphorylation	16	5.90E-04
	KEGG_PATHWAY	RNA transport	7	1.00E-03
	KEGG_PATHWAY	Protein processing in endoplasmic reticulum	5	2.70E-02
	KEGG_PATHWAY	Tight junction	4	6.60E-02
Proteinuria	GOTERM_BP_FAT	regulation of apoptotic process	11	7.70E-05
	GOTERM_BP_FAT	regulation of programmed cell death	11	8.40E-05
	GOTERM_BP_FAT	regulation of cell death	11	1.50E-04
	GOTERM_BP_FAT	angiogenesis	6	6.70E-04
	GOTERM_BP_FAT	negative regulation of cell death	8	7.10E-04
	GOTERM_BP_FAT	positive regulation of protein metabolic process	10	7.50E-04

Relevant Clinical Characteristics	Category	Term	Count	P-Value
	KEGG_PATHWAY	FoxO signaling pathway	3	2.90E-02
	KEGG_PATHWAY	Hepatitis B	3	3.40E-02
	KEGG_PATHWAY	Pathways in cancer	4	4.20E-02
Bp	GOTERM_BP_FAT	positive regulation of apoptotic process	5	1.70E-02
	GOTERM_BP_FAT	positive regulation of programmed cell death	5	1.70E-02
	GOTERM_BP_FAT	positive regulation of cell death	5	2.00E-02
	GOTERM_BP_FAT	ossification	4	2.40E-02
	GOTERM_BP_FAT	cellular response to nutrient levels	3	3.10E-02
	GOTERM_BP_FAT	regeneration	3	3.40E-02
	KEGG_PATHWAY	PI3K-Akt signaling pathway	2	4.00E-01
	KEGG_PATHWAY	Metabolic pathways	4	2.60E-01
	KEGG_PATHWAY	Hippo signaling pathway	2	2.00e-01

Secondly, KEGG pathways were analyzed on the same hub gene. For glomeruli, hub genes associated with Bp were enriched in central carbon metabolism in cancer and Rap1 signaling pathway; hub genes associated with BMI were enriched in type I diabetes mellitus and fatty acid degradation; hub genes associated with GFR were enriched in PI3K-Akt signaling pathway and endocytosis; genes significantly associated with proteinuria were enriched in PI3K-Akt signaling pathway and ECM-receptor interaction (Table 1). For tubulointerstitium, hub genes associated with Bp were enriched in PI3K-Akt signaling pathway metabolic pathways ($P > 0.05$); hub genes associated with GFR were enriched in RNA transport and protein processing in endoplasmic reticulum; hub genes associated with proteinuria were enriched in FoxO signaling pathway and hepatitis B (Table 2).

Protein-protein Interaction Network Analysis Of Hub Genes

In IgAN glomeruli, the PPI enrichment P-value of genes set associated with BMI was $1.78e^{-08}$, which those genes are at least partially biologically connected (Fig. 5). Moreover, the network PPI enrichment P-value of genes set associated with GFR and proteinuria is 0 (Fig. 6a). Furthermore, the P-value of genes set associated with BMI, GFR and proteinuria in tubulointerstitium was $7.33e^{-5}$ (Fig. 6b). In addition, in the PPI network, genes in one module or related to same clinical parameter are more likely to have interaction or be in a cluster, indicating that the WGCNA was successfully performed.

Discussion

A large number of studies have confirmed that BMI, hypertension, proteinuria and renal function contribute to the progression of IgAN, however, the genetic mechanism of these clinical characteristics leading to IgAN progression is not clear. The relationship and association between factors and IgAN also require more research and evidence of biological processes at the molecular level. Diseases involve thousands of gene expression changes with a huge and complex gene regulated network, which means research for a single gene is superficial and insufficient, it is hard to explain the mechanism of a disease. WGCNA is an advanced, successful and comprehensive algorithm for co-expression analysis, and it was not only used to construct gene co-expression network and screen gene modules. It served as a powerful tool to identify hub genes associated external information, clinical characteristics especially, and help researchers to understand the mechanism of the disease, providing a theoretical basis for the diagnosis and treatment of the disease.

