Increased Serum and Urinary MicroRNAs in Children with Idiopathic Nephrotic Syndrome

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BACKGROUND: MicroRNAs (miRNAs) are present in body fluids and may have the potential to serve as disease biomarkers. This study explored the clinical value of miRNAs in serum and urine as biomarkers for idiopathic childhood nephrotic syndrome (NS).

METHODS: We obtained serum samples from 159 NS children (24 steroid resistant and 135 steroid sensitive), 109 age/sex-matched healthy controls and 44 children with other kidney diseases. Serum miRNAs were analyzed with the TaqMan Low Density Array and then validated with a quantitative reverse-transcription PCR assay with 126 individual samples. Moreover, we collected paired serum samples from 50 patients before and after treatment to determine the value of these miRNAs for condition assessment. In addition, urine samples from these patients were examined for candidate miRNAs.

RESULTS: The concentrations of serum miR-30a-5p, miR-151-3p, miR-150, miR-191, and miR-19b were highly increased in NS children compared with controls (P < 0.0001). The urinary miR-30a-5p concentration was also increased in NS (P = 0.001). The area under the ROC curve and the odds ratio for the combined 5 serum miRNAs were 0.90 (95% CI, 0.86–0.94; P < 0.0001) and 40.7 (95% CI, 6.06–103; P < 0.0001), respectively. Moreover, the concentrations of the 5 serum miRNAs and urinary miR-30a-5p markedly declined with the clinical improvement of the patients.

CONCLUSIONS: We determined that 5 distinct serum miRNAs and urinary miR-30a-5p were increased in NS children. These circulating or urinary miRNAs may represent potential diagnostic and prognostic biomarkers for idiopathic pediatric NS.

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Childhood nephrotic syndrome (NS) is the most frequent glomerular disease that presents during childhood, primarily owing to a disturbed immune function. This disease is characterized by alterations in permselectivity at the glomerular capillary wall that lead to an inability to restrict the urinary loss of protein. Estimates on the annual incidence of NS range from 2 to 7 per 100 000 children, with a prevalence ranging from 12 to 16 per 100 000. More than 80% of children with idiopathic NS show minimal changes related to the disease and generally respond well to treatment with prednisone. The clinical hallmarks of NS are heavy proteinuria, edema, hypoalbuminemia, and hyperlipidemia. If untreated, NS is associated with an increased risk of life-threatening infections, thromboembolism, lipid abnormalities, and malnutrition. The molecular pathogenesis of NS is unclear, however, and relapse is a common problem, occurring in up to 90% of patients. Half of these patients experiencing frequent relapses. Currently, serum albumin, lipids, and proteinuria are the common diagnostic markers of childhood NS, but these markers may not accurately predict the outcome of individual patients because of the heterogeneity of the disease. Renal biopsy is more precise for establishing prognoses of renal outcome, but it has potential complications. Repeated monitoring is technically difficult, particularly for children. Therefore, there is still an urgent need to identify new noninvasive diagnostic and prog-

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Nonstandard abbreviations: NS, nephrotic syndrome; miRNA, microRNA; IgAN, IgA nephropathy; RT-qPCR, quantitative reverse-transcription PCR; AUC, area under the ROC curve; Cq, threshold cycle; OR, odds ratio.
nontrophic biomarkers and new therapeutic targets for this disease.

MicroRNAs (miRNAs), a family of small noncoding RNAs of approximately 22 nucleotides, play important roles in various biological processes through their regulation of posttranscriptional gene silencing via base pair binding to the 3′ untranslated region of their target mRNAs (7). Their expression patterns can indicate the pathophysiological status of a tissue and have been shown to be specific for particular disease states (7, 8). Recent studies by our group and others have shown that miRNAs have a relatively stable existence in the circulation and other body fluids, and their production patterns are tightly correlated with various diseases (9–19). Changes in circulating or urinary miRNAs have also been reported in a small number of patients with kidney diseases, such as lupus nephritis (20), IgA nephropathy (IgAN) (21, 22), and acute or chronic renal injury (23, 24). These results suggest that body fluid miRNAs may be informative with respect to human pathologies and may have great potential as a class of novel, noninvasive biomarkers of disease activity. Nonetheless, the global serum and urinary miRNA pattern in NS patients has not been reported.

