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Micro-RNA expression in the urinary sediment of patients with chronic kidney diseases

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Abstract. *Background*: Evidence indicates that microRNAs (miRNA) play a role in the pathogenesis of chronic kidney diseases (CKD). We explored the possibility of using urinary miRNA as non-invasive biomarkers for CKD.
Methods: We quantified miRNA expression in urinary sediment of 56 CKD patients who underwent kidney biopsy. Patients were followed for 16.2 ± 15.5 months.
Results: Patients with diabetic glomerulosclerosis had lower urinary miR-15 expression, while those with IgA nephropathy had higher urinary miR-17 expression, than other diagnosis groups. Baseline proteinuria had significant inverse correlation with urinary expression of miR-15, miR-192, and miR-216a; baseline renal function correlated with urinary expression of miR-15, miR-17, miR-192, and miR-217. The rate of renal function decline correlated with urinary expression of miR-21 ($r = 0.301$, $p = 0.026$) and miR-216a ($r = 0.515$, $p < 0.0001$). Patients with a high urinary expression of miR-21 and miR-216a had better dialysis-free survival than those with low expression (log rank test, $p = 0.005$ and $p = 0.003$, respectively).
Conclusions: Urinary miR-21 and miR-216a expression correlated with the rate of renal function decline and risk of progression to dialysis-dependent renal failure. Our results suggest that urinary miRNA profiling has the potential of further development as biomarkers of CKD.

Keywords: Proteinuria, glomerulonephritis, biomarker

1. Introduction

Chronic kidney disease (CKD) is a debilitating and costly medical condition. The clinical course is characterized by persistent proteinuria after an initial insult to the kidney, followed by progressive decline in renal function [1]. Reliable markers are much needed for the clinical management of CKD. Proteinuria and the severity of tubulo-interstitial scarring in kidney biopsy are the most commonly used prognostic indicators in CKD patients [1–3]. However, neither of them is an entirely satisfactory marker of CKD.

Micro-RNAs (miRNAs) are short noncoding RNA molecules that inhibit gene expression through incomplete base pairing with the 3'-untranslated region of target mRNAs [4,5]. Evidence indicates that miRNAs play a role in the pathogenesis of many human diseases [6,7]. Recently, miRNA control has also been found to have a critical regulatory role in epithelial-mesenchymal transition (EMT) [8], which may play an important role in the progressive kidney damage in CKD [9]. Recently, miRNA level could be measured in serum and other body fluids [10]. In urine, miRNA could be quantified in both the urinary sediment as well as the supernatant after centrifugation [10].

The role of urinary miRNA as biomarkers of kidney disease is under active research. There are several advantages of quantifying miRNA in urine as biomarkers. First, although the measurement of cytokines and

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growth factors in urine would seem logical means to assess the severity of kidney damage and provide prognostic information, measurement of urinary cytokine protein may not be clinically relevant because most of the cytokines exert paracrine effects and are not excreted in the urine, and the amount of cytokine protein found in urine may not correlate with the local concentration in tubulointerstitium [11]. Furthermore, there is a limited number of human miRNAs, with each individual miRNA modulating protein output from hundreds of target genes [12]. In addition, miRNA is relatively stable and resistant to degradation, making it an ideal substance to be tested for large scale clinical use or research with archive specimens.

We have previously reported that urinary expression of miR-200a, miR-200b and miR-429 were downregulated in patients with IgA nephropathy, and the degree of reduction correlated with disease severity and rate of progression [13], while patients with active lupus nephritis had lower urinary expression of miR-200a, miR-200c, miR-141, miR-429 and miR-192 than healthy controls [14]. In the present study, we explore the possibility of using urinary miRNA as non-invasive biomarkers for CKD.

2. Patients and methods

2.1. Case selection

We studied 56 consecutive patients who underwent kidney biopsy between January 2009 and June 2010 in the Prince of Wales Hospital, Hong Kong. Patients with acute renal failure or active glomerulonephritis were excluded. The study was approved by the Clinical Research Ethical Committee of the Chinese University of Hong Kong, all patients provided fully informed consent. A whole-stream early morning urine specimen was collected by each patient on the biopsy day for urinary miRNA expression study. Clinical data including serum creatinine and 24 hours urine protein were recorded. Glomerular filtration rate (GFR) was estimated by a standard equation [15].

