Expression profile of urinary exosomal miRNAs in patients with diabetic kidney disease and their associated with kidney damage

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Research Article

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Abstract

Purpose

Diabetic kidney disease (DKD) is the primary cause of end-stage renal disease. The aim of study is to seek noninvasive biomarkers for DKD at early stage or a target for the treatment of DKD through analysis of the urinary exosomal miRNAs expression profiles in DKD patients.

Methods

The urinary exosomes were isolated from type 2 diabetes (T2DM) patients with DKD confirmed by renal biopsy (DKD-Exo). Treatment of human podocytes and renal tubular epithelial cells (TECs) with DKD-Exo to observe the effects of DKD-Exo on podocyte apoptosis and epithelial-mesenchymal transition (EMT) of TECs. The urinary exosomal miRNAs expression profiles were detected using miRNA sequencing, and differentially expressed miRNAs were verified by real-time quantitative PCR. Target genes of these miRNAs and relevant pathways in DKD were analyzed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment.

Results

DKD-Exo induced the apoptosis of podocytes and EMT of TECs. A total of 40 differentially downregulated miRNAs were found, 17 of all were named and 23 were newly discovered, some differentially expressed miRNAs in DKD patients were reported for the first time. GO and KEGG pathway analysis suggest that these target genes were related to biological processes, molecular function and cellular component, and involved in 135 pathways.

Conclusion

Our study implies that the urinary DKD-Exo could deliver biological information to podocytes or TECs, which play an important role in pathogenesis of DKD.

1 Introduction

Diabetic kidney disease (DKD) is a common microvascular complication in patients with diabetes, and is the primary cause of end-stage renal disease (ESRD) around world. DKD not only seriously affects the quality of life but also significantly elevates the risk of cardiovascular diseases and mortality in diabetic patients. The clinical diagnosis of DKD is mainly based on a progressive decline of estimated glomerular filtration rate (eGFR<60 ml·min⁻¹·1.73 m⁻²), or/ and persistent albuminuria of urinary albumin creatinine ratio (UACR) ≥ 30 mg/g, along with clinical parameters, including diabetes duration and the presence of
diabetic retinopathy [1,2]. But nonproteinuric DN and DN without retinopathy are more common in type 2 DM patients. For some patients without typical clinical features, renal biopsy is need to clarify a diagnosis. Up to now, renal biopsy pathology is golden standard of DKD diagnosis, but cannot be performed routinely in clinic. Such, it is necessary to find a noninvasive biomarker for early diagnosis of DKD. DKD is a complicated disease process, inflammation, oxidative stress, mitochondrial dysfunction and impaired autophagy are involved in pathophysiologic mechanisms, eventually leading to kidney damage [1]. The pathological changes of DKD contain basement membrane thickening, mesangial cell hypertrophy, podocyte loss and apoptosis, tubulointerstitial fibrosis [2]. Particularly, podocytes apoptosis and renal tubular epithelial cell damage play an important role in the pathogenesis of DKD [3–5]. Due to requirement of high-energy and aerobic metabolism, renal tubules are more susceptible to the metabolic disturbances, leading to inflammation and fibrosis associated with DKD [6].

Although some novel biomarkers that capture the specific mechanism of DKD have been developed [7, 8], the prognostic significance of these biomarkers is not specific to DKD. Recently, the application of high-throughput sequencing approaches to analyze biological samples has emerged as a strong tool in biomarker discovery. Exosomes are a kind of extracellular vesicles produced by various cells and are present in all body fluids, including serum, urine, saliva, etc. Exosomes can carry different molecular constituents of the donor cells, such as proteins, lipids, mRNAs and microRNAs (miRNAs) into the recipient cells [9]. Because of double-layer membrane, exosomes can maintain signaling-molecule stability for long-distance transmission [10, 11]. Circulating exosomes are not filtered through the glomeruli, so urinary exosomes are thought to be secreted by natural cells of the urinary system and the biological information of urinary exosomes can reflect pathophysiologic changes in nephron under disease conditions. MiRNAs are small non-coding RNAs that directly interact with the 3'-untranslated region (3'-UTR) of their target messenger RNAs (mRNAs) to regulate gene expression by translational inhibition or degradation of target mRNAs. Emerging evidence shows that miRNAs play a key role in the pathogenesis of DKD [12, 13]. González-Palomo et al. observed the profile of urinary exosomal miRNAs in patients with T2DM and DKD, and found that the patients with DKD presented a significant increase in miR-126. Meanwhile, miR-146, miR-155, and miR-126 together, with some clinical parameters, can predict the development of DKD [14]. Zang et al. affirmed differential expression of miR-21-5p and miR-30b-5p in individuals with DKD, and these miRNAs maybe become potential biomarkers associated with the pathogenesis of DKD [15].

