Kidney microRNA-21 Expression and Kidney Function in IgA Nephropathy

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Rationale & Objective: Previous studies have suggested that microRNA-21 (miR-21) plays an important role in kidney fibrosis. We examined the relationship between intrarenal miR-21 level and rate of kidney function loss in immunoglobulin A nephropathy (IgAN).

Study Design: Prospective cohort study.

Setting & Participants: 40 patients with IgAN and 10 with hypertensive nephrosclerosis as controls.

Predictors: miR-21 levels in kidney biopsy specimen and urinary sediment, quantified as ratio to the housekeeping gene.

Outcomes: Kidney event–free survival and rate of kidney function decline.

Analytic Approach: Time-to-event and correlation analysis.

Results: The IgAN group had significantly higher intrarenal miR-21 expression compared with the hypertensive nephrosclerosis group (1.71 [IQR, 0.99-2.77] vs 0.31 [IQR, 0.25-1.32]; P < 0.0001), but urinary miR-21 levels were similar. Intrarenal miR-21 expression had significant but modest correlation with severity of glomerulosclerosis (r = 0.293; P = 0.05) and tubulointerstitial fibrosis (r = 0.341; P = 0.03). Patients with high intrarenal miR-21 expression had significantly higher risk for developing kidney end points compared with those with low expression (log-rank test, P = 0.017). Univariate Cox analysis showed that intrarenal miR-21 expression significantly predicted the development of kidney end points (unadjusted HR, 1.586; 95% CI, 1.179-2.134; P = 0.002). However, the result was just short of statistical significance after adjusting for the severity of histologic damage (P = 0.06). There was also a significant correlation between intrarenal miR-21 expression and the slope of kidney function decline by univariate analysis (r = −0.399; P = 0.02).

Limitations: Small sample size; uncertain cellular origin of miR-21.

Conclusions: We found that intrarenal miR-21 expression is increased in patients with IgAN, modestly correlated with the severity of histologic damage, and predictive of subsequent kidney function loss.

Immunoglobulin A (IgA) nephropathy (IgAN) is the most common type of primary glomerulonephritis.1 However, its clinical course can vary greatly. Within 10 years, around one-third of patients with IgAN progress to kidney failure and are dependent on dialysis,2 while many patients remain asymptomatic even with persistently abnormal urinalysis results.3 Unfortunately, the pathophysiologic mechanisms that govern the progression of IgAN are not completely understood.

In the past decade, the role of microRNA (miRNA) in the pathogenesis of chronic kidney disease (CKD) has been increasingly recognized.4,5 As the most extensively studied member of noncoding RNAs, miRNAs play a pivotal role in the regulation of gene expression.4,6,7 Urinary miRNA levels are associated with the degree of diabetic damage and rate of kidney function deterioration in diabetic kidney disease,8 hypertensive nephrosclerosis (HTN),9 and other nondiabetic CKDs.5 Numerous in vitro and animal studies have also shown that several miRNA targets are responsible for the regulation of kidney fibrosis.10,11

Among all miRNAs, miR-21 attracts the most attention from nephrologists. Analyses of miRNA expression profiles have shown that miR-21 is mostly expressed in the adult human kidney.12,13 In vitro studies have shown that transforming growth factor β1 (TGFβ1) directly increases mesangial miR-21 expression, which inhibits the tissue level of phosphatase and tensin homologue (PTEN), which in turn leads to activation of the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway, resulting in mesangial hypertrophy and accumulation of extracellular matrix.14,15 Moreover, a positive feedback loop between TGFβ1 and miR-21, through the miR-21 target Smad7 in mesangial cells, has been shown as a possible mechanism that amplifies TGFβ1 signaling during kidney fibrosis.16 In patients with IgAN, Hennino et al17 showed that intrarenal miR-21 expression is associated with fibrosis and kidney survival. However, only 24% of patients in this study were treated with inhibitors of the renin-angiotensin system. It remains uncertain whether tissue miR-21 level is associated with the rate of kidney function deterioration in patients with IgAN who receive state-of-the-art therapy.

METHODS

This study was approved by The Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee (reference number CREC-
Previous studies have suggested that microRNA-21 (miR-21) plays an important role in kidney fibrosis but that the effect may be disease specific. We measured intrarenal and urinary miR-21 levels in patients with immunoglobulin A nephropathy (IgAN) and those with hypertensive nephrosclerosis (HTN) and found that those with IgAN had significantly higher intrarenal miR-21 expression than those with HTN, but their urinary levels were similar. Intrarenal miR-21 level also correlated with the severity of histologic damage and, with univariate analysis, predicted the rate of subsequent kidney function decline. However, this prognostic role became insignificant after adjusting for the degree of histologic damage in multivariate analysis, further supporting the role of intrarenal miR-21 in the pathogenesis of progressive kidney damage in IgAN.