2.2. Quantification of urinary miRNA

The methods of urinary miRNA extraction and quantification have been described in our previous studies [13,14]. Briefly, urine specimen was collected and sent to laboratory for processing immediately or stored in 4°C overnight. Urine sample was centrifuged at 3000 g for 30 minutes and at 13000 g for 5 minutes at 4°C. Supernatant was discarded and the urinary cell pellet was lysed by RNA lysis buffer (Qiagen Inc, Ontario, Canada). Specimens were then stored at −80°C until use.

MirVana^{TM} miRNA isolation kit (Ambion, Inc. Austin, TX, USA) was used for the extraction of total RNA from urinary sediment according to the manufacturer's protocol. We confirmed the purity of urinary RNA by the relative absorbance at 260/280 nm ratio using a spectrometer (Hitachi, Japan). Our previous data have shown the integrity of RNA isolated from urinary sediment by this method is adequate for real time quantitative polymerase chain reaction (RT-QPCR).

TaqMan® miRNA reverse transcription Kit (Applied Biosystems, Foster City, CA, USA) and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) were used for reverse transcription. For miRNA, 5 μl total RNA was mixed with 3 μl specific primers, 0.15 μl 10mM dNTPs (with dTTP), 1.5 μl 10x reverse transcription buffer, 1 μl (50U) MultiScribe^{TM} Reverse Transcriptase 0.19 μl RNase inhibitor (20U/μl) and made up to 15 μl with H_2O. Reverse transcription was performed at 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes. For messenger RNA, 10 μl total RNA was mixed with 2 μl specific primers, 0.8 μl 10mM dNTPs (with dTTP), 2 μl 10x reverse transcription buffer, 1 μl (50U) MultiScribe^{TM} Reverse Transcriptase 1 μl RNase inhibitor (20U/μl) and made up to 20 μl with H_2O. Reverse transcription was performed at 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes. The resulting cDNA was stored in −80°C until use.

Urinary expression of miR-15, miR-17, miR-21, miR-30, miR-192, miR-216a, miR-217, and miR-377 were quantified by RT-QPCR using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). These targets were chosen because of their reported involvement in renal fibrosis and progression of CKD [16,17]. Commercially available Taqman primers and probes, including 2 unlabeled PCR primers and 1 FAM^{TM} dye-labeled TaqMan® MGB probe were used for all the targets (all from Applied Biosystems). Each sample was run in triplicate. RT-QPCR were performed at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Small RNA U6 (Applied Biosystems) was used as house-keeping genes to normalize the microRNA expression [18,19]. Results were analyzed with Sequence Detection Software version 2.0 (Applied Biosystems). In order to calculate the

Table 1
Demographic and clinical data of the study patients

Group	IgAN	DGS	HTN
No. of patients	17	17	22
Sex (M:F)	5:12	10:7	12:10
Age (years)	47.8 ± 12.6	52.5 ± 12.6	56.3 ± 14.0
Renal function			
Proteinuria (g/day)*	2.9 ± 2.1	6.6 ± 3.1	3.0 ± 3.0
Serum creatinine (μmol/l)	235.6 ± 193.9	292.9 ± 160.3	270.4 ± 223.7
GFR (ml/min/1.73 m^2)	46.7 ± 41.1	28.2 ± 21.5	34.5 ± 22.1
Histological scarring			
Glomerulosclerosis (%)	39.0 ± 31.3	28.4 ± 16.8	45.1 ± 31.4
Tubulointerstitial fibrosis (%)	36.3 ± 27.2	54.1 ± 14.1	39.9 ± 27.6
GFR decline (ml/min/1.73 m^2 per year)**	-10.2 ± 16.4	-14.1 ± 11.5	-3.6 ± 6.3

IgAN, IgA nephropathy; DGS, diabetic glomerulosclerosis; HTN, hypertensive nephrosclerosis; GFR, glomerular filtration rate.
*$p < 0.0001$ by one-way ANOVA; **$p = 0.005$ by Kruskal-Wallis test.

differences of expression level for each target among samples, the $\Delta\Delta C_T$ method for relative quantitation was used.

