Significance of microRNA-29a as a biological marker in evaluating the effect of mycophenolate mofetil treatment of IgA nephropathy.

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Abstract

Objective: This study was designed to analyse the urinary, serum and renal expression of *miR-29a* of patients diagnosed with IgAN, aiming to elucidate the effect of MMF upon the expression level of *miR-29a* in IgAN patients.

Methods: Thirty-three patients diagnosed with IgAN by biopsy were assigned in the IgAN group. Serum and urine sampling was collected from 15 healthy volunteers as healthy control group, normal renal tissue from the nephrectomy specimen of 15 patients with renal cell carcinoma in the control group. RT-PCR was performed to compare *miR-29a* level between three groups and explore the effect of MMF on expression level of urinary and serum *miR-29a*.

Results: Compared with control group, the urinary, serum and renal expression of *miR-29a* in IgAN group were decreased (P<0.05). Urinary and serum expression of *miR-29*a inversely correlated with proteinuria (r=-0.322, P<0.05; r=-0.356, P<0.05), and glomerulosclerosis (r=-0.427, P<0.05; r=-0.517, P<0.05) in IgAN group. Urinary expression of *miR-29a* positively correlated with evaluated Glomerular Filtration Rate (eGFR) (r=0.328, P<0.05), inversely correlated with diastolic blood pressure (r=-0.421, P<0.05) in the IgAN group. Serum and intra-renal expression of *miR-29*a was positively correlated with urinary expression of *miR-29a* (r=0.713, P<0.05; r=0.322, P<0.05). The serum and urinary levels of *miR-29a* in the MMF group were significantly higher than those in the IgAN group (both P<0.05).

Conclusion: Urinary and serum *miR-29a* level were closely correlated with the progression of IgAN. MMF could up-regulate the serum and urinary levels of *miR-29a* in IgAN patients. Serum and urinary *miR-29a* probably serve as biological markers to reflect the effect of MMF treated with IgAN.

Introduction

IgA nephropathy (IgAN) is regarded as a common glomerulonephritis with a prevalence of >50 % of primary glomerulonephritis diagnosed by pathological biopsy [1]. Approximate 15-40% of IgAN patients are likely to progress to the End-Stage Renal Disease (ESRD) [2,3]. At present, renal function and proteinuria are regarded as useful prognostic markers of IgAN. Nevertheless, these markers cannot precisely predict outcomes due to the heterogeneity of the disease [4]. Proliferation of mesangial cells with expansion of extracellular matrix in light microscopy, mesangial deposition of IgA in the immunofluorescence and paramesangial immune deposits in electron microscopy represent the histological features of IgAN. Assessment of the degree of glomerular and tubulointerstitial can be used to predict renal outcomes [5]. Renal biopsy has potential complications, and repeated renal biopsy is technically difficult. Exploring appropriate biomarkers which reflect disease severity and progression are urgently required in the management of IgAN patients [6].

Following the initial mesangial deposition of IgA, there is progressive glomerulosclerosis and tubulointerstitial fibrosis in IgAN [7]. Renal fibrosis plays an important role in IgAN progression to chronic renal failure, which are common pathological features of Chronic Kidney Disease (CKD) progress to ESRD. Available evidence shows that Transforming Growth Factor-β1 (TGF-β1) is the major mediator of progressive renal fibrosis in IgAN [8-10], which is largely *via* the SMAD-dependent pathway [11,12]. However, the molecular mechanisms underlying the link between TGF-β1/SMAD signaling pathway and disease progression in IgAN remain unknown.

MicroRNAs (miRNAs) are noncoding, single-stranded RNA molecules of approximately 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 many physiological and pathological processes [13-15]. miRNAs participate in each ring of cellular process, and dys-regulation of miRNA has been correlated with multiple human diseases including CKD [16,17]. Previous studies suggest that TGF-β1/Smad3 signaling promotes renal fibrosis by inhibiting *miR-29* [18]. In mouse models, the expression level of *miR-29* is down-regulated along with the progression of kidney fibrosis in obstructive nephropathy. Instead, mice with knockout of Smad3 presented with the up-regulated expression of *miR-29* over the lack of kidney fibrosis in obstruction mouse models [19].

