Evaluation of MicroRNAs miR-196a, miR-30a-5p, and miR-490 as Biomarkers of Disease Activity among Patients with FSGS

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Abstract

Background and objectives This study aimed to identify urinary microRNAs (miRNAs) as biomarkers for FSGS disease activity.

Design, setting, participants, & measurements Candidate urinary miRNAs were identified in pooled urine samples from patients with active FSGS (FSGS-A) and FSGS in remission (FSGS-CR), and were then validated using individual samples. Their levels were compared both under different treatment responses in a prospective study of FSGS and in patients with different membranous nephropathy (MN) and diabetic nephropathy (DN) disease activity. The prediction of these miRNAs for treatment responses was further analyzed in both retrospective and prospective cohorts of patients with FSGS.

Results All 54 miRNAs were included as candidate biomarkers, including those with high levels in patients with FSGS-A (n=9) under the TaqMan Low Density Array as well as those with conserved expression in kidneys and involved in immune response. TaqMan probe-based quantitative RT-PCR confirmed the higher levels of four miRNAs in patients with FSGS-A in two independent cohorts (n=18 and n=80). Urinary miR-196a, miR-30a-5p, and miR-490 discriminated FSGS-A from FSGS-CR, with an area under the curve of 0.63 ($P=0.03$). After steroid treatment, their levels were lower in steroid-responsive patients with FSGS (all $P<0.001$), but were unchanged in steroid-resistant patients. The levels of miRNAs were similar between active MN and MN in remission as well as active DN and incipient DN (all $P>0.05$). Urinary miR-30a-5p marginally predicted the response to steroid treatment in patients with FSGS-A, with an area under the curve of 0.63 ($P=0.03$).

Conclusions The levels of urinary miR-196a, miR-30a-5p, and miR-490 are associated with FSGS disease activity.


Introduction

Primary FSGS is a clinicopathologic entity characterized by heavy proteinuria and sclerotic changes in some glomeruli and represents a major cause of ESRD (1). Nephrotic syndrome in patients with FSGS can often recur because of poor responsiveness to currently available therapeutic agents, including steroids. Clinically, urine analysis remains a fundamental examination for the evaluation of FSGS states. Although the urinary protein level is the most commonly used parameter for FSGS, its use is limited by poor specificity and low sensitivity. Therefore, it is necessary to develop more specific and sensitive biomarkers for evaluating disease activity in patients with FSGS. The identification of biomarkers specific for the FSGS disease state may also provide insights into the pathomechanism of FSGS. Although increasing efforts have been made to identify such biomarkers, few novel noninvasive biomarkers have yet been identified (2–4).

MicroRNAs (miRNAs) are important regulators of gene expression that suppress the translation and/or promote the degradation of the transcripts of their target genes (5). The dysregulation of miRNAs results in various disease states, including renal dysfunction and injury (6–8). In recent studies, we (9–11) and others (12–14) found that miRNAs can be secreted by cells into body fluids and these circulating miRNAs are highly stable. Because the expression profiles of these circulating miRNAs are often associated with specific diseases, including cancers, they exhibit great potential as noninvasive or minimally invasive biomarkers for the diagnosis of various diseases (15). Urine, which is produced by the kidney and passes through almost all cell types of the kidney, may contain miRNAs that can serve as biomarkers for kidney diseases such as lupus nephritis and IgA nephropathy (16,17).

Whether urinary miRNAs can serve as biomarkers for disease activity of patients with FSGS, which is a common cause of proteinuric disease, is currently unknown (18). We conducted this study to address this question.
Materials and Methods

Enrollment of Patients and Controls
A total of 107 patients with biopsy-proven active FSGS (FSGS-A) and 103 patients with FSGS in remission (FSGS-CR) were retrospectively enrolled, and 105 healthy normal control (NC) participants were matched by age and sex. Independent 55 patients with FSGS-A were prospectively enrolled and treated with 60 mg glucocorticoid per day for 8 weeks. In addition, 29 patients with membranous nephropathy (MN) disease activity (MN-A), 26 patients with MN with complete remission (MN-CR), 23 patients with diabetic nephropathy (DN) with disease activity (DN-A), 27 patients with incipient DN (IDN), and 27 independent age- and sex-matched healthy controls were enrolled as disease control cohorts. The definitions of FSGS-A, FSGS-CR, MN-A, MN-CR, DN-A, IDN, and healthy controls, as well as detailed information regarding treatment and follow-up, are given in the Supplemental Methods. The baseline characteristics of patients enrolled in the study are shown in Supplemental Tables 1–6. The study protocol was approved by the ethics committee of Jinling Hospital (Nanjing, China) in accordance with the Declaration of Helsinki, and informed consent was obtained from all patients and controls.