In this study, we extracted urinary exosomes from T2DM patients with DKD which were confirmed by renal biopsy, and treated podocytes and renal tubular epithelial cells (TECs) with these urinary exosomes to observe the effects of urinary exosomes on podocyte apoptosis and epithelial-mesenchymal transition of TECs. Meanwhile, expression of urinary exosomal miRNAs were measured by miRNA sequencing, target genes of these miRNAs and relevant pathways were analyzed by bioinformatic data, with the objective of providing novel noninvasive biomarkers for DKD.

2 Materials and methods
2.1 The data of participants

40 diabetic patients hospitalized in the Second Hospital of Hebei Medical University from February 2022 to October 2022 were selected, including 20 patients without DKD (DM group) and 20 patients with DKD confirmed by renal biopsy (DKD group). 15 healthy subjects from medical examination department of the same hospital during the same period were selected as the normal control (NC group). All specimens were obtained with the consent of the patients and the protocols were approved by the Clinical Research Ethics Committee of the Second Hospital of Hebei Medical University, Ethical review number: 2022-R059. The clinical data were collected, including age, gender, body mass index (BMI). Fasting for 8 h, venous bloods of all participants were collected for biochemical indicators, including glycosylated hemoglobin (HbA1c), serum creatinine (SCr), total cholesterol (TC), triglyceride (TG). The morning urine of each participant were collected for the measure of ACR. 100 mL of first-morning urine were collected in sterile centrifuge tubes and centrifuged at 3,000 g for 30 min at 4°C to separate cells from debris, and 0.22µm filter for filtering to remove bacteria, residual cells and debris. The pellet was stored at −80°C for subsequent applications. All the samples were processed within 1 h after collection. In order to gain an expression profile of urinary exosomal miRNAs, 4 of DM group and 5 of DKD group respectively were randomly selected for miRNA sequencing.

2.2 Exosome extraction and identification

The urine samples were centrifuged at 15,000g for 20 min at 4°C. The 17,000 g supernatant was ultracentrifuged at 200,000g for 1 h at 25°C. The ultracentrifugation steps were repeated 3 times, adding new 17,000g supernatant volume each time to each of the 12 tubes. The suspensions were pooled together. The abundant urinary protein uromodulin or Tamm-Horsfall protein forms very high molecular weight complexes through disulfide linkages. These complexes sediment in the 200,000g spin unless denatured. The resuspended pellet was added to an ultracentrifuge tube, and isolation solution was added to increase the volume to 8 ml. The sample was centrifuged at 200,000g for 1 h at 25°C. The pellet was suspended in 50ul of isolation solution and frozen at -80°C. The exosome samples were diluted 5 times with PBS and then applied to 200-mesh nickel grids. Samples were stained with 2% phosphotungstic acid for 5 min at room temperature, and air-dried. The exosomes were identified by transmission electronic microscope analysis at Shanghai Umibio biotechnology Co Ltd. The exosomal surface markers CD63 and CD9 were detected via western blot. The exosomes from the NC, DM and DKD groups were respectively defined as the NC-Exo, DM-Exo and DKD-Exo.