2.3. Assessment of renal scarring

Analysis of renal fibrosis was determined on 4 μm paraffin-embedded sections stained by Periodic Acid Schiff (PAS) or Jones silver stain. The severity of renal fibrosis was scored subjectively by an experienced pathologist who was blinded to the results of molecular studies. The severity of glomerulosclerosis was represented by the percentage of sclerotic glomeruli in total glomeruli obtained from biopsy. For tubulointerstitial scarring, ten microscopic fields were viewed at magnification of 200$\times$ and scored subjectively from 0 to 100% for each patients. The severity of tubulointerstitial scarring was represented by the mean of ten scores.

2.4. Clinical management

After renal biopsy, all patients were followed every 2 months for at least 12 months Renal function and proteinuria levels were assessed at least every 4 months. Disease progression was measured by the rate of GFR decline, which was calculated by the least-square regression method [20]. Treatment for individual patient was determined by responsible physician and not affected by this study. All physicians were blinded from the results of RNA expression.

2.5. Statistical analysis

Statistical analysis was performed by SPSS for Windows software version 15.0 (SPSS Inc., Chicago, IL). All the results were presented in mean $\pm$ SD for data normally distributed and median (lower and upper quartiles) for the others. Since data of gene expression levels were highly skewed, either log transformation or nonparametric statistical methods were used. When no detectable level of a transcript was found (defined as no detectable level after 40 cycles of RT-QPCR) and there was zero value, a value equal to half of the minimum observed gene expression level was assigned.

We used Kruskal Wallis test or Mann-Whitney U test to compare gene expression levels between groups and Spearman's rank-order correlations to test associations between gene expression levels and clinical parameters. For the analysis of dialysis-free survival, Kaplan-Meier survival curves were constructed. Gene expression of each target was classified into high and low groups according to the median level of expression and then compared by the log rank test. A P value of below 0.05 was considered statistically significant. All probabilities were two-tailed.

3. Results

We studied 56 CKD patients. The underlying histological diagnosis were IgA nephropathy (17 cases), diabetic nephrosclerosis (17 cases), and hypertensive nephrosclerosis (22 cases). The baseline demographic and clinical data of the patients are summarized in Table 1. None of the patients with IgA nephropathy had necrotizing or crescentic lesion in renal biopsy. In short, most of the baseline clinical and biochemical parameters were highly comparable between the diagnosis groups, except patients with diabetic glomerulosclerosis had more proteinuria ($p < 0.0001$) and marginally more tubulointerstitial scarring ($p = 0.09$) than the other groups.

Table 2
Internal correlations between urinary expression of miRNA targets

	miR-17	miR-21	miR-30	miR-192	miR-216a	miR-217	miR-377
miR-15	$r = 0.619$, $p < 0.0001$	$r = 0.776$, $p < 0.0001$	$r = 0.364$, $p = 0.006$	$r = 0.541$, $p < 0.0001$	$r = 0.319$, $p = 0.017$	$r = 0.424$, $p = 0.001$	$r = -0.413$, $p = 0.002$
miR-17		$r = 0.500$, $p = 0.0001$	$r = 0.560$, $p < 0.0001$	$r = 0.607$, $p < 0.0001$	$r = 0.271$, $p = 0.04$	$r = 0.353$, $p = 0.008$	$r = -0.350$, $p = 0.008$
miR-21			$p = 0.636$, $p < 0.0001$	$r = 0.758$, $p < 0.0001$	$r = 0.423$, $p = 0.001$	$r = 0.595$, $p < 0.0001$	$r = -0.301$, $p = 0.024$
miR-30				$r = 0.903$, $p < 0.0001$	$r = 0.600$, $p < 0.0001$	$r = 0.629$, $p < 0.0001$	$r = -0.126$, $p = 0.4$
miR-192					$r = 0.630$, $p < 0.0001$	$r = 0.749$, $p < 0.0001$	$r = -0.166$, $p = 0.2$
miR-216a						$r = 0.394$, $p = 0.003$	$r = 0.113$, $p = 0.4$
miR-217							$r = -0.190$, $p = 0.16$

