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Gene expression-based analysis identified *NTNG1* and *HGF* as biomarkers for diabetic kidney disease

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Abstract

Diabetic kidney disease (DKD) is a leading cause of end-stage renal disease. Because the molecular mechanisms of DKD are not fully understood, exploration of hub genes and the mechanisms underlying this disease are essential for elucidating the pathogenesis and progression of DKD. Accordingly, in this study, we performed an analysis of gene expression in DKD. The differentially expressed genes (DEGs) included 39 upregulated genes and 113 downregulated genes in the GSE30528 dataset and 127 upregulated genes and 18 downregulated genes in the GSE30529 dataset. Additionally, functional analyses were performed to determine the roles of DEGs using glomeruli samples from patients with DKD and healthy controls from the GSE30528 dataset and using tubule samples from patients with DKD and healthy controls from the GSE30529 dataset. These DEGs were enriched in pathways such as the Wnt signaling pathway, metabolic pathways, and the mammalian target of rapamycin signaling pathway in the GSE30528 dataset and the longevity regulating pathway and Ras signaling pathway in the GSE30529 dataset. Moreover, a protein-protein interaction network was constructed using the identified DEGs, and hub gene analysis was performed. Furthermore, correlation analyses between key genes and pathological characteristics of DKD indicated that *CCR4*, *NTNG1*, *HGF* and *ISL1* are related to DKD, and *NTNG1* and *HGF* may server as diagnostic biomarkers in DKD using the receiver–operator characteristic (ROC) curve. Collectively, our findings established 2 reliable biomarkers for DKD.

Abbreviations: DEGs = differentially expressed genes, DKD = Diabetic kidney disease, DM = diabetes mellitus, ESRD = end-stage renal disease, GEO = Gene Expression Omnibus, GO = gene ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes, PPI = protein-protein interaction, ROC= receiver–operator characteristic.

Keywords: bioinformatic analysis, biomarker, diabetic kidney disease, gene expression

1. Introduction

The incidence of diabetes mellitus (DM) has risen dramatically over the past 2 decades, and this disease now affects over 300 million people worldwide.[1,2] Diabetic kidney disease (DKD) is a severe microvascular complication of DM, and approximately 20% to 40% of diabetic patients develop DKD, making this a leading cause of end-stage renal disease (ESRD).[3,4] Individuals with ESRD secondary to DKD have an increased risk of all-cause mortality and cardiovascular disease, which causes enormous medical and socioeconomic burdens on society.[5,6] DKD has 2 pathophysiological stages based on clinical manifestations and morphological abnormalities, namely, early diabetic kidney stage of glomerular hyperfiltration, microalbuminuria (30–300 mg/dl), glomerular hypertrophy and mesangial expansion, and advanced diabetic renal stage of progressive decline in glomerular filtration rate (GFR), macroalbuminuria (>300 mg/dl), glomerulosclerosis, and interstitial fibrosis.[7]

Despite extensive studies, the prognosis of individual patients with DKD is hard to predict owing to differences in disease progression and a lack of effective prognostic parameters. In addition, current treatments, including renin-angiotensin system blockade as well as stringent glycemic, lipid, and blood pressure control, are very limited, and none of these approaches can completely prevent the progression to ESRD. Hence, it is essential to explore the molecular mechanisms of DKD and thus establish accurate diagnostic tools and treatment regimens.

Transcriptomics is a promising approach for identification of biomarkers and monitoring disease activity.[8] Microarray technology facilitates the elucidation of mRNA profiles associated with human disease and provides a comprehensive, unbiased approach to systematically analyze disease processes.[9] Functional genomics explores gene interactions and cellular pathways involved in disease biology that may be potential targets of newer molecular therapeutics.[10] Ultimately, after more analysis, these biomarkers may be applied toward early diagnosis, prognosis, and prediction of therapeutic responses.