2019.363). All study procedures were in compliance with the Declaration of Helsinki.

Overall Study Design
We recruited 40 adult patients with biopsy-confirmed IgAN and 10 adults with biopsy-proved HTN as controls. All biopsy specimens had at least 10 glomeruli and 5 mm of cortex on histologic sections. We excluded patients with crescentic changes in the kidney biopsy specimen, IgA vasculitis, or concomitant urinary tract infection. After written informed consent, we retrieved the fresh frozen kidney biopsy specimen of the patient for RNA extraction. A whole-stream early morning urine specimen was also collected for RNA extraction. In addition, we reviewed patients’ demographic and clinical information, which included kidney function, proteinuria, and rate of glomerular filtration rate (GFR) decline as calculated using the least-squares method. Estimated GFR was calculated using the CKD Epidemiology Collaboration (CKD-EPI) equation and monitored every 4 months.

miRNA Extraction and Quantification
The RNA isolation kit was purchased from Ambion, Inc. The methods of RNA extraction have been described previously. Briefly, ten 10-μm sections were cut from the fresh frozen tissue blocks of the residual sample from the kidney biopsy using a microtome, pooled in a 1.5-mL microcentrifuge tube, and homogenized and dissolved in digestion buffer. Urine samples were centrifuged at 3,000g for 30 minutes and 13,000g for 5 minutes at 4 °C. Specimens were then stored at −80 °C until use.

Tissue and urinary miR-21 levels were quantified using real-time quantitative polymerase chain reaction (RT-QPCR) using the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Commercially available Taqman primers and probes, including 2 unlabeled PCR primers and 1 FAM dye-labeled TaqMan MGB probe, were used (all from Applied Biosystems). RNU48 (Applied Biosystems) was used as housekeeping genes. All RT-QPCR experiments were performed in triplicate. Results were analyzed using Sequence Detection Software, version 2.0 (Applied Biosystems). The ΔΔCT method for relative quantitation was used. Urinary miR-21 level represents the total amount excreted in an 8-hour overnight urine specimen, as compared with that of the housekeeping gene.

Assessment of Kidney Pathology
Kidney biopsy specimens were reviewed by a single experienced pathologist (FMML) and histologic lesions were classified using the revised Oxford classification. The severity of kidney fibrosis was further assessed semi-quantitatively by experienced pathologists who were blinded to results of molecular studies, as described previously. In brief, 4-μm paraffin-embedded sections were stained by Jones silver stain. The severity of glomerulosclerosis was represented by the percentage of sclerotic glomeruli in total glomeruli obtained from the biopsy

### Table 1. Demographic and Baseline Clinical Data of the IgAN and HTN Groups

| Group          | No. of patients | Sex (M:F) | Age, y ± SD | Blood pressure, mm Hg | Estimated GFR, mL/min/1.73 m² | Kidney scar, % |
|----------------|-----------------|-----------|-------------|------------------------|-------------------------------|---------------|
| **Group**      | **IgAN**        | **HTN**   |             |                        |                               |               |
| No. of patients | 40              | 10        |             |                        |                               |               |
| Sex (M:F)      | 14:26           | 5:5       | 45.0 ± 13.6 | 44.9 ± 15.1            | 0.98 b                       |               |
| Systolic       | 124.0 ± 14.8    | 124.4 ± 5.7|             |                        |                               |               |
| Diastolic      | 87.5 ± 13.2     | 87.4 ± 7.8|             |                        |                               |               |
| Proteinuria, g/d | 1.29 (0.87-2.85) | 0.67 (0.00-2.32) | 0.70 b | 61.1 (37.7-81.4) | 26.8 (5.2-40.8) | 0.006 b |
| Kidney scar, % |                 |           |             |                        |                               |               |
| Glomerulosclerosis | 20.7 (7.1-29.8) | 40.0 (32.5-43.8) | 0.008 b | 12.5 (5.0-31.3) | 32.5 (30.0-35.0) | 0.001 b |
| Tubulointerstitial fibrosis | 18 (45.0%) | — |             |                        |                               |               |
| Oxford classification, no. of cases (%) | Mesangial hypercellularity 15 (37.5%) | — |             |                        |                               |               |
| Endocapillary hypercellularity 4 (10.0%) | — |             |                        |                               |               |
| Segmental glomerulosclerosis 18 (45.0%) | — |             |                        |                               |               |
| Tubular atrophy/interstitial fibrosis | Mild 11 (27.5%) | — |             |                        |                               |               |
| Moderate to severe 9 (22.5%) | — |             |                        |                               |               |
| Cellular/fibrocellular crescents 1 (2.5%) | — |             |                        |                               |               |

