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Urinary miR-3137 and miR-4270 as potential biomarkers for diabetic kidney disease

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Abstract

Background: As one of the most prevalent diagnostic indicators of diabetic kidney disease (DKD), albumin-to-creatinine ratio (ACR) shows considerably limited predictive power in clinical application. We analyzed microarray expression profiling of urine to seek for differentially expressed miRNAs for potential biomarkers of DKD.

Methods: Urine samples from type 2 diabetes mellitus (T2DM) patients with (30 mg/g < ACR < 300 mg/g, DKD group) or without DKD (ACR < 30 mg/g, DM group) were collected for miRNA microarray analysis. The differentially expressed miRNAs were screened by bioinformatics analysis and validated by quantitative real-time PCR. Target genes of differentially expressed miRNAs were predicted in miRDB, Targetscan, and microRNA.org databases. We also conducted the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathways analysis to explore for potential mechanisms in DKD.

Results: Nine miRNAs were down-regulated and seventeen miRNAs were up-regulated in DKD group, compared to DM group. The levels of miR-3137 and miR-4270 in DKD group were 0.670 ± 0.505 and 2.116 ± 1.762 times than those in DM group, respectively, showing great significance. A total of 1076 target genes were simultaneously predicted by miRDB, Targetscan, and microRNA.org databases. According to the GO analysis results, disorders of endomembrane system may be one of the major pathological changes in DKD. In addition, Rap 1 signaling pathway is also altered obviously in DKD, discovered by the KEGG analysis.

Conclusion: MiR-3137 and miR-4270 show the potential for urinary biomarkers of DKD. The pathological changes of DKD may be related to disorders of endomembrane system and alternation of Rap1 signaling pathway.

KEYWORDS

bioinformatics analysis, biomarkers, diabetic kidney disease, miRNAs, urine

1 | INTRODUCTION

Diabetes mellitus (DM) is a growing worldwide issue. According to the latest 9th edition Diabetes Atlas of International Diabetes Federation, in 2019, 463 million people are estimated to have diabetes globally.[1] Approximately 20%-40% percent of diabetics will suffer from diabetic kidney disease (DKD), in which about one fifth will progress to stage III, even stage IV.[1,2]

DKD without timely and effective treatments gradually progress to end-stage renal disease and renal failure, requiring dialysis or kidney transplantation. Early identification and intervention are of great importance for DKD patients. Histological change is the golden standard for the diagnosis of DKD, but with quite narrow application for its invasion characteristics. Nowadays, albumin-to-creatinine ratio (ACR) is one of the most prevalent diagnostic indicators for DKD.[2] But its predictive power is limited and there are still some deficiencies in clinical application.[3] First, some patients display advanced renal pathological changes with absence of microalbuminuria. Second, microalbuminuria may remit of regress in some DKD patients, which provides a stern challenge for monitoring and diagnosis. Third, microalbuminuria is not a specific indicator for DKD. It is a predictor of progressive chronic kidney disease simultaneously.[3] It is extremely urgent to seek for a more specific, stable, and sensitive indicator for DKD.

Novel proteins or small molecules are being discovered to playing important role in DKD. Renalase, a kind of secreted flavoprotein, ameliorates renal injury and protects against the progression of DKD.[4] Interleukin 6 (IL-6) can serve as a therapeutic target for DKD. The IL-6 receptor antibody Tocilizumab decreases insulin resistance and inhibits the activation of inflammasomes.[5] The miRNA is a class of short noncoding RNA (20-22 nucleotides), acting as a kind of critical regulators of various cellular processes by posttranscriptional mechanisms.[6] MiRNAs repress the translation or accelerate the degradation through base pairing to 3′-untranslated regions of the target mRNAs. MiRNAs circulate widely in various biological fluids, such as plasma, urine, tears, and extracellular fluid.[6] Exosomes in urine contain plenty of miRNAs. It was noted that miRNAs in exosomes were stable and abundant even after shipping at 4°C for 24 hours, storing at −80°C for 12 months, or 5 repeat freeze-thaw cycles.[7] Based on the previous studies, miRNAs meet the criteria of eligible biomarkers, with qualified sensitivity, specificity, robustness, translatability, and concentration-responsiveness to disease pathology.[8] In addition, urinary miRNAs are non-invasive and easy to get, which provides a wider range of clinical applications.

In this research, we collected urine from DM and DKD patients to seek for differentially expressed miRNAs for potential biomarkers of DKD.