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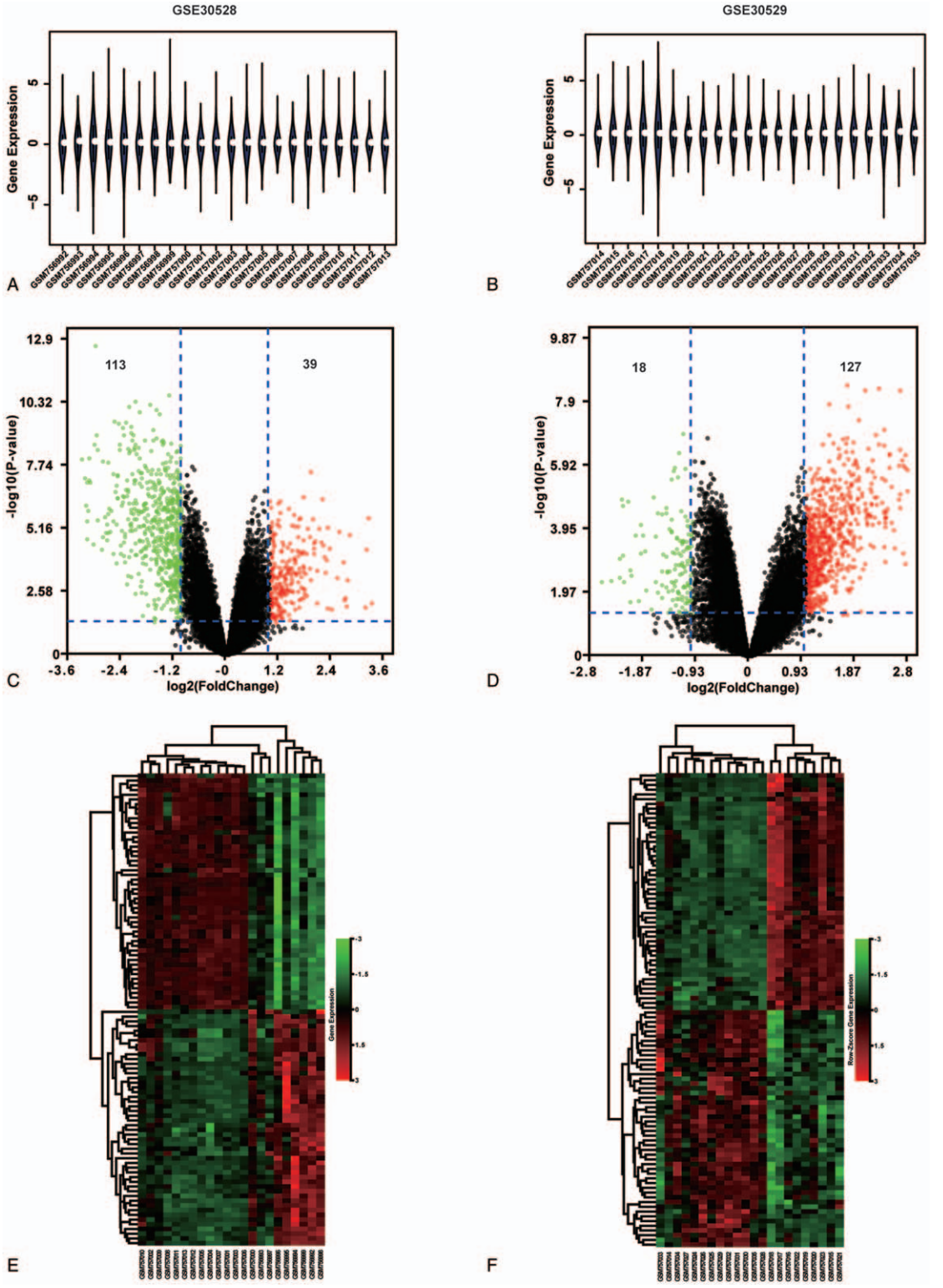

Figure 1. DEGs in the 2 datasets. (A and B) Standardization of GSE30528 and GSE30529 data. (C and D) Volcano plot of GSE30528 and GSE30529 data. (E and F) Hierarchical clustering heatmap of DEGs in GSE30528 and GSE30529 data. The upregulated genes are indicated as red dots; the downregulated genes are indicated as green dots; genes without significant differences are indicated as black dots. DEGs = differentially expressed genes.

In this study, we firstly analyzed differentially expressed genes (DEGs) in glomerular and tubule tissues from patients with DKD and healthy controls using data downloaded from the Gene Expression Omnibus (GEO). Then, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to explore the molecular mechanisms of DKD. Next, protein–protein interaction (PPI) analysis was performed and hub genes were identified. Besides,