In this study, we used TaqMan Low Density Array (Applied Biosystems) scanning followed by confirmation with a quantitative reverse-transcription PCR (RT-qPCR) assay to characterize the miRNA-production profile in the serum of children with idiopathic NS. We identified a panel of 5 serum miRNAs that may serve as novel diagnostic and prognostic indicators and thereby provide some useful information about the molecular pathogenesis of childhood NS.

Materials and Methods

PARTICIPANTS AND PROCESSING OF SERUM AND URINE SAMPLES

The study cohort consisted of 159 consecutive hospitalized proteinuric NS children and adolescents (daily urinary protein excretion ≥50 mg·kg⁻¹·day⁻¹), 44 children with other kidney diseases (18 with Henoch–Schönlein purpura nephropathy, 15 with IgAN, and 11 with lupus nephritis) who had been newly diagnosed according to the definition of the International Society of Kidney Disease in Children (25) and who were admitted to the Department of Pediatrics, Jinling Hospital, Nanjing, China, between 2011 and 2012. The inclusion criteria were an age of 1–14 years and an absence of clinical and laboratory findings of a systemic disease (including diabetes mellitus or liver disease), known familial hyperlipidemia, or malignant disease. All of the NS patients had idiopathic NS, and patients with secondary or congenital NS were excluded. Renal biopsies were performed in 23 NS patients: 5 with glomerular minimal change, 13 with membranoproliferative glomerulonephritis, 3 with podocyte lesions, and 2 with glomerular interstitial nephritis. The NS patients were treated with prednisone at 1.5–2 mg·kg⁻¹·day⁻¹ (≤60 mg/day) and antiinflammatory agents. Of the 159 NS children, 24 were steroid resistant (no response to the initial 8-week steroid treatment), and the others were steroid sensitive (remission obtained within 8 weeks of steroid therapy). Twenty-four patients were particularly sensitive to the steroids, and their proteinuria disappeared within 1 week. Blood and urine samples were collected before any therapeutic procedures. In addition, 50 paired serum samples and 15 paired urine samples were drawn from the patients over a 4- to 8-week treatment period before and after their regular daily dose of steroid therapy. For comparison, we selected 109 healthy age/sex-matched children who had visited the Nanjing Children’s Hospital, China, for a routine checkup. The study protocol was approved by the ethics committees of Jinling Hospital and Nanjing Children’s Hospital in accordance with the Declaration of Helsinki, and informed consent was obtained from the parents of all the children.

We collected 3 mL of venous blood from each study participant after 12 h of fasting overnight. Urine samples were collected in the morning. Each blood or urine sample was immediately centrifuged at 3000g for 10 min at room temperature and then centrifuged at 10 000g for 5 min at 4 °C. The samples were stored at −80 °C until analysis.

RNA ISOLATION, TaqMan LOW DENSITY ARRAY, AND RT-qPCR ASSAY

For the TaqMan Low Density Array, equal volumes of sera from 33 NS patients and 30 controls were pooled separately to form case and control sample pools. RNA was extracted from each pooled sample according to a previously described protocol (see Supplemental Methods in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue4) (11). The miRNA profile of 754 different human miRNAs was then examined with the TaqMan Low Density Array on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). For the RT-qPCR assay of serum and urine samples, total RNA was extracted from 100 μL serum or 200 μL urine with a 1-step phenol/chloroform purification protocol, as previously described (see Supplemental Methods in the online Data Supplement) (13). A hydrolysis probe–based RT-qPCR assay was performed according to the manufacturer’s instructions (7300 Sequence Detection System; Applied Biosystems), with a minor modification as described previously (11). We assessed the detection limits of the RT-qPCR assay and its dynamic range and calculated...
the absolute concentrations of the target miRNAs from calibration curves developed with corresponding synthetic miRNA oligonucleotides. We evaluated the repeatability of the extraction of RNA from urine. For the detailed methodology, see the Supplemental Methods online. The repeatability of RNA extraction from serum and the RT-qPCR assay for miRNAs was evaluated in a previous study (11).

