

Circulating MiR-29a, Possible Use as a Biomarker for Monitoring IgA Nephropathy

Haofei Hu,¹⁻⁵ Qijun Wan,¹⁻⁵ Tong Li,¹⁻⁵ Dongli Qi,¹⁻⁵ Xu Dong,¹⁻⁵ Yi Xu,¹⁻⁵ Hongtao Chen,¹⁻⁵ Hongping Liu,¹⁻⁵ Haogui Huang,¹⁻⁵ Cuimei Wei,¹⁻⁵ Wenxiong Zhou,¹⁻⁵ Shilun Jiang,¹⁻⁵ Zihe Mo,¹⁻⁵ Fupeng Liao,¹⁻⁵ Qitao Xu,¹⁻⁵ Yongcheng He,¹⁻⁵

¹Department of Nephrology, The First Affiliated Hospital of Shenzhen University, Shenzhen, Guangdong Province, China

²Department of Nephrology, The Affiliated Shenzhen Second People's Hospital of Anhui Medical University, Shenzhen, Guangdong Province, China

³Department of Nephrology, The Affiliated Shenzhen Second People's Hospital of Guangzhou Medical University, Shenzhen, Guangdong Province, China

⁴Department of Nephrology, Shenzhen Second People's Hospital, Shenzhen, Guangdong Province, China

⁵Center for Nephrology and Urology Shenzhen University, Shenzhen University Health Science Center, Shenzhen, Guangdong Province, China.

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Introduction. Previous studies have shown that TGF- β 1/Smad3 signaling promotes renal fibrosis by inhibiting miR-29. To date, only few studies have reported on circulating microRNAs in IgA nephropathy (IgAN). However, the plasma expression of miR-29a and its role in patients with IgAN remains unclear. In this study, we attempted to elucidate whether plasma miR-29a expression can be used as a biomarker for monitoring disease states.

Methods. For this study, 15 healthy subjects, 36 patients with untreated renal biopsy-proven IgAN, and 79 patients with IgAN, who were under treatment for a period of 1 year on an average, all of whom had similar age and gender distributions, were included. The plasma expression of miR-29a in each group was explored by real-time PCR, and the relationship between miR-29a expression and clinical, pathological, and prognostic indicators of IgAN was further evaluated.

Results. Relative plasma expression of miR-29a in patients with IgAN was significantly lower than that in healthy controls ($P < .001$), and these changes in plasma miR-29a could be suppressed by treatment ($P < .05$). Plasma miR-29a was positively correlated with eGFR and negatively correlated with proteinuria and serum creatinine, irrespective of whether or not the patients with IgAN accepted treatment ($P < .05$). Plasma miR-29a level was negatively correlated with primary pathological parameters such as crescent formation, Lee's and Oxford classification ($P < .05$). Kaplan–Meier analysis revealed that patients with high plasma expression of miR-29a had better renal function and better response to treatment compared to those with low expression ($P < .05$).

Conclusion. Plasma miR-29a could be considered as a biological marker that reflects renal damage and function, to predict the progression of IgAN.

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INTRODUCTION

Globally, IgA nephropathy (IgAN) is one of the most common glomerulonephritis, accounting for more than 50% of biopsy proven primary glomerulonephritis in Asia.¹ In fact, 15% to 40%

of patients with IgAN confirmed by biopsy would progress to end-stage renal disease (ESRD) within 10 to 20 years.^{2,3} Currently, renal function and proteinuria are common prognostic markers for IgAN used by clinicians. However, due to the

heterogeneity of the disease, these markers may not accurately predict the prognosis of individual patient.⁴ miRNAs are involved in almost every cellular process, and dysregulation of miRNAs is associated with many human diseases, including chronic kidney disease (CKD).^{5,6}

Previous studies have shown that TGF- β 1/Smad3 signaling promotes renal fibrosis by inhibiting the miR-29 family.⁷ The expression of mouse miR-29 decreases with the development of progressive renal fibrosis in obstructive nephropathy. In contrast, Smad3 knockout mice have increased expression of miR-29 and loss of renal fibrosis in the same occlusive model.⁸ A recent study explored urinary expression of miR-29 family in patients with IgAN. In this study, although the IgAN group had significantly lower level of urinary miR-29b and miR-29c when compared with healthy controls, the urinary miR-29a level was not altered significantly.⁹ Additionally, the plasma expression of miR-29a and its role in patients with IgAN remains unclear; therefore, it is necessary to assess the response of miR-29a to treatment. In the present study, we used real-time PCR to explore plasma and urinary expression of miR-29a and the relationship between these expressions a clinical, pathological, and prognostic indicators in IgAN. Furthermore, we aimed to elucidate whether plasma miR-29a expression could be used as a biological marker to predict the outcome of patients with IgAN and monitor the disease status.

MATERIALS AND METHODS

Subjects

For this study, we recruited 15 healthy subjects and 36 patients with untreated renal-biopsy proven IgAN with matched age and gender between March 2013 and November 2013 at the Second People's Hospital (Table 1). Blood and urine samples were collected at the time of performing renal biopsy. Patients with untreated IgAN displayed significantly elevated serum creatinine levels, increased proteinuria, and decreased eGFR (Table 1). Of these patients, 19 cases decided to receive treatment at our hospital for 3 months. Then blood and urine samples were collected. After treatment, patients with IgAN showed decreased serum creatinine levels and proteinuria and increased eGFR (Table 2). Further, to understand the relationship between plasma and urinary

Table 1. Basic Clinical Parameters of Healthy Subjects and Untreated IgAN Patients

Variable	Healthy Subjects (n = 15)	IgAN Patients (n = 36)
Age, year	34.93 \pm 1.409	34.94 \pm 1.577
Gender (Male), n (%)	5 (33.3)	16 (44.4)
Serum Creatinine, μ mol/L	64 \pm 2.217	141.9 \pm 12.66*
eGFR	119.7 \pm 3.179	64.69 \pm 6.235*
BUN, mmol/L	6.673 \pm 2.466	6.381 \pm 0.522

eGFR, evaluated glomerular filtration rate ($\text{mL}/\text{min}^{-1}/(1.73 \text{ m}^2)^{-1}$).

Data are presented at mean \pm SEM.