Note: Data are expressed as mean ± standard deviation or median [interquartile range].
Abbreviations: GFR, glomerular filtration rate; HTN, hypertensive nephrosclerosis; IgAN, immunoglobulin A nephropathy. Data compared using χ² test and Student’s t test.
specimen. For tubulointerstitial scarring, 10 microscopic fields were viewed at original magnification ×200 and scored subjectively from 0% to 100% for each patient. The severity of tubulointerstitial scarring was represented by the mean of 10 scores.

Clinical Outcome
After recruitment, the planned follow-up duration was 60 months. Clinical management was decided by individual nephrologists and not affected by the study. All patients were treated with the maximally tolerated dose of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, together with antihypertensive therapy with a target blood pressure <130/90 mm Hg. None of the patients received corticosteroid or immunosuppressive therapy. The primary end point was kidney event–free survival; kidney event was defined as 30% decrease in baseline GFR, need of long-term dialysis, or death from any cause. The secondary end point was the slope of GFR decline, which was calculated using the least-squares method.25,26

Statistical Analysis
Statistical analysis was performed using SPSS for Windows software, version 24.0 (IBM Corp). Data were expressed as mean ± standard deviation or median (interquartile range [IQR]), as appropriate. miRNA levels were compared using Mann-Whitney U test between groups, and correlation with clinical parameters was explored using Spearman rank correlation coefficient. P < 0.05 was considered statistically significant. All probabilities were 2 tailed.

RESULTS
Baseline clinical and demographic characteristics of patients are summarized in Table 1. All patients in the IgAN group received the maximally tolerated dose of angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker. The raw data for QPCR results are available in Table S1.

Relation With Clinical and Pathologic Parameters
The IgAN group had significantly higher intrarenal miR-21 expression as compared with the HTN group (1.71 [IQR, 0.99-2.77] vs 0.31 [IQR, 0.25-1.32]; P < 0.001) but their urinary miR-21 levels were similar (8.62 [IQR, 7.77-9.60] vs 9.45 [IQR, 7.65-9.69]; P = 0.30; Fig 1). For the entire study cohort, there was no significant correlation between intrarenal and urinary miR-21 levels (Spearman r = 0.211; P = 0.15). However, there was a highly significant correlation between intrarenal and urinary miR-21 levels in the HTN group (r = 0.831; P = 0.006), but not the IgAN group (r = 0.259; P = 0.11).

Intrarenal miR-21 expression had significant but modest correlation with the severity of glomerulosclerosis (r = 0.293; P = 0.05) and tubulointerstitial fibrosis (r = 0.341; P = 0.03). However, there was no significant correlation between intrarenal miR-21 expression and proteinuria (r = 0.102; P = 0.50) or baseline estimated GFR (r = 0.215; P = 0.14). There was also no significant association between intrarenal miR-21 expression and the presence of mesangial hypercellularity, endocapillary proliferation, segmental sclerosis, or fibrocellular crescent in the kidney biopsy specimen. In contrast, urinary miR-21 level had modest correlations with baseline GFR (r = −0.311; P = 0.05), severity of tubulointerstitial fibrosis (r = 0.361; P = 0.03), and glomerulosclerosis (r = 0.306; P = 0.05), although the last correlation did not reach statistical significance. Urinary miR-21 level did not correlate with degree of proteinuria (r = 0.263; P = 0.11).
Relation With Kidney Function Loss

The IgAN group was followed up for 65.0 (IQR, 52.6-101.2) months, with a total 714 estimated GFR measurements (average, 17.9 ± 14.1 times for each patient). During the follow-up period, 13 patients developed the kidney end point; no patient died. Patients with high intrarenal miR-21 expression had significantly higher risk for developing the kidney end point as compared with those with low expression (log-rank test, \( P = 0.02 \); Fig 2). By univariate Cox regression analysis, intrarenal miR-21 expression also significantly predicted the development of the kidney end point (unadjusted hazard ratio [HR], 1.586; 95% CI, 1.179-2.134; \( P = 0.002 \)), although the relation between urinary miR-21 level and kidney end point did not reach significance (unadjusted HR, 1.669; 95% CI, 0.958-2.908; \( P = 0.07 \)). After adjusting for the severity of glomerulosclerosis and tubulointerstitial fibrosis, there remained a relationship between intrarenal miR-21 expression and risk for developing the kidney end point, but the relationship did not reach statistical significance (adjusted HR, 1.841; 95% CI, 0.986-3.438; \( P = 0.06 \)). Elaborated multivariate Cox regression analysis was not performed in view of the small number of events.