Study Design
To identify biomarkers for disease activity, patients with FSGS and controls were randomly divided into three sets (Figure 1A): a screening set (FSGS-A, n=9; FSGS-CR, n=9; and NC, n=11), a confirmation set (FSGS-A, n=18; FSGS-CR, n=14; and NC, n=18), and a validation set (FSGS-A, n=80; FSGS-CR, n=80; and NC, n=76). The candidate miRNAs were further analyzed in two disease cohorts: MN controls (MN-A, n=29; MN-CR, n=26; and NC, n=27) and DN controls (DN-A, n=23; IDN, n=27; and NC, n=27). The urinary levels of miRNAs were prospectively evaluated in 55 patients with FSGS-A and were compared before and after steroid therapy. In addition, the urinary miRNAs associated with FSGS activity were then analyzed for predicting patient responses to steroid treatment in two sets (Figure 1B): a retrospective cohort (FSGS-A, n=139; 68 of these participants achieved complete remission [CR]) and a prospective cohort (FSGS-A, n=22; 11 of these individuals achieved CR).

Urine Collection and Processing and RNA Preparation
Random urine specimens collected from patients and controls were processed within 4 hours after collection. The urine samples were centrifuged at 1500 rpm for 10 minutes at 4°C, and the supernatant was stored at −80°C. One aliquot of each sample was used for protein measurement. To prepare pooled urine samples, equal volumes of urine from one patient or control were used. Extraction of RNA from urine was performed as previously described (10). The detailed methods are described in the Supplemental Material.

TaqMan Low Density Array and Quantitative RT-PCR
One microgram of the extracted RNA was reverse-transcribed followed by cDNA preamplification. The amplified CDNA samples were loaded onto a TaqMan Low Density Array (TaqMan Human MicroRNA Array v3.0 A

Figure 1. A flow diagram of the research design for identifying biomarkers of FSGS activity and predicting patient responses to steroid treatment. (A) The study of identifying biomarkers of FSGS activity. (B) The study of predicting patient responses to steroid treatment. *MicroRNAs with conserved expression in kidneys and involved in immune response were also included as candidates. CR, complete remission; DN-A, active diabetic nephropathy; FSGS-A, active FSGS; FSGS-CR, FSGS in remission; IDN, incipient diabetic nephropathy; miR, microRNA; miRNA, microRNA; MN-A, active membranous nephropathy; MN-CR, membranous nephropathy in complete remission; NC, normal control.
and B; Applied Biosystems, Foster City, CA). MiRNA abundance is presented as threshold cycle (Ct) values normalized to U6 snRNA. The relative abundance of different miRNAs in the samples was expressed as fold change calculated by the comparative Ct method (2^{-\Delta\Delta C_t}). A hydrolysis probe-based quantitative RT-PCR (qRT-PCR) assay was performed (7900 Sequence Detection System; Applied Biosystems) to quantify the levels of miRNAs in independent samples. The detailed method is described in Supplemental Figure 1.

Statistical Analyses
Statistical analysis was performed with SPSS 18.0 software. The miRNA data are presented as the mean±SD. One-way ANOVA followed by Student–Newman–Keuls analysis was used to determine the significance of the differences in miRNA levels between groups. For individual miRNAs, we constructed receiver operating characteristic (ROC) curves and calculated the area under the ROC curve (AUC) to evaluate the sensitivity and specificity of the data for evaluating disease activity and predicting treatment response. The combined diagnostic power of multiple miRNAs was calculated using ROC curve analysis previously described (20). The correlation between miRNA levels and clinical variables were assessed using Spearman rank correlation analysis. A P value <0.05 was considered statistically significant.