2.3 Cell culture

Both human tubular epithelial cells (HK-2) and podocytes are purchased from ATCC (American Type Culture Collection). Podocytes were cultured in 5.5 mM D-glucose RPMI-1640 (Gibco, USA) medium containing with 10% fetal bovine serum (Gibco, USA) and 0.5% penicillin/streptomycin 100X (Solarbio, China), then were incubated in the humidified incubator at 37°C with 5% CO₂. HK-2 cells were cultured in average DMEM and DMEM-F12 medium (DMEM; DMEM-F12, Gibco, USA), containing 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 10% L-glutamine, 0.5% penicillin/ streptomycin, with 5% CO₂
at 37°C. When cell fusion reached about 60%, the cells were respectively treated with NC-Exo, DM-Exo and DKD-Exo, namely, the NC-Exo, DM-Exo and DKD-Exo groups.

2.4 Exosome uptake assay

Exosomes were labeled with diluent C solution (50µl, Life Technologies) and PKH67 (4µl, Sigma-Aldrich Corp., USA) for 4 min at room temperature, then were added to exosomes suspension (DKD-Exo: 50ul) respectively, incubated for 5 min and resuspended in 1% BSA. HK-2 cells and podocytes were respectively treated with PHK67-labeled exosomes for 3 h, 7 h and 24 h, the uptake of exosomes was measured by using an immunofluorescence microscope (Nikon Instruments, Tokyo, Japan), and the pictures were taken using a Nikon inverted fluorescence microscope (Eclipse TE200 microscope).

2.5 Cell viability and apoptosis of podocytes

Cell viability was analyzed by cell counting kit (CCK-8, Dojindo, Japan) according to manufacturer's instructions. HPCs in the logarithmic growth were collected and inoculated into a 96 -well plate and incubated. After culturing for 24 h, experimental samples (NC-Exo; DM-Exo; DKD-Exo) were allocated to each group, with each group comprising six replicate wells. Urinary exosome concentrations were established at 20ug/ul, 40ug/ul, 60ug/ul, and 80ug/ul, respectively. Following a 48-hour incubation period, 10 µL of CCK-8 reagent was introduced to each well of the 96-well plate under sterile conditions, followed by a further 4-hour cell incubation. Optical density (OD) values for each group were determined at a wavelength of 450 nm using an automated microplate reader (Molecular Devices Corporation). Measurements were conducted at least thrice to obtain an average OD value for analytical calculations.

Apoptosis was analyzed using PE Annexin V/7-ADD detection kits. The study categorized samples into three groups: NC-Exo; DM-Exo;DKD-Exo. Following the experimental protocol, HPCs in each group underwent stimulation for 48 hours, were thrice washed with PBS, incubated with trypsin devoid of EDTA, and examined microscopically for round cell morphology prior to terminating digestion with fetal bovine serum-containing cell culture medium. HPC cells were harvested into EP tubes at a density of 1×10^5 cells per tube and subjected to two rounds of centrifugation with precooled PBS. After the addition of 1×binding buffer to each tube, 5ul of PE Annexin V and 7-ADD were gently pipetted into the tubes for mixing. The samples were incubated in the dark at room temperature for 15 minutes, followed by the addition of 400ul 1×binding buffer, thorough mixing, and transfer to flow cytometry tubes equipped with a filter. Apoptosis rates for each group were determined within one hour via flow cytometry (BD, Biosciences, USA), with subsequent data analysis conducted using FlowJo software.

2.6 Western blotting analysis

Total proteins were separated from podocytes and HK-2 cells or exosomes via using radio immunoprecipitation assay (RIPA) lysis buffer (Solarbio, China), the concentrations were estimated by BCA protein analysis kit. 30 ug denatured proteins from each sample were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) (Epizyme, China) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked by 5% skim milk (BioFroxx, Germany)
for 2 h at 24℃, then incubated with primary antibodies: CD63 (1:1000, Abcam), CD9 (1:1000, Abcam),
Bax (1:1000, Protein Tech), Bcl-2 (1:1000, Protein Tech), cleaved caspase-3 (1:500, Cell Signaling), α-
SMA(1:1000, Protein Tech), Collagen I (1:500, Immunoway), Fibronectin (1:2000, Immunoway), β-actin
(1:1000, Protein Tech), GAPDH (1:5000, Abcam) at 4℃ overnight. Next day, the membranes were
incubated with the horseradish peroxidase (HRP)-labeled goat anti-rabbit or anti-mouse secondary
antibody (1:5000, Affinity) for 1 h at 24℃. The protein bands were detected by using the chemiluminescence (ECL) reagents (General Electric, USA), and quantified by ImageJ software (Bio-Rad,
USA).