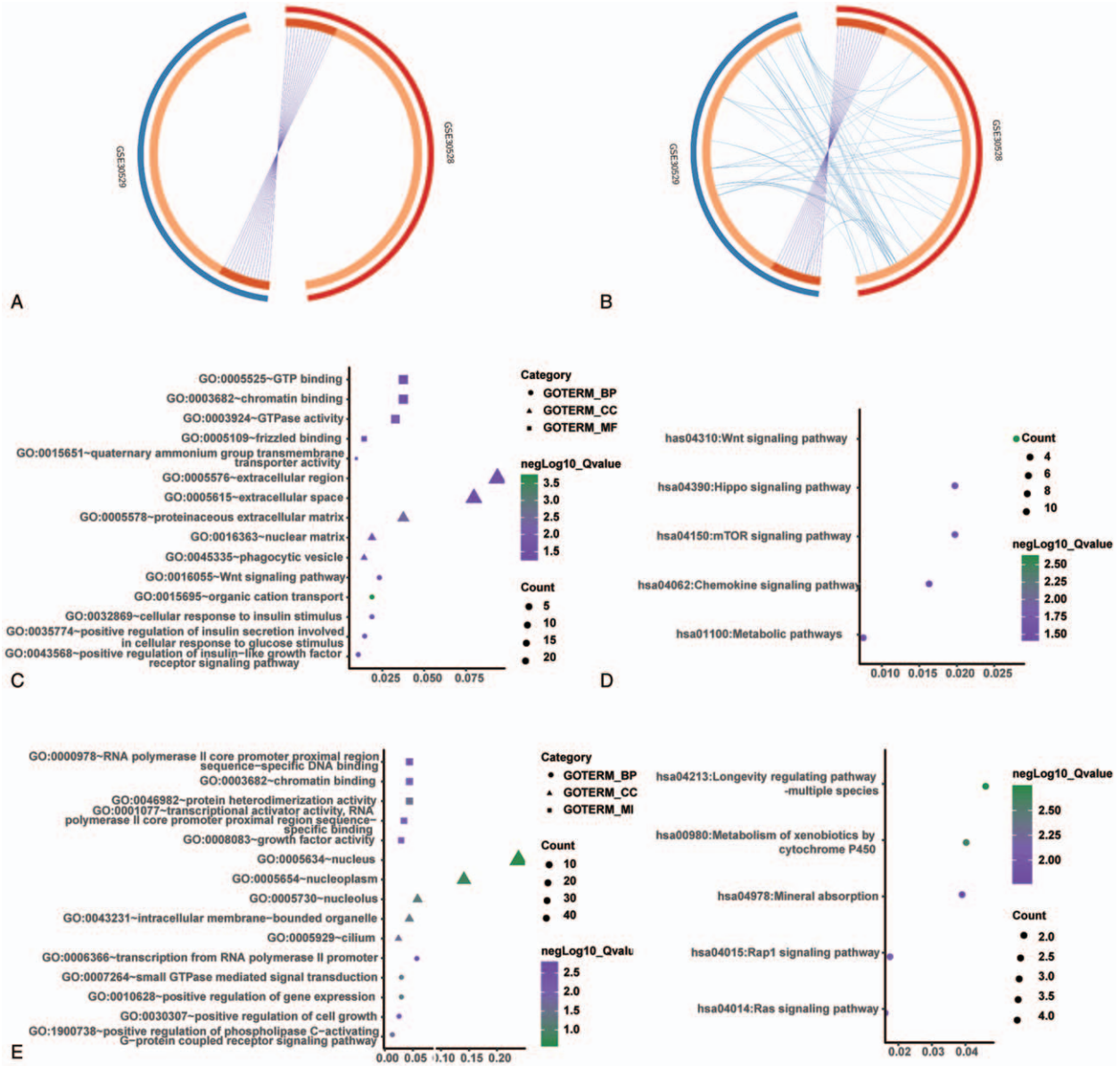

Figure 2. Functional enrichment analysis of DEGs in the 2 datasets. (A, B) Overlap analysis between gene lists and shared enriched ontologies. The outer circle represents the identity of the corresponding gene list, and the inner circle represents gene lists, where hits are arranged along the arc. Genes that hit 2 lists are indicated in dark orange, and genes unique to a list are shown in light orange. Purple curves link identical genes, and blue curves link genes that belong to the same enriched ontology term. (C and E) GO enrichment of DEGs in GSE30528 and GSE30529 data. GO analysis included 3 functional groups: molecular functions, biological processes, and cell components. (D and F) KEGG of DEGs in GSE30528 and GSE30529 data. DEGs = differentially expressed genes, GO = gene ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes.

correlation analysis between hub genes and clinicopathological features in patients with DKD and in mouse models was employed, and ultimately 4 hub genes were selected for diagnostic analysis. Finally, 2 hub genes (Nertin G1, *NTNG1*, and Hepatocyte growth factor, *HGF*) were identified as key biomarkers for DKD. The *NTNG1* gene is located on chromosome 1p13.3 and encodes a glycosylphosphatidylinositol protein anchored to the presynaptic membrane and *HGF* is known as scatter factor and tumor cytotoxic factor, is a large multidomain heterodimeric protein that belongs to the cytokine family. Our findings established 2 reliable biomarkers for DKD.

2. Materials and methods

2.1. Microarray data

Microarray data were downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo).[11] The GSE30528 dataset included data on glomeruli samples from patients with DKD (n = 9) and healthy controls (n = 13) and the GSE30529 dataset included data on tubule samples from patients with DKD (n = 10) and controls (n = 12) from *Homo sapiens* based on the GPL571 (Affymetrix Human Genome U133A 2.0 Array) platform. The R software package was applied to process the microarray data and to normalize the unqualified files.

2.2. Identification of DEGs

DEGs were analyzed using the limma package in Bioconductor (http://www.bioconductor.org/).[12] Samples with an absolute value of log fold change greater than 1 and *P* value less than .05 were considered DEGs. Probe sets without corresponding gene symbols or genes with more than 1 probe set were removed or averaged, respectively. Next, identified DEGs were used for further analysis.

2.3. Functional enrichment analysis of DEGs

To investigate the biological characteristics and functional enrichment of candidate DEGs, functional enrichment analysis was performed using the DAVID (https://david.ncifcrf.gov/) online database.[13] Results with *P* values of less than .05 were considered significant. In addition, Circos, an information aesthetic for comparative genomics, was applied to show how genes from the input gene lists overlapped and shared GO terms.[14]

2.4. PPI network integration and module analysis

PPI networks for DEGs were investigated with the STRING database (https://string-db.org/cgi/).[15] Cytoscape (version 3.7.1) is an open source bioinformatics software platform for visualizing molecular interaction networks.[16] CytoHubba, a Cytoscape plugin, was used to explore the hub genes in the PPI networks,[17] and the top 30 hub genes were displayed based on node degree.

2.5. Clinicopathological correlation analysis

We performed correlation analysis between hub genes and clinicopathological features in patients with DKD and in mouse models using the Nephroseq v5 database. Additionally, key hub gene expression in a DKD mouse model was analyzed using the same database.

2.6. Diagnostic analysis

We analyzed the diagnostic effectiveness of key hub genes for distinguishing patients with DKD and healthy individuals in those 2datasets using the area under the Receiver–operator characteristic (ROC) curve.

2.7. Statistical analysis

Values were depicted as the means ± standard deviations and were considered significant when the *P* value <.05. Statistical analysis was performed using unpaired *t* test. All statistical analyses were carried out using GraphPad prism 7.0 (GraphPad Software Inc. La Jolla, CA, USA).

2.8. Ethical statement

All the data of this paper was obtained from the open-access database, we did not get these data from patients or animals directly, nor intervene these patients. So the ethical approval was not necessary.