Genes sets, which associated with GFR in glomeruli, enriched in immune response, cellular response to chemical stimulus and PI3K-Akt signaling pathway, indicated that immune response and IgA stimulus were dominant in impaction of glomerular filtration. It is known that glycosylated IgA has a transferrin receptor on the surface of mesangial cells⁴ and abnormal glycosylated IgA immune complexes are specifically recognized and deposited in the mesangium, causing proinflammatory cytokines and angiotensin to be released⁵. Tumor necrosis factor alpha, derived from IgAN patients podocytes cells autocrine synthesis, caused TNF receptor 1, TNF receptor 2 and IL-6 to be up-regulated. Elevated expression of TNF receptor 1 leads to podocyte apoptosis and up-regulation of TNF receptor 2 expression, leading to chronic inflammation⁶; PI3K-Akt is an important intracellular pathway involved in cell metabolism, apoptosis, proliferation and differentiation^{7,8}. A study by Cox et al. reported that PI3K-Akt signaling pathway was hyperactive in IgAN patients and played an important role in IgAN⁹. Therefore, based on the results of WGCNA, we believed that PI3K-Akt signaling pathway specificity impacts the renal function in IgAN. For proteinuria, hub genes in glomeruli were enriched in the extracellular matrix organization, extracellular structure organization and PI3K-Akt signaling pathway, this means that these genes may play an important role in changing the extracellular matrix and potentially leading to proteinuria.

In tubulointerstitium group, there were 480 DEGs between IgAN and health control group. Among 480 DEGs, 6 hub genes associated with age, 15 hub genes associated with sex, 35 hub genes associated with Bp enriched in positive regulation of apoptotic process, cellular response to nutrient levels and regeneration. Moreover, 87 hub genes associated with GFR in tubulointerstitium enriched in negative regulation of macromolecule metabolic process, negative regulation of metabolic process and RNA transport, and 33 hub genes associated with proteinuria enriched in the regulation of apoptotic process, regulation of programmed cell death and FoxO signaling pathway. Proteinuria is closely associated with poor cardiovascular outcomes and progression of ESRD in patients with CKD^{10,11}. It is worth noting that since the expression pattern of genes is very similar, WGCNA only screens one turquoise module in the IgAN tubulointerstitium group and genes that cannot be clustered into one of the modules are assigned to the grey module which represents background genes outside of the modules. Thus, genes in the grey module could possibly be related to GFR or Scr but not belong to WGCNA modules.

Conclusions

In conclusion, we made a preliminary investigation of the molecular mechanisms of the relationship between IgAN and clinical characteristics. We identified hub genes and pathways closely related to BMI, GFR and proteinuria in IgAN through a series of bioinformatics analysis, but it still needs to further explored and validated.

Materials And Methods

IgAN samples and clinical parameters

A total of 66 human samples from the European Renal cDNA Bank¹² were collected from Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>)¹³ database. For consistent pre-processing and further analysis, we downloaded raw data (CEL file) from GSE37463 (the platform is Affymetrix GeneChip Human Genome HG-U133A), which was contributed by Berthier et al¹⁴. Clinical parameters including age, sex, Bp, BMI, GFR, Scr and proteinuria were collected from Nephroseq, a database which was used to store microarray datasets and clinical data of kidney disease (www.nephroseq.org, 11 2016, University of Michigan, Ann Arbor, MI).

Identification Of Differentially Expressed Genes

R (Version 3.3.4) programming language was applied to data quality assessment, normalization, and detection of DEGs. Based on “affyPLM”¹⁵ package, we conducted the quality assessment on microarray data using RNA degradation curve and Normalized Unscaled Standard Errors¹⁶. Then, we used RMA (Robust Multi-array Averaging)¹⁷ to preprocess the raw data and utilized the limma (Linear Models for Microarray Analysis) package to obtain genes differential expression. The false FDR for fold change between IgAN and healthy control was calculated by moderated t-test, and log₂ fold change ≥ 0.6 or $<$

-0.6 with FDR < 0.05 were considered as the threshold for select DEGs selection. Finally, probes were annotated by official annotations file (Affymetrix HG-U133A Annotations, Release 35).

