



Research paper

Increased urinary exosomal microRNAs in children with idiopathic nephrotic syndrome



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ABSTRACT

Background: Urinary exosomal miRNAs are gaining increasing attention for their potential as ideal non-invasive biomarkers for kidney diseases; however, little is known about their diagnostic ability for paediatric nephrotic syndrome (NS). This study explored the clinical value of urinary exosomal miRNAs for paediatric idiopathic NS. **Methods:** Urine samples were collected from 129 NS children and 126 age- and sex-matched healthy controls. The miRNA profile of urinary exosomes was analysed by high-throughput Illumina sequencing via synthesis (SBS) technology followed by verification with a quantitative reverse-transcription polymerase chain reaction (RT-qPCR) assay arranged in two independent cohorts. Additionally, paired urine samples from 65 of these patients were collected before and after treatment.

Findings: The Illumina SBS identified 30 markedly increased urinary exosomal miRNAs in NS children compared with controls (≥ 5 -fold, $P < .05$). Fifteen miRNAs were selected for further investigation, of which 5 (miR-194-5p, miR-146b-5p, miR-378a-3p, miR-23b-3p and miR-30a-5p) were verified by RT-qPCR to be significantly and steadily increased in NS (> 3 -fold, $P < .01$) and markedly reduced during the clinical remission period ($P < .001$). Moreover, the concentrations of miR-194-5p and miR-23b-3p were significantly positively correlated with the urine protein content and were markedly higher in the high urine protein group than in the low urine protein group ($P < .001$ and $P < .01$, respectively).

Interpretations: We identified 5 altered urinary exosomal miRNAs in NS children with disease progression and treatment. These urinary exosomal miRNAs could be promising and non-invasive potential biomarker candidates for diagnosing, monitoring and stratifying paediatric NS.

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Abbreviations: NS, nephrotic syndrome; miRNA, microRNA; RT-qPCR, quantitative reverse-transcription polymerase chain reaction; CKD, chronic kidney disease; FSGS, focal segmental glomerulonephritis; MCD, minimal change disease; MsPGN, mesangial proliferative glomerulonephritis; GML, glomerular minor lesion; NTA, nanoparticles tracking technology; Illumina SBS technology, Illumina sequencing via synthesis technology; TEM, transmission electron microscope; ROC, receiver operating characteristic curves; AUC, area under the ROC curve; Cq, quantification cycle; OR, odds ratio.

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1. Introduction

Paediatric idiopathic nephrotic syndrome (NS) is a chronic glomerular disease often encountered in children, which is predominantly attributed to immune-mediated inflammatory diseases and genetic mutations [1]. Approximately 1.15 to 16.9 per 100,000 children suffer from NS [2]. NS has severe complications, including bacterial infections, thromboembolisms and lipid abnormalities [1,3,4]. At present, the primary symptoms are severe proteinuria and hypoalbuminemia, as well as hyperlipidaemia and oedema, which are major clinical diagnostic indicators, but these symptoms may not be accurate predictors for patient outcome because of symptom heterogeneity and complications [5,6]. Renal biopsy, the standard method to judge pathological type and prognosis, is an invasive operation with latent risk and is normally not

Research in context

Evidence before this study

Childhood idiopathic nephrotic syndrome is the most frequent glomerular disease that presents during childhood. However, its molecular pathogenesis is unclear. Renal biopsy is the most precise approach for prognoses renal outcome, yet, it has potential complications, and repeated monitoring is technically difficult, especially for children. After a thorough literature search and review, we found that exosomal miRNAs have been proposed as more specific and excellent biomarker candidates for disease monitoring and prognosis; and several studies have highlighted the diagnostic and prognostic value of exosomal miRNAs in urine in adults with kidney disease. Nevertheless, the global urinary exosomal miRNA signature in paediatric idiopathic nephrotic syndrome patients and its clinical significance have not been reported.

Added value of this study

The major achievement from the research are summarized below: We identified and validated that five urinary exosomal miRNAs (miR-194-5p, miR-146b-5p, miR-378a-3p, miR-23b-3p and miR-30a-5p) were significantly elevated in the children with idiopathic nephrotic syndrome and markedly declined with the improvement of the patients.