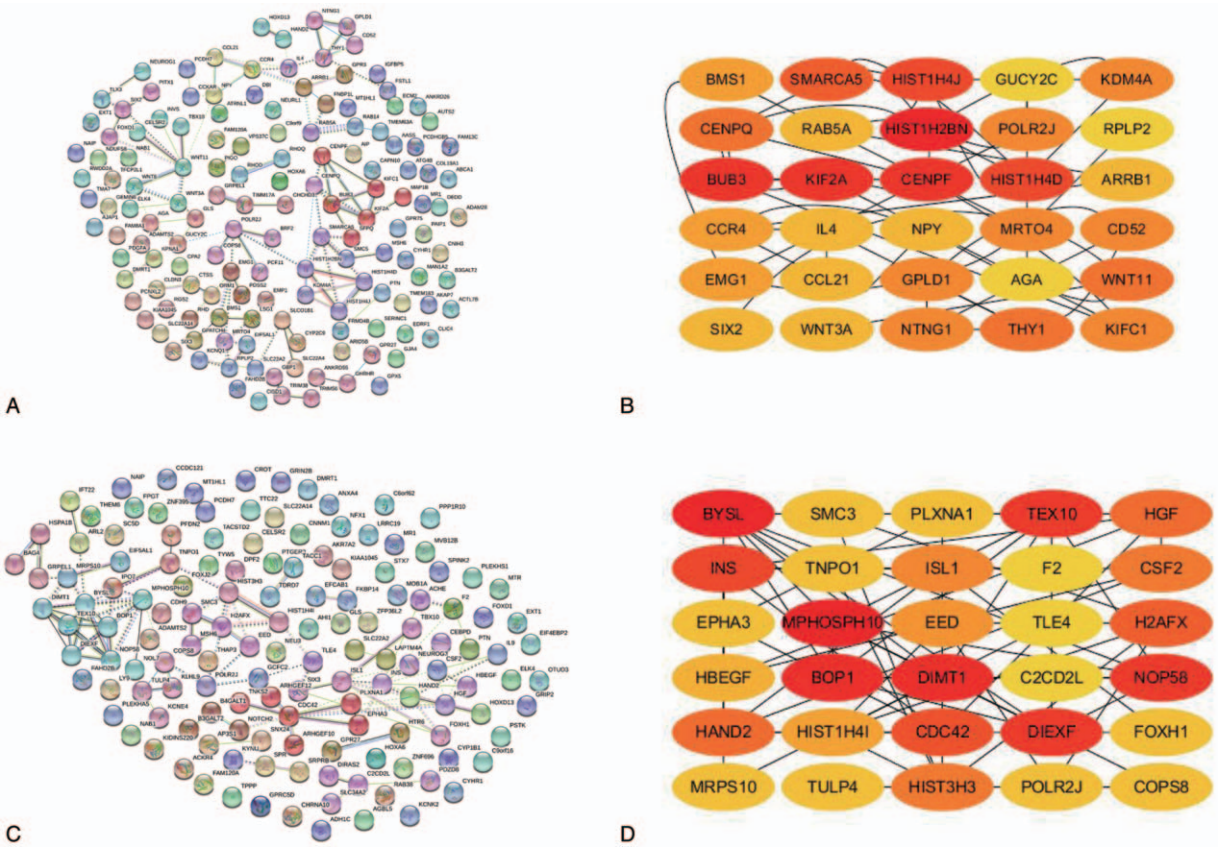

Figure 3. Hub gene identified from the PPI networks. (A and C) PPI network of DEGs in GSE30528 and GSE30529 data. (B and D) The top 30 hub genes of DEGs in GSE30528 and GSE30529 data. DEGs = differentially expressed genes, PPI = protein-protein interaction.

3. Results

3.1. Identification of DEGs in DKD

The microarray datasets GSE30528 and GSE30529 were standardized, and the results are shown in Figure 1A and 1B. After standardization of the microarray results, 152 DEGs were identified from the GSE30528 dataset, including 39 upregulated genes and 113 downregulated genes (Fig. 1C). Similarly, 145 DEGs were screened from the GSE30529 dataset, including 127 upregulated genes and 18 downregulated genes (Fig. 1D). The cluster heatmaps of the top 100 DEGs are shown in Figure 1E and 1F.

3.2. Functional enrichment analysis of DEGs

The overlap between ontology terms associated with DEGs in GSE30528 and GSE30529 was minimal (Fig. 2A and B); thus, it seemed logical to analyze the functional enrichment of these gene sets separately. In the GSE30528 dataset, GO analysis showed that DEGs were significantly enriched in cellular components (CCs), including proteinaceous extracellular matrix, extracellular region, phagocytic vesicle, nuclear matrix, and extracellular space. For molecular functions (MFs), DEGs were particularly enriched in GTPase activity, GTP binding, frizzled binding, chromatin binding, and quaternary ammonium group transmembrane transporter activity. Additionally, biological process (BP) and KEGG pathway analyses demonstrated that the DEGs were enriched in positive regulation of insulin secretion involved in cellular responses to glucose stimulus and positive regulation of the insulin-like growth factor receptor signaling pathway, Wnt signaling pathway, metabolic pathways, mammalian target of rapamycin (mTOR) signaling pathway, Hippo signaling pathway, and chemokine signaling pathway (Fig. 2C and D).

Similarly, functional enrichment analyses of 145 DEGs in GSE30529 were also performed using the DAVID database. GO analysis results showed that DEGs were significantly enriched in CCs, including cilium, nucleoplasm, nucleus, nucleolus, and intracellular membrane-bounded organelles. For MFs, DEGs were particularly enriched in RNA polymerase II core promoter proximal region sequence-specific DNA binding, growth factor activity, transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding, chromatin binding, and protein heterodimerization activity. Additionally, BP and KEGG pathway analyses demonstrated that the DEGs were enriched in positive regulation of cell growth, positive regulation of phospholipase C-activating G-protein coupled receptor signaling pathways, small GTPase-mediated signal transduction, longevity regulating pathways, metabolism of xenobiotics by cytochrome P450, the Rap1 signaling pathway, the Ras signaling pathway, and mineral absorption (Fig. 2E and 2F).

3.3. Identification of hub genes from the PPI networks

Here, PPI networks were generated with STRING database (Fig. 3A and C), and hub genes were identified using CytoHubba from the PPI network. The top 30 hub genes in 2 datasets were constructed using Cytoscape (Fig. 3B and D), and their corresponding node degrees were listed in Table 1. Finally, the top 20 hub genes in 2 datasets, including *HIST1H2BN, BUB3, CENPF, KIF2A, HIST1H4D, HIST1H4J, SMARCA5, THY1, CENPQ, WNT11, POLR2J, CD52, MRTO4, KIFC1, KDM4A, NTNG1, GPLD1, EMG1, BMS1,CCR4, MPHOSPH10, BYSL, BOP1, DIMT1, NOP58,DIEXF, TEX10, INS, CDC42, H2AFX, HAND2, HGF,CSF2, HIST3H3, ISL1, EED, HIST1H4I, HBEGF, POLR2J,* and *COPS8* were selected for following analysis.

