

Expression of microRNAs in the urinary sediment of patients with IgA nephropathy

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Abstract. *Background:* Micro-RNAs (miRNAs) regulate one-third of all protein-coding genes and are fundamental in the pathophysiology of a wide range of diseases. We studied the expression of several miRNA species (miR-200 family, miR-205 and miR-192) in the urinary sediment of patients with IgA nephropathy (IgAN).

Methods: We studied 43 patients with biopsy-proven IgAN. Urinary expression of miRNAs was determined and compared to that from 13 healthy controls.

Results: The levels of urinary miR-200a, miR-200b and miR-429, but not miR-200c, miR-141, miR-205, or miR-192, were down-regulated in patients with IgAN. Proteinuria significantly correlated with urinary expression of miR-200a ($r = -0.483$, $P < 0.001$), miR-200b ($r = -0.448$, $P = 0.001$) and miR-429 ($r = -0.466$, $P = 0.001$). Baseline renal function significantly correlated with urinary expression of miR-200b ($r = 0.512$, $P < 0.001$) and miR-429 ($r = 0.425$, $P = 0.005$). Urinary gene expression of ZEB2 inversely correlated with miR-200b ($r = -0.321$, $P = 0.017$); and vimentin expression inversely correlated with that of miR-200a ($r = -0.360$, $P = 0.007$), miR-200b ($r = -0.416$, $P = 0.002$) and miR-429 ($r = -0.375$, $P = 0.005$). After 33.4 ± 12.6 months, the rate of renal function decline significantly correlated with urinary expression of miR-200b ($r = 0.316$, $P = 0.034$).

Conclusions: Urinary expression of miR-200a, miR-200b and miR-429 were down-regulated in patients with IgAN, and the degree of reduction correlated with disease severity and rate of progression. The results suggested that these miRNA species might play important roles in the pathophysiology of IgAN. Further studies are needed to clarify the role of urinary miRNA repression as a non-invasive marker of IgAN.

Keywords: Glomerulonephritis, biomarker, proteinuria

1. Introduction

Immunoglobulin A nephropathy (IgAN) is the most common form of primary glomerulonephritis worldwide [1]. The clinical course of IgAN is highly variable. A small proportion of patients may completely recover without treatment, while others progress to

end-stage renal disease (ESRD) rapidly after presentation [2]. Currently, serum creatinine and proteinuria are common prognostic markers of IgAN used by clinicians. However, these markers might fail to accurately predict outcome in the single patient because of the heterogeneity of the disease [3]. Assessment of the degree of glomerular and tubulointerstitial lesions is more precise method to prognose renal outcome [4]. Yet, renal biopsy has potential complications, and repeated monitoring is technically difficult. Reliable non-invasive biomarkers reflecting disease severity and progression are urgently needed in the clinical manage-

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ment of patients with IgAN [5].

Our group and others have demonstrated the feasibility of urinary messenger ribonucleic acid (mRNA) expression in urinary sediment as non-invasive markers for various kidney diseases [6–8]. These previous studies suggest that the detection of urinary nucleic acid holds much promise for noninvasive assessment of renal lesions under pathological situations. Recently, another class of nucleic acid, known as micro RNAs (miRNAs), has attracted much interest. miRNAs are noncoding, single-stranded RNA molecules of about 21 to 23 nucleotides in length, it is involved primarily in the control of gene expression at post transcriptional level and plays important roles in a wide range of physiological and pathological processes [9–11]. Previous studies showed that a number of miRNAs species, such as miR-141 and miR-200b, miR-205 and miR-192, were regulated in renal cells that are undergoing epithelial-to-mesenchymal transition (EMT). These miRNA species may target ZEB1 and ZEB2, major transcription repressors of E-cadherin (a key marker of epithelial cells), and thereby regulate EMT [12–15]. In IgAN, activated tubular epithelial cell may develop EMT and change into activated fibroblast, the main effector of renal fibrosis. For example, the expression of fibroblast-specific protein 1 (FSP1), a mesenchymal marker protein, in tubular epithelial cells has been shown to correlate with renal function and disease progression of IgAN [16]. In this study, we investigated the expression of miR-200 family, miR-205 and miR-192, together with gene expression of molecules in the process of EMT, in the urinary sediment of patients with IgAN. We aim to explore the possibility of using these miRNAs as non-invasive markers for this disease.