Results
Identification of Candidate miRNAs
The TaqMan Low Density Array revealed distinct miRNA profiles among patients with FSGS-A, patients with FSGS-CR, and normal controls in the screening set. A total of 196 miRNAs with altered levels in urine of patients with FSGS-A were detected compared with other two groups (Supplemental Figure 2A). Among them, 27 miRNAs had Ct values <30, with a ≥2-fold in urine from patients with FSGS-A than that from patients with FSGS-CR and controls (Supplemental Figure 2B). These 27 miRNAs were considered a part of candidates. Moreover, miR-30 family (21,22) and miR-21 (23,24) were conserved expression in the kidneys of various species and are known to be involved in kidney disease. miR-155 (16,25) and miR-146a (25,26) were associated with immune response and their levels were altered in patients with kidney disease. The 25 miRNAs conserved expression in the kidneys (Supplemental Table 7) and 10 related with inflammation (Supplemental Table 8) were also selected as candidates. A total of 54 candidate miRNAs were included and subjected to confirmation and validation.

Evaluation of miR-196a, miR-30a-5p, and miR-490 for Disease Activity in FSGS
TaqMan probe-based qRT-PCR was performed to measure the urinary levels in the confirmation set. The urinary levels of six miRNAs (miR-135b, miR-490, miR-196a, miR-30a-5p, miR-320, and miR-155) were significantly higher in patients with FSGS-A compared with normal controls and patients with FSGS-CR (Table 1; detailed data are listed in Supplemental Table 9). The individual urinary levels of these six miRNAs were determined by quantitative RT-PCR in the validation set. The levels of miR-155, miR-196a, miR-30a-5p, and miR-490, but not of miR-135b and miR-320, were significantly higher in the urine of patients with FSGS-A relative to controls and patients with FSGS-CR (Table 1).

In the validation set, ROC curve analysis revealed that urinary miR-155, miR-196a, miR-30a-5p, and miR-490 levels discriminated patients with FSGS-A from normal controls, with AUC values of 0.86, 0.92, 0.82, and 0.96, respectively (Figure 2A). More importantly, these miRNAs can discriminate patients with FSGS-A from patients with FSGS-CR, with AUC values of 0.74, 0.90, 0.88, and 0.89, respectively (Figure 2B). Using an AUC cut-off of ≥0.80, urinary miR-196a, miR-30a-5p, and miR-490 levels well distinguished patients with FSGS-A. The combined 3-miRNA (miR-196a, miR-30a-5p, and miR-490) signature provided an increased AUC of 0.95 in discrimination.

In addition, the urinary levels of candidate miRNAs showed no differences in different histologic types of FSGS. The distribution of patients with different subtypes of FSGS in the study was shown in Supplemental Figure S6. Within the same type, the levels of miRNAs were significantly higher in patients with FSGS-A compared with patients with FSGS-CR (Supplemental Table 10).

Evaluation of miR-196a, miR-30a-5p, and miR-490 for Disease Activity in MN or DN
To test whether miR-196a, miR-30a-5p, and miR-490 are biomarkers unique to patients with FSGS, we enrolled other patients with proteinuria as disease controls, including patients with MN and DN (Figure 1A). Supplemental Tables 4 and 5 show the analyzed clinical parameters of patients with MN and DN, respectively. Compared with normal controls, the levels of urinary miR-196a, miR-30a-5p, and miR-490 were higher in patients with active MN (Table 2; all P<0.05). However, they were similar between patients with MN-A and patients with MN-CR (Table 2; all P>0.05). Urinary levels of these three miRNAs can discriminate patients with active MN from normal controls (Supplemental Figure 3A), but not from patients with MN-CR (Supplemental Figure 3B). Similar results were obtained in the study with the DN cohort (Table 2, Supplemental Figure 4).

Evaluation of miR-196a, miR-30a-5p, and miR-490 in a Prospective Study of FSGS
The urinary levels of three miRNAs before and after steroid therapy were analyzed in a prospective study with 55 patients with FSGS-A, and 33 patients presented steroid response (Figure 1A, Supplemental Table 6). For steroid-responsive patients, the urinary levels of miR-196a, miR-30a-5p, and miR-490 decreased significantly after steroid therapy (Figure 3A; all P<0.001). By contrast, the urinary levels of these three miRNAs were essentially unchanged in the steroid-resistant patients after treatment (Figure 3B; all P>0.001).

In the prospective study, ROC curve analysis showed that the urinary levels of miR-196a, miR-30a-5p, and miR-490 could serve as biomarkers to discriminate patients with FSGS-A from patients with FSGS-CR, with an AUC value >0.80 (Supplemental Figure 5A). The 3-miRNA signature could accurately discriminate the patients with FSGS-A from normal controls and patients with FSGS-CR (Table 1).
from the patients with FSGS-CR, with an AUC value of 0.94 (Supplemental Figure 5B).