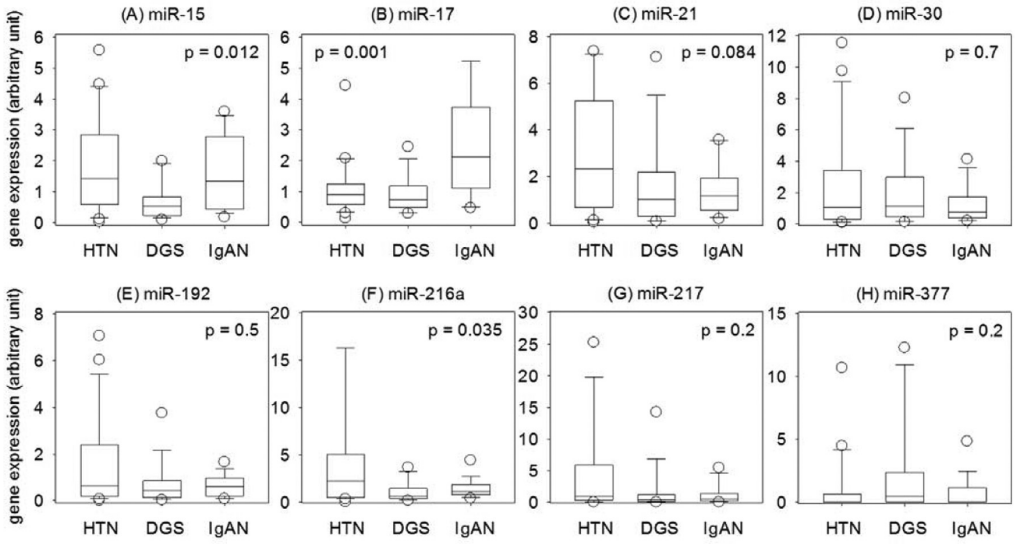

Fig. 1. Comparison of miRNA expression in urinary sediment between diagnosis groups: (A) miR-15; (B) miR-17; (C) miR-21; (D) miR-30; (E) miR-192; (F) miR-216a; (G) miR-217; and (H) miR-377. Whisker-box plot, with the boxes indicate median, 25 and 75 percentile; whisker caps indicate 5 and 95 percentile; circles indicate outliers. Data are compared by Kruskal Wallis test. (Key: HTN, hypertensive nephrosclerosis; DGS, diabetic glomerulosclerosis; IgAN, IgA nephropathy.)

3.1. Difference between groups

There was a close internal correlation between most of the urinary expressions of the miRNA targets except miR-377 (Table 2). Urinary expression of individual miRNA targets are compared between diagnosis groups and summarized Fig. 1. Briefly, we found that patients with diabetic glomerulosclerosis had lower urinary miR-15 expression than the other groups, when patients with IgA nephropathy had higher urinary miR-17 expression than other groups. Patients with hypertensive nephrosclerosis tended to have higher urinary expression of miR-216a and miR-21, although the latter did not reach statistical significance.

3.2. Relation with baseline characteristics

The relation between urinary expressions of miRNA targets and proteinuria, baseline renal function, degree of glomerulosclerosis and tubulointerstitial fibrosis is summarized in Table 3. In essence, baseline proteinuria had significant inverse correlation with urinary expression of miR-15, miR-192, and miR-216a, while baseline renal function positively correlated with urinary expression of miR-15, miR-17, miR-192, and miR-217. The degree of tubulointerstitial fibrosis positively correlated with urinary expression of miR-377, and inversely with urinary miR-217, while no significant correlation was observed between the degree of