Mycophenolate Mofetil (MMF) could significant reduce proteinuria, and improve renal function with patients of IgAN. In streptozotocin-induced diabetic nephropathy, the administration of MMF prevented the development of proteinuria, macrophage infiltration and glomerulosclerosis [20] and resulted in reduced expression level of Intercellular Adhesion Molecule-1 (ICAM-1), TGF-β1 and Monocyte Chemoattractant Protein-1 (MCP-1) [21,22].

Urinary level of *miR-29*a was decreased in IgAN patients [23]. However, serum, urinary and renal levels of *miR-29*a of patients with IgAN remain elusive. MMF has been applied in the treatment of IgAN and indicates that MMF can up-regulate the *miR-29*a expression and inhibit renal fibrosis in patients diagnosed with IgAN. In the present study, real-time PCR was performed to explore serum, urinary and intra-renal expression of *miR-29*a and its function in renal fibrosis of patients with IgAN, aiming to elucidate whether the changes of *miR-29*a expression are the mechanism underlying clinical efficacy of MMF in the management of IgAN.

Materials and Methods

Baseline data

Thirty-three consecutive patients with IgAN confirmed by kidney biopsy between March 2014 and November 2015 in Shandong Provincial Hospital affiliated To Shandong University were assigned in the IgAN group, and 47 patients with IgAN treated with MMF for 3 months were allocated into the MMF group. Serum and urine from 15 healthy volunteers as healthy control group, normal renal nephrectomy specimen from 15 patients with kidney cell cancer as control group. Patients complicated with alternative renal diseases were excluded. Study procedures were approved by the Ethics Committee of Shandong Provincial Hospital affiliated To Shandong University. Informed consents were obtained from all patients. Clinical and pathological data including serum creatinine, 24 h urine protein, hemoglobin, albumin, blood pressure, glomerulosclerosis, and crescent were recorded. Evaluated Glomerular Filtration Rate (eGFR) was estimated by the standard equation [24].

Patients in MMF group were treated with MMF combined with low-dose corticosteroids and Angiotensin Receptor Antagonist (ARB), MMF starting dose as 30 mg/kg/d, 1.5-2.0 g/d, orally

twice, appropriate reduction after 6 months of maintenance therapy, total treatment course for 1.5 y; initial dose of glucocorticoid prednisone was 0.5 mg/kg/d, appropriate reduction after six months of maintenance therapy, total treatment course for 1.5 y. All IgAN patients were treated with conventional dose of 100 mg/d losartan.

Sampling preparation

The urine sample and 5 ml of whole blood was prepared by each patient for urinary and serum *miR-29*a expression study. Blood and urine samples were kept at 4°C for 5 h. Subsequently, these obtained samples were centrifuged at 3000 g at 4°C for 30 min. The urine and serum supernatants were centrifuged at 12,000 g at 4°C for 15 min and kept at 80°C for subsequent experiment.

The kidney tissues were placed in 10% of neutral-buffered formaldehyde immediately after renal biopsy, and subsequently then soaked in the alcohol and embedded in paraffin for staining to analyse the expression level of renal miRNA. Five 10-mm formalin-fixed sections were obtained and handled using a microtome. These sections were soaked in xylene at 50°C for 3 min and rinsed twice with the absolute ethanol. Subsequently, the pellet was dried at room temperature for 30 min, and used for intra-renal *miR-29*a expression.

RNA extraction

Serum and urine RNA sampling was extracted by mirVana PARIS kit (Ambion) according to the manufacturer's instructions. In brief, 500 µl serum was mixed with an equivalent quantity of 2X denaturing solution, and total RNA was eluted. Total RNA derived from the obtained cells was isolated by the RNAiso Plus (Takara, Japan) according to the manufacturer's instructions.