The concentrations of urinary exosomal miR-194-5p and miR-23b-3p were significantly related with urine protein content and other renal function indexes. The two miRNAs could reflect the severity of the paediatric nephrotic syndrome disease.

Implications of all the available evidence

Urine is an ideal source of biomarkers for renal diseases. Exosomal miRNAs play important roles in occurrence and development of kidney disease, and urinary exosomal miRNAs identified in our study may potentially serve as novel liquid biopsy indicators for diagnosing, monitoring and stratifying paediatric nephrotic syndrome. Moreover, they may provide valuable insight on renal pathophysiology.

feasible for serial monitoring, especially in children [7,8]. Moreover, misdiagnosis may be caused by improper tissue sampling [9]. Therefore, new non-invasive diagnostic and prognostic biomarkers need to be found for the disease.

MiRNAs, small noncoding RNA molecules with a length of approximately 22 nucleotides, suppress gene expression by combining with the 3'-untranslated region (UTR) of target mRNAs and are involved in various physiological and pathological procedures [4,10]. In the kidneys, miRNAs not only maintain normal regulatory mechanisms but also play indispensable roles in renal dysfunction and structural damage [4]. We previously found a set of miRNAs that was dysregulated in the kidney tissues of children with nephropathy and was related to the pathogenesis of nephrosis in children [11]. Moreover, these miRNAs were also significantly increased in the serum or urine of children with NS and markedly decreased with the clinical remission of the patients, indicating that this specific set of circulating or urinary miRNAs could serve as a promising and non-invasive means in monitoring and stratifying children with severe complications [7,8].

Extracellular miRNAs are mainly composed of actively secreted miRNAs that are packaged into exosomes and microvesicles, passively released free miRNAs or the combination of miRNAs with RNA-binding proteins from tissues or cells [12]. Because they are actively

secreted by specific tissues or cells, exosomes may reveal the pathological or physiological conditions of the originating cells or tissues. Extracellular miRNAs embedded in exosomes (exosomal miRNAs), opposed to free miRNAs, have been proposed to be more specific and excellent biomarker candidates for disease monitoring and prognosis [13,14]. Several studies have reported that the expression levels of some exosomal miRNAs in urine were significantly changed in adults with some kinds of kidney diseases, such as IgA nephropathy, lupus nephritis, and chronic kidney disease (CKD) [15–19], suggesting that urinary exosomal miRNAs may have great potential as promising biomarkers of adult kidney disease activity. Nevertheless, the global urinary exosomal miRNA signature in paediatric idiopathic NS patients and its clinical significance have not been explored.

In this study, we used high-throughput Illumina sequencing via synthesis (SBS) technology scanning followed by verification with quantitative reverse-transcription polymerase chain reaction (RT-qPCR) assay to systematically evaluate a detailed urinary exosomal miRNA profile in a large cohort of children with idiopathic NS. We then assessed the diagnostic and monitoring value of the remarkably increased miRNAs in urinary exosomes as a promising liquid biopsy tool for idiopathic childhood NS.

2. Materials and methods

2.1. Participants and sample collection

A total of 129 children with idiopathic NS (24-h urinary protein >50 mg/kg) were enrolled in the present study. All patients were admitted to the Department of Paediatrics of Jingling Hospital in Nanjing, China, between February and November in 2017 and diagnosed according to the definition of the International Society of Kidney Disease in Children [20]. We established exclusion criteria to reduce the impact of other physical or pathological factors on urinary exosomal miRNA production, including congenital renal and urinary tract abnormalities, urinary tract infection, secondary nephrotic syndrome, other immune diseases, liver disease, cardiovascular disease and age > 15 years old. Of all the 129 NS patients in our study, 14 were newly diagnosed, and the others were in relapse. All the 14 newly diagnosed patients responded to prednisone initially, and 11 exhibited negative urine protein, while 3 experienced alleviation. All the NS children underwent prednisone therapy together with adjuvant treatment (including cyclophosphamide, cyclosporine or mycophenolate mofetil). Among them, 7 patients (5%) were treated with cyclosporine or/and mycophenolate mofetil. After 1 to 4 weeks of treatment, all patients showed negative or decreased protein in the morning urine samples. Renal biopsies were performed on 44 of these NS children: 13 with minimal change disease (MCD), 13 with mesangial proliferative glomerulonephritis (MsPGN), 8 with glomerular minor lesion (GML), and 10 with primary focal segmental glomerulonephritis (FSGS). Of the 26 patients with FSGS or MsPGN, 2 were treated with prednisone together with cyclosporine, 4 with cyclophosphamide, 3 with mycophenolate mofetil. We also selected 126 healthy age-/sex-matched children who had visited the Nanjing Children's Hospital in Nanjing, China, or Jinling Hospital, as the parallel controls. Details of the study were approved by the ethics committees of Jinling Hospital and Nanjing Children's Hospital based on the Declaration of Helsinki, and written informed consent was signed by the parents of all the children.