The median rate of GFR decline was \(-1.27 \) (IQR, \(-0.15 \) to \(-6.69 \)) mL/min/1.73 m² per year. There was a significant correlation between intrarenal miR-21 expression and the slope of GFR decline (Spearman \( r = -0.399 \); \( P = 0.02 \)). However, the correlation became insignificant after adjusting for the severity of glomerulosclerosis and tubulointerstitial fibrosis (adjusted rate of GFR decline, \(-0.512 \); 95% CI, \(-2.218 \) to \(1.193 \) mL/min/1.73 m² per year; \( P = 0.50 \)). Urinary miR-21 level did not correlate with the slope of GFR decline (\( r = -0.197 \); \( P = 0.31 \)).

**DISCUSSION**

In this study, we showed that intrarenal, but not urinary, miR-21 expression is significantly increased in patients with IgAN as compared with those with HTN despite a similar degree of decreased kidney function. In the IgAN group, intrarenal miR-21 expression had a modest correlation with severity of histologic damage and was predictive of subsequent kidney function loss.

In essence, our results are in line with the report by Hernino et al,\(^{17} \) which showed that intrarenal miR-21 expression is associated with fibrosis and kidney survival in IgAN. However, there was no control group in that previous study,\(^{17} \) and it was possible that intrarenal miR-21 expression was a nonspecific indicator of renal fibrosis. Our present study found that intrarenal miR-21 expression was increased in IgAN but not simple HTN despite a similar degree of kidney scarring, indicating that miR-21 expression is disease specific rather than a generic indicator of fibrosis.

However, it must be emphasized that we did not prove a diagnostic role of intrarenal miR-21 for IgAN; we simply showed that intrarenal miR-21 is not upregulated in every cause of renal fibrosis. The available literature shows that miR-21 is upregulated in a variety of kidney diseases, including (in addition to IgAN) diabetic nephropathy, interstitial disease, and mouse unilateral ureteric obstruction model.\(^ {27-32} \) Although the rate of kidney function decline may not be linear in advanced CKD, the simple linear approximation used in our study should be sufficient for patients with mild to moderate decreased kidney function.\(^ {25,26} \)

There was a marked difference in baseline kidney function between the IgAN and HTN groups, which is an inevitable consequence of the bias in selecting patients with kidney biopsy. Patients with microscopic hematuria and proteinuria were more likely to undergo kidney biopsy despite normal kidney function, whereas patients with hypertension and isolated proteinuria would only undergo kidney biopsy if there was substantial kidney function loss. However, we observed significantly higher intrarenal miR-21, which has a profibrotic property, in the IgAN group compared with the HTN group, suggesting that there was disease-specific and early upregulation of intrarenal miR-21 expression in IgAN.

In our present study, we did not distinguish glomerular from tubulointerstitial expression of miR-21. It is well recognized that miRNA expression is not uniform in the kidneys but varies across glomeruli and tubules, cortex, and medulla. The method we used could not differentiate the tissues that express miR-21 and may confound the

![Figure 2. Kaplan-Meier plot of kidney event–free survival of the immunoglobulin A nephropathy group. Patients were divided into high and low intrarenal microRNA-21 (miR-21) levels as compared with the median level. Data are compared using log-rank test.](image-url)
result. A previous report by Bao et al. suggested that miR-21 was upregulated in both glomerular and tubular interstitial tissues of patients with IgAN, but little is known regarding the pathogenic role of miR-21 in individual nephron segments. Although inhibition of miR-21 prevents fibrogenic activation in both podocytes and tubular cells in IgAN, transfection with miR-21 mimic to kidney tubular cells does not affect the endogenous megalin, cubulin, or aquaporin 1 messenger RNA expression. However, Xu et al. showed that in patients with IgAN, miR-21 expression is upregulated in T lymphocytes, which might induce T-helper cell 17 polarization and favor IgA1 overproduction. Taken together, the available evidence suggests that glomerular (either in podocytes or mesangial cells) rather than tubulointerstitial miR-21 expression is an important mediator of progressive glomerulosclerosis and kidney function loss in IgAN. This notion is further supported by the observation that despite a similar degree of renal fibrosis, the IgAN group had significantly higher intrarenal miR-21 expression than the HTN group. However, further studies are needed to further clarify the cellular origin of miR-21 and its downstream mechanisms. Because therapeutic silencing of miR-21 is a foreseeable reality, it would be important to test whether miR-21 inhibition could reduce the rate of disease progression of IgAN.