Gene Enrichment Analysis And Pathway Analysis

Biological significance of hub genes was explored by Gene ontology (GO)¹⁹ enrichment analysis and KEGG (Kyoto Encyclopedia of Genes and Genomes)²⁰ analysis based on DAVID²¹ (Database for Annotation, Visualization and Integrated Discovery). Biological Process (BP), the most representative sub-ontologies of GO enrichment analysis, was used to find the critical biological function in hub genes closely related to BMI, GFR and proteinuria in tubulointerstitium and glomeruli of IgAN respectively. A $P < 0.01$ was considered statically significant. PPI information was acquired from STRING (Search Tool for the Retrieval of Interacting Genes, <http://www.string-db.org/>)²² and 0.4 medium confidence was chosen to be the minimum required interaction score to screen the interactions among hub genes. Cytoscape²³ (version 3.4.0) software was used for visualization of PPI network.

Abbreviations

BMI

Body Mass Index; Bp: blood pressure; GFR: glomerular filtration rate; IgA: Immunoglobulin A; ESRD: end-stage renal disease; CKD: In chronic kidney disease; Scr: serum creatinine; WGCNA: Weighted Gene Co-expression Network Analysis; GEO: Gene Expression Omnibus; RMA: Robust Multi-array Averaging; Limma: Linear Models for Microarray Analysis; GO: Gene Ontology; BP: Biological Process; KEGG: Kyoto Encyclopedia of Genes and Genomes; DAVID: Database for Annotation, Visualization and Integrated Discovery; STRING: Search Tool for the Retrieval of Interacting Genes; GS: Gene Significance; GFB: glomerular filtration barrier; GBM: glomerular base membrane; ESL: endothelial surface layer; SPS: subpodocyte space; Cyr61: cysteine-rich angiogenic inducer 61; DEG: differentially expressed gene; IgAN: Immunoglobulin A nephropathy; LD: Healthy Living Donor; FDR: False discovery rate.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing Interest

The authors declare that they have no competing interests.

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Authors' contributions

Chengyu Yang^{1*}, Chenyu Li^{1*}, Long Zhao¹, Bin Zhou¹, Congjuan Luo¹, Wei Jiang¹, Hong Luan¹, Yan Xu^{1*}

CYY, CYL and YX designed the studies; CYY, CYL, LZ, BZ, CJL, WJ and HL performed the study; CYY, CYL and YX discussed the results; CYY and CYL wrote the paper. All authors have read and approved the manuscript.

