

Original Article

Expression of microRNA-141 in the urine, serum, and kidneys of patients with IgA nephropathy

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Abstract: Previous studies have shown that members of the miR-200 family (for example, miR-141) can repress epithelial-mesenchymal transition (EMT) via downregulation of ZEB1 and ZEB2, two major transcriptional repressors of E-cadherin. In this study, the urinary, serum, and intra-renal expression levels of miR-141 in patients with IgA nephropathy (IgAN) were studied. The study included 33 patients with biopsy-proven IgAN, as well as serum and urine samples from 15 healthy volunteers and normal renal tissue from nephrectomy specimens of 15 patients with renal cell carcinoma as a control group. The urinary, serum, and intra-renal expression levels of miR-141 in each of the groups were examined by using real-time PCR. Serum miR-141 expression levels were higher in the IgAN group than in the healthy control group ($P = 0.000$), and the intra-renal miR-141 expression was lower in the IgAN group than in the healthy control group ($P = 0.000$). The difference between urinary miR-141 levels in the IgAN and control groups was not statistically significant ($P = 0.260$). Urinary and serum miR-141 levels were inversely correlated with the glomerular filtration rate (eGFR) in the IgAN group ($r = -0.368$, $P = 0.049$; $r = -0.389$, $P = 0.024$). Serum expression of miR-141 was positively correlated with proteinuria ($r = 0.452$, $P = 0.014$) in the IgAN group. Serum expression of miR-141 was inversely correlated with intra-renal expression of miR-141 ($r = -0.473$, $P = 0.015$). The serum expression of miR-141 was significantly increased and intra-renal expression of miR-141 was decreased in patients with IgAN. Urinary and serum miR-141 levels were highly correlated with renal function, and serum miR-141 levels were highly correlated with proteinuria. Serum and urinary miR-141 might play important roles in the progression of IgAN. Serum and intra-renal expression levels of miR-141 were highly correlated, and this relationship could be an important indicator of EMT in IgAN. Further studies are needed to clarify the role of urinary and serum microRNA as a biological marker of IgAN.

Keywords: IgA nephropathy, microRNA-141, epithelial mesenchymal transition, renal fibrosis

Introduction

IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis worldwide, and an important cause of end-stage renal disease (ESRD) [1]. In fact, 15-40% of patients with biopsy-proven IgAN progress to ESRD in 10-20 years [2]. At present, serum creatinine and proteinuria are common prognostic markers of IgAN used by clinicians. However, these markers might fail to accurately predict patient outcomes owing to the heterogeneity of the disease [3]. An assessment of the degree of glomerular and tubulointerstitial lesions is a more precise method to predict renal outcomes [4].

However, renal biopsy has potential complications and repeated monitoring is technically difficult. Reliable biomarkers reflecting disease severity and progression are urgently needed for the clinical management of patients with IgAN [5].

Following the initial mesangial deposition of IgA, progressive glomerulosclerosis and tubulointerstitial fibrosis occur in IgAN [6]. Renal interstitial fibrosis (RIF) plays an important role in IgAN progression to chronic renal failure, and it is a common pathological feature of chronic kidney disease progression to ESRD. Available evidence shows that epithelial-mesenchymal

transition (EMT) is an important process in RIF [7].

MicroRNAs (miRNAs) are noncoding, single-stranded RNA molecules of about 21 to 23 nucleotides in length. They are primarily involved in the control of gene expression at the post-transcriptional level and play important roles in a wide range of physiological and pathological processes [8, 9]. They are involved in almost every cellular process, and miRNA dysregulation is associated with many human diseases, including IgAN [10, 11].

Previous studies have shown that members of the miR-200 family (for example, miR-141) can repress the EMT process via downregulation of zinc finger E-box-binding homeobox (ZEB)-1 and ZEB2, the two major transcriptional repressors of E-cadherin, which is a key marker of epithelial cells [12, 13].