2.7 MiRNA sequencing

The experimental procedures were conducted by Kangchen Biotech Co., Ltd. (Shanghai, China) as
follows: Total RNA was extracted from tissues using TRizol® Reagent, according to Invitrogen's
instructions. Degradation and contamination were assessed on 1% agarose gels, and RNA concentration
was measured using the ND-2000 spectrophotometer (NanoDrop Technologies). RNA integrity was
subsequently assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A total of
3 µg of RNA per sample was used for the small RNA library preparation. Sequencing libraries were
generated using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA), following
the manufacturer's recommendations, and index codes were added to each sample. PCR amplification
was carried out using LongAmp Taq 2X Master Mix, SR Primer for Illumina, and index (X) primer. Library
quality was evaluated using the Agilent Bioanalyzer 2100 system with DNA High Sensitivity Chips.
Following cluster generation, the libraries were sequenced on an Illumina platform, generating 50bp
single-end reads. The hairpin structure characteristics of miRNA precursors can aid in predicting novel
miRNAs. The expression levels of miRNAs were quantified using the transcripts per million reads (TPM)
method. Significantly differentially expressed (DE) miRNAs were identified using DEseq2, with criteria of
|log2FC| >1 and FDR < 0.05.

2.8 Real-time quantitative polymerase chain reaction (RT-
qPCR)

Total RNA was extracted using RNA-easyTM Isolation Reagent (Vazyme, Nanjing, China). cDNA was
synthesized using Bulge-LoopTM miRNA RT-qPCR Starter kit (Ribobio, guangzhou, China). RT-qPCR was
performed on a CFX96 PCR system (Bio-Rad, USA) using GoTaq® qPCR Master Mix (Promega, USA). The
relative expression of miRNAs were normalized by internal references U6, and then quantified using by
2−ΔΔCt method. The primers were as follows:

miR-371a-3p: Forward: 5’-ACUCAAACUGUGGGGCACU-3’, Reverse: 5’-AAGUGCCGCAUCUUUGAGUGU-3’;
miR-483-5p: Forward: 5’-GCGAAGACGGGAGGAAAGA-3’, Reverse: 5’-AGTGCAGGGTCCGAGGTATT-3’;
miR-124-3p: Forward: 5’-CGUGUUCACACGGGACCUUGAU-3’, Reverse: 5’-UAAGGCACGCUGAUGCCAA-
3’;
miR-371a-5p: Forward: 5’-ACUCAAACUGUGGGGGCACU-3’, Reverse: 5’-AAGUGCCGCAUCUUUUGAGUGU-3’;

U6: Forward: 5’-CTCGCTTCGGCAGCACAU-3’ Reverse: 5’-AACGCTTCACGAATTTGCGT-3’.

## 2.9 Target gene analyses of differential miRNAs

Target gene predictions for miRNAs were conducted using the miRanda algorithm (http://www.miranda.org/). Predicted target genes were aligned using BLAST (http://blast.ncbi.nlm.nih.gov/) and annotated using the GO (http://www.geneontology.org/) and KEGG (http://www.genome.jp/kegg/) databases. Functional-enrichment analyses, including those involving GO and KEGG, were conducted to identify targets significantly enriched in GO terms and metabolic pathways, with a Bonferroni-corrected P-value ≤ 0.05, compared to the whole-reference gene background. GO functional enrichment and KEGG pathway analyses were conducted using Goatools (https://github.com/tanghaibao/Goatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do).

## 3.0 Statistical analysis

Statistical analysis was performed using Prism (GraphPad 8.0 Software). All data were tested for normality and presented as mean ± standard error of the mean (SEM). The data between the two groups were analyzed by independent-samples t-test. The data in multiple groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey’s test. A level of p<0.05 was identified statistically significant.