SERUM LIPIDS, RENAL FUNCTION PARAMETERS, AND BLOOD AND URINE PROTEIN MEASUREMENT
The serum concentrations of total protein, albumin, urea, creatinine, uric acid, total cholesterol, and triacylglycerols, as well as the urinary protein content, were measured on a Hitachi 7600 analyzer with commercial reagents (Randox Laboratories).

STATISTICAL ANALYSIS
The statistical analyses were performed with SAS software (version 9.1.3; SAS Institute). The miRNA data were presented as the mean (SE), urinary protein values were expressed as the median (interquartile interval), and other variables were expressed as the mean (SD). The nonparametric Mann–Whitney U-test was used to compare differences in variables between groups. For miRNAs, we constructed ROC curves and calculated the area under the ROC curve (AUC) to identify their associations with NS. In addition, forward stepwise binary logistic regression analysis was conducted to evaluate the influences of miRNAs on NS, controlling for other variables. A $P$ value $< 0.05$ was considered statistically significant. Correlations between miRNAs and clinical features were calculated by using Spearman rank correlation analysis.

Results

MICROARRAY ANALYSIS OF SERUM miRNAs IN NS
A multiphase, case control study was designed to identify markedly increased serum miRNAs in NS children (an overview of the strategy is shown in Fig. 1). We used a TaqMan Low Density Array with pooled serum samples to initially identify miRNAs that were present at significantly different concentrations in NS patients and healthy control individuals (see Table 1 in the online Data Supplement). miRNA concentrations are presented as threshold cycle (Cq) values and normalized to an internal control recommended by the manufacturer. The relative concentration was calculated by the comparative Cq method ($2^{-\Delta\Delta\text{Cq}}$). miRNAs were considered upregulated if their Cq values were $< 35$ in the control sample and the production of the miRNAs in serum showed at least a 2-fold increase in the NS sample, compared with the control sample. Of the 754
miRNAs scanned, 30 were upregulated in the NS group (see Table 2 in the online Data Supplement).

**INCREASES IN SERUM miRNA CONCENTRATIONS CONFIRMED BY RT-qPCR**

To validate the TaqMan Low Density Array results, we performed an RT-qPCR assay of the upregulated miRNAs in both the same patient cohort and a larger cohort (126 patients and 79 controls). Table 1 summarizes the clinical features for all of the participants. No significant differences between the NS and the controls in age or sex were identified. The serum total protein and albumin concentrations, however, were significantly decreased in NS patients compared with controls, and the urine protein content and the serum concentrations of total cholesterol, triacylglycerols, and uric acid were markedly increased (Table 1; see Table 3 in the online Data Supplement).

The inclusion criteria for significantly upregulated miRNAs were as follows: a mean change >1.5-fold, a P value <0.05 for comparison of the case and control groups, a Cq value <35, and a detection rate >75% in either the cases or controls. Consequently, we identified 5 miRNAs (miR-30a-5p, miR-151-3p, miR-150, miR-191, and miR-19b) that were significantly increased in the NS samples (Table 2; see Table 4 in the online Data Supplement).

The concentrations of the selected miRNAs were also measured in serum samples obtained from children with other kidney diseases. Only miR-30a-5p, miR-150, and miR-19b were significantly increased in Henoch–Schönlein purpura nephropathy, and the concentrations of the 5 miRNAs in IgAN and lupus nephritis patients were not different from those of the controls (see both Table 5 and Fig. 3 in the online Data Supplement).