In IgAN, activated tubular epithelial cells may undergo EMT and change into activated fibroblasts, the main effectors of RIF. However, the clinical-pathophysiological link between IgAN and expression of miR-200 family members is not clear, and additional studies are needed to clarify the role of miR-141 in IgAN. In this study, real-time PCR was used to explore urinary, serum, and intra-renal expression of miR-141 and its function in RIF in patients with IgAN.

Materials and methods

Subjects

The study included 33 consecutive patients with IgAN confirmed by kidney biopsy between March 2013 and August 2013 at Shenzhen Second People's Hospital. Patients with other coexisting renal pathologies were excluded. The study was approved by the Clinical Research Ethical Committee of the Shenzhen Second People's Hospital; all patients provided fully informed consent. Clinical and pathological data, including serum creatinine, 24 h urine protein, hemoglobin, albumin, blood pressure, glomerulosclerosis, and crescent formation, were recorded. The glomerular filtration rate (eGFR) was estimated by using a standard equation [14]. Urine and blood samples from 15 healthy subjects were collected as healthy controls. Normal renal tissue from the nephrectomy specimens of 15 patients with renal cell

carcinoma (clear cell type) were also obtained as controls.

Sample processing

A whole-stream, early-morning urine specimen and 5 mL of whole blood were collected from each patient on the day of biopsy for the study of urinary and serum miR-141 expression. Blood and urine samples were stored at 4°C and processed within 5 h of collection. The whole blood and urine specimens were centrifuged at 3000 × g for 30 min at 4°C. The serum and urine supernatant aliquots were then transferred to Eppendorf tubes and centrifuged at 12,000 × g for 10 min at 4°C and stored at 80°C until use.

Immediately after the kidney biopsy, the renal tissue samples were placed in 10% neutral-buffered formaldehyde overnight, dehydrated in ethanol, and embedded in paraffin for the intra-renal miR-141 expression analysis. Ten 10 mm sections were cut from the formalin-fixed and paraffin-embedded tissue blocks by using a microtome and pooled in a 1.5 mL microcentrifuge tube. The sections were then treated with xylene for 3 min at 50°C and washed twice with 100% ethanol. The pellet was air-dried for 30 min at room temperature and used for the intra-renal miR-141 expression study.

RNA extraction

Serum and urine RNA was extracted by 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 by using RNAiso Plus (TaKaRa, Otsu, Japan) according to the manufacturer's protocol.

Measurement of miRNA levels

The S-Poly (T) miRNA qPCR assay (Cat. No. AB-MAS100001-AB-MAS86581) was used for reverse transcription (RT) and RT-qPCR. For miRNA, 5.5 µL of tailed RNA was mixed with 2.5 µL of 4 × Reaction Buffer Mix, 1.0 µL of PolyA/RT Enzyme Mix, 0.5 µL of miRNA RT primer (1

Urinary, serum and intra-renal miR-141 in IgAN

Table 1. Demographic and baseline clinical and pathological data of the subjects

	IgAN	Healthy control	Statistics (T/X ²)	P
No. of case	33	15		
Sex (M:F)	16:17	6:9	0.299	0.584
Age (year)	36.60±9.34	35.60±7.32	0.403	0.689
SBP (mmHg)	136.52±20.06	-		
DBP (mmHg)	85.97±15.15	-		
Proteinuria (g/day)	1.92±1.75	-		
Hemoglobin (g/L)	129.34±19.46	-		
Albumin (g/L)	36.10±5.45	-		
Serum creatinine (μmol/l)	149.65±74.62	-		
EGFR (ml/min/1.73 m ²)	59.61±34.83	-		
Glomerulosclerosis (%)	32.11±26.00	-		
Crescent (%)	21.36±19.22	-		

IgAN, immunoglobulin A nephropathy; eGFR, evaluated glomerular filtration rate; SBP, systolic blood pressure; DBP, diastolic blood pressure.