* $P < 0.001$, Mann-Whitney test (one-tail)

Table 2. Pre- and Post-treatment Clinical Parameters in IgAN Patients (n = 19)

Variable	Pre-treatment	Post-treatment
Serum creatinine, μ mol/L	138.20 \pm 17.29	116.10 \pm 12.61*
eGFR	63.80 \pm 8.30	70.61 \pm 7.13*
24 h mTP, mg/24h	2042.00 \pm 500.90	922.00 \pm 209.60**
SBP, mmHg	135.70 \pm 3.78	125.40 \pm 2.42**
DBP, mmHg	83.95 \pm 3.10	76.63 \pm 2.24*
Serum Albumin, g/L	35.81 \pm 1.48	39.58 \pm 1.63***

eGFR, evaluated glomerular filtration rate ($\text{mL}/\text{min}^{-1}/(1.73 \text{ m}^2)^{-1}$);

SBP, systolic blood pressure; DBP, diastolic blood pressure.

Data are presented at mean \pm SEM.

* $P < .05$, ** $P < .01$, *** $P < .001$, Mann-Whitney test (one-tail)

expression of miR-29a and progression of the disease, 79 patients with biopsy proven IgAN, who were undergoing treatment, were enrolled into our study during the same period. The initial dosage of MMF was 1.0 g/d (body weight < 50 kg) or 1.5 g/d (body weight > 50 kg). The dosage was reduced to 0.75~1.0 g/d after 6 months of treatment, the maintenance dosage was 0.5~0.75 g/d after 12 months. The initial oral dosage of prednisone was 0.8 mg/kg/d, the dosage was reduced regularly. The total course of treatment lasted for at least 12 months. On an average, these patients were treated for 1 year during the time of collection of plasma and urine samples. Written informed consent was obtained from each patient and healthy subjects. Ethnic committee of the First Affiliated Hospital of Shenzhen University approved this study. Clinical and pathological data, including serum creatinine, 24 hours urine protein, hemoglobin, albumin, blood pressure, glomerulosclerosis, mesangial cell proliferation, endothelial cell proliferation, segmental glomerular sclerosis, tubular atrophy/interstitial fibrosis, and crescent were recorded. Evaluated glomerular filtration rate (eGFR) was estimated using a standard equation.¹⁰ All patients

were followed up every 3 months until the study's endpoint or deadline time. The deadline of the study was June 30, 2016. The primary outcome was therapeutic effect with complete remission, which was defined as a value for urinary protein excretion that was below 0.3 g/24h and a normal serum creatinine (Scr) level. The second outcome was a composite endpoint, which included ESRD or 50% decrease in eGFR.

Sample Processing

A whole-stream early morning urine sample and 5 ml whole blood was collected from each patient for performing urinary and plasma miR-29a expression study. Blood and urine samples were stored at 4°C and processed within 5h after collection. The whole blood and urine specimens were centrifuged at 3000 g for 30 min at 4°C. The plasma and urine supernatant aliquots were then transferred into Eppendorf tubes and centrifuged at 12,000 g for 10 min at 4°C and stored at 80° C until use.

RNA Extraction

Plasma and urinary RNA was extracted using the mirVana PARIS Kit (Ambion, Foster City, CA, USA) based on the manufacturer's instructions. Briefly, 500 µL of serum from each sample was mixed with an equal volume of "2 × denaturing solution", and total RNA was eluted in 100 µL of RNase-free water. Total RNA from cultured cells was isolated using RNAiso Plus (TaKaRa, Otsu, Japan) according to the manufacturer's protocol.

Measurement of MiR-29a Level

The S-Poly (T) miRNA qPCR-assay (Cat. No. AB-MAS100001-AB-MAS86581) was used for reverse transcription (RT) and RT-QPCR. For miR-29a, 5.5 µL tailed RNA was mixed with 2.5 µL of 4 × reaction buffer mix, 1.0 µL of PolyA/RT enzyme mix, 0.5 µL miRNA RT primer (1 µM), 0.5 µL of internal control RT primer (1 µM), and H₂O (to a total volume of 10 µL). The reaction conditions were 42°C for 60 min and 75°C for 10 min, after which samples were quickly placed on ice for 2 min. Samples were diluted 4-15 times by adding 30-140 µL of water. In the 20 µL PCR, 8 µL was used as the template.

Urinary and plasma miR-29a were quantified using RT-qPCR according to the RT-qPCR Kit instructions. RNU48 (Applied Biosystems, Waltham,

MA, USA) was used as a housekeeping gene to normalize microRNA expression.¹⁹ Results were analyzed using Sequence Detection Software version 2.0 (Applied Biosystems).

Assessment of Pathological Data

Analysis of renal pathological damages was performed using 4 µm paraffin-embedded sections stained with periodic acid-Schiff (PAS) or Jones silver stain. An experienced pathologist who was blinded to the results of molecular studies scored the severity of renal pathological damage subjectively. The severity of glomerulosclerosis and crescent formation was estimated as the percentage of sclerotic glomeruli and crescents in total glomeruli obtained from biopsy; Lee's classification and MEST classification, which included mesangial cell proliferation, endothelial cell proliferation, segmental glomerular sclerosis, and tubular atrophy/interstitial fibrosis were also used to assess the severity of renal pathological damage.

Statistical Analysis

Statistical analysis was performed by SPSS for Windows software version 17.0. However, because the data of miRNA expression levels were highly skewed, either log transformation or nonparametric statistical methods were used. For data description, normally distributed quantitative variables were expressed as mean ± SD. For non-normally distributed variables, medians and interquartile ranges were used. Categorical data were summarized as absolute frequencies and percentages. The normal variables were compared using *t*-test for unpaired data, if skewed variables were compared by rank-sum test, and the discontinuous variables were tested for trend by the χ^2 test. Pearson's correlation for the normal variables or Spearman's correlation for skewed variables was used to assess associations between the study parameters. For survival analysis, Cox proportional hazards models were used. The impact of plasma and urinary miR-29a levels on predicting IgAN progression was assessed by the ROC curve. A one-tailed *P* value < .05 was considered statistically significant.