## 3 Results

### 3.1 Clinical data of participants

As shown in Table 1. HbA1c levels of the DM and DKD groups were observably higher than that of the NC group, and HbA1c levels of the DKD group were significantly higher than that of the DM group. Compared with the NC and DM groups, eGFR, SCr, TC, TG and urine ACR were significantly increased in the DKD group. There were no differences in age, gender, BMI and UA among three groups.
Table 1
Characteristics of participants

<table>
<thead>
<tr>
<th></th>
<th>NC (15)</th>
<th>DM (20)</th>
<th>DKD (20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.33 ± 5.26</td>
<td>54.85 ± .91</td>
<td>57.05 ± 5.19</td>
</tr>
<tr>
<td>Sex (female/male)</td>
<td>7/8</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.38 ± 17</td>
<td>24.68 ± .17</td>
<td>24.86 ± 1.97</td>
</tr>
<tr>
<td>ACR (mg/g)</td>
<td>7.45 ± 4.21</td>
<td>10.15 ± 6.13</td>
<td>5443.8 ± 1940.28**</td>
</tr>
<tr>
<td>SCr (µmol/L)</td>
<td>66.72 ± 91</td>
<td>61.75 ± 12.46</td>
<td>120.1 ± 43.42**</td>
</tr>
<tr>
<td>eGFR (mL/min)</td>
<td>100.78 ± 7.40</td>
<td>103.85 ± 13.65</td>
<td>59.15 ± 19.69**</td>
</tr>
<tr>
<td>UA (µmol/L)</td>
<td>335.06 ± 0.22</td>
<td>345 ± 54.28</td>
<td>376.75 ± 73.09</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>4.53 ± 0.43</td>
<td>4.54 ± 0.56</td>
<td>5.15 ± 0.59**</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.155 ± 0.30</td>
<td>1.12 ± 0.42</td>
<td>1.36 ± 0.28**</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.5 ± 0.15</td>
<td>8.4 ± 2.35#</td>
<td>9.53 ± 1.49**</td>
</tr>
</tbody>
</table>

The data are expressed as the mean ± SEM, #P<0.05 vs. NC group; **P<0.01 vs. DM and NC groups.

Notes: BMI, Body Mass Index; ACR, Urinary albuminuric creatinine ratio; eGFR: glomerular filtration rate; SCr, serum creatinine; UA, serum uric acid; TC, total cholesterol; TG, triglycerides; HbA1c, glycosylated hemoglobin.

The structures of normal renal tissues from renal tumor patients without DKD and the renal tissues of DKD patients were shown in Fig. 1A. Compared with normal glomeruli (NC), DKD patients had severe abnormalities in renal pathology, which manifested as: glomerular basement membrane thickening, mesangial cell and stromal hyperplasia, and inflammatory cell infiltration. The nephropathological manifestations of the 5 DKD patients sented for testing ranged from grade II-IV to diabetic nephropathy, ranging from mild to severe: From proliferation of the mesangium and stroma to the formation of K-W nodules with tubular atrophy and interstitial cell infiltration.

3.2 Urinary exosome features

The diameters of vesicles in the DM and DKD groups ranged from 40 to 100 nm, the most were approximately 80 nm. These vesicles expressed CD63 and CD9 proteins, suggesting that exosomes were successfully extracted from participants urinary samples (Fig. 1B and 1C).

3.3 Exosome uptake
To observe whether podocytes and HK-2 cells could take up exosomes, exosomes were labeled with PKH67. Treatment of podocytes and HK-2 cells with exosomes for 3 h, 7 h and 24 h, it was observed that these cells began to absorb exosomes at 3 h point, and an absorption peak at 24 h point. It was shown that exosomes were located in the cytoplasm by laser confocal microscopy (Fig. 1D and 1E).

### 3.4 Effects of DKD-Exo on the viability and apoptosis of podocytes

Compared with the NC-Exo and DM-Exo groups, the cell viability of podocytes in the DKD-Exo groups were significantly decreased, but there were no differences in the cell viability of podocytes between the NC-Exo and DM-Exo groups (Fig. 2A).