Evaluating Prediction of miR-30a-5p for Steroid Response in FSGS

Regarding all analyzed patients with FSGS-A, data on steroid responses were retrospectively available for 139 patients (Figure 1B); 68 of these patients were classified as having CR after an 8-week follow-up. Only miR-30a-5p levels before treatment were significantly different between the groups with and without CR (Supplemental Table 11; $P=0.03$). Multiple logistic regression analyses showed that the levels of urinary miR-30a-5p at baseline were directly related to the occurrence of CR after treatment. Urinary miR-30a-5p levels can distinguish patients with CR from patients without CR with moderate

<table>
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<th>microRNAs</th>
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<th>Fold Change for FSGS-A versus FSGS-CR</th>
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<td></td>
<td>Controls</td>
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</table>

Data are expressed as the mean±SD unless otherwise indicated. $P$ values were calculated by one-way ANOVA analysis followed by Student–Newman–Keuls analysis. miRNAs, microRNAs; FSGS-CR, FSGS in remission; FSGS-A, active FSGS.

Table 1. The concentrations of urinary miRNAs in patients with FSGS and controls in the validation cohort

Figure 2. | Receiver operating characteristic curve analysis of the ability of urinary miRNAs to serve as biomarkers in the validation set for discriminating FSGS-A from controls and from FSGS-CR. (A) ROC curve of discriminating FSGS-A from controls. (B) ROC curve of discriminating FSGS-A from FSGS-CR. 95% CI, 95% confidence interval; AUC, area under the curve.
To confirm the predictive value of urinary miR-30a-5p levels, changes in this parameter were further prospectively analyzed in 22 patients with FSGS-A (Figure 1B). Eleven of these patients presented with CR after 8 weeks of treatment (Supplemental Table 12). Before treatment, the levels of proteinuria and miR-30a-5p were similar between the groups with and without CR. After 8 weeks of treatment, both proteinuria and miR-30a-5p were decreased significantly in the two groups ($P < 0.001$ and $P = 0.003$, respectively; Figure 4B). Interestingly, after 4 weeks of treatment, miR-30a-5p levels differed significantly between patients with and
without CR ($P=0.003$), whereas the level of proteinuria did not ($P=0.29$).

Discussion

In this study, we utilized multiple strategies to identify urinary miRNA-based biomarkers for FSGS disease activity. We directly screened the distinct expression profile of urinary miRNAs in patients with FSGS-A using a TaqMan Low Density Array, and candidate miRNAs were selected for further analysis. Because previous studies reported that kidney-enriched miRNAs such as the miR-30 (21,22,27) and miR-10 families (28) as well as miRNAs involved in immune responses and inflammation, including miR-155 (29,30) and miR-146a (26,31), have roles in the modulation of renal function, these kidney-specific and immune response-associated miRNAs are also included in the candidate list.

A TaqMan probe-based qRT-PCR assay was used to validate these miRNAs in individual urine samples. Three miRNAs (miR-196a, miR-30a-5p, and miR-490) thus serve as biomarkers for the discrimination of patients with FSGS-A from healthy controls and patients with FSGS-CR, with higher levels in patients with FSGS compared with healthy controls and patients with FSGS-CR. We compared the urinary levels of miR-196a, miR-30a-5p, and miR-490 in patients with FSGS with those in patients with MN and DN, two other renal diseases that are characterized by nephrotic proteinuria. Importantly, we found that although the urinary levels of these three miRNAs in patients with proteinuria with MN and DN were significantly higher than in healthy controls, the levels did not decrease when the diseases were in remission. In addition, patients in the MN-A, MN-CR, DN-A, and IDN groups showed no differences in the levels of these miRNAs. This suggests that the urinary levels of miR-196a, miR-30a-5p, and miR-490 serve as biomarkers of disease activity that might be unique to FSGS. However, because the sample of patients with MN and DN in our study was small, further studies are needed to validate the relationship between these miRNAs and MN and DN.