RESULTS

The basic clinical parameters of healthy subjects and untreated IgAN patients are summarized in Table 1. Pre- and post-treatment clinical

parameters in patients with IgAN are summarized in Table 2. Patients with untreated IgAN displayed significantly elevated serum creatinine levels, increased proteinuria, and decreased eGFR (Table 1). After treatment, IgAN patients showed decreased serum creatinine levels and proteinuria and increased eGFR (Table 2).

Alteration of Levels of Urinary and Plasma MiR-29a

Urine and plasma expression of miR-29a was compared and the results are summarized in Figure 1. Plasma relative expression of miR-29a of patients with IgAN was significantly lower than

that of controls (miR-29a (A): 3.33 ± 0.45 versus 8.49 ± 1.26 , $P < .001$); urinary expression of miR-29a did not differ significantly between the IgAN group and healthy control group (miR-29a (B): 0.56 ± 0.08 versus 0.70 ± 0.22 , $P > .05$). This result suggests that it is possible for plasma miR-29a to be a biological marker for monitoring IgAN. However, it is not yet clear that the altered plasma level of miR-29a could be the cause or consequence of renal damage in IgAN.

Were Plasma and Urinary MiR-29a Levels Responding to Treatments?

Treatment is effective in lowering serum

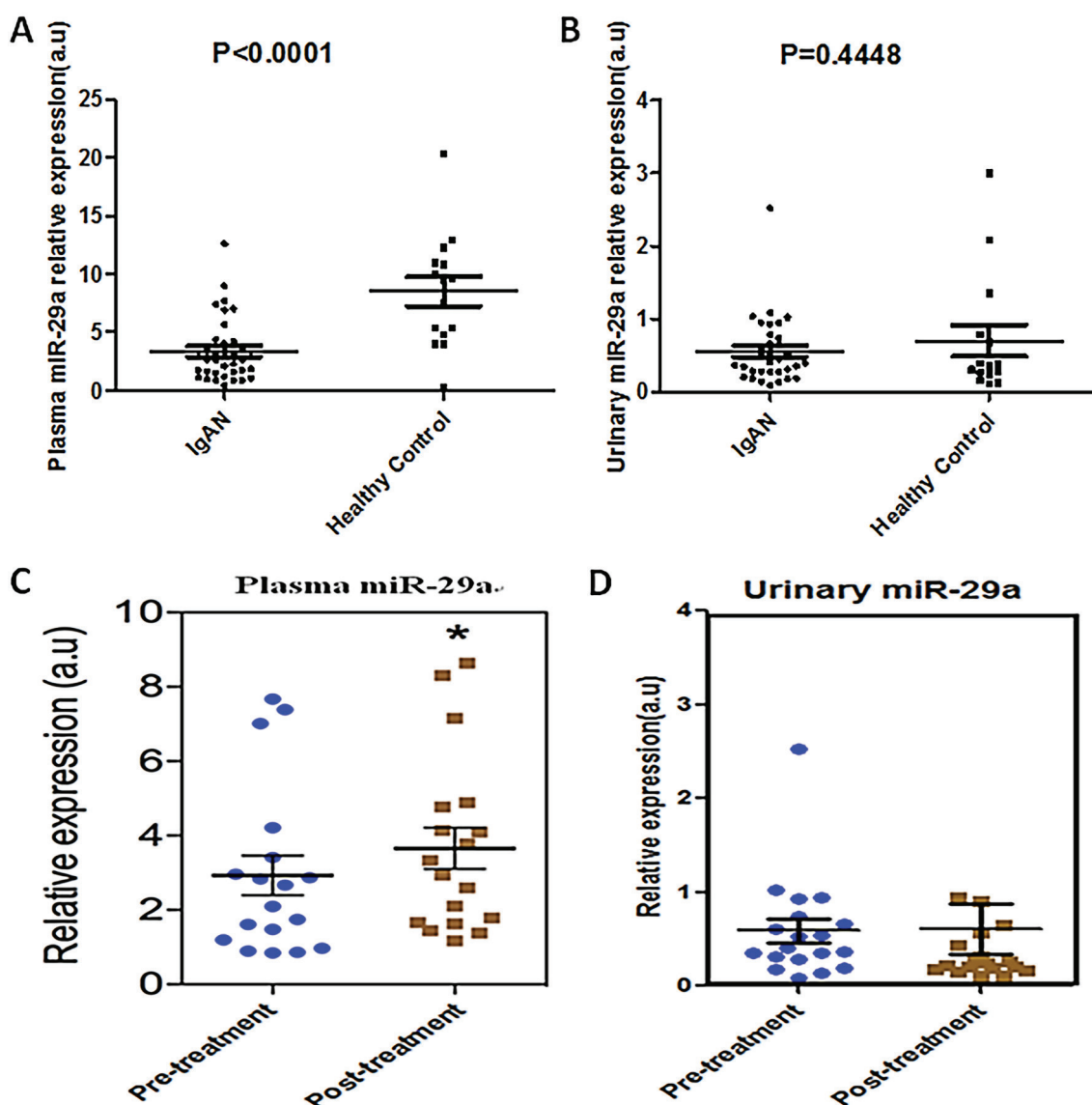


Figure 1. It shows plasma and urinary miR-29a levels in IgAN patients and healthy controls (A, B); IgAN treatments altered plasma miR-29a level, but did not alter urinary miR-29a (C, D).

creatinine levels and proteinuria and increasing eGFR (Table 2). Plasma miR-29a level, which was changed because of IgAN, was reversed by treatment, suggesting miR-29a may exert a protective effect. Hence, physicians might use the level of plasma miR-29a for reflecting curative effects of drug in the treatment of IgAN. Unfortunately, urinary miR-29a level was not responding to treatment (Figure 1).

Correlation of Plasma MiR-29a to Proteinuria, eGFR, and Serum Creatinine Levels

Plasma miR-29a correlate with eGFR, proteinuria and serum creatinine levels (Figure 2), and urinary

miR-29a correlate with eGFR either in patient with untreated or treated IgAN. However, urinary miR-29a did not correlate with proteinuria and serum creatinine levels either in patient with untreated or treated IgAN. As expected, plasma miR-29a positively correlates with eGFR, and negatively correlates with proteinuria and serum creatinine, irrespective of whether or not the IgAN patients accepted treatments. Notably, the correlation between plasma miR-29a and primary clinical parameters is not influenced by treatments. These results suggest that plasma miR-29a was closely related to renal damages induced by IgAN (Figure 2).