2 | MATERIALS AND METHODS

2.1 | Patients included and urine sample preparation

Morning urine samples were gathered in Tianjin Medical University Chu Hsien-I Memorial Hospital. The study project has been approved by the Medical Ethics Committee of Tianjin Medical University Metabolic Diseases Hospital, and it is conform to the Declaration of Helsinki. The inclusion criteria were as follows: (a) aged over 18 years old; (b) with a course of type 2 diabetes mellitus (T2DM) duration for more than 10 years. Patients were excluded according to the following criteria: (a) with type 1 diabetes mellitus; (b) with malignant tumor; (c) with other chronic kidney diseases except for DKD, such as glomerulonephritis and IgA nephropathy; (d) with systemic autoimmune disease; (e) with congestive heart failure (NYHA class III or IV) and/or left ventricular ejection fraction ≤40%; (f) with a major cardiovascular history within 3 months: myocardial infarction, coronary angioplasty or bypass surgery, unstable angina, transient ischemic attack or cerebrovascular accident; (g) with immunodeficiency, such as a history of organ transplantation, or diagnosis of Acquired Immune Deficiency Syndrome (AIDS); (h) in pregnancy or lactation.

A total of 20 patients contributed their urine samples to this study. All the included patients were informed of the benefits and risks of this trail. The informed consents were obtained before enrollment. Ten patients suffered from DKD (30 mg/g < ACR < 300 mg/g) were included in the DKD group. Another ten volunteers were T2DM patients without DKD (ACR < 30 mg/g), regarded as the DM group. The collected urine samples were immediately frozen at −80°C. Two patients in the DM group and four patients in the DKD group randomly contributed their urine samples for miRNA microarray analysis.

2.2 | RNA isolation and miRNA microarray analysis

Total RNA was isolated from urine samples for miRNA microarray analysis. The quality and quantity of RNA were identified by NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific) and Agilent Bioanalyzer 2100 (Agilent technologies). The microarray analysis was conducted using Agilent human miRNA (Version 21.0) by Biotechnology Corporation. MiRNAs in the total RNA were labeled and then hybridized with miRNA Complete Labeling and Hyb Kit (Cat#5190-0456, Agilent technologies) according to manufacturer's instructions. Results were scanned by Agilent Microarray Scanner (Cat#G2565CA, Agilent technologies) and normalized by Quantile algorithm, part of R package AgiMicroRna.[9]

2.3 | Hierarchical clustering analysis

Hierarchical clustering analysis was performed based on the microarray results. Statistics analysis was conducted by R package Limma. MiRNAs were supposed to be significantly differential expressed, when $P < .1$ and absolute value of fold change >2. The heatmap and volcano plot were operated by R package pheatmap and ggplot, respectively.

2.4 | Quantitative real-time PCR validation

RNA from 20 volunteers (10 in DM group and 10 in DKD group) was isolated for validation. An equal volume of the urine specimen was

centrifuged at 2000 ***g*** for 10 minutes at 4°C. Supernatant was transferred to another sterile tube, and a certain volume of TRIzol reagents (Invitrogen) was added. Then 500 fmol/L *Caenorhabditis elegans* miR-39 (cel-miR-39, UCACCGGGUGUAAAUCAGCUUG) was spiked into urine samples for normalization. Total RNA was isolated and purified based on the manufacturer's protocol. Reverse transcriptions were carried out with Reverse transcription kit (Thermo Fisher Scientific). Primer sequences are listed in the Table S1. Quantitative RT-PCR was performed with SGExcel Fast SYBR Mixture (Sangon Biotech) and the levels of cel-miR-39 were detected for normalization. Relative expressions of miRNAs were calculated by $2^{-\Delta\Delta Ct}$ methods. We utilized Student's *t* test to compare the differences between two groups by GraphPad Prism (Version 5, GraphPad Software). Data are shown as mean ± SEM) And it is deemed to be significantly different when $P < .05$.

2.5 | Prediction of miRNA targets

Target genes of the differentially expressed miRNAs were predicted using miRDB (http://mirdb.org/), Targetscan (http://www.targetscan.org/vert_72/) and microRNA.org (http://www.microrna.org/microrna/home.do) database. Target genes simultaneously appeared in all the three databases were picked out by Venny (Version 2.1.0, http://bioinfogp.cnb.csic.es/tools/venny/index.html) and incorporated in the following analysis.

2.6 | Bioinformatics analysis and network generation

Target genes of the differentially expressed miRNAs were subjected to Gene Ontology (GO) analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathways analysis. We applied Cytoscape (Version 3.7.0) software (https://cytoscape.org/) with the ClueGO Plugin (Version 2.5.4)[10] and CluePedia (Version 1.5.4)[11] apps. Enriched pathways in Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) were explored. Processes or pathways were considered to be meaningful when *P* value (corrected with Bonferroni step down) <.05.

