MicroRNA-29c in urinary exosome/microvesicle as a biomarker of renal fibrosis

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Progressive chronic kidney disease (CKD) is a leading cause of death worldwide. The histopathological feature is tubulointerstitial fibrosis, which leads to progressive loss of organ function. Consequently, assessment of the degree of tubulointerstitial fibrosis by renal biopsy is used to predict renal outcome (9). However, renal biopsy is invasive with potential complications, and repeated monitoring is practically difficult. Hence, reliable noninvasive biomarkers reflecting disease severity are urgently needed in the clinical management of patients with CKD.

In kidneys, micro (mi)RNAs are indispensable for its development and homeostasis (12). Aberrant miRNA expression is observed in the mouse models of kidney fibrosis. Dicer is a key enzyme involved in the production of mature miRNAs, and its ablation in podocytes led to proteinuria and glomerulosclerosis (11, 13). Transforming growth factor-β (TGF-β)-regulated miRNA families, miR-21, miR-200, and miR-29, have been shown to modulate renal fibrosis. miR-21, through a feedback loop, amplifies TGF-β signaling and promotes fibrosis. Conversely, miR-200 and miR-29 reduce fibrosis by inhibiting epithelial-to-mesenchymal transition and preventing the deposition of extracellular matrix, respectively. The identification of those miRNAs in renal fibrosis may lead to breakthroughs in the development of novel diagnostic tools and therapeutic targets for the treatment of kidney fibrosis (3, 22, 26).

Exosomes are small membrane vesicles with a size of 30–120 nm that are released by different cell types (4, 30) and can be isolated from various body fluids (10, 15). In addition to exosomes, other types of microvesicles (larger in size) can also be included because of the overlap in size when they are isolated. In this study, the pellet isolated using ultracentrifugation was collectively termed the exosome because the majority of the pellet consisted of the exosome (4). Exosomes contain molecules that reflect the physiological state of their cells of origin and consequently provide a rich source of potential biomarker (29).

More recently, Valadi et al. (28) demonstrated that exosomes contain both mRNA and microRNA. The findings triggered the hypothesis that extracellular miRNA from exosome may function in cell to cell communication (20, 31) and may be a potential biomarker for disease. A few pilot studies have demonstrated that miRNA in serum exosome could be potential markers for particular cancer. Taylor and Gercel Taylor (27) demonstrated that miRNA profiling of circulating tumor exosomes could potentially be used as surrogate diagnostic markers for biopsy profiling. Tanaka et al. (25) showed that exosomal miR-21 expression is upregulated in serum from patients with esophageal squamous cell cancer vs. serum from patients who have benign diseases and was positively correlated with tumor progression and aggressiveness. Bala et al. (2) shown that circulating miRNAs may serve as biomarkers to differentiate between hepatocyte injury and inflammation. Exosomes are normally secreted into the urine from all nephron segments (23), and it is reasonable to hypothesize that it may contain miRNAs associated with renal diseases. To our knowledge, there is no published literature addressing the role of miRNAs in urinary exosome, specifically its association with renal function and histopathological changes.

Here we sought to observe whether members of miR-29 and miR-200 family could be detected within urinary exosome and whether it could serve as a novel biomarker for renal fibrosis.

Materials and Methods

Research Subjects

All of the studies were approved by the Ethical Committee of Affiliated Zhongda Hospital of Southeast University. Written informed consent was obtained from all of the subjects to use their urine for research purposes. Thirty-two patients with biopsy proven were studied (diabetic nephropathy, n = 3; focal segmental glomerulosclerosis, n = 7; IgA nephropathy, n = 13; membranous nephropathy,
$n = 6$; and mesangial proliferative glomerulonephritis, $n = 3$). The exclusion criteria were as follows: patients younger than 18 yr old or patients with urinary tract infection, cancer, lupus, organ transplantation, signs, or symptoms of severe complications including cardiovascular disorder and use of steroids or immunosuppressive medications. Estimated glomerular filtration rate (eGFR) was calculated by the Modification of Diet in Renal Disease (MDRD) equation as suggested by National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) guidelines.

A group of healthy volunteers ($n = 7$) was also enrolled in the study as controls. Clinical data including albuminuria, blood urea nitrogen (BUN), and serum creatinine were recorded at baseline for each of the groups.

**Sample Collection and Exosome Purification**

A whole-stream, early morning urine specimen was collected from patients and healthy volunteers. The exosomes were isolated from the human urine samples using differential centrifugation. The urine was centrifuged at 2,000 g for 20 min, followed by ultracentrifugation at 200,000 g for 60 min. The pellet was stored at $-80^\circ C$ for subsequent applications.