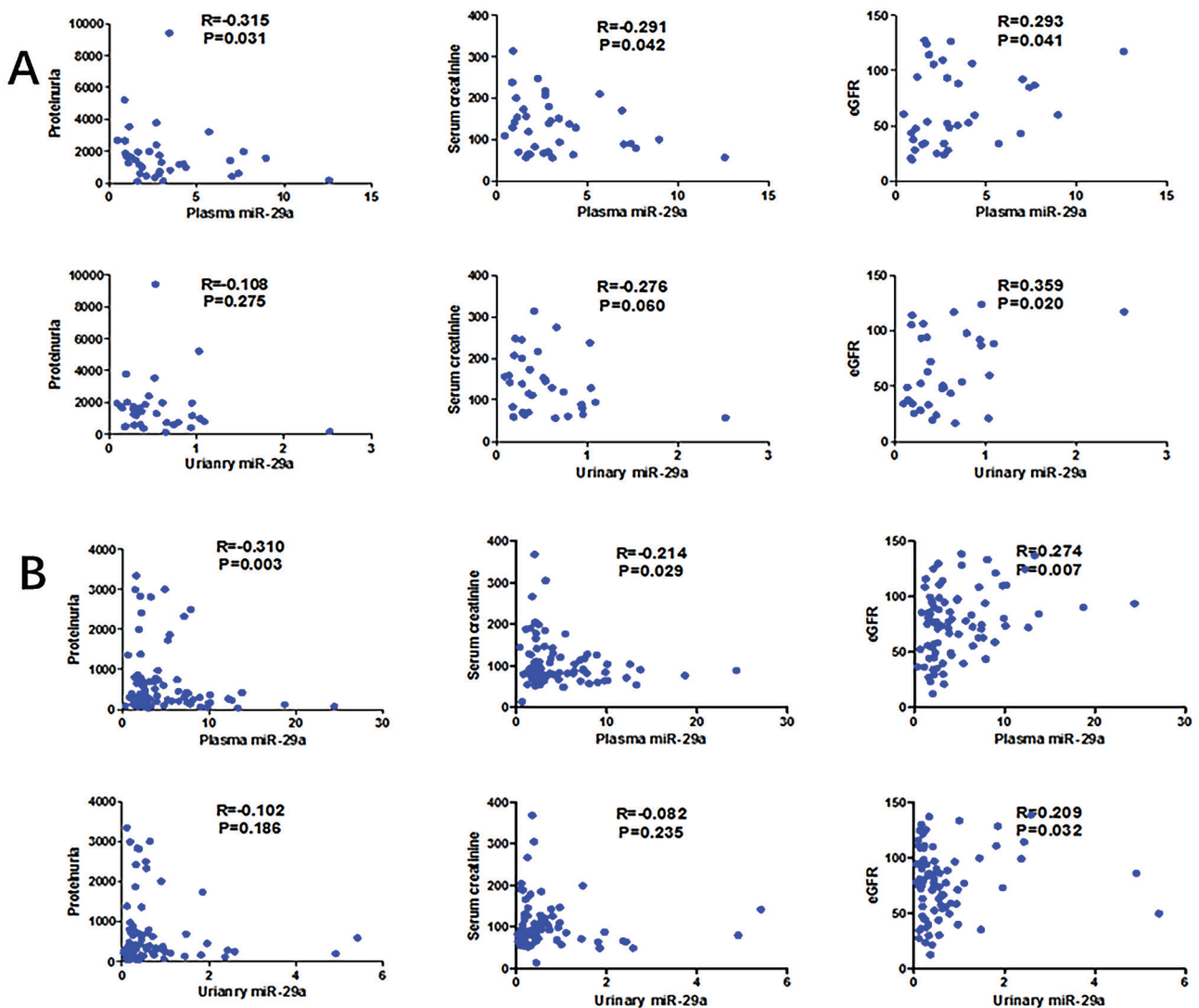


Figure 2. Plasma miR-29a level correlated with Scr, eGFR and proteinuria, urinary miR-29a level correlated with eGFR but not with proteinuria nor with serum creatinine levels in untreated IgAN patients (n = 36, 2A); Plasma miR-29a level correlated with Scr, eGFR and proteinuria, urinary miR-29a level correlated with eGFR but not with proteinuria nor with serum creatinine levels in treated IgAN patients (n = 79, 2B).

Correlation of Plasma MiR-29a with Pathological Damages

Some studies suggest that the pathological parameters mentioned in this article previously are all closely related to the progression of IgAN.¹²⁻¹⁴ We analyzed plasma and urinary miR-29a levels in patients at different stages of IgAN, based on a renal-biopsy conducted using Lee's classification. From the 79 patients with IgAN, 13 were diagnosed at stage 2, 40 at stage 3, 17 at stage 4, and 9 at stage 5. We found that plasma miR-29a decreases with progression of the IgAN stage (Figure 3A). Urinary miR-29a displayed a similar pattern but was less clear compared to plasma miR-29a ($P > .05$ for trend, Figure 3B). We also explored plasma and urinary miR-29a levels in patients at different stages of Oxford classification, which displayed a pattern similar to Lee's classification stages; the miR-29a levels decreased with the progression of Oxford classification stages. Moreover, plasma miR-29a level also decreased with the progression of pathological damages with crescent formation. However, the relationship between urinary miR-29a and primary pathological parameters was not notable ($P > .05$ for trend, Figure 4 and 5). These results suggest that plasma miR-29a level could reflect the degree of renal pathological damages, and therefore, its level was closely related to the progression of IgAN, and this relation would not be influenced by treatments. Thus, it is possible for plasma miR-29a to serve as a biological marker to predict progression of IgAN.