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Figures

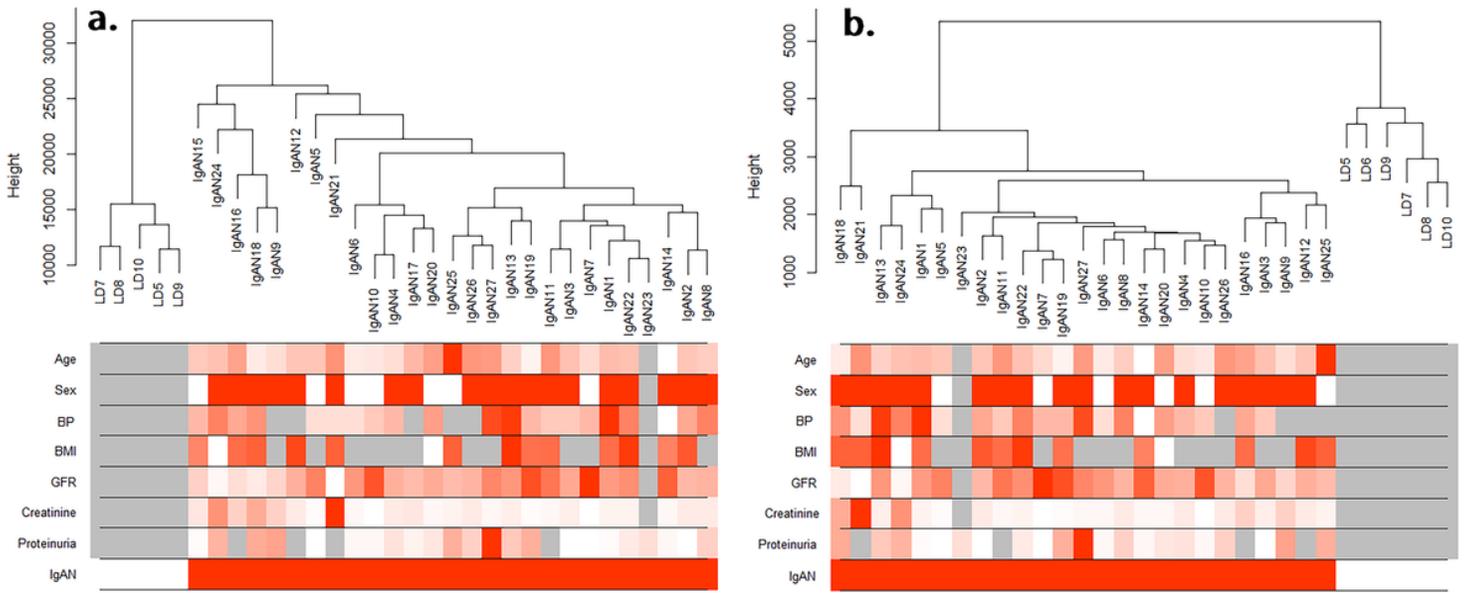


Figure 1

Sample of hierarchical clustering dendrogram and traits heatmap In tubulointerstitium group or glomeruli group, dendrogram mainly yielded two clusters, where living donors became one cluster, and IgA nephropathy yielded the other one. In heatmap, each column represents a sample from the dendrogram above and each row corresponds to a clinical characteristic that is color-coded: white represents low value, red represents high value and grey represents missing data; for sex, red represents male and white represents female. a. Tubulointerstitium of clustering dendrogram and traits heatmap. b. Glomeruli of clustering dendrogram and traits heatmap.