In this work, we further performed a prospective study to evaluate the correlation between the urinary levels of miR-196a, miR-30a-5p, and miR-490 and disease activity in patients with FSGS receiving steroid treatment. Our results clearly demonstrated that at the end of therapy, the urinary levels of miR-196a, miR-30a-5p, and miR-490 were greatly decreased in patients with FSGS-CR, whereas they remained unchanged in patients with FSGS without remission. Through these strategies, we demonstrated that the urinary levels of miR-196a, miR-30a-5p, and miR-490 can be used as novel noninvasive biomarkers for evaluating FSGS disease activity. To our knowledge, this is the first report of a urinary miRNA-based biomarker for FSGS disease activity.

Finally, we analyzed the predictive value of these candidate miRNAs for treatment response of FSGS. On the basis of retrospective analysis, urinary miR-30a-5p at baseline was the only biomarker that distinguished patients with CR from those without. When changes in urinary levels of miR-30a-5p were prospectively tracked, differences in urinary miR-30a-5p levels between patients with and without CR were observed at the fourth week of therapy, before changes in proteinuria. The levels of proteinuria at 8 weeks of treatment were usually taken as a marker for treatment response. Urinary levels of miR-30a-5p seemed to predict the response at 4 weeks of treatment. It will give clinicians a more option to judge the treatment response on time. Although these findings suggest that urinary levels of miR-30a-5p can be used to predict the responses of individual patients with FSGS to treatment, a study that includes a well-designed prospective validation cohort is needed to confirm this finding.

Previous studies suggested that urinary miRNAs, like circulating miRNAs in other body fluids, are derived from tissues and cells in the presence of ongoing disease (32,33). In general, cellular miRNAs can passively leak from damaged cells or can be actively secreted into the extracellular space or circulation via the microvesicle pathway (34,35) or within HDL protein complexes (36,37). The expressions and functions of miR-196a, miR-30a, and miR-490 miRNAs in their respective tissues also support a role for these miRNAs as potential biomarkers of FSGS disease activity. Previous studies, including ours (22,27), showed that the miR-30 family is strongly expressed in podocytes and that its levels vary with disease state. Dysfunction and/or

![Figure 4](image-url)  
**Figure 4.** | Prediction value of urinary miR-30a-5p in steroid-responsive patients. (A) Receiver operating characteristic curve analysis of the urinary miR-30a-5p in predicting steroid-responsive patients in a retrospective study ($n=139$). (B) The percentage reduction in the level of proteinuria and the miR-30a-5p in a prospective study ($n=22$).
decrease in miRNAs of the miR-30 family could lead to podocyte apoptosis and depletion, glomerulosclerosis, and proteinuric renal disease. In other work, we found that the miR-30 family is expressed in renal tubular cells (J. Wu unpublished observations), and miR-196a was also shown to be expressed predominantly in the kidney (38). miR-196a was previously shown to correlate negatively with disease activity in SLE and has critical roles in cancer pathogenesis, viral immunity, and cell differentiation (39) as well as a role in kidney development. No studies that reveal the distribution of miR-490 in the kidney are currently available. Thus, although progress has been made in elucidating the cellular functions of miR-30a-5p, miR-196a, and miR-490, it is difficult to understand the molecular mechanisms that govern the urinary expression levels of these miRNAs in different states of FSGS until more in vivo and in vitro studies are performed. Thus, further studies are needed to help uncover the mechanism underlying these relationships.

This study has some limitations. First, the cohort consisted of a relatively small number of patients with FSGS, which prevented us from using more strict criteria for sample separation. The preliminary results did not reveal any differences in the urinary miRNA levels between active MN and MN in remission or between active DN and incipient DN. However, because the sample size in this study is small, further studies are needed to validate our findings and to explore the relationship of urinary miRNAs to MN and DN. Second, because this study was conducted in Chinese patients, further studies are required to validate whether the findings can be extrapolated to patients of other races with FSGS. With this in mind, a full understanding of the molecular mechanisms by which specific urinary miRNAs are linked to the disease status of patients with FSGS may have broad clinical applications.

In summary, this is the first study to report a link between urinary miRNAs, disease activity, and treatment response in patients with FSGS. The data presented here demonstrate that urinary levels of miR-196a, miR-30a-5p, and miR-490 are positively associated with FSGS disease activity, and they might be promising as biomarkers for evaluating FSGS disease activity. In addition, the change in the urinary level of miR-30a-5p could be used to predict the treatment response in patients with FSGS.

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Disclosures

None.

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