Table 1

The top 30 hub genes in PPI networks.

GSE30528		GSE30529	
Gene symbol	Degree	Gene symbol	Degree
HIST1H2BN	15	MPHOSPH10	724
BUB3	13	BYSL	724
CENPF	13	BOP1	722
KIF2A	13	DIMT1	722
HIST1H4D	12	NOP58	720
HIST1H4J	12	DIEXF	720
SMARCA5	10	TEX10	720
THY1	8	INS	21
CENPQ	8	CDC42	14
WNT11	8	H2AFX	12
POLR2J	6	HAND2	11
CD52	6	HGF	11
MRTO4	6	CSF2	10
KIFC1	6	HIST3H3	10
KDM4A	6	ISL1	9
NTNG1	6	EED	8
GPLD1	6	HIST1H4I	7
EMG1	5	HBEGF	6
BMS1	5	POLR2J	4
CCR4	5	COPS8	4
SIX2	4	TULP4	4
IL4	4	MRPS10	4
ARRB1	4	EPHA3	4
RAB5A	4	PLXNA1	4
CCL21	4	FOXH1	4
NPY	4	TNPO1	4
WNT3A	4	SMC3	4
AGA	3	C2CD2L	3
GUCY2C	3	TLE4	3
RPLP2	3	F2	3

PPI = protein-protein interaction.

3.4. Clinicopathological correlation analysis of hub genes

The aforementioned hub genes associated with DKD were further analyzed with Nephroseq v5 database. We found that the mRNA levels of C-C motif chemokine receptor 4 (CCR4) and NTNG1 were positively related to GFR in patients with DKD, whereas the mRNA levels of HGF and Insulin gene enhancer binding protein-1 (ISL1) were negatively related to GFR in DKD patients (Fig. 4). CCR4 is the receptor 2 two CC chemokine ligands (CCLs)-CCL17 (also called thymus- and activation-regulated chemokine) and CCL22 (macrophage-derived chemokine). ISL1 is a subtype of the LIM homologous domain transcription factors. The rest of hub genes mRNA levels were not significantly correlated to GFR (data not shown). Additionally, we verified the transcriptional levels of CCR4, NTNG1, HGF, and ISL1 in DKD mice model using the same database. Compared with those in non-DKD mice, expression of NTNG1 and CCR4 were down-regulated in eNOS-deficient C57BLKS db/db mice (Fig. 5A and B). Furthermore, we analyzed the correlations between NTNG1 and CCR4 mRNA levels and other clinical parameters, such as weight, fasting blood glucose (FBG), and urinary albumin to creatinine