2. Patients and methods

2.1. Subjects

We studied 43 consecutive patients with IgAN confirmed by kidney biopsy between 2004 and 2007 in the Prince of Wales Hospital, Hong Kong. Patients with other coexisting renal pathology 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 and mRNA expression study. Clinical data including serum creatinine and 24 hours urine protein were recorded.

Glomerular filtration rate (GFR) was estimated by a standard equation [17]. We also studied the urine from 13 healthy subjects as controls.

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 [18]. 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.

2.2. Sample preparation

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.

2.3. Measurement of miRNA and mRNA levels

MirVana™ 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 100mM dNTPs (with dTTP), 1.5 µl 10x reverse transcription buffer, 1 µl (50U) MultiScribe™ Reverse Transcriptase, 0.19 µl RNase inhibitor (20U/µl) and made up to 15 µl with H₂O. 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 100mM dNTPs (with dTTP), 2 µl 10x reverse transcription buffer, 1 µl (50U) MultiScribe™ Reverse Transcriptase, 1 µl RNase inhibitor (20U/µl) and made up to 20 µl with H₂O. Reverse transcription was performed at 25°C for 10 min-

Table 1
Demographic and baseline clinical data of the subjects

	IgAN	Healthy control
No. of case	43	13
Sex (M:F)	27:16	8:5
Age (year)	48.37 ± 12.64	31.77 ± 4.02
Proteinuria (g/day)	1.13 ± 1.13	0.00
Serum creatinine (μmol/l)	175.07 ± 123.03	—
GFR (ml/min/1.73m ²)	50.44 ± 29.50	—

IgAN, immunoglobulin A nephropathy; GFR, glomerular filtration rate.

utes, 37°C for 120 minutes and 85°C for 5 minutes. The resulting cDNA was stored in −80°C until use.

Urinary expression of microRNA 200 family (miR-200a, miR-200b, miR-200c, miR-141, miR-429), miR-205 and miR-192, together with messenger RNA of ZEB1, SIP1, E-cadherin and vimentin were quantified by RT-QPCR using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Commercially available Taqman primers and probes, including 2 unlabeled PCR primers and 1 FAMTM dye-labeled TaqMan[®] MGB probe were used for all the targets (all from Applied Biosystems). For messenger RNA expression, the primer and probe set was deliberately designed across the intron-exon boundary so as not to detect probable genomic DNA. For RT-QPCR, 10 μl universal master mix, 1 μl primer and probe set, 1.33 μl cDNA and 7.67 μl H₂O were mixed to make a 20 μl reaction volume. 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. β-Glucuronidase (GUSB, Applied Biosystems) and RNU48 (Applied Biosystems) were used as house-keeping genes to normalize the messenger RNA and microRNA expression respectively [19,20]. Results were analyzed with Sequence Detection Software version 2.0 (Applied Biosystems). In order to calculate the differences of expression level for each target among samples, the $\Delta\Delta C_T$ method for relative quantitation was used. Average expression level of normal subjects was used as calibrator for urinary expression and the expression level of targets was a ratio relative to that of the controls.

2.4. 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 molec-

ular 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 200x and scored subjectively from 0 to 100% for each patients. The severity of tubulointerstitial scarring was represented by the mean of ten scores.

2.5. Statistical analysis

Statistical analysis was performed by SPSS for Windows software version 13.0 (SPSS Inc., Chicago, IL). All the results were presented in mean ± 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. We used 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. 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. A P value of below 0.05 was considered statistically significant. All probabilities were two-tailed.