**DIAGNOSTIC VALUE OF THE SELECTED SERUM miRNAs**

ROC curve analyses were then conducted to determine the diagnostic usefulness of the 5 selected miRNAs for NS. For the NS and control groups, the AUCs ranged from 0.833 to 0.919 (Fig. 2, A–E; see Table 6 in the online Data Supplement). To further evaluate the diagnostic value of the 5-miRNA profiling system, we used a risk score formula to calculate the risk score function for patient and control samples, as previously described (see Supplemental Methods in the online Data Supplement) (11). The frequency table and the ROC curves were then used to evaluate the diagnostic effect of the combined panel of the 5 miRNAs. The ROC curve for the 5-miRNA panel revealed a high diagnostic accuracy (AUC, 0.902; 95% CI, 0.860–0.944; P < 0.0001), which was better than that of most individual miRNAs (Fig. 2F; see Table 6 in the online Data Supplement). With an optical cutoff value of 0.805, the specificity was 84.8%, and the sensitivity was 86.5%.

### Table 1. The clinical features of the NS children and controls in the RT-qPCR assay.\(^a\)

<table>
<thead>
<tr>
<th>Variable(^b)</th>
<th>Controls (n = 79)</th>
<th>NS (n = 126)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female), n</td>
<td>61/18</td>
<td>103/23</td>
<td>0.542</td>
</tr>
<tr>
<td>Age, years</td>
<td>5.04 (2.87)</td>
<td>5.44 (3.10)</td>
<td>0.418</td>
</tr>
<tr>
<td>Total protein, g/dL</td>
<td>6.80 (0.49)</td>
<td>4.70 (0.87)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Albumin, g/dL</td>
<td>4.71 (0.22)</td>
<td>2.78 (0.87)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urea, mg/dL</td>
<td>25.6 (6.54)</td>
<td>28.8 (23.6)</td>
<td>0.711</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.36 (0.08)</td>
<td>0.34 (0.13)</td>
<td>0.019</td>
</tr>
<tr>
<td>Uric acid, mg/dL</td>
<td>4.40 (1.08)</td>
<td>5.26 (1.80)</td>
<td>0.001</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>156 (27.5)</td>
<td>339 (150)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triacylglycerols, mg/dL</td>
<td>87 (39)</td>
<td>217 (169)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine protein, g/dL(^c)</td>
<td>0</td>
<td>0.11 (0.07)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^a\) The data are expressed as the median (interquartile interval).

\(^b\) To convert total protein, albumin, and urine protein concentrations from g/dL to g/L, multiply by 10. To convert urea concentrations from mg/dL to μmol/L, multiply by 0.1665. To convert creatinine concentrations from mg/dL to μmol/L, multiply by 88.402. To convert uric acid concentrations from mg/dL to μmol/L, multiply by 0.05286. To convert triacylglycerol concentrations from mg/dL to mmol/L, multiply by 0.01129.

\(^c\) The data are expressed as the mean (SD), unless noted otherwise.

### Table 2. Serum miRNA concentrations in NS children and healthy controls.\(^a\)

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Controls (n = 79), fmol/L</th>
<th>NS (n = 126), fmol/L</th>
<th>Fold change</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-30a-5p</td>
<td>540 (4.9)</td>
<td>1353 (5.5)</td>
<td>2.51</td>
<td>5.90 × 10(^{-18})</td>
</tr>
<tr>
<td>miR-151-3p</td>
<td>29.3 (0.35)</td>
<td>74.9 (0.39)</td>
<td>2.56</td>
<td>2.37 × 10(^{-12})</td>
</tr>
<tr>
<td>miR-150</td>
<td>39.0 (0.37)</td>
<td>101 (0.49)</td>
<td>2.59</td>
<td>7.94 × 10(^{-15})</td>
</tr>
<tr>
<td>miR-191</td>
<td>21.7 (0.18)</td>
<td>71.3 (0.31)</td>
<td>3.29</td>
<td>1.13 × 10(^{-21})</td>
</tr>
<tr>
<td>miR-19b</td>
<td>12.2 (0.10)</td>
<td>29.6 (0.15)</td>
<td>2.43</td>
<td>6.39 × 10(^{-13})</td>
</tr>
</tbody>
</table>