3 | RESULTS

3.1 | Characteristics of patients

Characteristics of 2 DM patients and 4 DKD patients who were included for the miRNA microarray analysis are described in Table 1. A total of 2549 miRNAs were measured after normalization. The box and whisker plot was applied to display the distribution (Figure S1). Boxes which covered interquartile ranges showed that most miRNAs were expressed at extremely low levels in urine samples. Only a small part of miRNAs were expressed highly and scattered above the third quartile. In addition, we employed the scatter plot to present the distribution and correlativity between the DM group and DKD group (Figure S2).

3.2 | Differentially expressed miRNAs

A total of 26 miRNAs (9 down-regulated and 17 up-regulated) were differentially expressed, with $P < .1$ and the absolute value of $\log_2$ fold changes >1. Six miRNAs (miR-193b-5p, miR-6809-5p, miR-3137, miR-6831-5p, miR-4270, and miR-7846-3p) were significantly changed and showed the absolute value of $\log_2$ fold changes >1.58, along with $P < .05$ (Table 2). The results of hierarchical clustering analysis were exhibited by heat map after scaling (Figure 1A). Then we applied volcano plot to seek for the most significantly changed miRNAs. As shown in the figure, miR-3137, miR-4270, and miR-7846-3p were the top three significantly changed miRNAs, which were chosen for the following analysis (Figure 1B).

TABLE 1 Characteristics of patients included for miRNA microarray analysis

	DM (n = 2)	DKD (n = 4)	*P* value
Male, n (%)	1 (50%)	5 (50%)	—
Age (y), median (range)	60.5 (56, 65)	52.5 (46, 58)	.1792
HbA1c (%)	12.3 (1.3)	13.1 (3.9)	.7912
BMI (kg/m^2)	24.0 (3.5)	25.3 (5.5)	.7727
ACR (mg/g)	21.8 (8.7)	153.4 (56.3)	.0359
24 h-UMA (mg/24 h)	13.4 (8.8)	265.7 (66.8)	.0074
BUN (mmol/L)	7.5 (1.4)	6.4 (2.6)	.6075
SCr (μmol/L)	57.3 (16.2)	67.9 (23.0)	.5998
SBP (mm Hg)	147.5 (17.7)	142.5 (17.1)	.7544
DBP (mm Hg)	85.0 (21.2)	87.5 (12.6)	.8587
ALT (U/L)	19.4 (1.5)	13.6 (5.5)	.2418
AST (U/L)	21.4 (11.8)	16.6 (6.2)	.5266
UA (umol/L)	395.7 (30.1)	394.5 (100.5)	.9880
TG (mmol/L)	1.6 (0.5)	0.5 (0.5)	.8788
TC (mmol/L)	3.8 (2.6)	5.0 (1.4)	.4738

Note: Data are shown as mean (SD) unless otherwise indicated.

Abbreviations: ACR, albumin-to-creatinine ratio; ALT, alanine aminotransferase; BMI, body mass index; AST, aspartate aminotransferase; BUN, blood urea nitrogen; DBP, diastolic blood pressure; HbA1c, glycosylated hemoglobin A1c; SBP, systolic blood pressure; SCr, serum creatinine; TC, total cholesterol; TG, triglyceride; UA, serum uric acid; UMA, urinary microglobulin.

3.3 | Validation with RT-qPCR and target genes research

We applied RT-qPCR to validate the changes of the three miRNAs. Characteristics of 10 DM patients and 10 DKD patients, who were included for the miRNA microarray analysis, were described in Table 3.

After normalizing by cel-miR-39, the levels of miR-3137 and miR-4270 in the DKD group were 0.670 ± 0.505 ($P < .05$) and 2.116 ± 1.762 ($P < .01$) times than those in the DM group. The expressions of miR-3137 were down-regulated in DKD group, while the levels of miR-4270 were elevated in DKD group. But there was no significant difference in expression of miR-7846-3p in different groups (Figure 2A). We searched the target genes of the identified two miRNAs (miR-3137 and miR-4270) in three prediction databases mentioned above. There were 3251, 357, and 6170 target genes of miR-4270 predicted by TargetScan, miRDB and microRNA.org respectively, as well as 5729, 989, and 9319 target genes of miR-3137. A total of 1076 target genes were simultaneously predicted by all the three databases (Figure 2B).