Flow cytometry results showed that a significant increase in podocyte apoptosis of the DKD-Exo group when compared with the DM-Exo and NC-Exo groups (Fig. 2B and 2C). Meanwhile, the expression of apoptosis-related proteins including Bax, Bcl-2 and cleaved caspase-3 were measured. Compared with the NC-Exo and DM-Exo groups, the ratio of Bax to bcl-2 and the expression of cleaved caspase-3 were observably increased in the DKD-Exo group (Fig. 3A and 3B).

### 3.5 Effects of DKD-Exo on transdifferentiation of HK-2 cells and collagen synthesis

Compared with the NC-Exo and DM-Exo groups, the expression of COI-I, and FN were significantly increased in the DKD-Exo group, but there were no differences in the expression of Collagen I and Fibronectin between the NC-Exo and DM-Exo groups. Compared with the NC-Exo group, the expression of α-SMA were significantly increased in the DM-Exo and DKD-Exo groups, but there were no differences in α-SMA expression between the DM-Exo and DKD-Exo groups (Fig. 3C and 3D).

### 3.6 Exosome sequencing

The hierarchical clustering of differentially expressed miRNAs was shown by Heatmap and volcano plot (Fig. 4A and 4B) and a total of 40 differential miRNAs were recorded, of which 17 were known and 23 were newly discovered in this study. These known miRNAs were downregulated in the DKD group, including miR-371a-3p, miR-483-5p, miR-373-3p, miR-9-5p, miR-145-5p, miR-372-3p, miR-371a-5p, miR-1260b, miR-222-3p, miR-1224-5p, miR-1246, miR-124-3p, miR-4516, miR-150-5p, miR-6739-5p, miR-1253 and miR-1260a. The miRNAs were also shown in descending order according to fold changes (Table 2). To validate the significant changes of miRNA expression, 4 of the most differentially expressed miRNAs were assessed by real-time qPCR (Fig. 4C).
<table>
<thead>
<tr>
<th>MATURE-ID</th>
<th>DKD/DM foldchange</th>
<th>DKD/DM P value</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-371a-3p</td>
<td>0.003</td>
<td>6.25E-05</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-483-5p</td>
<td>0.003</td>
<td>0.00033401</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-373-3p</td>
<td>0.007</td>
<td>0.000760455</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-9-5p</td>
<td>0.007</td>
<td>0.001490016</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-145-5p</td>
<td>0.022</td>
<td>0.002430467</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-372-3p</td>
<td>0.023</td>
<td>0.006679351</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-371a-5p</td>
<td>0.007</td>
<td>0.009622864</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-1260b</td>
<td>0.058</td>
<td>0.014072619</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-222-3p</td>
<td>0.089</td>
<td>0.015364562</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-1224-5p</td>
<td>0.006</td>
<td>0.018645958</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-1246</td>
<td>0.152</td>
<td>0.019286976</td>
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<tr>
<td>hsa-miR-124-3p</td>
<td>0.035</td>
<td>0.027010338</td>
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<td>hsa-miR-4516</td>
<td>0.04</td>
<td>0.030078458</td>
<td>down</td>
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<tr>
<td>hsa-miR-150-5p</td>
<td>0.088</td>
<td>0.031630516</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-6739-5p</td>
<td>0.013</td>
<td>0.035177044</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-1253</td>
<td>0.012</td>
<td>0.044285116</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-1260a</td>
<td>0.122</td>
<td>0.047376458</td>
<td>down</td>
</tr>
</tbody>
</table>

3.7 GO enrichment and KEGG pathway analyses

GO analysis showed that the target genes of down-regulated miRNAs were related to biological processes, molecular function and cellular component. KEGG pathway analysis showed that these target genes were involved in 135 pathways, including Toll-like receptor, MAPK, PIK3-Akt, p53 and calcium signaling pathway as well as FOXO and apoptosis signaling pathways, etc. (Fig. 4D and 4E). The pathways associated with cell apoptosis include Bcl-2 binding component 3 and PI3K-Akt signaling pathways. The pathways associated with fibrosis include NF-κB and MAPK signaling pathways.