\(^a\) The data are expressed as the mean (SE).
Fig. 2. ROC curves for the capacity of the serum miRNAs to differentiate the NS cases (n = 126) from the control individuals (n = 79) (A–F) and the NS onset period from the remission period (n = 50) (G–K).
We next performed a forward stepwise binary logistic regression analysis to further weigh the usefulness of the selected miRNAs. We used NS status as the dependent variable and controlled for kidney function parameters and serum lipid concentrations, including the serum concentrations of total protein, albumin, creatinine, urea, total uric acid, and triacylglycerols, as well as urine protein content. Consequently, the odds ratios (ORs) for miR-150 (OR, 40.056; 95% CI, 10.935–146.737; \( P = 2.536 \times 10^{-10} \)), miR-191 (OR, 32.552; 95% CI, 14.860–71.308; \( P = 2.536 \times 10^{-10} \)), and miR-30a-5p (OR = 17.241, 95% CI, 8.504–34.953; \( P = 2.875 \times 10^{-15} \)) were statistically significant in the NS group when the concentration cutoff values for these miRNAs were 763.793, 59.253, and 30.59 fmol/L, respectively. Furthermore, the combined panel of the 5 miRNAs had the highest OR for NS (OR, 40.695; 95% CI, 6.056–103.145; \( P = 5.99 \times 10^{-15} \)). These results suggest that these miRNAs are potent diagnostic markers for pediatric NS.

ALTERATION OF THE SELECTED SERUM miRNAs BEFORE AND AFTER STEROID TREATMENT

Next, to identify the potential of the selected miRNAs to predict condition improvement, we compared the concentrations of these miRNAs in paired serum samples obtained from 50 patients both before and after treatment. After 4–8 weeks of steroid therapy, these patients tested negative for urinary protein, and values for other kidney function and lipid markers had improved (see Table 7 in the online Data Supplement). All 5 of the selected serum miRNAs decreased significantly after treatment (\( P < 0.0001 \), compared with the values before treatment), and the miR-19b concentration was nearly restored to the normal concentration (\( P = 0.069 \), compared with healthy controls) (Fig. 3, A–E; see Table 7 in the online Data Supplement). For the pretreatment and posttreatment samples, the AUCs of these miRNAs ranged from 0.814 to 0.908 (Fig. 2, G–K; see Table 6 in the online Data Supplement), suggesting that these
miRNAs can serve as additional indicators for evaluating the prognosis of NS.

**COMPARISON OF SUBTYPES FOR THE SELECTED SERUM miRNAs**

Histologic subtypes showed no significant differences with respect to the serum concentrations of these miRNAs (see Table 8 in the online Data Supplement).

In addition, to evaluate the ability of the selected serum miRNAs for predicting the response of patients to steroid therapy, we compared the serum concentrations of these miRNAs in particularly steroid-sensitive and steroid-assistance groups; however, no significant differences were found (see Table 9 in the online Data Supplement).

**URINARY CONCENTRATIONS OF THE SELECTED miRNAs IN NS**

We also compared the concentrations of these miRNAs in urine samples from 30 NS patients and 30 controls. The RT-qPCR assay for measuring the urine miRNA concentration was reliable and reproducible (see Supplemental Methods in the online Data Supplement). The Cq values of replicate assays for urinary miRNAs were similar \((r^2 = 0.966)\), indicating that the RNA extraction method was reproducible (see Supplemental Methods and Fig. 2 in the online Data Supplement).