TABLE 2 Significantly changed miRNAs with meaningful *P* value and fold change

	log_2FC	FC	Regulation	*P* value
miR-193b-5p	−1.601	0.330	Down	.000311
miR-6809-5p	−2.121	0.230	Down	.00083
miR-3137	−2.751	0.149	Down	.008623
miR-6831-5p	1.984	3.957	Up	.010763
miR-4270	2.017	4.047	Up	.020534
miR-7846-3p	2.360	5.133	Up	.044289

Abbreviation: FC, fold change.

3.4 | Bioinformatics analysis

Assuming that these 1076 screened genes were the target genes of miR-3137 and miR-4270, we carried out the GO and KEGG analysis to seek for the potential processes or pathways (Figure 3). There are several processes enriched in Biological Process (BP), such as nervous system development, system development, trans-synaptic signaling, regulation of localization, skeletal system development, and positive regulation of nucleic acid-templated transcription (Figure 3A, Table S2). When it comes to Cellular Component (CC), neuron part, endomembrane system, plasma membrane part, and bounding membrane of organelle are processes changed remarkably (Figure 3B, Table S3). In addition, enriched progresses in Molecular Function (MF) are transcription regulatory region DNA binding, protein domain specific binding, protein kinase binding, protein tyrosine kinase activity, and phosphotransferase activity, alcohol group as acceptor (Figure 3C, Table S4).

What's more, we found that five pathways significantly altered in two groups, thyroid hormone signaling pathway, Rap1 (Ras-associated protein 1) signaling pathway, melanogenesis, Cushing syndrome, and prostate cancer (Figure 4). The *P* value of the five pathways is all smaller than 10^{-3} (Table S5).

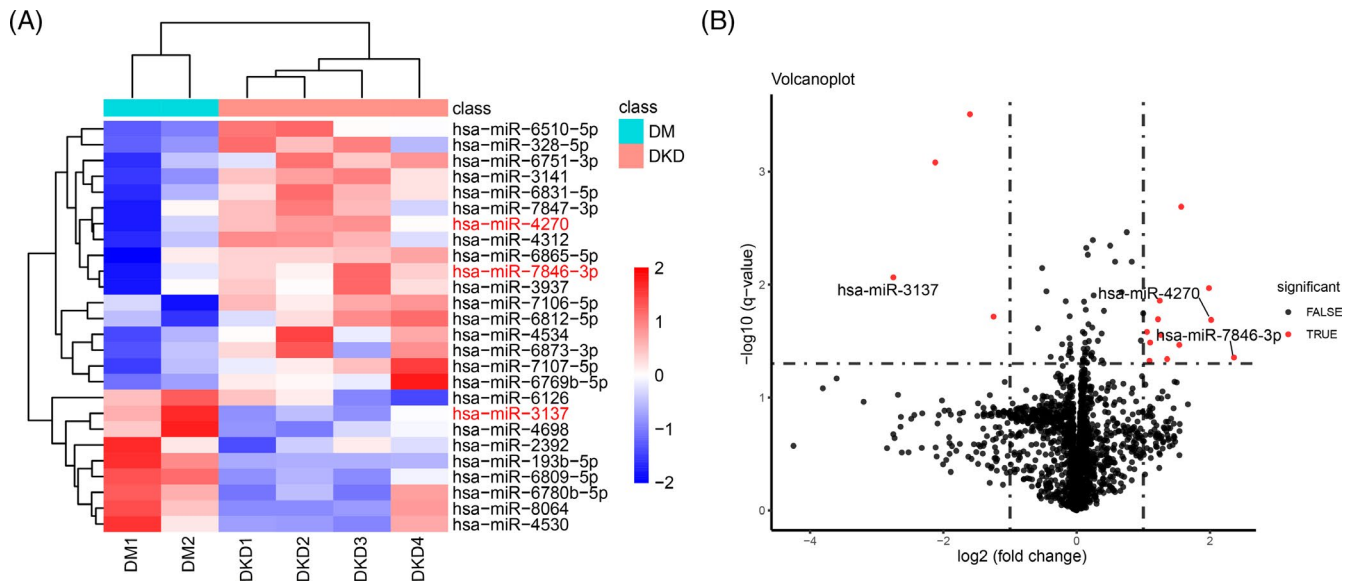

FIGURE 1 Identification of miRNAs from microarray expression profiling analysis. A, Heatmap of 26 differently expressed miRNAs between DM group and DKD group. Hierarchical clustering analyses of samples and miRNas were shown on the top and on the left, respectively. Red blocks represent relatively high expressions whereas blue blocks represent relatively low levels of miRNAs. B, Volcano plot demonstrating the fold change degree and statistical significance of all the detected miRNAs. The vertical dotted lines stand for fold change values of ±2.0, while the horizontal dotted line stands for *P* value of .05. Points in red indicate significantly different change (absolute value of fold change >2 and $P > .05$) between DM and DKD group. DM, diabetes mellitus; DKD, diabetic kidney diseases