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 put on ice for 2 min. Samples were diluted 4-15 times and 30-140 μL of water was added. In the 20 μL PCR, 8 μL was used as template.

Urinary, serum, and intra-renal miR-141 levels were quantified by using RT-qPCR. For RT-qPCR, 8 μL of diluted cDNA (5 × dilution) was mixed with 2 μL of 10 × Taq Buffer, 2 μL of 2 mM dNTP mix, 0.5 μL of Self Taq Polymerase, 0.4 μL of 10 μM forward primer, 0.4 μL of 10 μM reverse primer, 0.5 μL of 10 μM probe, 0.2 μL of ROX Reference Dye (100 ×, Sigma-Aldrich, St. Louis, MO, USA), and 6.0 μL of H₂O to make a 20-μL reaction volume. Each sample was run in triplicate. RT-qPCR was performed at 95°C for 30 s, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. RNU48 (Applied Biosystems, Waltham, MA, USA) was used as a house-keeping gene to normalize microRNA expression [15]. The results were analyzed by using Sequence Detection Software version 2.0 (Applied Biosystems). In order to calculate the differences in expression level for each target among samples, the 2^{-ΔCt} method for quantitation was used, where ΔCt = AVG (miR-141 Ct) - AVG (house-keeping gene Ct).

Assessment of pathological data

An analysis of renal fibrosis was performed by using 4-μm paraffin-embedded sections sta-

ined with periodic acid-Schiff (PAS) or Jones silver stain. The severity of renal pathological damage was scored subjectively by an experienced pathologist who was blinded to the results of molecular studies. The severity of glomerulosclerosis and crescent formation was estimated as the percentage of sclerotic glomeruli and crescents in the total glomeruli obtained from the biopsy.

Statistical analysis

A statistical analysis was performed in SPSS for Windows version 17.0. Results

are presented as means ± SD for normally distributed data and medians (lower and upper quartiles) for other data. The *t*-test was used to compare group means for normally distributed data, and the χ² test was used for other data. Since miRNA expression levels were highly skewed, nonparametric statistical methods were used. The Mann-Whitney U test was used to compare gene expression levels between groups and Spearman's rank-order correlations were used to test associations between gene expression levels and clinical parameters. A *P*-value of less than 0.05 was considered statistically significant. All probabilities are two-tailed.

Results

Clinical and pathological data

The demographic and baseline clinical and pathological data of the study subjects are summarized in **Table 1**. As compared with the control group, the IgAN group had significantly higher levels of proteinuria and worse renal function. Age and gender did not differ significantly between the two groups.

Level of urinary, serum, and intra-renal miR-141

The urinary, serum and intra-renal miR-141 expression levels are summarized in **Figure 1**. Urinary expression of miR-141 did not differ significantly between the IgAN group and healthy

Urinary, serum and intra-renal miR-141 in IgAN

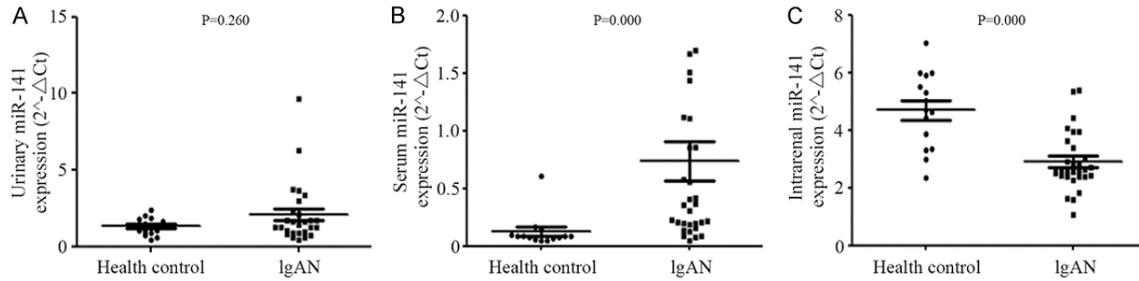


Figure 1. Comparison of urinary, serum and intrarenal expression of miR-141 between patients with IgAN group and healthy control group. Data were compared by Mann-Whitney U test.