The urinary concentrations of miR-30a-5p in the NS patients were significantly higher than in the controls \((P = 0.001; \text{Table 3})\). The case and control groups, however, were not significantly different with respect to urinary miR-150 and miR-191 concentrations, and miR-151-3p and miR-19b were undetectable in the urine samples from the 2 groups (Table 3). An ROC curve analysis showed that the AUC for urinary miR-30a-5p was 0.749 (95% CI, 0.628–0.870; \(P = 0.001\)). Subsequently, we compared urine miR-30a-5p concentrations in paired urine samples obtained from patients before and after treatment and found that they had decreased markedly in 13 of 15 patients after steroid therapy \((P = 0.002, \text{compared with before treatment}; \text{Fig. 3F})\).

Furthermore, our evaluation of the relationship between the urine and serum concentrations of miR-30a-5p in matched samples revealed a positive correlation between urinary and serum miR-30a-5p concentrations \((r = 0.443; P = 0.014)\).

**CORRELATIONS BETWEEN miRNA CONCENTRATIONS AND CLINICAL PARAMETERS**

We then evaluated whether the patients’ clinical features were related to miRNA abundance. Proteinuria and other kidney function and lipid parameters showed no significant effects on serum or urinary miRNA concentrations (see Tables 10 and 11 in the online Data Supplement). The only exception was serum triacylglycerols, which showed inverse correlations with serum miR-30a-5p, miR-151-3p, and miR-150 concentrations (see Table 10 in the online Data Supplement). These results indicate that these 5 miRNAs are independent indicators for NS.

**Discussion**

Current evidence suggesting that body fluid miRNAs may be informative regarding human pathology has kindled wide interest in their diagnostic potential \((9–22)\). A number of studies have shown that the imbalanced production of some miRNAs is also present in the circulation in a variety of kidney diseases. The circulating levels of total and specific miRNAs are reduced in patients with severe chronic renal failure, compared with patients with mild renal impairment or normal renal function \((24)\). The plasma of patients with acute kidney injury showed miR-16 and miR-320 downregulation, compared with healthy and disease control individuals, and miR-210 showed significant upregulation \((23)\). The detection and quantification of circulating miRNAs may thus represent a novel noninvasive tool to detect and monitor kidney disease activity. In the present study, we systematically examined the serum miRNA profile in pediatric NS by means of a TaqMan Low Density Array analysis, followed by an RT-qPCR validation assay. Our results demonstrate that pediatric NS is associated with striking perturbations in serum miR-30a-5p, miR-151-3p, miR-150, miR-191, and miR-19b. These 5 serum miRNAs were significantly increased in NS patients and decreased markedly as the patient attained remission. ROC curves and logistic regression analyses also revealed a strong relationship between these miRNAs and NS. Such information will not only increase the number of novel biomarkers for molecular diagnostics and assessment in pediatric NS but also provide mechanistic insight regarding the pathogenesis and progression of this disease.
The cause and pathogenesis of idiopathic childhood NS remain unknown. Recent evidence suggests that the primary defect in NS is at the level of glomerular visceral epithelial cells (podocytes), which are key cells in the selective filtering action of the glomerular capillary wall (26). Recently, several studies with mice have demonstrated an important role for miRNAs in the development and function of podocytes and glomeruli (27, 28). The podocyte-specific deletion of Dicer or Drosha, 2 key enzymes that function in a stepwise manner to generate a mature miRNA, during development leads to proteinuric renal disease, multiple abnormalities (including podocyte apoptosis and depletion), and collapsing glomerulopathy. This result confirms that the mutant phenotype is due to the loss of miRNAs (27, 28). Thus, normal generation of miRNAs is required for the normal function of mature podocytes. Identifying these miRNAs may provide new insight into disease pathogenesis in and novel therapeutic targets for various podocytopathies, and recent observations have suggested a role for miR-30a in the homeostasis and podocytopathies of podocytes (27, 29). Dicer-knockout mouse podocytes were found to be incapable of synthesizing miR-30a, and these mice developed proteinuria by 3 weeks after birth and progressed rapidly to end-stage kidney disease (27, 28). Furthermore, the knockdown of miR-30a-5p phenocopied most of the phenotypic defects observed after the global inhibition of miRNA biogenesis by the knockdown of Dicer and another key component, Dgcr8, in Xenopus (30). A molecular analysis revealed that miR-30a regulates the LIM-class homeobox factor Xlim1/Lhx1, a major transcriptional regulator of kidney development, and restricts its activity (30). Therefore, miR-30a-5p is an important genomic regulator of molecular podocyte homeostasis and is therefore involved in the pathogenesis of childhood NS. In addition, strong evidence suggests that immune dysregulation is involved in the physiopathology of idiopathic NS, chiefly involving cell-mediated immunity (1) and primary T-cell disorders in particular (31). Several of the miRNAs selected in our study are involved in cell-mediated immunity. For example, miR-150 is highly produced in T and B cells and can be detected at high concentrations in lymph nodes, spleen, and thymus (32). Mice models with a podocyte-specific loss of Dicer have shown miR-150 to be specific to lymphoid cells. Furthermore, miR-150 may have an important impact on T-cell development and physiology by targeting NOTCH3, a member of the Notch receptor family that plays important roles in both T-cell differentiation and leukemogenesis (33). miR-19b, a member of the miR-17-92 cluster, is the key player controlling Th1 responses through multiple coordinated biologic processes, including suppressing inducible regulatory T-cell differentiation by regulating the functionally important target phosphatase and tensin homolog (34). All of these findings support a possible role for these miRNAs in the pathogenesis of NS.