4 | DISCUSSION

It is not the first time that urinary miRNAs draw great attention as biomarkers. In 2018, Assmann TS reviewed the changes of miRNAs in DKD patients, in which eleven researches aimed at urinary miRNAs.[12] Several miRNAs differentially expressed in urine of DKD patients, but not consistently regulated in different researches. Levels of five miRNAs (miR-21-5p, miR-29a-3p, miR-126-3p, miR-214-3p, and miR-342-3p) were elevated, and expressions of one miRNA (miR-192-5p) were reduced in DKD patients, compared to DM patients or healthy volunteers.[12]

In this study, we explored changes of miRNAs in urine of DKD patients by microarray analysis and found that two miRNAs (miR-3137 and miR-4270) were significantly changed in DKD group. Previous research also detected elevated expressions of miR-4270 in urine of DKD patients, compared to that of healthy controls and T2DM patients, supporting the results in ours.[13] Another study aimed at platelet miRNA profiles and demonstrated that miR-3137 was differently expressed with high reselection probabilities in diabetic and non-diabetic patients.[14] MiR-3137 and miR-4270 may serve as potential biomarkers in the monitoring and diagnosis of DKD.

The levels of miR-3137 and miR-4270 may differ greatly in other diseases. MiR-3137 may indicate the course of small intestine neuroendocrine tumors.[15] And it is reported that miR-4270 can serve as a potential biomarker for Helicobacter pylori infection,[16] immune thrombocytopenic purpura (ITP),[17] sepsis-induced acute kidney injury,[18] breast cancer,[19] hepatocellular carcinoma,[20] brain metastasis in lung adenocarcinoma,[21] and peritoneal metastasis of gastric cancer.[22] It should be noted that neither miRNA is specific enough for DKD. The variation may be owing to the alternation of metabolic levels. In accordance with our results, miR-3137 and miR-4270 do show great potential as biomarkers in urine of DKD patients. But more comprehensive basic researches and cohort studies with larger sample should be conducted before clinical application.

TABLE 3 Characteristics of patients for validation by RT-qPCR

	DM (n = 10)	DKD (n = 10)
Male, n (%)	5 (50%)	5 (50%)
Age (y), median (range)	58.5 (36, 65)	55.7 (28, 74)
HbA1c (%)	11.7 (1.8)	11.1 (3.4)
BMI (kg/m^2)	25.6 (2.7)	25.5 (4.9)
ACR (mg/g)	16.0 (9.0)	99.7 (58.1)*
24 h-UMA (mg/24 h)	8.1 (6.4)	276.1 (50.3)*
BUN (mmol/L)	6.5 (2.3)	7.1 (2.5)
SCr (μmol/L)	63.2 (27.3)	70.5 (21.0)
SBP (mm Hg)	134.0 (20.3)	140.0 (14.9)
DBP (mm Hg)	78.5 (10.6)	86.0 (9.7)
ALT (U/L)	17.5 (6.5)	15.1 (5.5)
AST (U/L)	26.1 (14.3)	18.3 (7.8)
UA (μmol/L)	311.0 (105.0)	338.8 (105.9)
TG (mmol/L)	1.7 (0.8)	1.6 (0.8)
TC (mmol/L)	4.6 (1.9)	4. 9 (1.6)

Note: Data are shown as mean (SD) unless otherwise indicated.

Abbreviations: ACR, albumin-to-creatinine ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; DBP, diastolic blood pressure; HbA1c, glycosylated hemoglobin A1c; SBP, systolic blood pressure; SCr, serum creatinine; TC, total cholesterol; TG, triglyceride; UA, serum uric acid; UMA, urinary microglobulin.

*$P < .05$, compared to DM group.