Table 3
Relation between urinary expression of miRNA targets and baseline clinical and pathological parameters

	Proteinuria	Estimated GFR	Glomerulosclerosis	Tubulointerstitial fibrosis
miR-15	$r = -0.355, p = 0.008$	$r = 0.277, p = 0.04$	$r = -0.032, p = 0.8$	$r = -0.256, p = 0.06$
miR-17	$r = -0.228, p = 0.1$	$r = 0.359, p = 0.007$	$r = -0.142, p = 0.3$	$r = -0.228, p = 0.1$
miR-21	$r = -0.160, p = 0.2$	$r = 0.163, p = 0.2$	$r = 0.006, p = 0.9$	$r = -0.137, p = 0.3$
miR-30	$r = -0.075, p = 0.6$	$r = 0.226, p = 0.09$	$r = -0.046, p = 0.7$	$r = -0.137, p = 0.3$
miR-192	$r = -0.284, p = 0.04$	$r = 0.370, p = 0.005$	$r = -0.119, p = 0.4$	$r = -0.261, p = 0.06$
miR-216a	$r = -0.430, p = 0.001$	$r = 0.131, p = 0.3$	$r = 0.149, p = 0.3$	$r = -0.200, p = 0.15$
miR-217	$r = -0.261, p = 0.06$	$r = 0.398, p = 0.002$	$r = -0.010, p = 0.9$	$r = -0.354, p = 0.009$
miR-377	$r = 0.097, p = 0.5$	$r = -0.145, p = 0.3$	$r = 0.020, p = 0.9$	$r = 0.313, p = 0.02$

GFR, glomerular filtration rate.

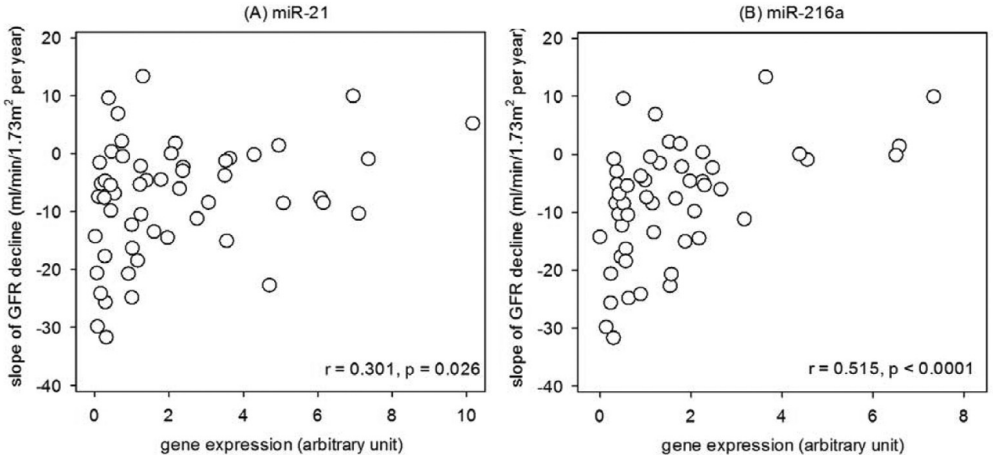

Fig. 2. Relation between the rate of glomerular filtration rate (GFR) decline and urinary expression of (A) miR-21; and (B) miR-216a. Data are compared by Spearman's rank correlation coefficient.

glomerulosclerosis and the urinary expression of any miRNA target examined in this study.

3.3. Relation with renal function decline

Patients were followed for an average of 16.2 ± 15.5 months. All patients received angiotensin converting enzyme inhibitor or angiotensin receptor blocker therapy; none of the patient with IgA nephropathy received corticosteroid or immunosuppressive treatment. The average rate of GFR decline was −8.8 ± 12.4 ml/min/1.73m^2 per year. Patients with hypertensive nephrosclerosis had a significantly slower rate of GFR decline as compared to other diagnosis groups (see Table 1). When all patients were pooled and analyzed, the rate of GFR decline positively correlated with the urinary expression of miR-21 ($r = 0.301, p = 0.026$) and miR-216a ($r = 0.515, p < 0.0001$) (Fig. 2). When the three diagnosis groups were analyzed separately, urinary miR-216a expression correlated with the rate of GFR decline in patients with hypertensive nephrosclerosis ($r = 0.588$, $p = 0.005$) and diabetic glomerulosclerosis ($r = 0.605, p = 0.010$), but not IgA nephropathy ($r = 0.042, p = 0.9$). Urinary miR-21 expression did not correlate with the rate of GFR decline for any diagnosis group.