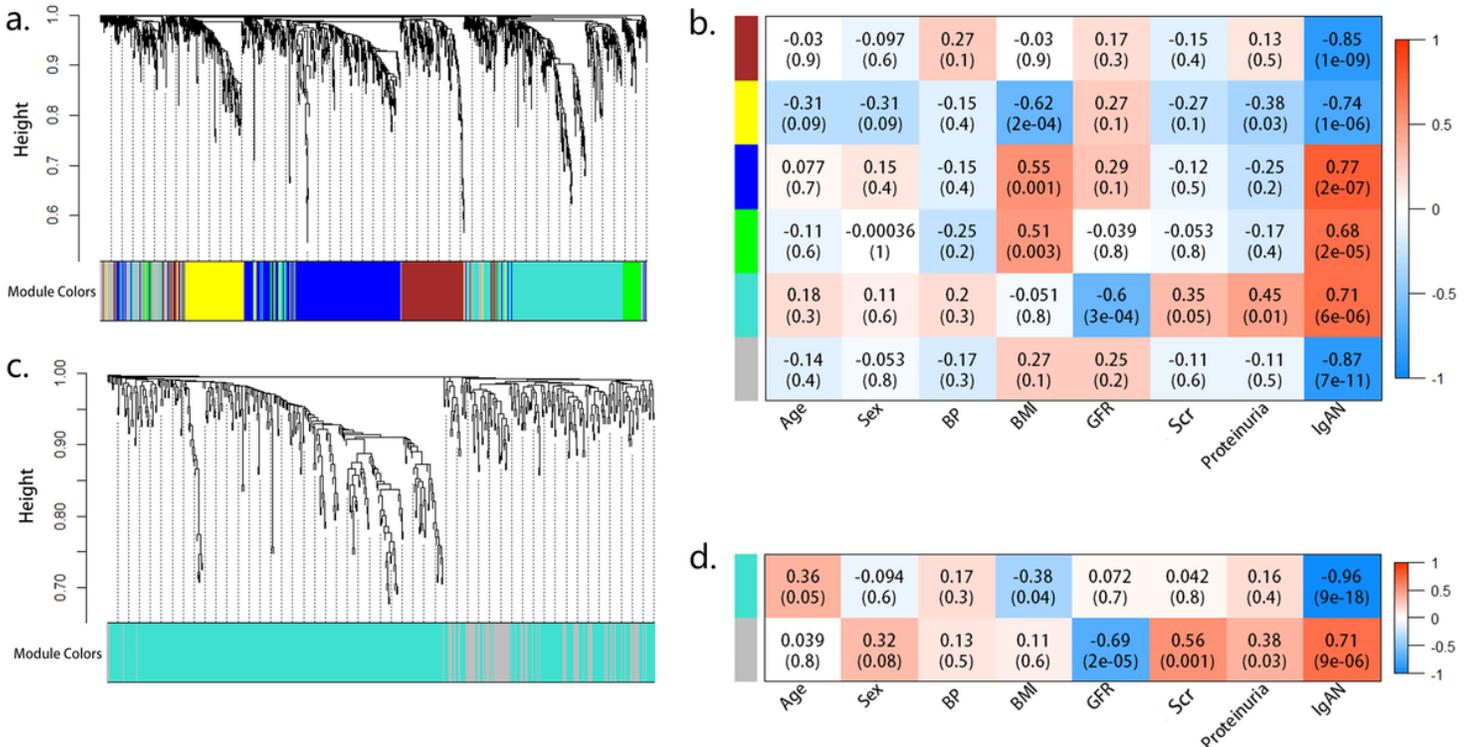


Figure 2

Weighted gene co-expression network analysis in IgAN glomeruli and tubulointerstitium a. Clustering dendrogram of genes in glomeruli, with dissimilarity based on the topological overlap, and module colors assigned. b. Associations of module and traits in glomeruli. Each column corresponds to a trait, each row to a module eigengene. Each cell contains the corresponding correlation, which are color-coded according to the color legend, and P-value in brackets. c. Clustering dendrogram of genes and assigned module in tubulointerstitium. d. Associations of module and traits in tubulointerstitium.