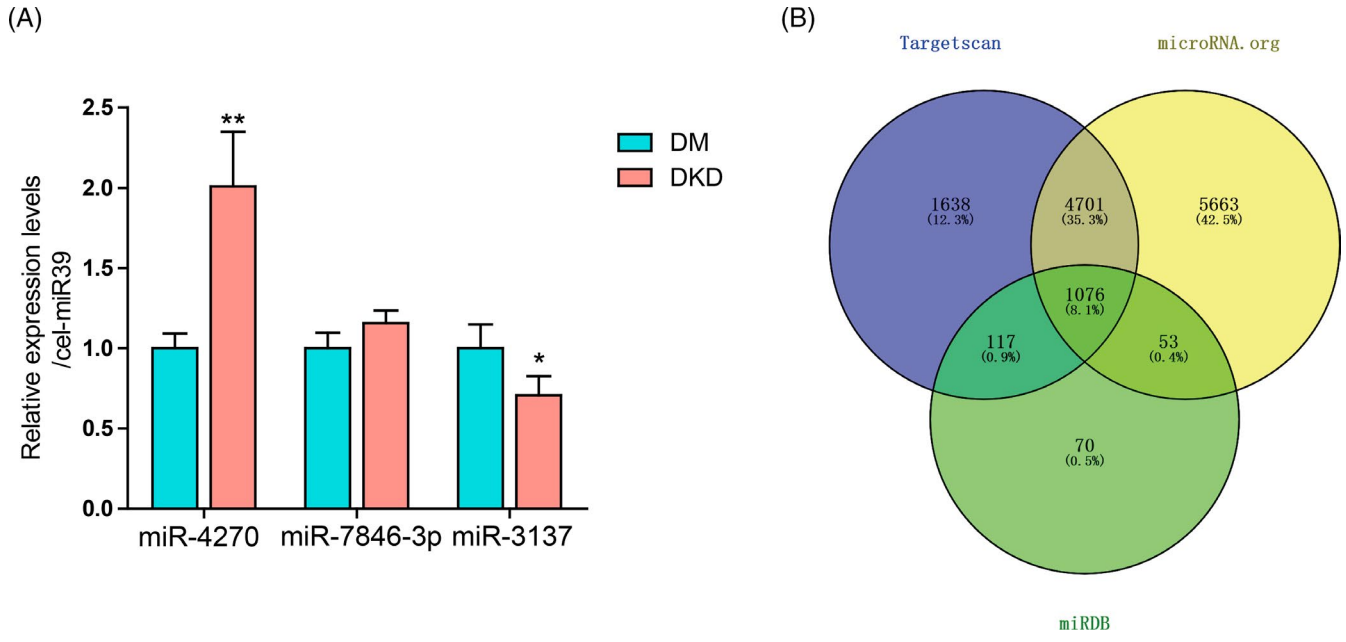

FIGURE 2 A, Validation of differently changed miRNAs by RT-Qpcr. The expressions of cel-miR-39 were detected for normalization. B, Venn diagram of target genes predicted by three databases. DM, diabetes mellitus; DKD, diabetic kidney diseases. *$P < .0.01$; **$P < .001$