Relationship of Plasma MiR-29a with Treatment Efficacy and the Protective Effect of MiR-29a Against IgAN-induced Renal Damages

Plasma expression of miR-29a in patients with complete remission was significantly higher compared to that in patients without complete remission ($P < .05$); urinary expression of miR-29a did not differ significantly between the patients with or without complete remission ($P > .05$) (Figure 6A). Similarly, plasma expression of miR-29a in patients with $eGFR \geq 60$ mL/min was significantly higher compared to that in patients with $eGFR < 60$ mL/min ($P < .05$); urinary expression of miR-29a did not differ significantly between the patients with or without renal function damages ($P > .05$) (Figure 6B). Based on these results, it is possible to verify if plasma miR-29a level could reflect treatment efficacy of IgAN, and miR-29a can protect from renal damages in patients with IgAN.

Plasma But Not Urinary MiR-29a Expression Levels Predict the Progression and Treatment Efficacy of IgAN

46 patients progressed to complete remission. Kaplan-Meier analysis showed that patients with a high plasma expression of miR-29a had a better treatment efficacy survival compared to those with low expression (log rank test, $P < .05$; Figure 7A). Furthermore, during follow up, 10 patients progressed to the composite endpoint. Kaplan-Meier analysis showed that patients with high plasma expression of miR-29a had better renal function

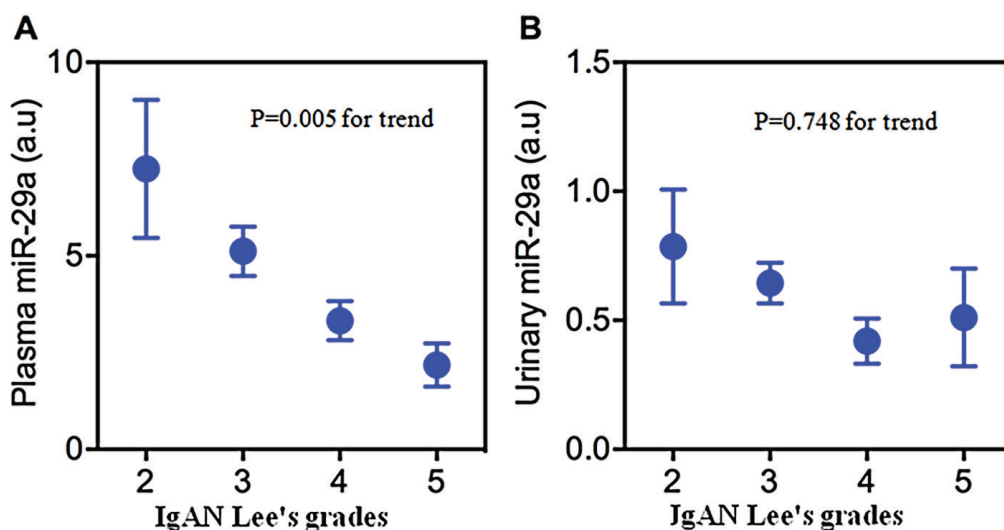


Figure 3. Plasma (A) and urinary (B) miR-29a levels were changing when Lee's grades of IgAN progressing.

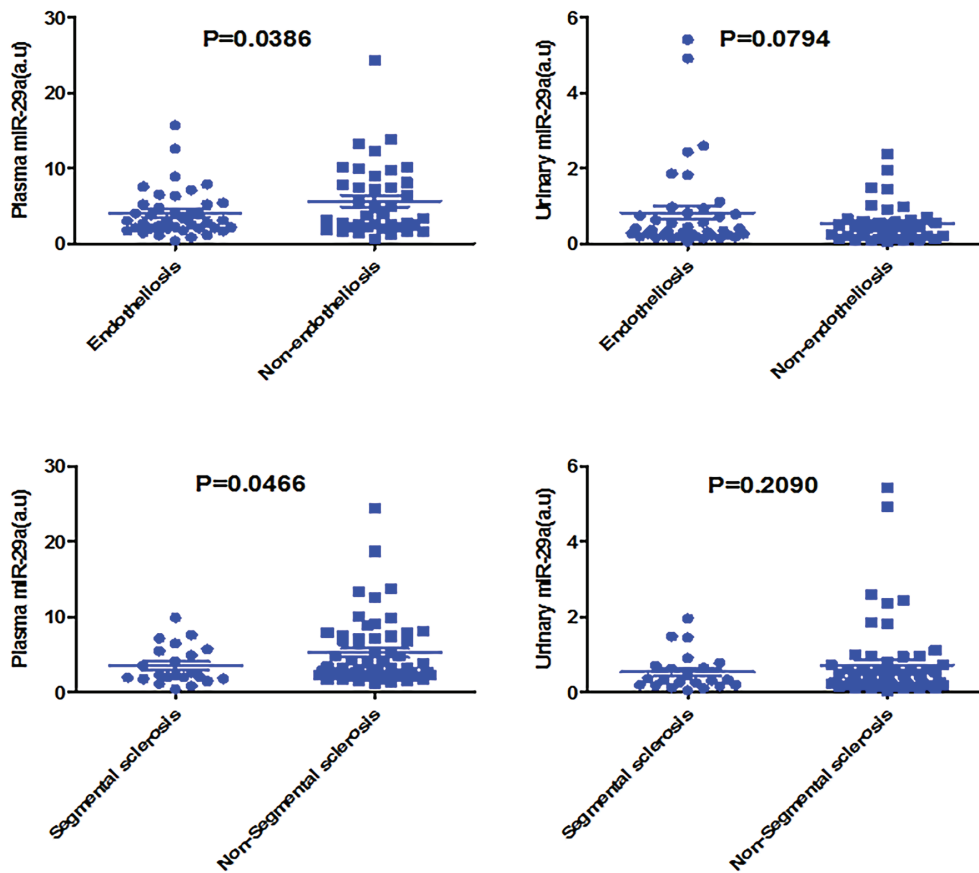


Figure 4. Plasma miR-29a level was decreased when pathological damages of IgAN with endothelial cell proliferation or segmental glomerular sclerosis, but the changes of urinary miR-29a was not obvious.