**Immunoelectron Microscopy**

The exosome suspensions from CKD patient were mixed 1:1 with 4% paraformaldehyde and were then applied to 200-mesh nickel grids. After being blocked with 1% BSA and washed, the grid was incubated with a primary antibody recognizing aquaporin-2 (AQP-2; Santa Cruz, CA, Biotechnology) and CD9 (Santa Cruz Biotechnology) in 0.02% Triton X-100 for 1 h at room temperature. After being washed, the grids were exposed to species-specific anti-IgG antibodies conjugated to colloidal gold particles (5 nm; Boster, Wuhan, China). Then, the membranes were washed with PBS once and water twice. Control labeling was performed in an identical experiment, but the diluting solution was substituted for the primary antibody.

**RNA Isolation and microRNA Measurement**

**RNA isolation.** The microRNA was isolated using the miRNeasy micro kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The exosomes were disrupted and homogenized in 700 μl QIAzol lysis reagent, and the rest of the procedure was performed according to the manufacturer’s protocol. The RNA concentration and purity were confirmed by measuring the relative absorbance ratio at 260/280 on a Nanodrop 2000 (Thermo, Wilmington, DE).

Reverse-transcription quantitative real-time PCR. Detection of the mature miRNAs was performed by reverse transcription using the Takara One Step PrimeScript miRNA cDNA synthesis kit (Takara, Dalian, China) according to the manufacturer’s instructions, and the quantitative RT-PCR reaction was performed using SYBR Premix Ex Taq II. The primer mix for RNU6B, hsa-miR-29a, hsa-miR-29b, hsa-miR-29c, hsa-miR-200a, hsa-miR-200b, and hsa-miR-200c was obtained from Takara Biotechnology (Takara, Dalian, China). Non-template controls were included for each miRNA to assess for contaminants. The expression data were normalized to the expression of housekeeping gene RNU6B.

**Table 1. Primary clinical and laboratory characteristics of the study subjects**

<table>
<thead>
<tr>
<th></th>
<th>Control ($n = 7$)</th>
<th>CKD ($n = 32$)</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>41.6 ± 16.3</td>
<td>46.9 ± 14.9</td>
<td>0.305</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>3/4</td>
<td>19/13</td>
<td>0.677</td>
</tr>
<tr>
<td>Scr, mmol/l</td>
<td>84.0 ± 26.6</td>
<td>102.0 ± 53.8</td>
<td>0.442</td>
</tr>
<tr>
<td>Proteinuria, g/24 h</td>
<td>3.0 ± 2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUN, mmol/l</td>
<td>4.6 ± 1.2</td>
<td>6.2 ± 2.5</td>
<td>0.133</td>
</tr>
<tr>
<td>eGFR, ml/min·1.73 m$^{-2}$</td>
<td>92.8 ± 27.9</td>
<td>78.4 ± 28.5</td>
<td>0.421</td>
</tr>
</tbody>
</table>

Values are means ± SE. CKD, chronic kidney disease; Scr, serum creatinine; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate.

**Table 2. Levels of miRNA in urinary exosome between CKD and healthy controls**

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>CKD ($n = 32$)</th>
<th>Controls ($n = 7$)</th>
<th>Fold Change</th>
<th>AUC</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-29a</td>
<td>0.42 ± 0.46</td>
<td>3.28 ± 2.65</td>
<td>7.89</td>
<td>0.951</td>
<td>0.000</td>
</tr>
<tr>
<td>miR-29b</td>
<td>0.97 ± 1.64</td>
<td>6.41 ± 5.30</td>
<td>6.59</td>
<td>0.902</td>
<td>0.001</td>
</tr>
<tr>
<td>miR-29c</td>
<td>5.12 ± 6.21</td>
<td>116.09 ± 138.85</td>
<td>22.69</td>
<td>0.982</td>
<td>0.000</td>
</tr>
<tr>
<td>miR-200a</td>
<td>2.39 ± 1.98</td>
<td>31.03 ± 20.33</td>
<td>13.01</td>
<td>0.996</td>
<td>0.000</td>
</tr>
<tr>
<td>miR-200b</td>
<td>2.37 ± 2.09</td>
<td>26.80 ± 21.71</td>
<td>11.29</td>
<td>0.991</td>
<td>0.000</td>
</tr>
<tr>
<td>miR-200c</td>
<td>3.19 ± 4.39</td>
<td>86.86 ± 114.56</td>
<td>27.24</td>
<td>1</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Values are means ± SE. AUC, area under the curve; mi, micro.
Assessment of Renal Scarring

Analysis of renal fibrosis was determined on paraffin-embedded sections stained by Masson trichrome. Serial 3-μm sections were acquired from each paraffin block. The severity of renal fibrosis was scored subjectively by two experienced pathologists who were blinded to the results of molecular studies. For tubulointerstitial scarring, the whole tissue were viewed at magnification of ×100 and scored subjectively from 0 to 100% for each patients. The severity of tubulointerstitial scarring was represented by the mean of scores from the two pathologists.