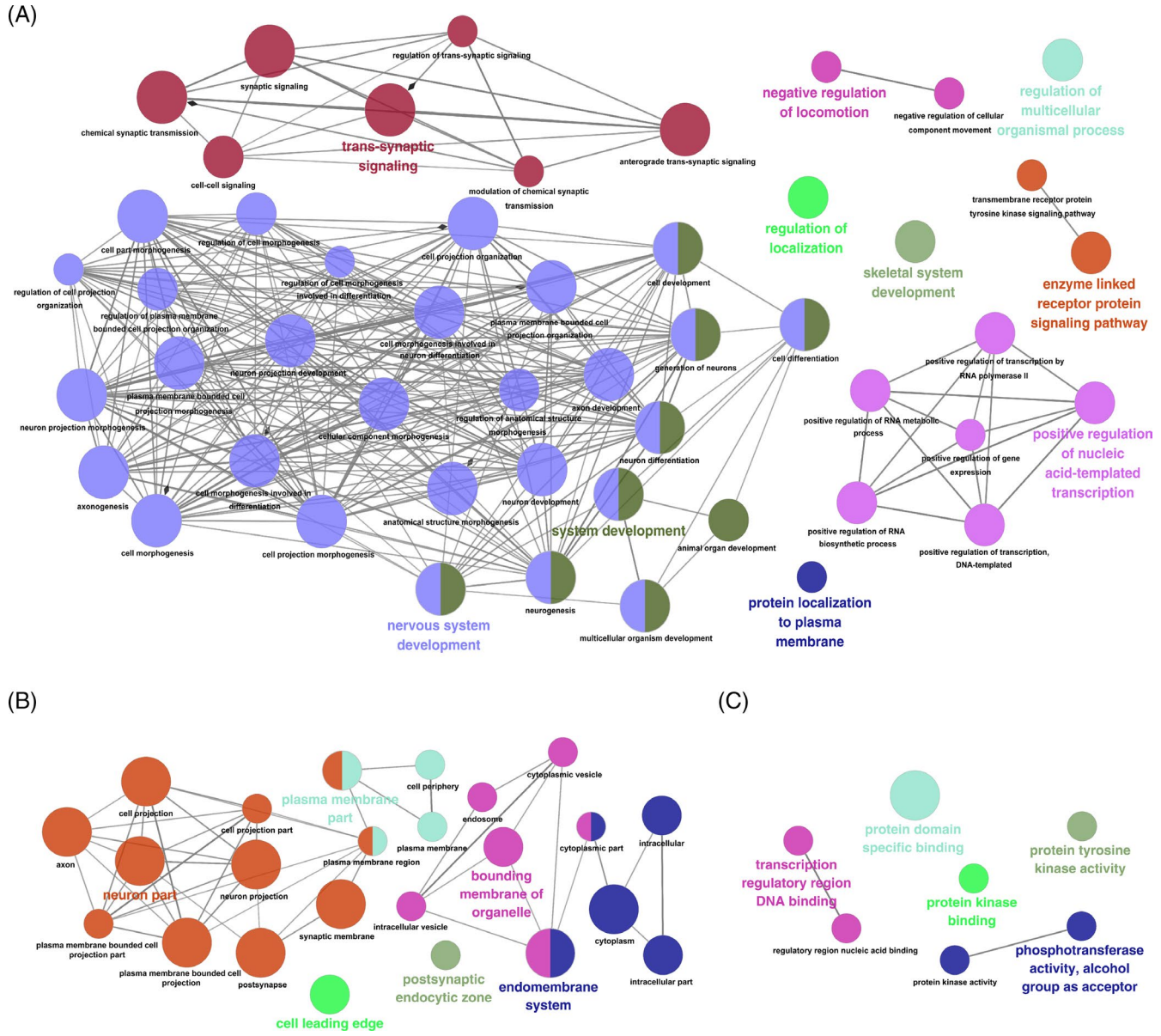

FIGURE 3 Gene ontology (GO) processes classification of target genes. A, biological process (BP). B, cellular component (CC). C, molecular function (MF)

MiRNAs not only act as biomarkers for monitoring or diagnosing DKD, but also work as therapeutic targets.[23] Several basic researches have proved that certain miRNAs are correlated with autophagy, fibrosis, inflammation, epithelial-to-mesenchymal transition, and so on.[24-27] MiRNAs are closely involved in the occurrence and development of DKD. And it is of great significance to probe the role of miRNAs in DKD.

As important regulators of metabolism, miRNAs can affect several functions or processes. Variation of miRNAs can disturb multisystem developments, like nervous system, skeletal system, and so on.[28,29] Based on our results, disorders of endomembrane and bounding membrane systems (endosomes, intracellular vesicles, cytoplasmic vesicles, and cytoplasms) are also closely associated with the pathologic changes in DKD.

As shown above, Rap1 signaling pathway is strongly associated with the progression of DKD. As a small GTPase, Rap 1 competes with Ras for Raf 1 and antagonizes the activity of Ras.[30] Rap1 regulates numerous biological processes, such as cell adhesion, integrin function, vesicle trafficking, nuclear transport, cytoskeletal rearrangement, and cell cycle progression.[30] In 2014, Xiao et al[31] discovered that Rap1b ameliorated renal tubulointerstitial fibrosis by modulation mitochondrial fission and fusion proteins. In addition, researches on Rap1 demonstrated that the regulation of Rap1 might be correlated with inflammation, oxidative stress, and deregulated metabolism. But detailed mechanisms are still uncertain and need further researches.[32]

The latest epidemiologic evidence demonstrated that DM reduces the risk of prostate cancer by lowering circulating testosterone