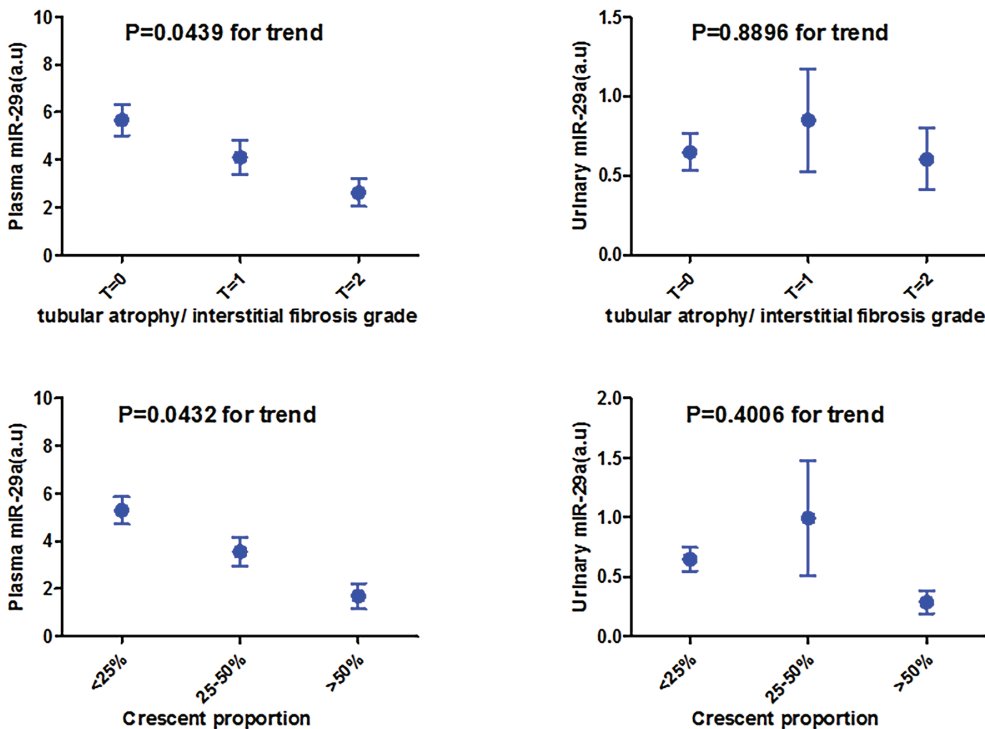


Figure 5. Plasma miR-29a level was decreased along with the progression of pathological damages of IgAN with crescent formation or tubular atrophy/ interstitial fibrosis, but the changes of urinary miR-29a was not obvious.

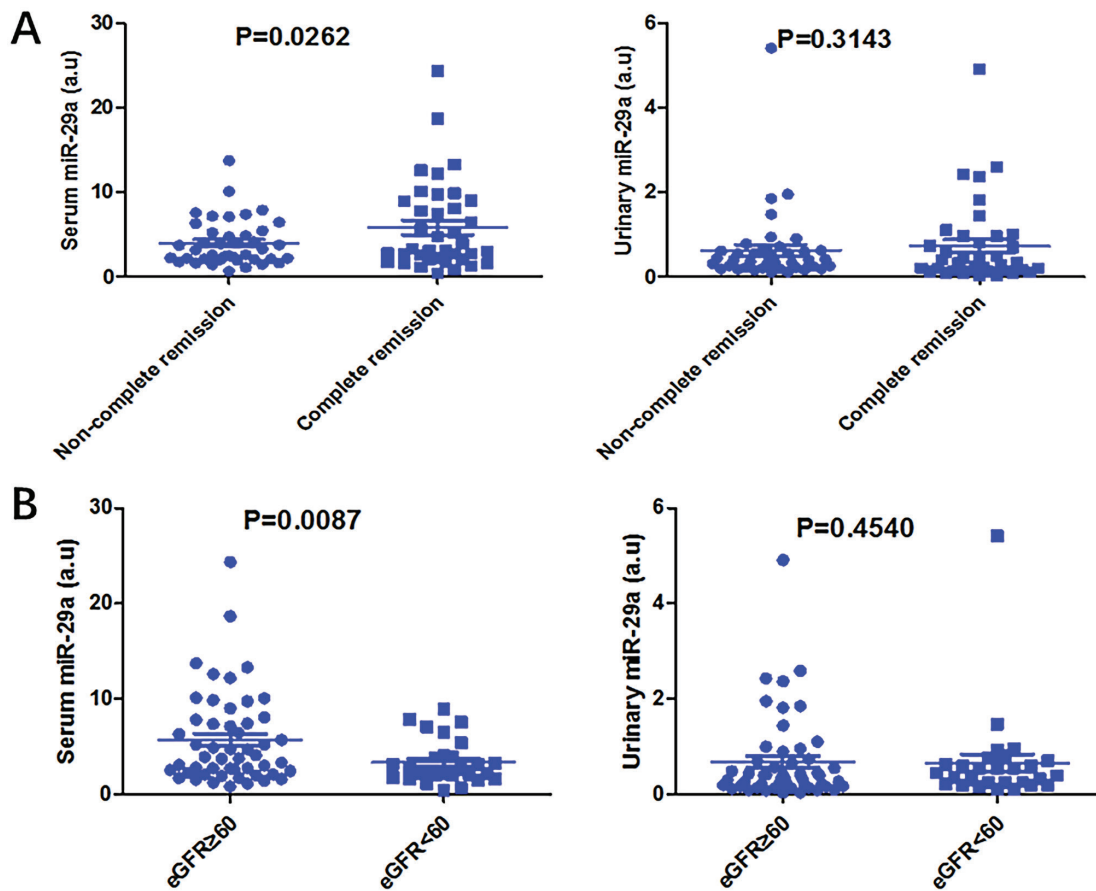


Figure 6. Plasma miR-29a level was lower in Non-complete remission group, compared with complete remission group, suggested that plasma miR-29a level is closely related to efficacy of treatments, but the changes of urinary miR-29a was not obvious (6A); Plasma miR-29a level was higher in eGFR ≥ 60 mL/min group, compared with eGFR < 60 mL/min group, suggested that plasma miR-29a plays a protective effect against IgAN induced renal damages, but the changes of urinary miR-29a was not obvious (6B).