3. Results

The demographic and baseline clinical data of the study subjects were summarized in Table 1. As compared with controls, patients with IgAN had significantly higher level of proteinuria and worse renal function. Histological studies showed that the percentage of glomerulosclerosis and tubulointerstitial scarring of patients were 28.33 ± 26.97% and 25.60 ± 24.06% respectively.

3.1. Levels of miRNAs

The urinary expression of various miRNA species are compared and summarized in Fig. 1. Urinary expression of miR-200a, miR-200b and miR-429 of patients with IgAN was significantly lower than that of controls (miR-200a: 0.22 [0.08–0.65] versus 1.06 [0.50–1.86], $P = 0.002$; miR-200b: 0.48 [0.23–0.89] versus 1.32 [0.48–2.04], $P = 0.049$; miR-429: 0.30 [0.09–0.62] versus 0.85 [0.48–2.50], $P = 0.002$). Urinary expression of miR-200c, miR-141, miR-205 and miR-192 were similar between patients with IgAN and controls (details not shown).

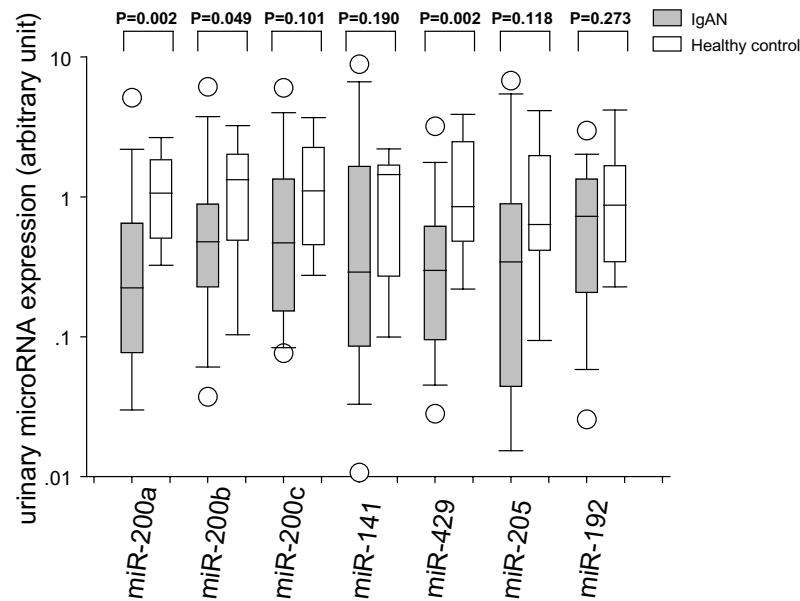


Fig. 1. Comparison of urinary expression of miRNAs between patients with IgA nephropathy (IgAN) and healthy controls. Data are compared by Mann-Whitney U test. Levels are represented as ratio to the average of controls.