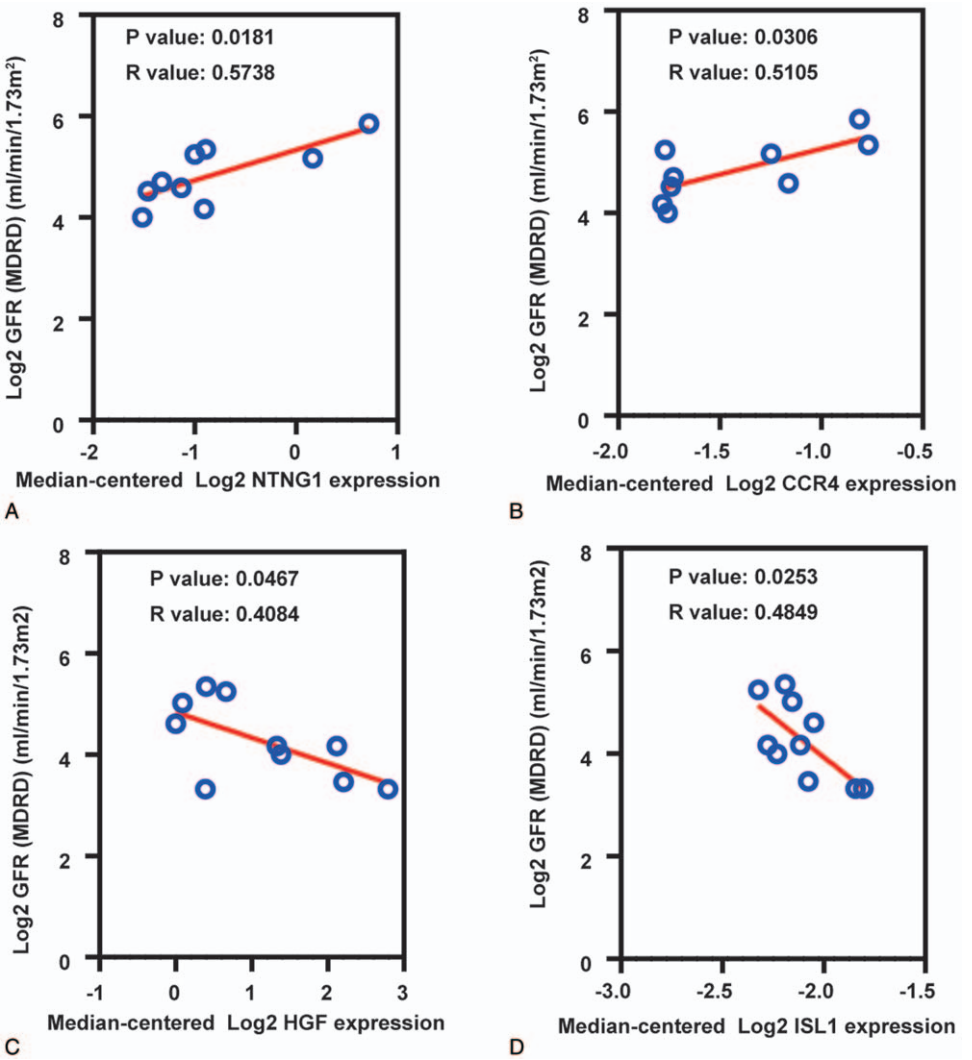

Figure 4. Correlation analysis between the expression of hub genes and GFR in patients with DKD. (A) *NTNG1* was positively correlated with GFR ($P = .0181$, $r = 0.5738$). (B) *CCR4* was positively correlated with GFR ($P = .0306$, $r = 0.5105$). (C) *HGF* was negatively correlated with GFR ($P = .0467$, $r = 0.4084$). (D) ISL1 was negatively correlated with GFR ($P = .0253$, $r = 0.4849$). DKD = diabetic kidney disease; GFR = glomerular filtration rate.

ratio (UACR). Correlation results showed that NTNG1 mRNA levels was positively related to FBG and negatively related to weight (Fig. 5C and D), while CCR4 mRNA levels was negatively related to FBG and positively related to weight (Fig. 5E and F). NTNG1 and CCR4 mRNA levels were not significantly correlated to UACR (data not shown). The data of HGF and ISL1 in mice models were not available.

3.5. Diagnostic analysis

We finally tested whether these 4 hub genes associated with GFR can server as diagnostic biomarkers using ROC curves. ROC curve analysis revealed that the AUC was 0.9658 (95%CI, 0.8995–1.032) for *NTNG1*, 0.5897 (95%CI, 0.315–0.8645) for *CCR4*, 0.8 (95%CI, 0.6039–0.9961) for *HGF*, 0.65 (95% CI, 0.4166–0.8834) for *ISL1* (Fig. 6), suggesting *NTNG1* and *HGF* can serve as diagnostic biomarkers for distinguishing patients with DKD from healthy controls with the AUC exceeded 0.80.

4. Discussion

DKD is a heterogeneous disease with various clinicopathological stages and therapeutic responses. Approximately half of patients with type 2 diabetes and one-third of patients with type 1 diabetes will develop DKD.[18,19] Moreover, it is difficult to precisely identify DKD in epidemiology or clinical practice, particularly in patients with type 2 diabetes. Indeed, high mortality rates associated with type 1 and type 2 diabetes are largely confined to those with DKD.[20–22] Consequently, there is a need for innovative treatment strategies for preventing, treating, and even reversing DKD. Current evidence has revealed that genetic factors may explain why some individuals develop and some do not.[23] In addition, transcriptional and bioinformatics analyses have expanded our understanding of the molecular mechanisms of disease pathogenesis and progression, which is essential for identifying genetic alternations and establishing potential diagnostic biomarkers. However, the exact molecular mechanisms of DKD have not been fully elucidated. Accordingly, in the