Detection of miR-29a level

For *miR-29*a, 5.5 µl Tailed RNA was mixed with 2.5 µl 4X reaction buffer, 1.0 µl PolyA/RT enzyme, 0.5 µl miRNA RT primer (1 µM), 0.5 µl Internal control RT primer (1 µM) and made up to 10 µl with H_2O at 42°C for 60 min, 75°C for 10 min, diluted for 4-15 times, added with 30-140 µl water. Urinary, serum and intra-renal *miR-29*a were quantified by RT-QPCR. For RT-QPCR, 8 µl Diluted cDNA (5 times dilution) was mixed with 2 µl 10X self Taq buffer, 2 µl 2 mM dNTP mix, 0.5 µl Self Taq Polymerase, 0.4 µl 10 µM Forward primer, 0.4 µl 10 µM Reverse primer, 0.5 µl 10 µM Probe, 0.2 µl ROX reference dye (100X, Sigma) and 6.0 µl H_2O were mixed to obtain 20 µl reaction volume. Each sample was run in triplicate. RT-qPCR was performed at 95°C for 30 s for 40 cycles at 95°C for 10 s and 60°C for 30 s. RNU48 (Applied Biosystems) was used as house-keeping genes to normalize the miRNA expression [25]. To investigate the expression for all targets of varying sampling, the $2^{-\Delta Ct}$ method was utilized for quantitation. ΔCT=AVG(*miR-29a* CT)-AVG (house-keeping genes CT).

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Pathological examination

The severity of kidney fibrosis was assessed by Periodic Acid Schiff staining (PAS). The severity of renal pathological damage was scored by experienced pathologists who were blinded to the results of this experiment. The severity of glomerulosclerosis and crescent was represented by the proportion of sclerotic glomeruli and crescent in all glomeruli.

Statistical analysis

SPSS 19.0 statistical software was adopted for data analysis (SPSS Inc., Chicago, IL). All data were expressed in mean ± standard deviation. T-test was used for comparison between the two groups for data normally distributed, and χ^2 test for the others. Mann-Whitney U test was used to compare the expression levels between groups and Spearman's correlation to analyse the relationship between gene expression and clinical indexes. A P value of less than 0.05 was considered statistical significance.

Results

### Baseline data

Demographic and baseline clinical and pathological data of the study subjects were illustrated in Table 1. The difference of clinical and pathological data between IgAN and MMF group was not statistically significant, which matched the results between two groups (all P>0.05).

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

	IgAN	MMF	T/ χ^2	P value
No. of case	33 47			
Gender/male/female	16:17	20:27	0.276	0.6
Age/year	35.55 ± 9.66	36.02 ± 10.53	-0.206	0.837
SBP/mmHg	136.52 ± 20.06	140.28 ± 17.44	-0.892	0.375
DBP/mmHg	85.97 ± 15.15	87.70 ± 11.88	-0.573	0.569
Proteinuria/g/day	1.92 ± 1.75	2.10 ± 1.57	-0.494	0.623
Hemoglobin/g/L	129.34 ± 19.46	128.31 ± 18.51	0.239	0.812
Albumin/g/L	36.10 ± 5.45 37	56 ± 5.72	-1.147	0.255
Serum creatinine/μmol/L	149.65 ± 74.62	138.48 ± 75.02	0.657	0.513
eGFR/ml/min/1.73m^2	59.61 ± 34.83	67.09 ± 43.99	-0.813	0.418

Abbreviation: IgAN: Immunoglobulin A Nephropathy; eGFR: Evaluated Glomerular Filtration Rate; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure.
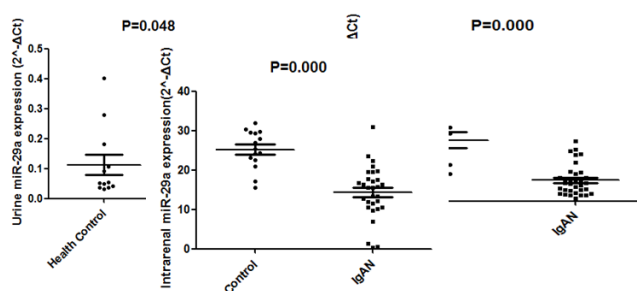
Figure 1. Comparison of urinary, serum and intra-renal expression of miR-29a between IgAN group and healthy controls.