An increasing incidence of primary steroid resistance has recently been identified in childhood NS (35). The prognosis for these patients can be considered very favorable, and renal biopsy is unnecessary unless indicated by the subsequent clinical course. Unfortunately, the selected 5 miRNAs failed to distinguish steroid-resistant patients from steroid-sensitive patients; however, our miRNAs were chosen by comparing NS patients with healthy individuals rather than by comparing steroid-sensitive cases with steroid-resistant cases. Further studies are necessary to identify miRNAs that have prognostic value in steroid resistance.

The reasons and underlying mechanisms of increased concentrations of serum miRNAs remain unknown. miR-30a-5p is strongly produced in the pronephros and increases as kidney development progresses (30, 31). Given these findings, we suspect that the production of these miRNAs may change simultaneously during the course of NS, particularly miR-30a-5p in podocytes as a response to podocyte damage, leading to either apoptosis or the stimulation of proliferation and some form of repair. These miRNAs will then be easily released from apoptotic podocytes into the circulation. Further studies are needed, however, to investigate the production of these miRNAs in specific renal cell types, because such studies might help determine the mechanism of increased serum miRNAs.

In this study, we also evaluated miRNAs in urine, because aberrant levels of some urinary miRNAs have been reported in patients with IgAN (26, 27). We detected miR-30a-5p and found it markedly increased in urine samples from NS patients. Furthermore, we observed that the urinary miR-30a-5p concentration tended to decrease with the remission of patients, demonstrating that the urinary miR-30a-5p concentration is altered with disease progression and therapy. In addition, urinary miR-30a-5p concentration was positively correlated with serum miR-30a-5p concentration in our patients. Urinary miRNAs most likely originate from deciduous tubular epithelial cells and podocytes (36). Further study is necessary to clarify the mechanism and role of increased urinary miR-30a-5p in NS.

In summary, we have defined a distinctive serum miRNA signature in NS children. Specifically, we have demonstrated that the serum concentrations of miR-30a-5p, miR-151-3p, miR-150, miR-191, and miR-19b, as well as the content of miR-30a-5p in urine, are altered with disease progression and therapy. These results suggest that these miRNAs play important roles in the pathogenesis and progression of pediatric NS and
that they have potential as novel diagnostic and prognostic indicators of childhood NS.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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**References**