Discussion
Exosomes are a type of vesicles in the form of cup-shaped lipid bilayers approximately 40–160 nm in diameter. Studies on urinary exosomes as markers for early diagnosis and treatment of diabetic nephropathy have shown the value of urinary exosomes in the early diagnosis of diabetic nephropathy, but there are relatively few studies on the correlation between diabetic nephropathy and urinary exosomes with clear histopathological diagnosis by renal biopsy \cite{16-18}. Studies have shown the mechanism of glomerular cell crosstalk and the transmission of information between cells by exosomes in early diabetic nephropathy \cite{19-22}. MiRNAs hoarded within exosomes are more stable and specific than mRNAs and are not easily degraded \cite{23}. Such, exosomal miRNAs hold promise as a biomarker for some diseases \cite{24}. Exosomal miRNAs stemed from different cells possess specific expression profiles of miRNAs, and these miRNAs can be transferred to target cells, subsequently regulating the physiological and pathological processes of the human body \cite{25}. Because the circulating exosomes secreted from the different tissue cells in the body cannot cross the glomerular filtration barrier, urinary exosomes can only be generated from the kidney cells including podocytes, TECs and epithelial cells, etc \cite{26}. Some studies have found that the expression changes in miRNAs of urinary exosomes could reflect the progression of DN \cite{27,28}. The pathological manifestations of DKD contain podocyte damage, basement membrane thickening, mesangial cell hypertrophy and tubulointerstitial fibrosis. Podocyte loss and apoptosis as well as renal TEC damage are thought to play a critical role in the pathogenesis of DKD, the renal interstitial fibrosis contributes to the deterioration of DKD \cite{29,30}. In this study, we observed that urinary exosomes from T2DM patients with DKD induced human podocyte apoptosis, including increased apoptosis rates of podocytes and activated mitochondrial apoptotic pathway. Similarly, we also found that collagen synthesis in TECs and epithelial-mesenchymal transdifferentiation (EMT) were markedly increased, these suggest that urinary exosomes from T2DM patients with DKD participated in the occurrence or progress of DKD.

In order to understand the underlying mechanism about the action of urinary exosomes on DKD, we observed the expression profile of exosomal miRNAs, and found a total of 40 miRNAs expressed differently in DKD patients, all of them were observably downregulated, including named miR-371a-3p, miR-483-5p, miR-373-3p, miR-9-5p, miR-145-5p, miR-372-3p, miR-371a-5p, miR-1260b, miR-222-3p, miR-1224-5p, miR-1246, miR-124-3p miR-4516, miR-150-5p, miR-6739-5p, miR-1253 and miR-1260a. Recent studies have shown that miR-483-5p is involved in renal TEC injury and interstitial fibrosis. Liu et al confirmed that circulating miR-483–5p was significantly downregulated in DKD patients, and could distinguish DKD from T2DM patients. Such, it is possible that circulating miR-483–5p would become a noninvasive biomarker for diagnosis of DKD \cite{31,32}. Su et al. analyzed miRNA expression profiles in kidney tissues from DKD patients through miRNA microarray, and found that miR-483–5p was observably downregulated \cite{33}. Numerous studies are in favor of serum miR-130b and miR-21 and urinary miR-192, let-7c-5p, miR-29c-5p and miR-15b-5p as potential diagnostic biomarkers for DKD \cite{34,35,36,37}. It is reported that 4 differentially expressed serum miRNAs, such as miR-21-3p, miR-378-3p, miR-16-5p and miR-29a-3p are closely correlated with the pathological grade of patients with DKD \cite{38}. Recent research suggested that miR-372-3p might be considered as a potential glucose responsive miRNA participating in
high glucose (HG)-induced glomerular endothelial cell dysfunction \[39\]. In addition, some differentially expressed miRNAs were not reported in DKD patients, such as miR-371a-3p, miR-371a-5p, miR-1260b, miR-222-3p, miR-1224-5p and miR-1253, and 23 miRNAs were newly discovered in our study, which may provide new clues to the pathogenesis of DKD.