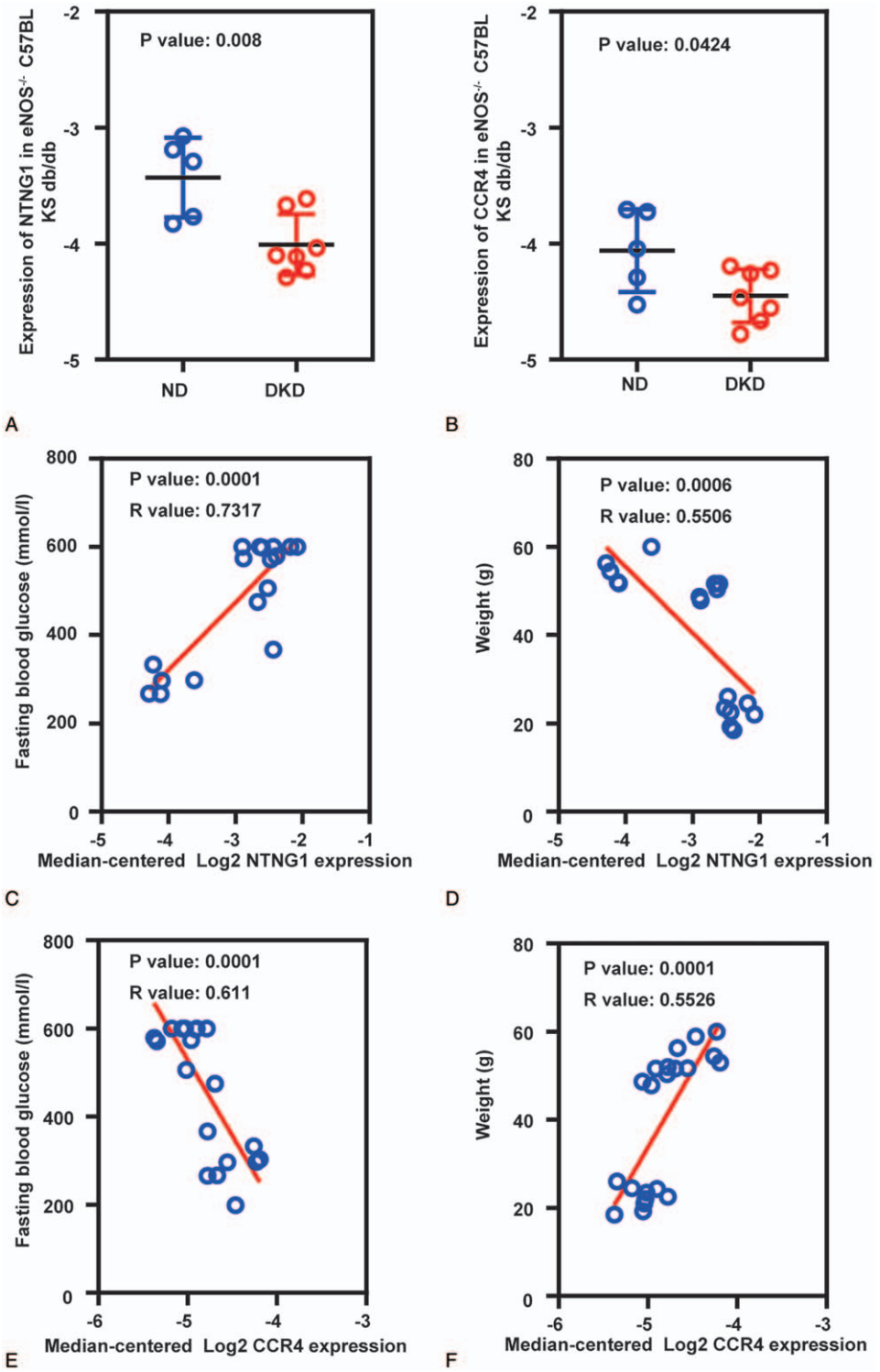

Figure 5. Correlation analysis between the expression of hub genes and FBG and weight in DKD model mice. (A) Expression of *NTNG1* in eNOS-/- C57BLKS db/db mice. (B) Expression of *CCR4* in eNOS-/- C57BLKS db/db mice. (C) *NTNG1* was positively correlated with FBG ($P = .0001$, $r = 0.7317$). (D) *NTNG1* was negatively correlated with weight ($P = .0006$, $r = 0.5506$). (E) *CCR4* was negatively correlated with FBG ($P = .0001$, $r = 0.611$). (F) *CCR4* was positively correlated with weight ($P = .0001$, $r = 0.5526$). DKD = diabetic kidney disease, FBG = fasting blood glucose.

current study, 2 mRNA microarray datasets (GSE30528 and GSE30529) were analyzed to obtain DEGs from glomerular and tubule tissues of patients with DKD and healthy controls. We identified many nonoverlapping DEGS in the 2 datasets and showed that DEGs in GSE30528 were significantly enriched in extracellular space, organic cation transport, Wnt signaling pathway, metabolic pathways, mTOR signaling pathway, Hippo signaling pathway, and chemokine signaling pathway.

Some evidences have demonstrated that Wnt/β-catenin signaling plays an essential role in the development of DKD,

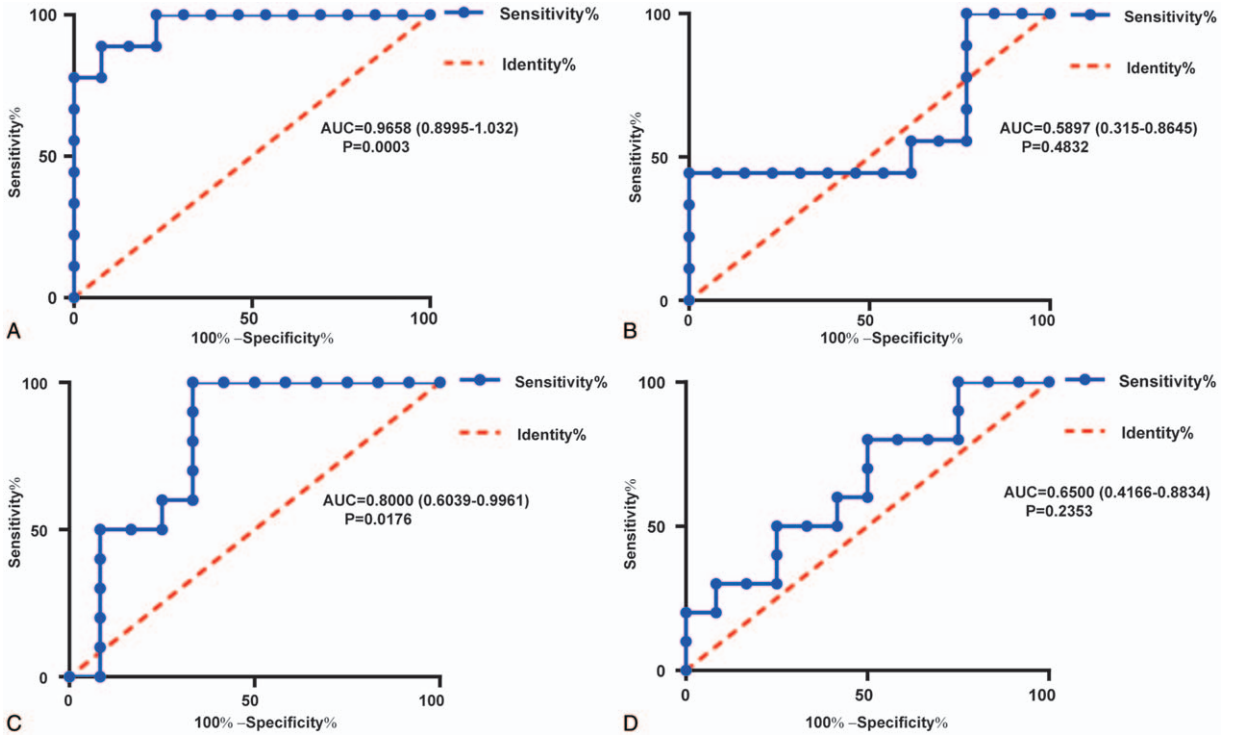

Figure 6. ROC curves of *NTNG1*, *CCR4*, *HGF*, and *ISL1* for DKD diagnosis. (A) ROC curve of *NTNG1* (AUC = 0.9658, *P* = .0003). (B) ROC curve of *CCR4* (AUC = 0.5897, *P* = .4832). (C) ROC curve of *HGF* (AUC = 0.8, *P* = .0176). (D) ROC curve of *ISL1* (AUC = 0.65, *P* = .2353).