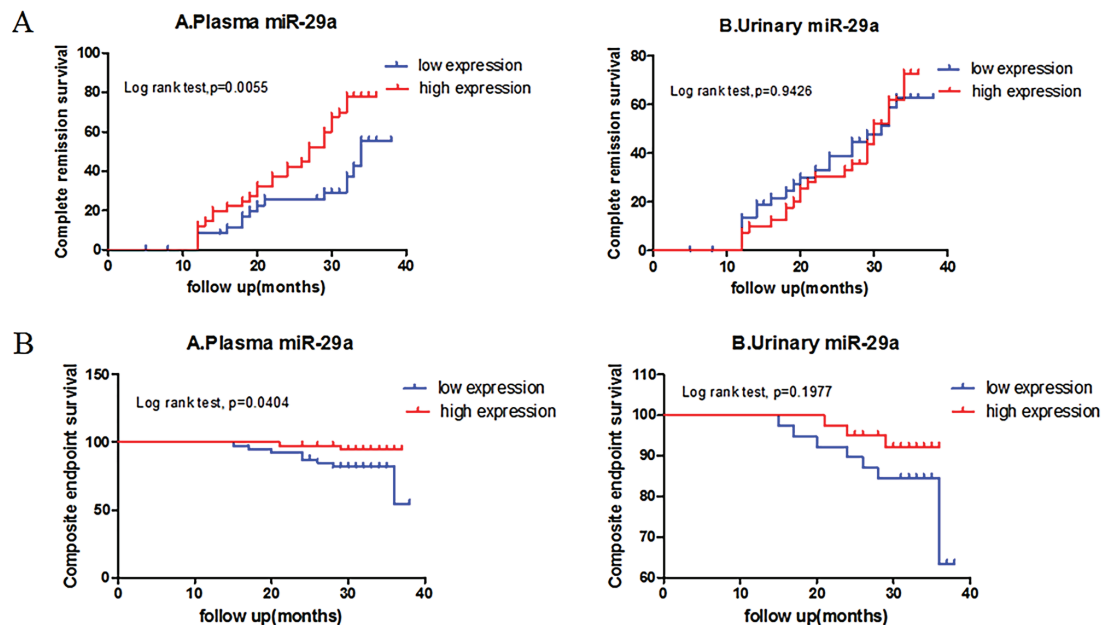


Figure 7. Kaplan-Meier plot of complete remission survival with respect to the plasma and urinary expression levels of miR-29a. For each miRNA, patients were divided into two groups according to the median level of expression. Data are compared by log rank test. The result suggests that plasma miR-29a level could predict long-term treatment efficacy and progression of IgAN.

survival compared to those with low expression (log rank test, $P < .05$; Figure 7B). Urinary miR-29a tested in this experiment was not associated with progression and treatment efficacy of IgAN (log rank test, $P > .05$ and $P > .05$; respectively) (Figure 7). However, because the number of event was small, extensive multivariate survival analysis was not performed. These results indicate that plasma miR-29a could predict the progression and treatment efficacy of IgAN.

Cutt-off Point of Plasma MiR-29a for Predicting the Progression and Treatment Efficacy of IgAN

We further analyzed the performances and optimal value of cut-off point of plasma miR-29a in predicting the progression and treatment

efficacy of IgAN. The area under the curve (AUC) for predicting the progression of IgAN was 0.764 (95% CI: 0.566 to 0.868, $P < .001$), the optimal value of cut-off point of plasma miR-29a was 2.55, sensitivity was 0.826, and specificity was 0.364. The AUC for predicting the treatment efficacy was 0.745 (95% CI: 0.590 to 0.900, $P < .05$), the optimal value of cut-off point of plasma miR-29a was also 2.55, sensitivity was 0.696, and specificity was 0.200. These results suggest that plasma miR-29a had consistently better performance for predicting the progression and treatment efficacy of IgAN (Figure 8, Table 3).

DISCUSSION

Unlike many other studies that only compared

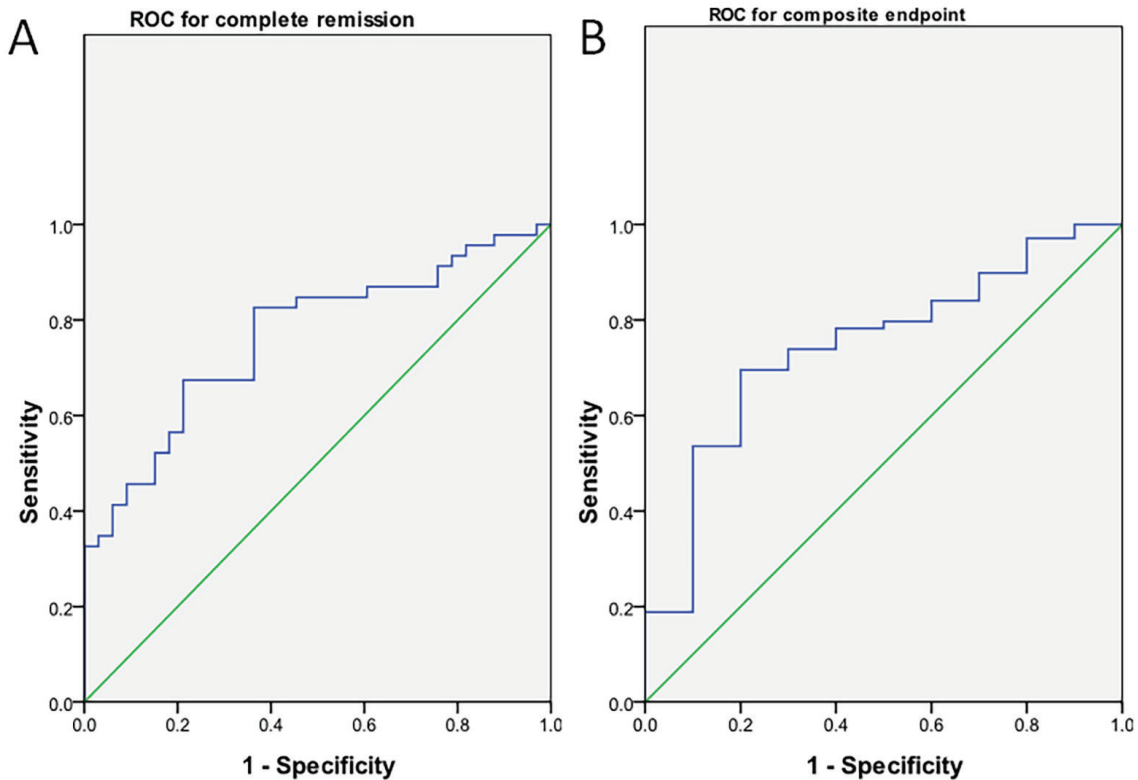


Figure 8. Receiver operating characteristics (ROC) curves with composite outcome and complete remission as the status variable. The areas under the receiver operating characteristics curve (AUCs) were plasma miR-29a. Using composite endpoint and complete remission as status variable.