During follow up, 18 patients (5 patients with IgA nephropathy, 8 with diabetic glomerulosclerosis; and 5 with hypertensive nephrosclerosis) progressed to end stage renal disease and were put on dialysis. Kaplan-Meier analysis showed that patients with a high urinary expression of miR-21 and miR-216a had better dialysis-free survival than those with low expression (log rank test, $p = 0.005$ and $p = 0.003$, respectively) (Fig. 3). Other miRNA targets tested in this experiment were not associated with dialysis-free survival (details not shown). Because the number of event was small, extensive multivariate survival analysis was not performed.

4. Discussion

In the present study, we found that urinary expression of miR-15, miR-17, and miR-216a were significantly

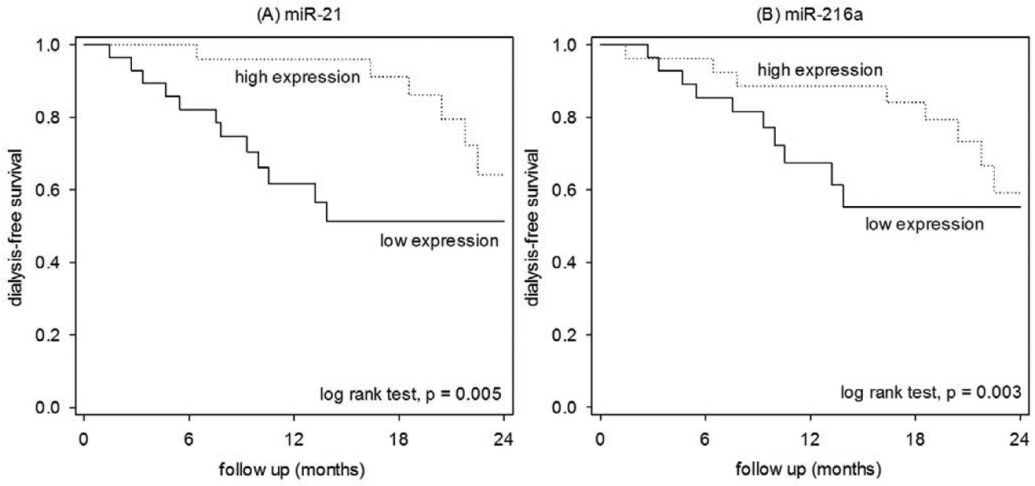

Fig. 3. Kaplan-Meier plot of dialysis-free survival with respect to the urinary expression of (A) miR-21; and (B) miR-216a. For either miRNA, patients were divided into two groups according to the median level of expression, which was 25 copies per 100,000 copies of the housekeeping gene. Data are compared by log rank test.

different between diagnosis groups. The expression of some miRNA targets in the urinary sediment correlated with proteinuria, renal function, and the degree of tubulointerstitial fibrosis. In addition, urinary miR-21 and miR-216a expression correlated with the rate of renal function decline as well as the risk of progression to dialysis-dependent renal failure. Our results suggest that urinary miRNA profiling has the potential of further development as biomarkers of chronic kidney diseases.