Level of urinary, serum and intra-renal miR-29a

The urine, renal and serum expression of *miR-29*a were compared and summarized in Figure 1. Urinary expression of *miR-29*a of IgAN patients was significantly lower than those in the control group (*miR-29*a (A): 0.047 (0.027-0.071) versus 0.052 (0.038-0.162), P<0.05); serum expression of *miR-29*a of patients with IgAN was also significantly down-regulated than that in the control group (*miR-29*a (B): 0.355 (0.170-0.546) versus 1.254 (0.687-1.491), P<0.05); intra-renal expression of *miR-29*a of patients with IgAN was significantly lower than that of controls (*miR-29*a (C): 15.608 (10.458-18.110) versus 25.304 (22.491–29.532), P<0.05).

MiR-29a level and clinical, pathological data

Since serum, urinary and renal expression of *miR-29*a significantly differed between the study and control groups, the study analysed the relationship between *miR-29*a level and clinopathological parameters. The urinary expression of *miR-29*a was significantly correlated with proteinuria (r=-0.336, P<0.05), eGFR (r=0.358, P=0.023), glomerulosclerosis (r=-0.537, P<0.05) and DBP (r=-0.471, P<0.05). Similarly, serum expression of *miR-29*a significantly correlated with proteinuria (r=-0.365, P<0.05) and glomerulosclerosis (r=-0.487, P<0.05). There was no significant correlation between intra-renal expression of *miR-29*a and clinical and pathological data.

### Relationship among urinary, serum and intra-renal expression of miR-29a

Since urinary, serum and intra-renal expression of *miR-29*a of patients with IgAN were decreased consistently, the study further to explore the relationship of urinary, serum and intra-renal *miR-29*a level in IgAN group. It found that urinary expression of *miR-29*a positively correlated with serum expression of *miR-29*a (r=0.733, P<0.05), and intra-renal expression of *miR-29*a (r=0.360, P<0.05).There was no significant relationship between serum and intra-renal expression levels of *miR-29*a (r=0.152, P <0.05) (Figure 2).
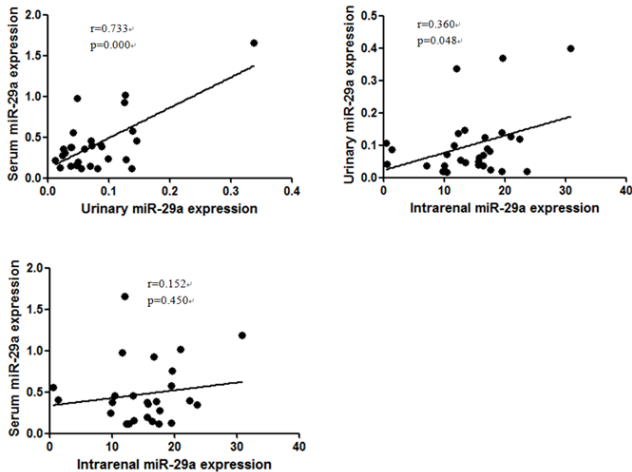
Figure 2. Relationship among urinary, serum and renal expression levels of miR-29a.