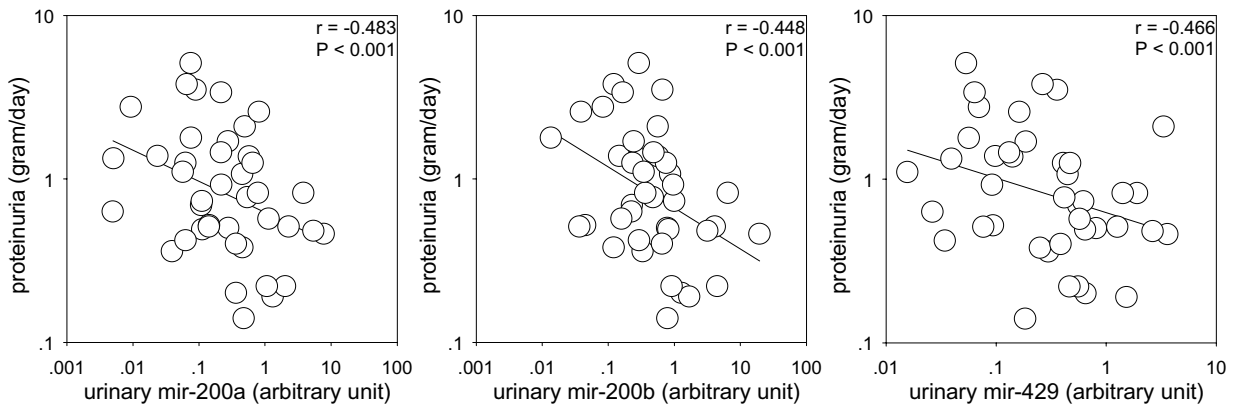


Fig. 2. Relation between proteinuria and urinary expression of (A) miR-200a; (B) miR-200b; and (C) miR-429. Data are compared by Spearman's rank correlation coefficient.

3.2. MicroRNA levels and clinical data

Since urinary expression of miR-200a, miR-200b, and miR-429 were significantly different between cases and controls, we further explore the relation between their levels and clinical parameters. We found that proteinuria significantly correlated with urinary expression of miR-200a ($r = -0.483$, $P < 0.001$), miR-200b ($r = -0.448$, $P = 0.001$) and miR-429 ($r = -0.466$, $P = 0.001$) (Fig. 2). Similarly, GFR significantly correlated with urinary expression of miR-200b ($r = 0.512$, $P < 0.001$) and miR-429 ($r = 0.425$, $P = 0.005$), but not miR-200a ($r = 0.246$, $P = 0.112$)

(Fig. 3). The relation between urinary miRNA expression and the degree of histological damage is summarized in Table 2. In short, the degree of glomerular scar inversely correlated with urinary expression of miR-429 ($r = -0.321$, $P = 0.038$), but not other targets. There was no significant correlation between the degree of tubulointerstitial scar and any of the studied miRNAs (see Table 2).

3.3. MiRNA levels and markers of EMT

Since the miRNA targets we identified may affect the process of EMT, we explore the relation between

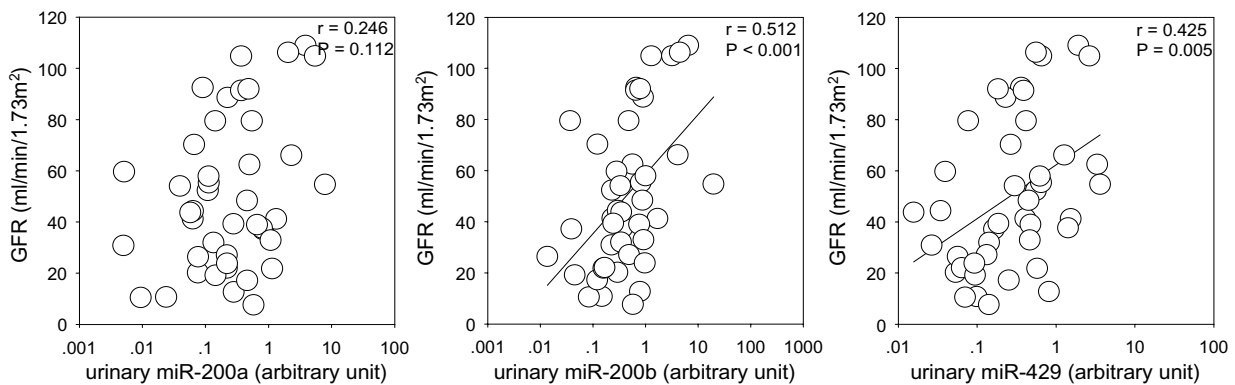


Fig. 3. Relation between baseline estimated glomerular filtration rate (GFR) and urinary expression of (A) miR-200a; (B) miR-200b; and (C) miR-429. Data are compared by Spearman's rank correlation coefficient.