including induction of podocyte dysfunction.[24,25] Activation of Wnt signaling in podocytes contributes to glomerular basement membrane abnormalities, decreases nephrin expression, induces detachment of podocytes, and disrupts the epithelial-mesenchymal transition, ultimately causing albuminuria and glomerulosclerosis.[26–28] The mTOR signaling cascade has effects on cellular growth, survival, and metabolism.[29] Podocyte-specific loss of mTOR causes proteinuria and progressive glomerulosclerosis, and podocyte-specific genetic inhibition of mTOR activation prevents progressive glomerular diseases.[30] The Hippo pathway regulates cell proliferation, apoptosis, and stemness in response to a wide range of extracellular and intracellular signals, including cell-cell contact, mechanical cues, ligands of G-protein-coupled receptors, and cellular energy status.[31] A previous study indicated that crosstalk between the epidermal growth factor (EGF) receptor signaling pathway and the Hippo pathway may be an important underlying mechanism mediating the development and progression of DKD.[32]

Although most studies have focused on glomerular damage, tubules are also known play a pivotal role in the pathogenesis of DKD.[33,34] Moreover, there is a good correlation between tubulo-interstitial changes and renal function.[35,36] Functional analysis using the GSE30529 database demonstrated that DEGs were mainly enriched in growth factor activity, the longevity regulating pathway, metabolism of xenobiotics by cytochrome P450, the Rap1 signaling pathway, the RAS signaling pathway, and mineral absorption. Rap1 is a small GTPase that regulates cell adhesion, migration, proliferation, and cell survival.[37] The expression and activity of Rap1 were decreased in patients with DKD and in animal models, and activation of Rap1 ameliorates renal tubular injury by regulation of mitochondrial dysfunction.[38] Interestingly, hyperactivity of intrarenal RAS has been strongly implicated in both the onset and progression of DKD, and pharmacological RAS inhibition has been reported to prevent microtubular changes.[39,40]

Next, the top 30 hub genes were identified from PPI networks, and correlation analysis was performed between transcriptional levels of hub genes and clinicopathological parameters. Among these genes, only NTNG1, CCR4, HGF, and ISL1 mRNA levels were significantly correlated to clinical parameters. NTNG1, an axon guidance molecule, contains laminin-type EGF-like domains and is bound to the plasma membrane through a GPI anchor.[41] Till now, evidences associated with *NTNG1* were focused on mental and neurological disorders,[42,43] and association between *NTNG1* and DKD is unknown. Chemokines and their receptors play a crucial role in the immune homeostasis and inflammatory response of renal diseases.[44] CCR4, highly expressed T-helper type 2 (Th2) cells and regulatory T cells, involves in Th1/Th2 regulation.[45]

A recent study suggests that CCR4 and its ligand CCL22 axis facilitated Th22 cells recruited into mesangial cells and tubular epithelial cells, and thus contributes to proteinuria in IgA nephropathy.[46] The functional role of CCR4 in DKD still await to be elucidated. In the present study, NTNG1 and CCR4 mRNA level was decreased in DKD patients and mouse models, and positively related to GFR in DKD patients. In DKD mice model, NTNG1 mRNA level was positively related to FBG and negatively related to weight; CCR4 mRNA level was negatively related to FBG and positively related to weight. These results suggest that *NTNG1* and *CCR4* may server as a predictive biomarker in glomerular injury.

HGF, a protein that binds to the hepatocyte growth factor receptor to regulate cell growth, cell motility and tissue regeneration, is extensively investigated in diabetes and diabetic

complications, such as DKD. Evidences revealed that exogenous HGF can attenuate proteinuria and tubulointerstitial fibrogenesis via inhibiting TGF-β expression and accelerating kidney repair in murine models.[47–49] In concordance with previous studies, our results shown that HGF mRNA level was downregulated in patients with DKD, seemingly suggesting *HGF* plays a renoprotective role in DKD. ISL-1, a member of the LIM/homeodomain family of transcription factors, regulates insulin gene expression.[50] Our results shown that ISL-1 mRNA level was overexpression and negatively correlated to GFR in DKD patients compared to healthy samples, and further researches are needed to unravel its mole mechanisms in DKD.

Furthermore, we examined whether *NTNG1*, *CCR4*, *HGF*, and *ISL-1* may server as reliable biomarkers using ROC curve. Finally, *NTNG1* and *HGF* were defined as diagnostic biomarkers with high sensitivity and specificity. Significant progress has been made to broaden our understanding about the mechanisms of DKD based on transcriptomic analysis.

Several limitations should also be considered in interpreting study results. First, transcriptional alterations and downstream changes in protein function do not always correspond, and thus predicted hub genes at protein level should be detected using western blot or immunohistochemistry. Second, hub genes and pathways were identified based on small sample sizes by bioinformatic analysis. Therefore, further research with large sample sizes is needed to verify the molecular mechanisms of the identified genes in DKD using loss-of-function and gain-of-function study.

5. Conclusion

Taken together, our findings provided an integrative analysis of candidate genes and pathways in human DKD and identified 2 hub genes (*NTNG1* and *HGF*) may server as reliable biomarkers in DKD.

Author contributions

Data curation: Yun-Liang Tang.
Methodology: Xiao-Yang Dong.
Supervision: Yun-Liang Tang.
Writing – original draft: Zhen-Guo Zeng.
Writing – review and editing: Zhen Feng.

Correction

The affiliation for Drs. Xiao-Yang Dong and Zhen Feng is now appearing as affiliation a.

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