Effect of MMF treatment on serum and urinary expression of miR-29a

Since urinary, serum and intra-renal expression of *miR-29*a of patients with IgAN was decreased, It also found the serum and urinary level of *miR-29*a in MMF group was significantly higher than IgAN group (P=0.032, P<0.05), as illustrated in Figure 3.
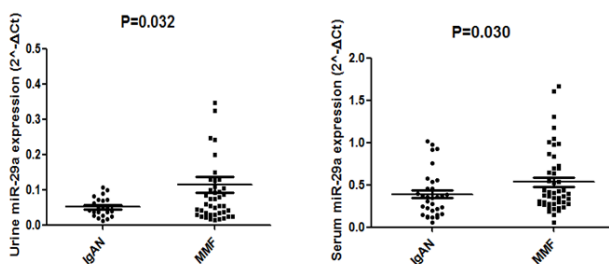
Figure 3. Comparison of urinary and serum expression of miR-29a between IgAN group and MMF group.

Discussion

IgAN is frequently encountered in Asian countries. Poor clinical prognosis is obtained in the patients diagnosed with massive hematuria, renal dysfunction and histological lesions,

etc. [26]. The degree of renal fibrosis is closely related to prognosis of IgAN. Although many studies investigated urinary protein and mRNA as potential biological markers for kidney diseases, the expression of miRNA in serum with IgAN has been rarely investigated. However, urinary and serum miRNAs have several theoretical advantages to be utilized as biological markers for renal illness. First, global miRNA quantification approach is more efficient compared with the detection of conventional protein markers [27]. Secondly, miRNAs are more stable and abundant compared with traditional mRNAs [28,29]. Third, a single miRNA species is able mediate the expression of different genes [30]. This study aimed to analyse the serum, urinary and renal expression of *miR-29*a of IgAN patients, and investigate whether *miR-29*a expression is associated with the clinical efficacy of MMF in the treatment of IgAN.

*MiR-29*a is one of the *miR-29* family members, including *miR-29*a, *miR-29*b, and *miR-29*c. Previous studies suggested that *miR-29* family was closely related renal fibrosis mediated by TGF-β1/Smad3 signaling pathway [19].

In the present study, it examined the expression level of *miR-29*a in urine, serum and kidney biopsy of patients with IgAN and found that urinary, serum and renal expression of *miR-29*a of IgAN patients was significantly lower compared with those in the control group. And the relation among urinary, serum and intra-renal expression of *miR-29*a was highly consistent, and intra-renal *miR-29*a was closely related to kidney tissue injury. These results suggest that serum and urinary *miR-29*a level had the potential to be used as biological marker for kidney tissue injury of IgAN.

During the procession of renal fibrosis of IgAN, recent studies have found that TGF-β1/Smad3 signaling pathway is the main and final common pathway for renal fibrosis [31]. Previous studies demonstrate that TGF-β1/Smad3 signaling promotes renal fibrosis by inhibiting *miR-29*a [18]. Additionally, *miR-29*a could inhibit renal fibrosis by inhibiting TGF-β1/Smad3 signaling [18]. Another study suggest that , in the cultured *in vitro* proximal tubular cells, primary mesangial cells, and podocytes, after exposed to TGF-β1, which targets the expression of collagen gene and up-regulates the expression of ECM proteins, reduced the expression of the *miR-29*a. For the resting cells and cells treated with TGF-β1, abnormal expression of *miR-29* inhibited the expression levels of collagens I and IV mRNA and protein. Furthermore, they demonstrated a low level of *miR-29* in rat models with early and advanced-stage renal fibrosis [19]. Another study found in Hepatocellular Carcinoma (HCC), *miR-29*a is able to mediate the EMT induced by TGF-β through influencing the DNA Methyltransferases (DNMT) [32]. This might be the specific pathogenesis among *miR-29*a with renal fibrosis.

The findings of current investigation indicated that the renal expression level of *miR-29*a of IgAN patients was considerably down-regulated than compared with those in the control group, which was in accordance with above previous findings.