Table 2. Correlations between urinary and serum expression of miR-141 and clinical, pathological data in IgAN group

	Proteinuria	eGFR	SBP	DBP	Crescent	Glomerulosclerosis
Urinary miR-141	$r = 0.056$, $P = 0.769$	$r = -0.368$, $P = 0.049$	$r = -0.166$, $P = 0.355$	$r = -0.050$, $P = 0.782$	$r = 0.001$, $P = 0.998$	$r = -0.176$, $P = 0.336$
Serum miR-141	$r = 0.452$, $P = 0.014$	$r = -0.398$, $P = 0.024$	$r = 0.097$, $P = 0.603$	$r = 0.052$, $P = 0.782$	$r = -0.101$, $P = 0.595$	$r = 0.241$, $P = 0.199$
Intrarenal miR-141	$r = 0.290$, $P = 0.120$	$r = -0.082$, $P = 0.666$	$r = -0.182$, $P = 0.337$	$r = 0.010$, $P = 0.959$	$r = 0.316$, $P = 0.089$	$r = 0.062$, $P = 0.743$

eGFR, evaluated glomerular filtration rate; SBP, systolic blood pressure; DBP, diastolic blood pressure. *Statistical significance with $P < 0.05$.

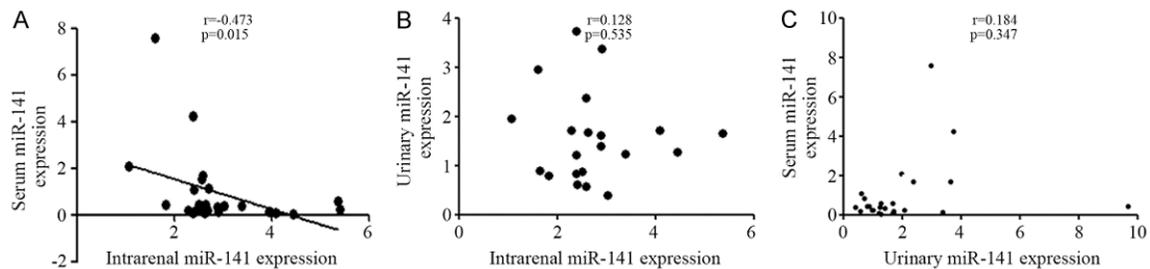


Figure 2. Relation between urinary, serum and intrarenal expression of miR-141. Data were compared by Spearman's rank correlation coefficient.

control group (1.402 [0.891-2.378] versus 1.376 [0.904-1.762], $P = 0.260$). Serum expression of miR-141 was significantly high in patients with IgAN than in healthy controls (0.359 [0.191-0.979] versus 0.083 [0.060-0.113], $P = 0.000$). Intra-renal expression of miR-141 was significantly lower in patients with IgAN than in controls (2.632 [2.388-3.440] versus 4.692 [3.341-5.893], $P = 0.000$).

miR-141 levels and clinical pathological data

The relationships between miR-141 levels and clinical and pathological parameters in the IgAN group are summarized in **Table 2**. Urinary expression of miR-141 was significantly corre-

lated with eGFR ($r = -0.386$, $P = 0.049$). Similarly, serum expression of miR-141 was significantly correlated with proteinuria ($r = 0.452$, $P = 0.014$) and eGFR ($r = -0.398$, $P = 0.024$). There were no significant correlations between intra-renal expression of miR-141 and any clinical and pathological parameters ($P > 0.05$).