Morning urine samples were collected from all participants, immediately centrifuged at 3000g for 10 min at room temperature and then stored at –80 °C until analysis. Additionally, paired urine samples were collected from 65 of the enrolled patients before and after 1 to 4 weeks of treatment.

2.2. Urinary exosome isolation

For Illumina SBS technology, exosomes were extracted from 5 pooled NS urine samples and 4 pooled healthy urine samples. The

pooled samples were collected from 5 individuals with 5 mL urine from each person. We conducted ultracentrifugation to isolate exosomes according to the standard method with a slight modification as previously depicted [21]. Simply put, for the purpose of eliminating urine sediment and cell debris, pooled urine samples were initiated with low-speed centrifugation (500g followed by 1500 g) at 4 °C for 20 min. After transferring into a sterile centrifuge tube, the cell-free supernatant was centrifuged at 10,000 g for 30 min. Subsequently, supernatant was obtained and filtered with 0.22- μ m filters (Millipore, Massachusetts, USA) to remove microvesicles (100–1000 nm in diameter) and apoptotic bodies (800–5000 nm in diameter). Eventually, the supernatant that originated from the previous procedure was centrifuged at 110,000g for 70 min at 4 °C in an ultracentrifuge (Beckman Coulter, FL, USA) to pellet exosomes from urine. After the supernatant was extracted, the exosomal pellet was suspended with 200 μ L of phosphate-buffered saline (PBS) again.

For the RT-qPCR assay, exosomes isolated from each individual urine sample was conducted utilising the ExoQuick-TC™ Exosome Precipitation Solution (System Biosciences, CA, USA) [9]. According to the manufacturer's indication, we centrifuged urine sample at 3000g for 15 min at 4 °C to remove cells and cell debris, and then transferred 500 μ L of supernatant to a sterile vessel. After 100 μ L Exosome Precipitation Solution was added, the admixture was mixed well by inverting and then refrigerated overnight at least 12 h at 4 °C with an attention that the tube should remain upright during incubation without shaking. Centrifuging ExoQuick-TC/urine mixture at 1500g for 30 min at 4 °C, the exosomes appeared as pellets at the bottom of the tube, followed by aspirating supernatant. Centrifugation at 1500g for 5 min at 4 °C continued to yield residual ExoQuick-TC solution and all residual fluid was removed by aspiration, taking great care not to touch the precipitated exosomes in pellet form.

2.3. Nanoparticle tracking analysis

Exosomes isolated from urine were processed for nanoparticle tracking analysis (NTA) with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and with its corresponding software. Briefly, exosomes isolated freshly from urine were resuspended in 200 μ L 1 x PBS (Gibco, NY, USA). Diluted in 1 mL 1 x PBS, the sample was loaded into the cell, and then we regulated the instrument pre-acquisition parameters as follows: the temperature was 23 °C; the sensitivity was 85; the frame rate was 30 frames per second (fps); the shutter speed was 100; and the laser pulse duration was equal to that of shutter duration. We measured each sample at 11 different positions in the whole procedure, with two cycle readings per position. Post-acquisition parameters were adjusted to 25 for minimum brightness, 200 pixels for maximum size and 5 pixels for minimum size. Prior to measuring, we applied polystyrene particles with a diameter of 100 nm produced by ThermoFisher Scientific to align the instrument. We also carried out automatic quality control, such as, qualified cell detection and instrument calibration as well as focus. After analysing all 11 positions and removing all aberrant positions, measuring parameters, including median, mean, diameter and the density of the sample, were computed with the optimisational instrument software. Data gained from measurement were handled using the corresponding software, ZetaView 8.02.28, and Microsoft Excel 2013 (Microsoft Corp., Seattle, WA, USA).