One intriguing observation we made was the lack of correlation between intrarenal and urinary miR-21 levels in the IgAN cohort but a correlation in HTN controls. Because of the small sample size, it is entirely possible that the apparently strong correlation is a type 1 statistical error. However, our result may suggest that in hypertension, urinary loss of miR-21 is a surrogate of intrarenal miR-21 level, whereas in the scenario of IgAN, intrarenal miR-21 is specifically activated (eg, in a different tissue compartment within the kidney) and this upregulated portion is not excreted into urine. This hypothesis would need to be confirmed by further studies.

In our present study, urinary miR-21 level had weak correlations with baseline kidney function and histologic scarring but not progression of kidney function loss. The result is consistent with previous reports from our group and others. Taken together, urinary miR-21 does not appear to be a promising biomarker for IgAN. Nonetheless, the alteration in urinary miR-21 level may be disease specific. Other studies have reported that urinary miR-21 level may have prognostic value in hypertensive kidney injury and kidney allograft recipients.

The major limitation of study was the small sample size. As an exploratory study, the original sample size was based on a 4-fold difference in miR-21 expression as compared with the control group (and our result revealed an ~6-fold difference). Nonetheless, as an outcome study, a total of 13 events was very low and it was not possible to perform an elaborated multivariate analysis to determine an independent prognostic effect of intrarenal miR-21. Specifically, we did not take into account several established clinical factors that predict kidney function decline, including baseline kidney function, blood pressure, and proteinuria. Because intrarenal miR-21 expression correlates with the severity of histologic damage and the prognostic effect of intrarenal miR-21 became insignificant after adjusting for histologic parameters, our results suggest that intrarenal miR-21 contributes to the pathogenesis of chronic kidney damage in IgAN.

In addition to the small sample size, lack of other glomerular diseases as control, and not distinguishing glomerular from tubulointerstitial miR-21 expression, there are several other limitations to our study. The degree of glomerulosclerosis and tubulointerstitial fibrosis were quantified semi-quantitatively, although it would be ideal to have fully quantitative assessment of renal fibrosis (eg, by morphometric study of Picro Sirus Red or silver-stained histologic sections). Also, >70% of our patients had little renal fibrosis (ie, T score of zero by the Oxford classification) and it could be argued that the role of miR-21 may be limited to patients at the early stage of disease. Unfortunately, it is practically difficult to obtain a kidney biopsy from patients with IgAN with moderate to several renal fibrosis because the kidneys are often shrunken. In this study, we excluded patients with a crescentic form of IgAN, which may introduce selection bias by excluding the most severe group of the disease. Nonetheless, crescentic IgAN is difficult to distinguish from Henoch-Schönlein purpura and parainfectious glomerulonephritis, which would make the result of the gene expression study difficult to interpret. Last, intrarenal and urinary miR-21 levels in this study were not quantified in absolute terms but represented as the ratio to the expression level of housekeeping gene. Although this approach is suitable for hypothesis exploration, batch-to-batch and interlaboratory comparisons are difficult and it is not possible to derive a “cutoff” expression level that allows clinical use for patient risk stratification.

In summary, we found that intrarenal miR-21 expression is increased in patients with IgAN and is predictive of subsequent kidney function loss. Our results support the role of miR-21 in progressive renal fibrosis in IgAN. Further studies are needed to determine the cell origin and molecular pathways involved in miR-21-related renal fibrosis and explore the therapeutic value of miR-21 silencing.

**SUPPLEMENTARY MATERIAL**

**Supplementary File (PDF)**

Table S1: Raw data of real-time quantitative polymerase chain reaction (RT-QPCR).

**ARTICLE INFORMATION**

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Do higher miR-21 levels in the kidney and urine predict worse outcomes in IgA nephropathy?

**Conclusion:** Intrarenal miR-21 expression is increased in patients with IgAN, had modest correlation with the severity of histological damage, and is predictive of subsequent kidney function loss.

**Reference:** Szeto CC, Ng JK, Fung WWS, et al. Kidney microRNA-21 expression and kidney function in IgA nephropathy. *Kidney Medicine*, 2021.

**Visual Abstract by Caitlyn Vlaescheart, MD, MSc**