Our analysis showed that the target genes of these urinary exosomal miRNAs were related to biological processes, molecular function and cellular component, and were involved in apoptosis and fibrosis signaling pathways. Zhang et al. found that miR-124 expression was dramatically downregulated in renal tissues from STZ-induced diabetic mice and in HK-2 cells treated with HG \[40\], and miR-124 might lead to renal fibrosis through TLR4/NF-κB pathway \[41\]. Recent study was different from our results, and showed that miR-145-5p was upregulated in HG-induced HK-2 cells and dual-specificity phosphatase 6 (DUSP6) was identified as a target downstream of miR-145-5p. The regulation of miR-145-5p/DUSP6 axis decreased the inflammatory response and fibrosis of HG-stimulated HK-2 cells \[42\]. But Wei B et al indicated that miR-145-5p was downregulated in HK-2 cells in HG conditions. miR-145-5p overexpression attenuates HG-induced cell apoptosis by targeting the Notch signaling pathway in podocytes \[43\]. It is reported that downregulation of miR-150-5p in HG-treated HK-2 cells was involved in tubular damage in DKD by suppressing mitophagy, and DRP1 was a target gene of miR-150-5p \[44\].

To sum up, our study suggests that the urinary exosomes from DKD patients could carry biological information such as miRNAs and deliver them to podocytes or TECs, which play an important role in pathogenesis of DKD. Due to superior stability and specificity of exosomal miRNAs, urinary exosomal miRNAs can become noninvasive diagnosis biomarkers for DKD or therapeutic target of DKD, however, these require further researches.

**Declarations**

**Ethics approval and consent to participate:**

All specimens were obtained with the informed consent of the patients and the protocols were approved by the Clinical Research Ethics Committee of the Second Hospital of Hebei Medical University, Ethical review number: 2022-R059.

**Consent for publication:** Not applicable.

**Availability of data and materials**

The dataset supporting the conclusions of this article is included within the article and its additional files.

**Competing interests:**

The authors declare that they have no conflict of interest.

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Author Contribution

Juan Li: data analysis, manuscript writing/editing; Hong Zhou: protocol development manuscript editing; Lulu Han: manuscript editing; Ting Wang: manuscript editing, Lin Yang: image acquisition.

References


Figures
Renal pathology and characterization of urinary exosomes. (A) The structures of normal renal tissues from renal tumor patients without DKD and the renal tissues of DKD patients (PASM+MOSSON×400). (B) The morphology of exosomes was observed under a transmission electron microscope. (magnification = ×30.0 k; scale bar: 200 nm). (C) The exosomal surface markers CD63 and CD9 were detected by Western blotting. (D-E) Immunofluorescence microscope observed the uptake of exosome in HPC and HK-2.
Figure 2

Effects of DKD-Exo on the viability and apoptosis of HPC cells. (A-B-C) HPC cell viability and apoptosis were evaluated by CCK-8 assay and flow cytometry analysis. N=3, *P 0.05 vs. DM, #P 0.05 vs. NC.

Note: NC: normal control exosomes; DM: diabetes mellitus exosomes; DKD: diabetic kidney disease exosomes.
Figure 3

Effects of DKD-Exo on the apoptosis of HPC cells and fibrosis of HK-2 cells. (A-B) The protein levels of Bax, Bcl-2 and Cleaved Caspase-3 in HPC cells were examined by Western blot. Full-length blots/gels are presented in Supplementary Figure 1. (C-D) The expression of α-SMA, Collagen I, Fibronectin in HK-2 cells was detected by Western blot. Full-length blots/gels are presented in Supplementary Figure 2. N=3, *P 0.05 vs. DM #P 0.05 vs. NC.

Note: NC: normal control exosomes; DM: diabetes mellitus exosomes; DKD: diabetic kidney disease exosomes.
Figure 4

The miRNA expression profiles in urine exosome. (A) Heat map of differentially expressed miRNAs. (B) Volcano Plot of differentially expressed miRNAs. (C) Verification of differentially expressed miRNAs using real-time PCR. N=3. **** P<0.001 vs. DM. (D-E) GO analysis and KEGG pathway analysis of differentially expressed miRNAs.

Supplementary Files

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

- SupplementaryFigure1.zip
• SupplementaryFigure2.zip