2.4. Transmission electron microscopy (TEM) and western blotting

The exosomes isolated from pooled urine samples by ultracentrifugation were resuspended in 1 x PBS and applied to a carbon coated 200 mesh copper grids for 20 min. Excess liquid at the edge was wicked off using filter paper. Then, 2% phosphotungstic acid solution (HT152-250ML, Sigma, Germany) was added to yield negative staining for 10 min at room temperature. After removing the unnecessary liquid by filter paper again, the copper grids were dried with the incandescent lamp. The microphotographs were obtained using a JEM-1011 scanning

transmission electron microscope (Hitachi, Tokyo, Japan). Moreover, we extracted exosomal protein and performed western blot analysis as previously described [22]. Briefly, we isolated exosomes from pooled urine samples by ultracentrifugation. Exosomal protein was extracted with radioimmunoprecipitation assay (RIPA) buffer. Equal loading of extracted protein was separated on a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), followed by being transferred to a polyvinylidene difluoride (PVDF) membrane under the condition of 300 mA for 1 h (Tannon, Shanghai, China). We purchased antibodies as follows: anti-CD63 (Absin, Shanghai, China), CD9 (Absin, Shanghai, China), CD81 (Santa Cruz Biotechnology, CA, USA). After blocking with 5% non-fat milk in 1 x TBST (TBS, 0.1% Tween 20) buffer for 1 h, the membrane was incubated with primary antibody prepared with 5% non-fat milk in 1 x TBST (CD63, 1:1000; CD9, 1:1000; CD81, 1:500) overnight at 4 °C. After washing with 1 x TBST for 4 times, 15 min each time, secondary antibody incubation at dilution of 1:10,000 was performed for 1 h at room temperature. The membrane was washed again. Proteins of interest were detected on a gel imaging system using ECL western blotting substrate (Thermo Fisher Scientific, MA, USA) and band density was analysed with ImageJ software.

2.5. RNA extraction from urinary exosomes

Exosomal total RNA extraction was performed with the Trizol Reagent (Invitrogen, MA, USA) according to our previous report [22]. We added 2 magnetic beads and 1 mL of Trizol reagent (Invitrogen, MA, USA) to freshly isolated exosomes. The exosomes were sufficiently broken in Trizol using a tissue homogeniser with 120 HZ for 120 s. The mixture was vortex-mixed sufficiently and stood at room temperature for 5–10 min. After adding 200 μ L of chloroform, the mixture was immediately vortex-mixed and stood again, followed by centrifugation at 16,000g for 20 min at 4 °C. After the supernatant was transferred to sterile and RNase-free tube, equal volume of isopropyl alcohol was added and blended. This solution was set at -20 °C for at least 1 h. The RNA was precipitated by centrifugation at 16,000g for 20 min at 4 °C. We discarded the supernatant, washed the pellet with 1 mL of 75% ethanol and precipitated RNA again. The resulting RNA pellet was dried for 15 min at room temperature, dissolved in 20 μ L of RNase-free water and stored at -80 °C until further analysis.

For the repeatability of exosomal RNA extraction from urine samples, 2 equal portions were taken from pooled urine samples composed of 20 healthy individuals (500 μ L for each). The contents of 10 miRNAs (miR-508-3p, miR-509-3-5p, miR-509-3p, miR-196a-5p, miR-140-3p, miR-194-5p, miR-378a-3p, miR-23b-3p, miR-30a-5p, miR-146b-5p) were quantified by the RT-qPCR assay after exosomes and exosomal RNA were extracted from the 2 portions separately. Triplicate analysis was made for each sample and miRNA levels were represented as quantification cycle (Cq) values, which were plotted against each other, and then, the data were fitted to a straight line. As a result, Cq values of repetitive analysis were highly similar between the two portions ($R^2 = 0.946$), suggesting that the exosomal RNA extraction technique used in this study was reproducible (Supplemental Fig. 1).