Relationships among urinary, serum, and intra-renal expression of miR-141

Serum miR-141 expression was significantly correlated with intra-renal expression of miR-141 ($r = -0.473$, $P = 0.015$). However, no significant correlations between urinary and serum miR-141 or between urinary and intra-renal

expression of miR-141 were detected ($r = 0.184, P = 0.347; r = 0.128, P = 0.535$) (Figure 2).

Discussion

Some published studies have evaluated miRNA expression in urinary sediment and kidney biopsy samples in patients with IgAN. However, no studies to date have analyzed serum miRNA expression in patients with IgAN. Urinary and serum miRNAs have advantages with respect to their use as biological markers for kidney diseases because they are more stable and probably more abundant than ordinary mRNAs [16, 17]. In this study, urinary, serum, and intra-renal expression of miR-141 was examined in patients with IgAN, and the relationships between clinical and pathological parameters and miR-141 were assessed to facilitate the development of molecular biological markers for IgAN.

In the process of renal fibrosis associated with IgAN, EMT is an important step [7]. In addition to miR-141, the miR-200 family includes miR-200a, miR-200b, miR-200c, and miR-429. Previous studies have indicated that a loss of expression of miR-200 family members (for example, miR-141) might have a critical role in the repression of E-cadherin by ZEB1 and ZEB2 during EMT, as it appears to enhance cell migration and invasion during metastasis [12, 13]. Overexpression of these miRNAs prevents EMT. Additionally, miR-141 is necessary for the development and progression of TGF- β 1-dependent EMT and fibrosis [18]. The results of the present study indicated that intra-renal expression of miR-141 in patients with IgAN is significantly lower than that of controls, which is in accordance with previous findings. Accordingly, intra-renal levels of miR-141 are closely related to the degree of EMT.

In a study of urinary expression of miR-200 family members by Wang et al., miR-141 expression in patients with IgAN was not significantly different than that of healthy controls ($P = 0.190$), consistent with the results of this study [19]. The results of this study further indicated that serum miR-141 expression is upregulated in patients with IgAN. Previous studies [20] have shown that serum miR-141 levels are increased in bladder cancer patients, consistent with the results of this study.

Based on the results of this study, serum expression of miR-141 in patients with IgAN was significantly higher than that of controls, intra-renal expression of miR-141 in patients with IgAN was lower than that of controls, and serum and intra-renal miR-141 expression levels were highly correlated. Therefore, serum miR-141 levels have potential for use as biological markers for kidney tissue injuries in IgAN patients.

These results suggested that lower miR-141 expression in tubular epithelial cells in patients with IgAN is associated with repression of E-cadherin during EMT, and elevated expression of miR-141 can suppress EMT and potentially treat renal fibrosis. Taken together, these results suggested that miR-141 is renal-protective. Serum expression of miR-141 has potential as a biological marker of EMT in IgAN, and its upregulation may have a protective function.

Urinary and serum miR-141 expression levels were inversely correlated with eGFR, and serum miR-141 expression was positively correlated with proteinuria in IgAN. These results strongly suggested that increased urinary and serum miR-141 expression is closely related to the progression of renal function in patients with IgAN, and serum miR-141 expression is closely related to the degree of renal damage in IgAN.

There were a few limitations of our study. First, urinary and serum miR-141 levels were detected without determining the cellular source. These may have been derived from necrotic or apoptotic cells or from active cells. This requires further study. Additionally, many factors affect serum expression miR-141 levels, and its specific mechanism and function in patients with IgAN requires additional research.

In summary, serum expression of miR-141 was upregulated and intra-renal expression of miR-141 was downregulated in patients with IgAN. The urinary and serum miR-141 levels were correlated with renal function, and serum miR-141 levels were correlated with the degree of proteinuria. These results suggested that urinary and serum expressions of miR-141 play important roles in the pathogenesis and progression of IgAN. Serum and intra-renal miR-141 levels were closely related, and serum expression of miR-141 may be developed as a biological marker of RIF in IgAN.

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Disclosure of conflict of interest

None.

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