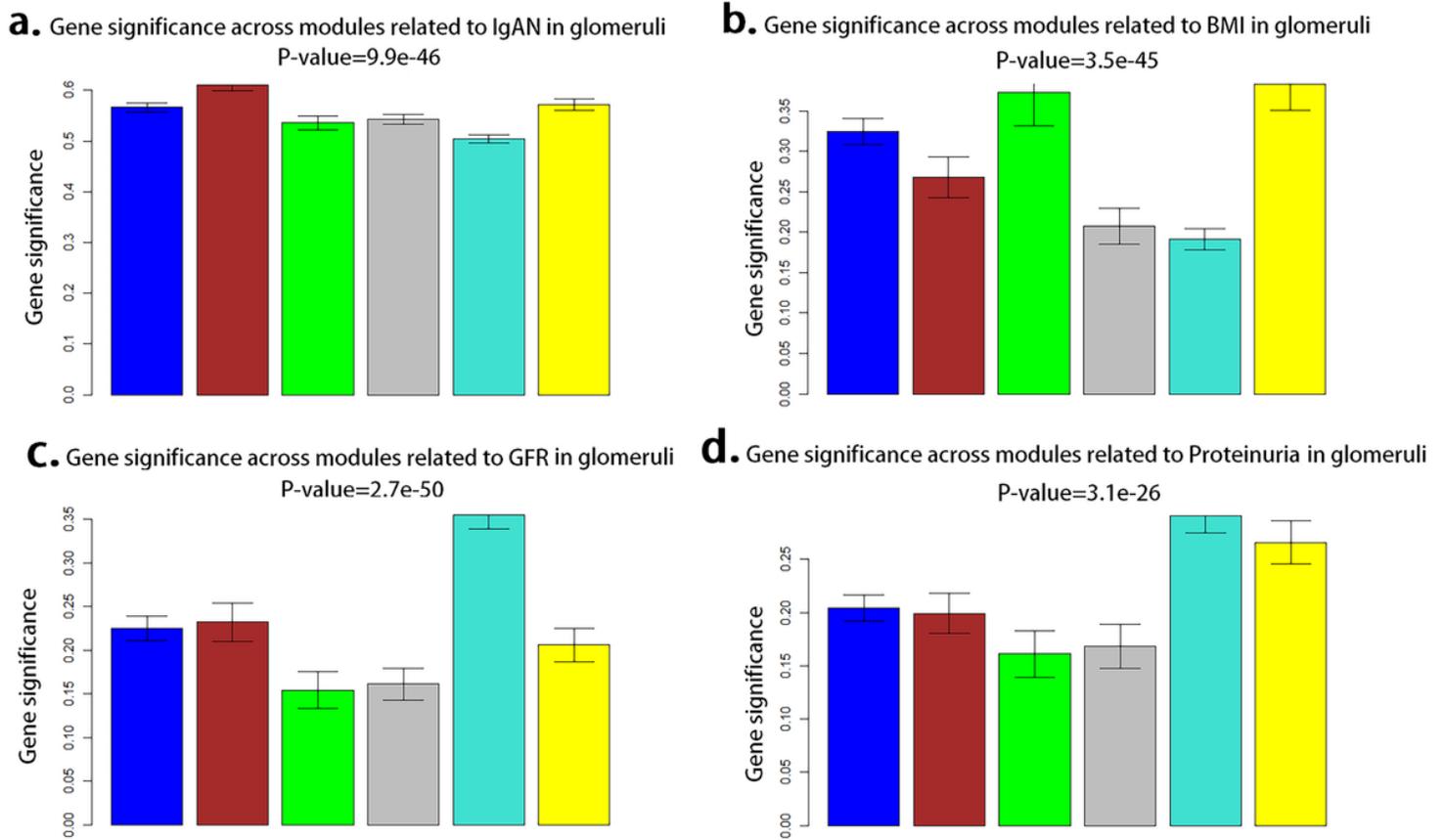


Figure 3

Module GS related to clinical characteristics in glomeruli a. Module GS related to IgAN. b. Module GS related to BMI. c. Module GS related to GFR. d. Module significance related to proteinuria.

Gene significance across modules related to IgAN, GFR and Proteinurie in tubulointerstitium

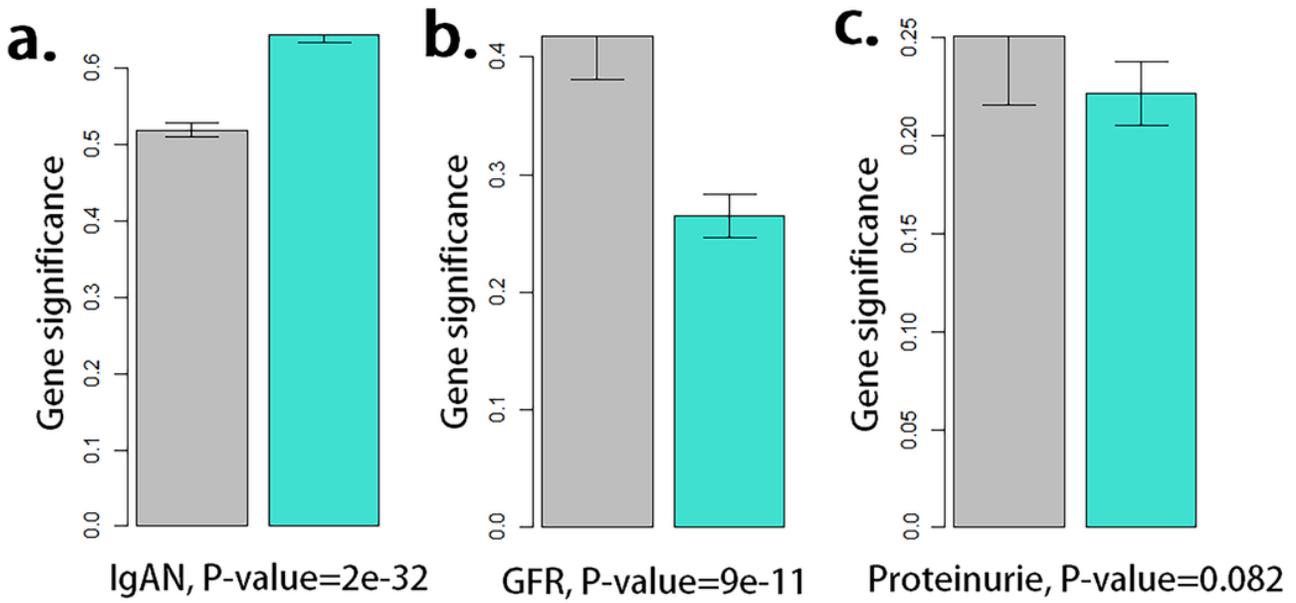


Figure 4

Module GS related to clinical characteristics in tubulointerstitium a. Module GS related to IgAN. b. Module GS related to GFR. c. Module GS related to proteinuria.