Statistical Analysis

Statistical analysis was performed by SPSS for Windows software version 11.0. Briefly, raw threshold cycles (Ct) values were imported from ABI7300 SDS software and relative expression levels for each miRNA were calculated using the comparative Ct method. The relationship between levels of miRNA in urinary exosome and estimated glomerular filtration rate (eGFR) is shown for the chronic kidney disease (CKD) patients, along with Spearman’s rank-order correlation (r). A significant positive relationship between the levels of miRNA-29c and eGFR was found. miRNA-200a showed correlation with marginal statistical significance. miRNA levels calculated using the equation $2^{-\Delta\Delta C_t}$ normalized for RNU6B are shown.

Fig. 2. Correlation between levels of micro (mi)RNA in urinary exosome and estimated glomerular filtration rate (eGFR). The relationship between levels of miRNA in urinary exosome and eGFR is shown for the chronic kidney disease (CKD) patients, along with Spearman’s rank-order correlation ($r$). A significant positive relationship between the levels of miRNA-29c and eGFR was found. miRNA-200a showed correlation with marginal statistical significance. miRNA levels calculated using the equation $2^{-\Delta\Delta C_t}$ normalized for RNU6B are shown.

Fig. 3. CKD patients was divided into 2 groups based on tubulointerstitial scarring score. CKD was divided as mild and moderate to severe according to the mean grades scored subjectively by 2 experienced pathologists. Left: scores of tubulointerstitial fibrosis. Microphotographs demonstrate representative Masson trichrome staining of renal sections from each group (top right, mild fibrosis; bottom right, moderate fibrosis).
relative amount of each miRNA to RNU6B (14, 16) was described by using the equation $2^{-\Delta C_t}$, where $\Delta C_t = (C_{t_{miRNA}} - C_{t_{U6}})$. All the results are presented in means $\pm$ SD for data. We used Mann-Whitney U-test to compare gene expression levels between groups and Spearman's rank-order correlations to assess associations between gene expression levels and clinical parameters. $P < 0.05$ was considered statistically significant. All probabilities were two tailed. The diagnostic performance of biomarkers was evaluated by calculating their sensitivity and specificity using the receiver operating characteristics (ROC) curves.

RESULTS

Confirmation of Pellet Urinary Exosome

To confirm that the pellet structures were indeed exosomes, they were examined using electron microscopy and immunogold staining. Quantitative analysis of the electron micrographs revealed the average diameters of vesicle is 65.14 $\pm$ 25.9 nm, consistent with a small size for the exosomes (23). Immunogold-staining analysis with antibodies against AQP-2 and CD9 shows that the majority of the vesicles were immunogold-labeled with anti-AQP-2 and anti-CD9 (Fig. 1).

Levels of Urinary Exosomal miRNAs in CKD Compared with Controls

We studied a total of 39 subjects. The baseline demographic and clinical data of the patients and controls are summarized in Table 1. There are no significant differences in age, sex, serum creatinine, BUN, and eGFR between CKD group and healthy controls. eGFR was calculated with MDRD formula. The result showed that 12 of the patients were diagnosed with stage 1 CKD, 12 with stage 2, 5 with stage 3, and 3 with stage 4 CKD.

We first compared the levels of miRNAs expression between the CKD group and the healthy controls. With the use of the expression values for each miRNA relative to RNU6B, all 6 miRNAs were found to be reduced in CKD group compared with healthy controls. Members of miR-29 showed 7.89-, 6.59-, and 22.69-fold decrease ($P < 0.01$) and members of miR-200 showed 13.01-, 11.29-, and 27.24-fold decrease in the CKD samples compared with the controls ($P < 0.01$). The ability of the urinary miRNA to discriminate CKD from healthy controls was analyzed by ROC. In Table 2, miR-200c displayed the highest area under the curve (AUC) of 1 ($P = 0.000$), followed by miR-200a, miR-200b, miR-29c, miR-29a, and miR-29b (AUC $0.996$, $0.991$, $0.982$, $0.951$, and $0.902$, respectively; Table 2).