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Above results showed that the intra-renal expression of *miR-29*a decreased in patients with IgAN associated with TGF-β1/Smad3 mediated signal inhibit expression of *miR-29*a in the process of renal fibrosis, and elevated expression of *miR-29*a could also inhibit the TGF-β1/Smad3-signaling pathway, to achieve the purpose of the treatment of renal fibrosis. Available results suggested that *miR-29*a were renal-protective. As urinary, serum and intra-renal expression of *miR-29*a in IgAN patients was considerably down-regulated than compared with those in the control group, and their level was highly consistent. So urinary and serum expression of *miR-29*a have the potential as biological markers of renal fibrosis in IgA nephropathy.

In the present study, urinary and serum expression of *miR-29*a significantly inversely correlated with proteinuria and glomerulosclerosis, and urinary expression of *miR-29*a positively correlated with eGFR. These result highly suggest that urinary and serum decreased expression of *miR-29*a is closely related to the degree of renal damage in patients with IgAN, and urinary expression of *miR-29*a was closely related to the progress of renal function of IgAN. Urinary and serum expression of *miR-29*a could also be considered as an important indicator of degree of glomerularsclerosis in IgAN. Therefore, restoring *miR-29*a expression level might be an important treatment target of IgAN. Currently, research on the drugs effecting *miR-29*a expression is lacking. Therefore, finding a clinical available drug that can regulate the *miR-29*a expression can enhance the clinical efficacy for IgAN.

MMF is a potent immunosuppressant, elective inhibition of lymphocyte proliferation and antibody formation were regarded as the mechanism underlying the clinical efficacy. Previous study suggested that MMF could reduce proteinuria, and improve renal function in IgAN patients. Chen et al. [33] treated, either by MMF (1-1.5 g/d) or oral prednisone (0.8 mg/kg/d), 62 Chinese adult IgAN patients with advanced pathological lesions and proteinuria>2 g/d. One year after initiation of treatment, MMF-treated patients showed significant reduction of proteinuria and serum levels of cholesterol and triglyceride. A repeat renal biopsy performed in five patients after 9.8 ± 2.3 months of MMF treatment showed improvement of interstitial lesions. The authors concluded that MMF monotherapy for $\geq$ 12 months was superior to prednisone in reducing proteinuria in patients with an unfavorable histological grading. Previous study indicated that MMF could down-regulate the expression levels of MCP-1, ICAM-1 and TGF-β1 [21,22]. Regarding that *miR-29*a is crucial to the renal fibrosis and pathogenesis of IgAN, it hypothesized that *miR-29*a level may account for the function of MMF efficacy.

Nevertheless, the effect of MMF on *miR-29*a has been rarely performed. Considering that the process of renal fibrosis leads to decreased *miR-29*a. Hence, this study was designed to evaluate the influence of MMF on serum, urinary *miR-29*a expression.

MMF treatment could significantly increase the serum and urinary expression level of *miR-29*a, along with the decline of proteinuria. After MMF treatment, elevated expression of *miR-29*a could inhibit the TGF-β1/Smad3-signaling pathway, to inhibit the progress of renal fibrosis. We hypothesized that IgAN patients could restore the *miR-29*a expression after MMF administration. The serum, urinary and renal expression levels of *miR-29*a were highly consistent. So serum and urinary level of *miR-29*a is regarded as biological marker, which can assess the effect of MMF upon IgAN. But the specific mechanism needed further study.

Taken together, the present study found that the serum, urinary and renal expression of *miR-29*a were down-regulated of patients with IgAN. The urinary and serum *miR-29*a level is related with the severity of proteinuria and glomerularsclerosis, and urinary *miR-29*a level correlated with renal function. The results suggested urinary and serum expression of *miR-29*a might play a vital role in reflecting the pathogenesis and development of IgAN. As urinary, serum and intra-renal expression of *miR-29*s was highly consistent, so urinary and serum expression levels of *miR-29*a serve as biological markers of renal fibrosis in IgAN. MMF could significantly up-regulate the serum and urinary levels of *miR-29*a in IgAN. It may be a mechanism underlying the clinical efficacy of MMF in treating IgAN.

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