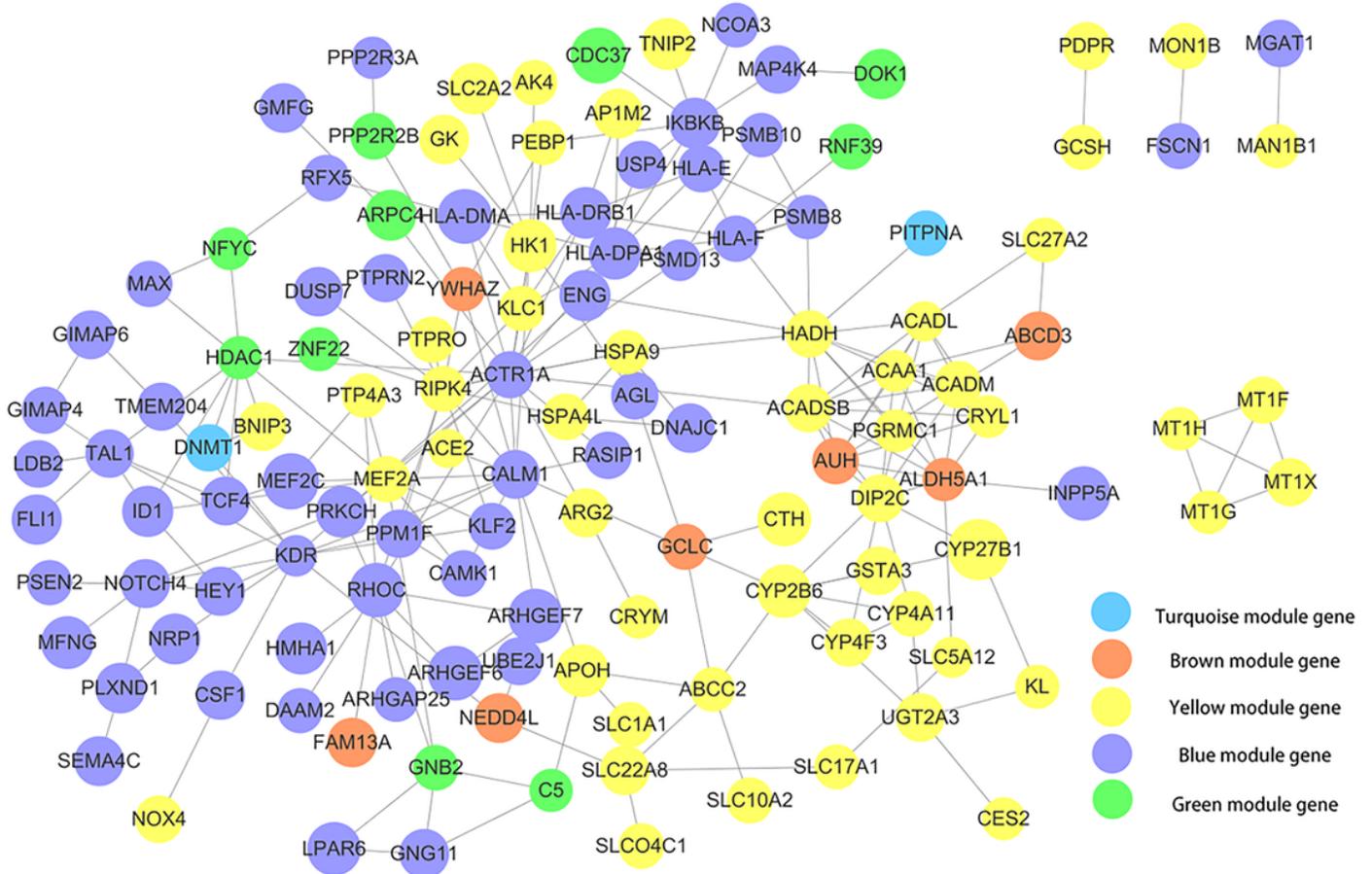
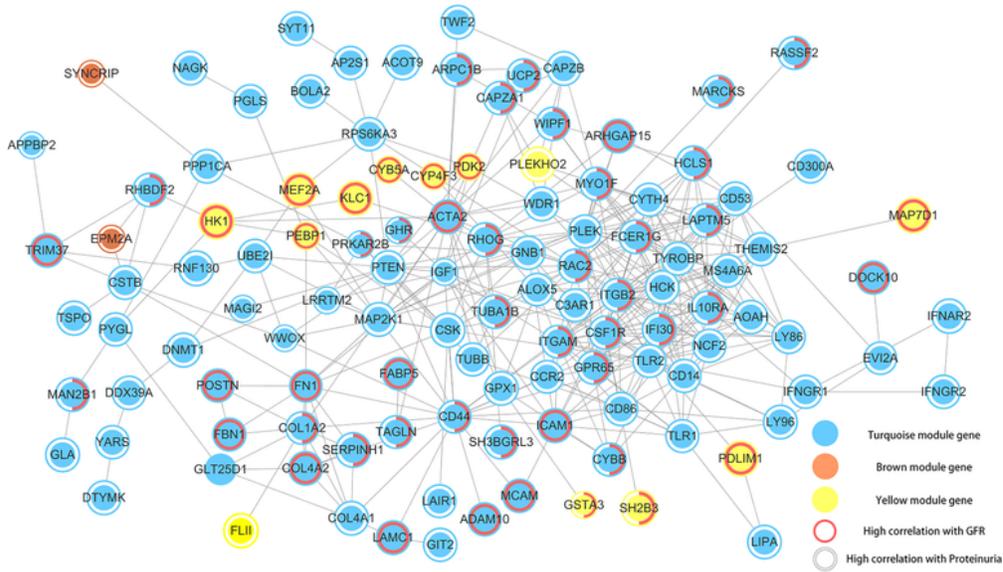