2.6. Illumina SBS technology and quantification of miRNAs by RT-qPCR analysis

Illumina SBS technology for exosomal miRNAs from pooled urine samples was performed as previously reported [23]. Briefly, following filtration of small RNA (18–30 bp) by PAGE gel, ligation of 3' adaptor system and 5' adaptor mix system under optimal reaction condition, we amplified the small RNA molecules in 17 cycles with the adaptor primers. Then, the PCR products, fragments approximately 90 bp, were purified with PAGE gel, and the average molecule length was determined using the Agilent 2100 bioanalyser instrument (Agilent DNA 1000 Reagents) and quantified by real-time quantitative PCR (qPCR) (hydrolysis probes). The eligible libraries were amplified on cBot to

produce the cluster on the flow cell, which was sequenced single end with the HiSeq 2000 System, following the manufacturer's illustration. The digital-quality data were produced by processing image files obtained by the sequencer. After covering the adaptor sequences and discarding polluted reads, we assessed the reads with silico analysis.

A hydrolysis probe-based RT-qPCR assay for exosomal miRNAs from individual urine sample was performed as previously reported [7]. As there is currently a lack of consensus on which the reference gene for the RT-qPCR analysis of exosomal miRNAs is optimal, we normalised exosomal miRNAs concentrations to urine volume in the present study. The reverse transcription was performed in a reaction system (2 μ L of extracted RNA, 3.5 μ L of diethyl pyrocarbonate (DEPC) water, 2 μ L of 5 \times reverse transcription buffer, 1 μ L of 10 mmol/L deoxyribonucleotide triphosphate (dNTPs), 0.5 μ L of avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa) and 1 μ L of a stem-loop RT primer (Applied Biosystems)) with a favourable procedure at 16 $^{\circ}$ C for 30 min, 42 $^{\circ}$ C for 30 min, 85 $^{\circ}$ C for 5 min and then held at 4 $^{\circ}$ C. For real-time PCR, a total of 20 μ L of reaction containing 1 μ L of cDNA, 0.3 μ L of Taq, 0.33 μ L of hydrolysis probe (Applied Biosystems), 1.2 μ L of 25 mmol/L MgCl₂, 0.4 μ L of 10 mmol/L dNTPs, 2 μ L of 10 \times PCR buffer, and 14.77 μ L of MilliQ water was carried out with 1 cycle of 95 $^{\circ}$ C for 5 min, 40 cycles of 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 min using a LightCycler@96 (Roche). All reactions, including no-template controls, were analysed in triplicate. We set a fixed threshold to determine the resulting Cq values, which was blinded to distinguish between cases and controls in experimenters. To reduce batch effects, the samples of patients and controls were analysed simultaneously on the 96-well plates. To calculate the absolute concentrations of candidate miRNAs, we made calibration curves with respective synthetic miRNA oligonucleotides (miR-194-5p, miR-146b-5p, miR-378a-3p, miR-23b-3p and miR-30a-5p synthesised by TaKaRa, Dalian, China) at 10-fold serial dilution from 1 fmol/L to 10⁹ fmol/L, and the levels of the synthetic miRNAs were detected in triplicate by RT-qPCR assay. The final Cq values were plotted versus the log₁₀ of the amount of the synthetic miRNAs. To ensure the reliability of the method, we evaluated the detection limit of the RT-qPCR assay for each miRNA and its dynamic range. Consequently, the limit of detection and dynamic range for each miRNA was 10 fmol/L and 10 fmol/L–1 \times 10⁴ pmol/L (miR-194-5p), 1 fmol/L and 1 fmol/L–1 \times 10⁵ pmol/L (miR-146b-5p), 10 fmol/L and 10 fmol/L–1 \times 10⁴ pmol/L (miR-378a-3p), 10² fmol/L and 10² fmol/L–1 \times 10³ pmol/L (miR-23b-3p), 10 fmol/L and 10 fmol/L–1 \times 10⁴ pmol/L (miR-30a-5p) (Supplemental Fig. 2).