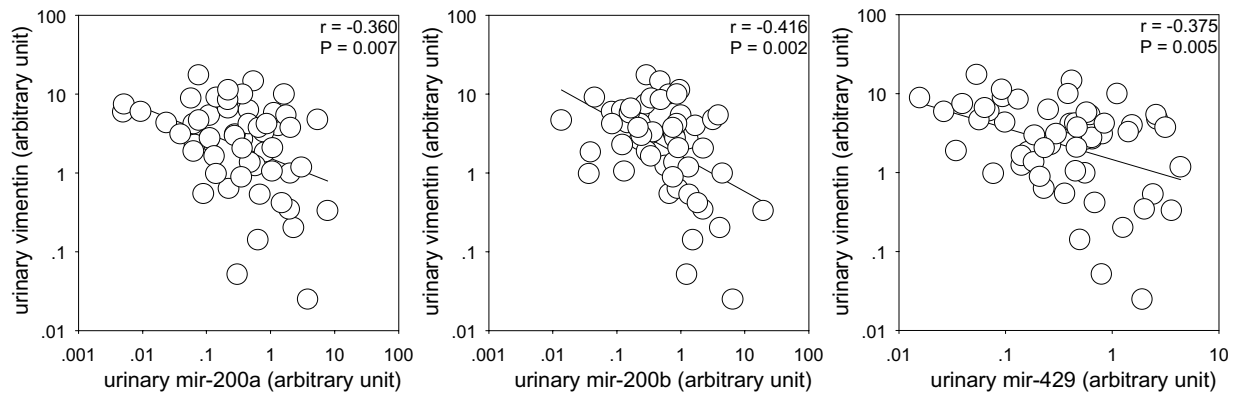


Fig. 4. Relation between urinary expression of vimentin and the expression of (A) miR-200a; (B) miR-200b; and (C) miR-429. Data are compared by Spearman's rank correlation coefficient.

Table 2

Correlations between urinary expression of miRNAs and histological data

	Glomerular scar	Tubulointerstitial scar
miR-200a	$r = -0.080, P = 0.616$	$r = -0.204, P = 0.240$
miR-200b	$r = -0.255, P = 0.103$	$r = -0.283, P = 0.099$
miR-429	$r = -0.321, P = 0.038^*$	$r = -0.236, P = 0.173$

urinary miRNA level and the gene expression of targets related to EMT. We found that urinary gene expression of vimentin inversely correlated with urinary expression of miR-200a ($r = -0.360, P = 0.007$), miR-200b ($r = -0.416, P = 0.002$) and miR-429 ($r = -0.375, P = 0.005$) (Fig. 4). Urinary gene expression of ZEB2 inversely correlated with urinary expression of miR-200b ($r = -0.321, P = 0.017$), but not other targets. Urinary gene expression of E-cadherin and ZEB1, however, did not correlate with urinary expression of any miRNA tested in this study (details not shown).

3.4. MicroRNA levels and renal function decline

The average duration of follow up was 33.4 ± 12.6 months; the average rate of GFR decline was -0.24 ± 0.62 ml/min/month. The rate of GFR decline had a modest but significant correlation with urinary expression of miR-200b ($r = 0.316, P = 0.041$), but not other targets (Fig. 5), indicating that the lower the urinary expression of miR-200b, the faster the renal function decline.

4. Discussion

Although there are many studies investigating urinary protein and mRNA as potential non-invasive markers for kidney diseases, there is no published study on the expression of miRNA in urinary sediment. However, urinary miRNAs have several theoretical advan-