Correlation Analysis with Clinical Parameters

Within the CKD group, the eGFR significantly correlated with urinary exosomal levels of miR-29c ($r = 0.362$, $P = 0.042$). miR-200a showed correlation with the eGFR with marginal statistical significance ($P = 0.068$). For miR-29a,
miR-29b, miR-200b, and miR-200c, no significant correlation was observed with eGFR (Fig. 2).

No significant correlation was observed between the BUN and miRNAs \( (P > 0.05) \) in the CKD group. Moreover, the urine protein excretion did not correlate with the expressions of miRNA in urinary exosome.

**MicroRNA Levels and Renal Fibrosis**

We further studied the correlations between histological fibrosis grading and the expression of the target miRNA in urinary exosome. The CKD group is divided into two groups: mild fibrosis (fibrosis area <25%; \( n = 16 \)) and moderate to severe fibrosis group (fibrosis area >25%; \( n = 16 \)) based on the severity of tubulointerstitial fibrosis. Histological studies show that the percentages of tubulointerstitial scarring are \( 9.25 \pm 5.43 \) and \( 48.34 \pm 16.44 \% \) for mild and moderate to severe group, respectively; Fig. 3 shows the representative Masson trichrome staining of renal sections from each group.

The expression of miRNA is shown in Fig. 4. The moderate group showed significant lower levels of miR-29a \( (P = 0.000) \), miR-29c \( (P = 0.023) \), miR-200b \( (P = 0.000) \), and miR-200c \( (P = 0.000) \) compared with mild group. The other members of miRNA also showed decreased levels although without statistically significance. ROC analysis showed miR-29a and miR-29c could discriminate mild fibrosis group from moderate to severe group with AUC of 0.883 (95% confidence interval, 0.748–1.017, \( P = 0.000 \)) and AUC of 0.738 (95% confidence interval, 0.556–0.920, \( P = 0.022 \)), respectively (Fig. 5). Other members of miRNAs could not discriminate mild fibrosis group from moderate to severe group effectively \( (P > 0.05) \). We also calculated the cut-off value for urinary exosomal miRNA to predict mild and moderate to severe tubulointerstitial scarring. miR-29a displayed sensitivity 93.8% and specificity 81.3% at the optimal cut-off value of 0.172 (relative gene expression level). The optimal cut-off value for miR-29c was 3.907 (sensitivity 68.8%, specificity 81.3%).

Besides the ability of discriminating different levels of fibrosis, we also explored the correlation of miRNAs with severity of fibrosis. There was a significant negative correlation between the level of miR-29c in urine exosome and tubulointerstitial fibrosis as showed in Fig. 6 \( (r = -0.359; P = 0.044) \). Levels of other members of miR-29 and miR-200 did not correlate with the degree of histological scarring.

Proteinuria is a well-established risk factor for the progression of CKD, and its correlation with renal fibrosis was analyzed as a comparison with miRNA identified in this study. The correlation analysis showed that proteinuria had no association with renal fibrosis \( (r = 0.108; P = 0.556) \).

**DISCUSSION**

Recent findings indicated that miRNAs are important mediators of renal fibrosis and might be potential biomarkers and therapeutic targets for CKD \( (17) \). Interestingly, recent studies have indicated that miRNAs are incorporated into exosomes and are more stable than their cellular counterparts. miRNAs can resist degradation through protection in vesicles released from cultured cells or during circulation in the body \( (18) \). Our previous study has shown that miRNA confined inside urine exosome is resistant to RNase digestion and showed high stability under shipping conditions and repeated frozen and freeze cycles (data not published). This apparent stability and its origination from functional cells make exosomal miRNA as attractive, noninvasive biomarkers.

Besides high stability, the result from the present study suggests that miRNA could be readily detected from urinary exosome for various kidney disease including diabetic nephropathy, focal segmental glomerulosclerosis, membranous nephropathy, and IgA nephropathy. In addition, we observed a striking reduction in the levels of all members of miR-29 and miR-200 families in these CKD patients compared with controls. A number of studies have reported that intrarenal miR-29
and miR-200 may have specific roles in kidney diseases by reducing renal fibrosis (5, 7, 32). In the present study, we observed reduced level of miR-29 and miR-200 in urinary exosome in CKD patients, which further support the protective role of those miRNAs in renal disease. Also, more importantly, the detection of miRNA in urinary exosome suggests its potential for translating to clinic biomarkers. The result also was consistent with intrarenal findings from Wang et al. (33, 34) who showed that the level of intrarenal miR-200c was downregulated and miR-29c levels from whole urine sediment were decreased in patients with IgA nephropathy. Neal et al. (21) detected miR-16, miR-21, miR-155, miR-210, and miR-638 from whole urine, but they did not find any association between urinary miRNA level and kidney function.