Our result is, in general, consistent with previous reports. For example, Kato et al. [21] showed that miR-192 levels were enhanced significantly in glomeruli isolated from streptozotocin-injected diabetic mice as well as diabetic db/db mice, and miR-192 contributes to the TGF-beta-induced collagen expression via inhibition of E-box repressors. The same group also found that miR-216a and miR-217 target phosphatase and tensin homologue (PTEN), which in turn regulates Akt activation and TGF-beta-induced fibrosis [22]. With the same animal models of diabetic nephropathy, Zhang et al. [23] showed that miR-21 expression was down-regulated in response to early diabetic nephropathy, and over-expression of miR-21 inhibited proliferation of mesangial cells and decreased urine albumin excretion rate in diabetic db/db mice. Wang et al. [24] reported that overexpression of miR-377 in diabetic nephropathy leads to increased fibronectin production via reduced expressions of p21-activated kinase and superoxide dismutase. Similarly, Lee et al. [25] found that miR-15 contributes to the cystogenesis of polycystic kidney disease, while, in a similar model, Sun et al. [26] showed that overexpression of miR-17 may promote cell proliferation via post-transcriptionally repression of target genes. Taken together, our results further support the hypothesis that the above-mentioned miRNA targets may contribute to the progression of renal failure in CKD.

In addition to shedding light on the pathogenic role of miRNA in progressive CKD, another important reason to explore miRNA expression in urinary sediment of CKD patient is biomarker development. In fact, there are several advantages of quantifying miRNA in urine as compared to the measurement of messenger RNA. First, there is a limited number of human miRNAs, with each individual miRNA modulating protein output from hundreds of target genes [12]. Second, miRNA is relatively stable and resistant to degradation, making it an ideal substance to be tested for large scale clinical use or research with archive specimens. Finally, effective means of *in vivo* introduction of miRNA is being developed [27], and any relevant miRNA species may have a therapeutic potential in the future.

A number of previous studies from our group had explored the role of urinary miRNA level as the biomarker for various forms of kidney diseases [13,14,28,29]. Notably, our previous study showed that urinary levels of miR-146a and miR-155 correlated with the levels of various inflammatory markers in IgA nephropathy [28] and lupus nephritis [29]. In the present study, we focused on the miRNA targets that are reported to be involved in the development and progression of renal

fibrosis in CKD in general [16,17], rather than those miRNA targets relevant for immune responses (such as miR-146a and miR-155) in specific inflammatory glomerulonephritis.

There are a few inadequacies of our study. First, we detected the expression levels of the studied miRNAs using urine sediment without determining the cellular sources for each of them. Although not directly proved, our previous experience suggests that urinary miRNAs in the present study are most likely from deciduous tubular epithelial cells and podocytes [28]. Previous studies, however, showed that miR-21, miR-192, miR-216a, and miR-377 are largely expressed in mesangial cells, while miR-15 and mR-17 are probably expressed in tubular epithelial cells [16,17]. Future studies would be necessary to investigate miRNA expression level in specific renal cell type. To further delineate the role of individual miRNA species in the progression of CKD, it would also be necessary to study their intra-renal expression in renal biopsy specimens. Since we aimed to determine the relation between urinary miRNA level and CKD progression, we did not include a control group with normal kidney function for comparison. Recently study, however, showed that urinary miR-21 levels are elevated in patients with microalbuminuria and early CKD as compared to healthy schoolchildren [30].

Second, the scope of our present study is limited, both in terms of renal diagnosis and miRNA targets that were tested. Since we only studied three common renal diseases, our result may not be extrapolated to CKD in general. In addition, the overall sample size was small, so that we might not have sufficient statistical power for a proper multivariate analysis and determine the prognostic role of urinary miRNA expression. Notably, proteinuria is one of the best prognostic indictor of CKD progression. Our present study did not confirm that urinary miRNA quantification provides additional prognostic information after adjusting for the degree of proteinuria, and further validation study is necessary on this respect.

Also because of the small sample size of our study, therapeutic intervention of the recruited patients could have be heterogeneous. Nonetheless, we put the utmost effort to study a homogeneous population of CKD patients treated with standard therapy (blood pressure control, renin-angiotensin-axis blockade, and no immunosuppressive therapy). In this study, we chose specific miRNA targets, based on their reported involvement in renal fibrosis and progression of CKD [16,17]. Further study is also needed to screen for the expression of all possible miRNA species by a hypothesis-free technology (for example, microarray).

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