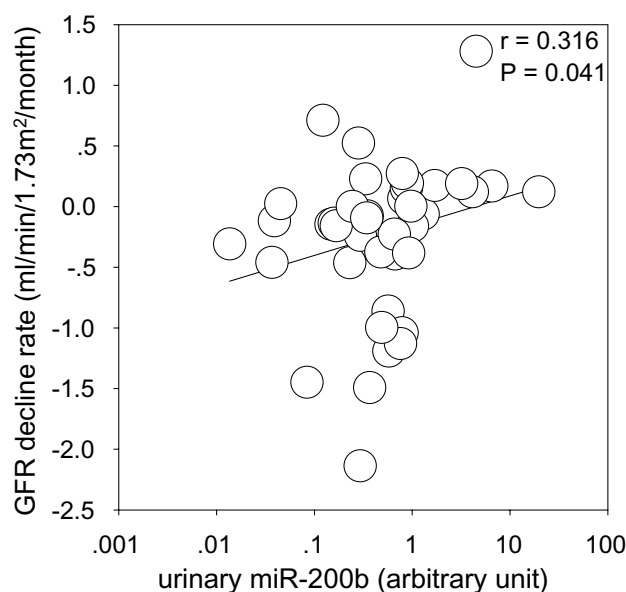


Fig. 5. Relation between the rate of change in estimated glomerular filtration rate (GFR) and urinary expression of miR-200b. Data are compared by Spearman's rank correlation coefficient. Negative change in GFR indicates worsening of renal function.

tages to be used as non-invasive markers for kidney diseases. First, compared to validation method of traditional protein marker, the approaches for global miRNA characterization and quantification (e.g., microarray and RT-QPCR) are more efficient [21]. Secondly, miRNAs have been proved to be more stable and probably more abundant than ordinary mRNAs [22, 23]. Finally, a single miRNA species could regulate the expression of multiple genes, indicating important biological relevance of this pathway [24].

In the present study, we examined the expression levels of miR-200 family, miR-205 and miR-192 in the urine sediment of patients with biopsy-proven IgAN and found that urinary expression of miR-200a, miR-200b and miR-429 of patients with IgAN was significantly lower than that of controls and correlated with proteinuria and renal function. Urinary expression of miR-429 correlated with the degree of glomerular scar and miR-200b correlated with the rate of renal function decline. These results, for the first time, suggest that specific miRNA species in the urinary sediment could reflect the severity and progression of IgAN and therefore have the potential to be used as non-invasive markers.

A number of studies have reported that various intrarenal miRNA species may have specific roles in different kidney diseases [15,25–28]. In the present study, we observed correlations between clinical data and urinary expression of miR-200a, miR-200b and miR-

429, suggesting that these miRNA species may play an important role in the kidney. However, the clinicopathophysiological link between IgAN and urinary expression of miR-200a, miR-200b and miR-429 is not clear, and further studies would be needed to clarify the role of miRNAs in kidney diseases. Available preliminary data suggest that miRNA 200 family can suppress EMT by directly inhibiting E-cadherin repressor: ZEB1 and ZEB2 [12]. In this study, we also found inverse correlations between urinary expression of vimentin and miR-200a, miR-200b and miR-429, but we observed little relation between urinary miRNA expression and E-cadherin, ZEB1 or ZEB2. Nonetheless, the results are consistent with the hypothesis that these miRNA species may have important roles in the prevention of EMT.

There are a few shortcomings 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. The miR-200 family and miR-205 were suggested to be expressed in an epithelial-specific manner and miR-192 expression was previously found in mesangial and tubular epithelial cells [12,15]. Although not directly proved, it seems most possible that urinary miRNAs in the present study are most likely from deciduous tubular epithelial cells and podocytes [29]. Future studies would be necessary to investigate miRNA expression level in specific renal cell type. In this study, we did not examine free

miRNAs in the urine supernatant, which probably has a different physiological implication from the miRNAs in urinary sediment.

Secondly, the present study is only cross-sectional, and it is possible that miRNA expression levels may alter with disease progression and therapy. Future studies are needed to investigate the serial change in urinary expression of miRNAs as the disease progresses or following various therapy strategies.

In summary, we found in the present study that the urinary expression of miR-200a, miR-200b and miR-429 were down-regulated in patients with IgA nephropathy, and the degree of down-regulation correlated with disease severity and rate of progression. The results suggested these miRNAs may play important roles in the pathogenesis and progression of IgA nephropathy, and urinary expression of miRNAs has the potential of further development as non-invasive markers of kidney diseases.

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