Table 3. Impact Effect of Plasma MiR-29a on Predicting Progression and Treatment Efficacy of IgAN

Variable	Area Under Curve (95% CI)	P	Cut-off Point	Sensitivity	Specificity
P-miR-29a for:					
Complete Remission	0.76 (0.57 - 0.87)	< .001	2.55	0.83	0.36
Composite Endpoint	0.75 (0.59 - 0.90)	< .05	2.55	0.70	0.20

microRNA expression in healthy subjects with that in patients with IgAN, in the present study, we used a step-by-step approach to explore plasma miR-29a that may have potential biomarker value in reflecting renal damages, evaluating treatment efficacy, and predicting progression in IgAN. First, we found that plasma miR-29a has different plasma levels in patients with IgAN as compared to healthy subjects. miR-29a also responded to the treatments and displayed different pre- and post-treatment level. Additionally, plasma miR-29a correlated with serum creatinine levels, eGFR and proteinuria, irrespective of whether or not the patients with IgAN accepted treatments. Therefore, plasma miR-29a level did not only reflect renal damages, but also reflected the efficacy of the treatments in IgAN. Plasma miR-29a was closely related to the degree of renal pathological damages. Furthermore, high plasma expression of miR-29a had better treatment efficacy and renal function survival than those with low expression, and this further demonstrated that miR-29a plays an important role in protecting the kidney from damages induced by IgAN. This indicates that plasma miR-29a could be used as markers to monitor IgAN. Although many studies have investigated urinary protein and mRNA as potential biomarkers for kidney diseases, a study on the expression of miRNAs in urinary sediment and kidney biopsy with IgAN has also been published. To date, only a limited number of studies have been conducted on circulating miRNAs in IgAN. Similar to urinary miRNAs, plasma miRNAs have the advantage to be used as biomarkers for kidney diseases as miRNAs have been proved to be more stable and possibly more abundant than ordinary mRNAs.^{15,16} Therefore, in the current study, we aimed to investigate the urinary and plasma expression of miR-29a in IgAN patients and explore the roles of miR-29a in renal damages, treatment efficacy assessment, and progression prediction in patients with IgAN. MiR-29a belongs to the miR-29 family, which includes miR-29a, miR-29b, and miR-29c. Members of miR-29 family play important roles in chronic kidney disease (CKD). They suppress the expression of collagen and many extracellular matrix proteins, and have been demonstrated to protect the kidney from fibrosis both *in vivo* and *in vitro*.^{7,17,18} Intra-renal miR-29c levels are reduced in renal interstitial fibrosis in rat models and patients with IgAN.¹⁹ A recent study

reported that decreased miR-29a expression is concomitantly associated with attenuated Wnt/ β -catenin signaling in the glomeruli of diabetic mice, and enhanced miR-29a expression increases Wnt/ β -catenin signaling in diabetic mice.²⁰ Another study found that in hepatocellular carcinoma (HCC), miR-29a could regulate TGF- β -induced EMT by affecting DNA methylation by suppressing DNA methyl transferases (DNMT).²¹ However, the role of plasma miR-29a in IgAN has not yet been elucidated. In the present study, we found that plasma expression of miR-29a in patients with IgAN was significantly lower compared to that in healthy controls; urinary expression of miR-29a did not differ significantly, which was consistent with the findings of previous studies.

Regarding the renoprotective roles in CKD, plasma miR-29a level positively correlated with renal function, negatively correlated with proteinuria, and found to be reduced in IgAN patients. Glomerular sclerosis, mesangial cell proliferation, endothelial cell proliferation, segmental glomerular sclerosis, and tubulointerstitial fibrosis occur when IgAN progresses and adversely affect renal function; plasma miR-29a negatively correlated with these pathological parameters. Following treatment, plasma miR-29a level enhanced and continued to positively correlate with renal function and negatively correlate with proteinuria in patients with IgAN. Therefore, it is possible that increased miR-29a levels after treatments for IgAN suppresses fibrosis and glomerular sclerosis, and protects patients with IgAN from renal damage. Collectively, it can be inferred that a high plasma expression of miR-29a had better treatment efficacy and renal function survival compared to those with low expression, which further demonstrated the protective role of miR-29a in IgAN-induced renal damages. Accordingly, miR-29a may not be a specific marker for IgAN but rather a marker for all kinds of CKD. Additionally, it can provide a different approach to assess renal damages in patients with IgAN, and may help the clinicians to select therapeutic modalities. We also found that 2.55 is the cut-off point of plasma miR-29a level to predict progression and treatment efficacy of IgAN; therefore, it could be used to help the clinicians to evaluate the degree of renal damages and choose the best therapy for IgAN. This implies that the control plasma miR-29a level above 2.55

would slow down the progression of renal damage and ensures a better treatment efficacy for IgAN patients. This study has a few limitations. First, we detected the urinary and plasma miR-29a level without determining their cellular source; it may have been expressed in multiple cell types, and future studies would be necessary to investigate the expression level in specific cell types. Additionally, many factors affect plasma expression miR-29a levels, its underlying specific mechanism, and function in IgAN, and these factors need to be further investigated.

CONCLUSION

In summary, plasma miR-29a could be considered as a biological marker that reflects renal damages and function, to evaluate the efficacy of drugs used for treating IgAN and predicts the progression of IgAN. miR-29a could have a specific mechanism underlying the therapeutic effect of drugs in the treatment of IgAN; however, additional studies need to be conducted to elucidate this mechanism.

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CONFLICTS OF INTEREST

None declared.

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Correspondence to:

Qijun Wan

Department of Nephrology, The First Affiliated Hospital of
Shenzhen University, Shenzhen, Guangdong Province, China

E-mail:wanqijunsz@aliyun.com

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