Although urinary miRNAs can be analyzed directly from whole urine, it may be possible to increase both the sensitivity and the specificity of miRNA biomarker through the enrichment of exosomes. Szeto et al. (24) measured miRNA from urine sediment and found that urinary miR-21 and miR-216a expression correlated with the rate of renal function decline and risk of progression to dialysis-dependent renal failure. Although miRNA could be detected from both whole urine and urine sediment, exosome miRNA might show its unique superiority since exosome was released by functional cells. Moreover, Miranda et al. (18) have demonstrated that RNA profile was better preserved in urinary microvesicles/exosome compared with whole cells isolated from urine, suggesting the advantage of RNA markers from urinary microvesicles/exosome. Also, exosomes have the capacity to shuttle their cargo between kidney cells and may represent a mechanism for cell-to-cell signaling along the nephron (4). Therefore, the molecules contained within exosome should provide more valuable information of the kidney compared with whole urine/urinary sediment. In the present study, we also find that urinary levels of miR-29c positively correlated with eGFR. This suggests that urinary miRNA might be a new, noninvasive marker for detecting deterioration of renal function.

It has been shown that, in response to various pathophysiological stimulations, cells can actively package miRNA into exosome and release them into circulation. It is reasonable to hypothesize that urinary exosome could be used as a mirror to reflect the renal histopathological change. More importantly, unlike renal biopsy that provides only a small sample from the kidney, urinary exosomes provide a full representation of the entire urinary system (1). The result in this study demonstrated that levels of urinary exosomal miR-29c could distinguish mild and moderate to severe fibrosis of the kidney. Moreover, levels of miR-29c correlated negatively with tubulointerstitial fibrosis score. Interestingly, proteinuria did not show correlation with renal fibrosis, which indicates the superiority of exosome as a marker for detecting deterioration of renal function.

Thus, from what have discussed above, we draw that levels of miR-29c in urinary exosome could be used to reflect both renal function and degree of histological fibrosis. Interestingly, more recently, Fang et al. (8) also found that miR-29c is downregulated in renal interstitial fibrosis in humans and rats and restored by activation of hypoxia-inducible factor-α that attenuates fibrosis. Therefore, miR-29c play an important role in the development of renal fibrosis and its measurement from whole urine/urinary sediment. Additionally, Miranda et al. (18) have demonstrated that RNA profiles from urinary microvesicles/exosome might be useful to predict the severity of renal histological lesions and might be useful to predict the development and progression of renal histological lesions.

Fig. 6. Relationship between scores of tubulointerstitial fibrosis with miRNA expression in urinary exosome. A significant inverse relationship between the levels of miR-29c and scores of tubulointerstitial fibrosis was found, but not other miRNAs. miRNA levels calculated using the equation $2^{-\Delta C_{t}}$ normalized for RNU6B are shown.
miR-29c in renal fibrosis. Exosome miRNA is a very recent interest being studied as disease biomarkers (6) this pilot study demonstrates for the first time the feasibility of using urinary exosome miRNA as markers reflecting both changes of kidney structure and function. However, there are a few limitations with this study. First, the sample size is relatively small. Further studies are necessary to validate these results in a large cohort study. Second, we have no data on the follow-up of the patients. It would be of interest to find the additional clinical and prognostic clues by continuous monitoring of the miRNA during the follow-up. Finally, we did not study the intrarenal expression of these miRNA for comparison, which is important to determine whether the information provided by urine exosome was consistent with changes of the kidney.

Conclusions

In summary, the levels of miR-29 and miR-200 family in urinary exosome are significantly downregulated in CKD patients. The levels of miR-29c in urinary exosome could be used to reflect both renal function and degree of histological fibrosis, suggesting it as a novel, noninvasive marker for renal fibrosis. In addition, the universally applicable assays for quantitation of microRNA and the stability of exosomal miRNA further underscore their unique potential as novel, noninvasiveness biomarkers for CKD.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.-L.L. and B.-C.L. conception and design of research; L.-L.L., H.L., and P.-S.C. acquisition of data; L.-L.L., D.L., and P.-S.C. interpretation of results; L.-L.L. and B.-C.L. writing of the manuscript; L.-L.L. and B.-C.L. editing and revising the manuscript.

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