Figure 5

PPI enrichment analysis of hub genes associated with BMI in glomeruli Color of each node represents the module that the genes belong to. PPI enrichment analysis parameters: number of nodes: 218; number of edges: 227; average node degree: 2.08; avg. local clustering coefficient: 0.346; expected number of edges: 153; PPI enrichment p-value: 1.78e-08.

a.



b.

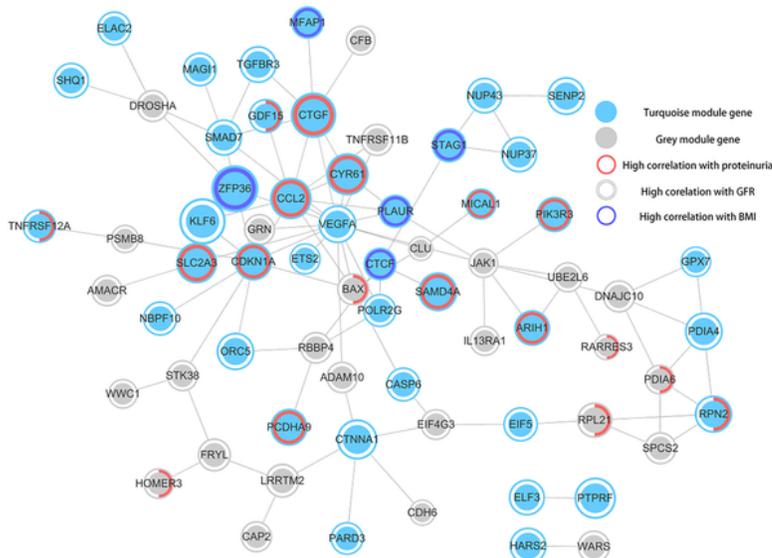


Figure 6

PPI enrichment analysis a. Hub genes associated with GFR and proteinuria in glomeruli. Color of nodes represents the module that genes belong to and the color of the circle represents gene relevant clinical characteristics. PPI enrichment analysis parameters: number of nodes: 168; number of edges: 390; average node degree: 4.46; avg. local clustering coefficient: 0.375; expected number of edges: 174; PPI enrichment p-value: 0. b. Color of nodes represents the module that genes belong to and the color of the circle represents gene relevant clinical characteristics. PPI enrichment analysis parameters: number of nodes: 111; number of edges: 94; average node degree: 1.69; avg. local clustering coefficient: 0.361; expected number of edges: 62; PPI enrichment p-value: $7.33e-05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.csv](#)
- [Additionalfile2.csv](#)
- [Additionalfile3.csv](#)
- [Additionalfile4.csv](#)
- [Additionalfile5.csv](#)