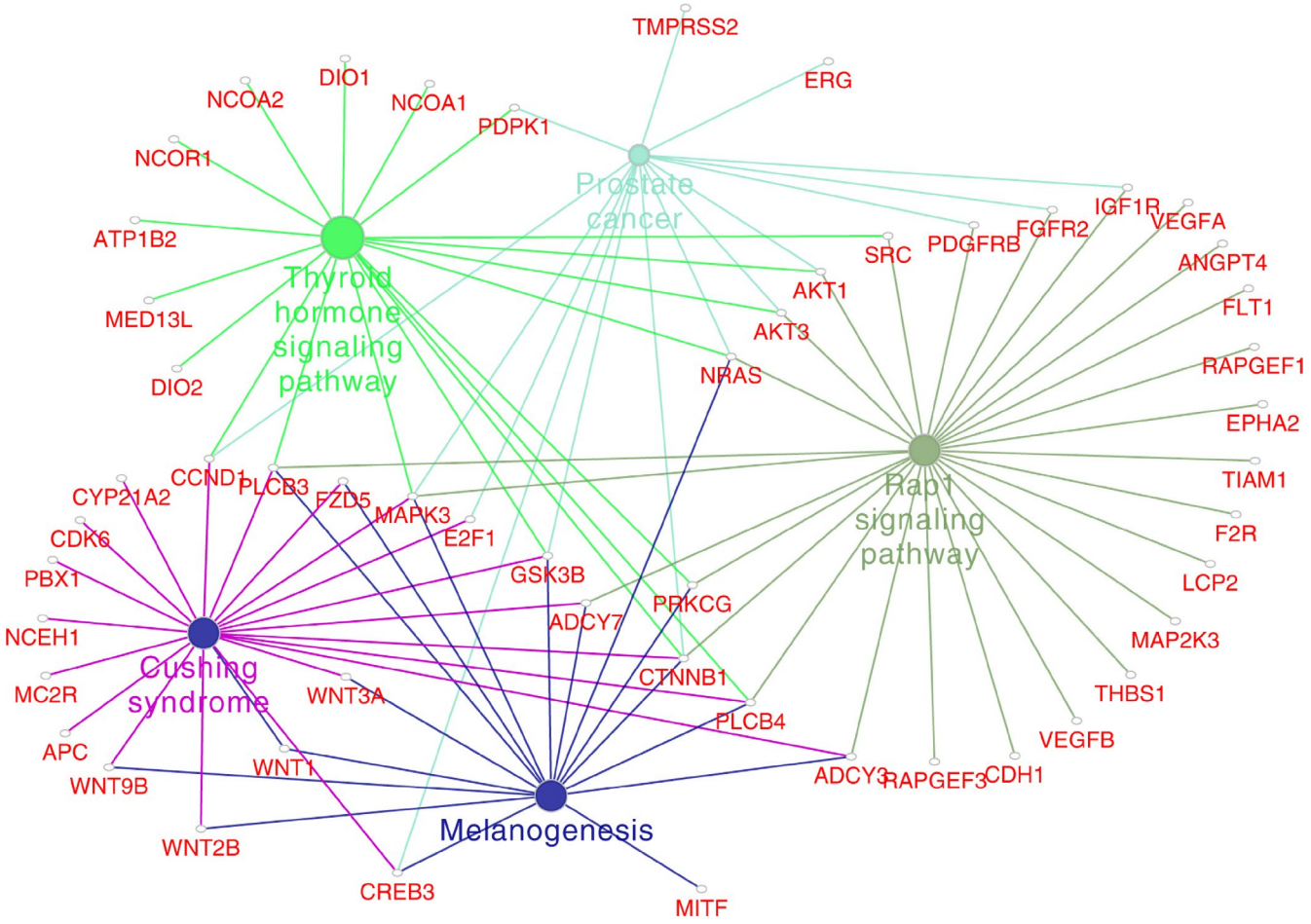

FIGURE 4 Kyoto encyclopedia of genes and genomes (KEGG) pathways enriched of target genes

concentrations,[33] but correlates with elevated all-cause mortality of prostate cancer.[34] What's more, the levels of thyroid hormone may contribute to the origination of DM. A longitudinal study for 7 years considered that thyroid was an additional risk factor of T2DM, but there were still a few issues to solve.[35] On account of our study, there are several significantly changed pathways associated with DKD, like Cushing syndrome and melanogenesis. The mechanisms underlying may be related to changes of miRNAs but require further researches.

Nevertheless, there are still some limitations of this study which cannot be ignored. First, the differential expressed miRNAs were detected from urinary samples of 6 diabetic patients. The final conclusion should be validated in a larger sample of different populations. Second, whether the expression levels of these two miRNAs linear-correlated with the severity of DKD is lack of further experiment. Third, as mentioned above, the two differential miRNAs may be not specific enough for diagnosis of DKD. More comprehensive basic researches and cohort studies with a larger sample should be conducted before clinical application. Seeking for alternative biomarkers of DKD received great interest in recent years. However, few newfound biomarkers are indeed applied to routine use for DKD in clinical practice to date.[36] Regardless, the focus on urinary biomarkers for DKD still reveals great potential because of the characteristics of its non-invasive manner. Current researches driven by biomarkers exploration have enriched the body of knowledge of DKD to some extent.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

LC, BS, and CL conceived and designed the experiments. XYLi, RX, XYLiu, and LX performed the experiments. MX and YC interpreted data. XYLi, TL, XY, and YW wrote and revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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