2.7. Blood biochemistry index and urine protein measurement

The Hitachi 7600 analyser was used to measure the serum concentrations of total protein and albumin (Leadman, Beijing, China), the serum concentrations of urea, creatinine and uric acid (Maker, Chengdou, China), the serum concentrations of total cholesterol and triacylglycerols (Randox, Crumlin, United Kingdom) with commercial reagents. Twenty-four-hour urine protein was quantified by the biuret method.

2.8. Statistical analysis

Statistical analysis was carried out with GraphPad Prism 5 (GraphPad Software, CA, USA) and SPSS 22.0 (IBM, NY, USA). We used the mean (SE) to represent miRNA concentration, the median (interquartile interval) for 24-h urine protein content and the mean (SD) for other variables. The nonparametric Mann–Whitney test and Kruskal–Wallis test were respectively utilised to identify the differences in variables among groups and groups with different histological subtypes. We utilised receiver operating characteristic curve (ROC) analysis to estimate the diagnostic value of urinary exosomal miRNAs. The correlations between miRNAs and clinical indicators were confirmed by Spearman correlation analysis. Binary logistic

regression analysis was performed to assess the risk of each miRNA on NS.

Additionally, risk score analysis was performed to evaluate the ability of the combinations of urinary exosomal miRNAs to predict paediatric idiopathic NS as previously described [24]. We described the risk score of each miRNA as *s*. When the expression level was above the upper 95% reference interval for the corresponding miRNA level in controls, the risk score was defined as 1 and as 0 otherwise. Moreover, we established a risk score function (RSF) to predict NS in accordance with a linear combination of the expression level of each miRNA. For example, *S*_{*i*} denotes the risk score for miRNA *j* on sample *i*, and *W*_{*j*} denotes the weight of the risk score of miRNA *j*. Then, the RSF for sample *i* using information from five miRNAs was calculated with the formula: $RSF_i = \sum_{j=1}^5 W_j \cdot S_{ij}$. To compute the *W*s, five univariate logistic regression models were conducted on the basis of the disease status generated by each of the risk scores. The regression coefficient for each risk score served as the weight to identify the contribution of each miRNA to the RSF. After being ranked according to their RSFs, all samples were divided into two groups, a high-risk group, representing the predicted NS cases and a low-risk group, representing the predicted control individuals. Frequency tables and ROC curves were then used to evaluate the diagnostic effects of the profiling and to find the appropriate cut-off point. A *P*-value <.05 was regarded as statistically significant.

3. Results

3.1. Characterisation of urine exosomes

Because efficiency and purity of exosome extraction are prerequisites for variability in the analysis of exosomal miRNAs, exosomes isolated from urine samples with ultracentrifugation were first characterised. We visualised exosomes by TEM and observed exosomes of the typical size and rounded membrane-bound morphology (Fig. 2a). We then assessed the concentration and size distribution of the exosomes by NTA and found that they were approximately 100 nm in diameter (Fig. 2b and Supplemental Table 1). We further performed western blots to confirm the presence of some established markers for exosomes. As shown in Fig. 2c, the isolated exosomes had markedly detectable CD63, CD9 and CD81. Furthermore, we evaluated the urine exosomes isolated by the ExoQuick exosome precipitation solution, and consistently, the obtained exosomes were also approximately 100 nm in size (Fig. 2b and Supplemental Table 1). These results demonstrated that urine exosomes were extracted successfully.

3.2. Urine exosomal miRNAs profile analysed by Illumina SBS technology

We designed a multiphase, case-control study to identify markedly increased urinary exosomal miRNAs in NS children as shown in an overview of the protocol in Fig. 1. Supplemental Table 3 shows the clinical features of NS patients. Illumina SBS technology was first performed on exosomal miRNAs and measured approximately 2000 miRNAs in 9 pooled urine samples, including 4 control samples (male/female: 13/7; mean age (SD): 5.50 (3.55)) and 5 NS samples (male/female: 17/8; mean age (SD): 6.16 (3.51)). No significant difference was found in age and sex distribution among the groups. A heat map and volcano plot showed markedly different profiles of urinary exosomal miRNAs between the NS groups and control groups (Fig. 3 and Supplemental Fig. 3). A miRNA was regarded as markedly upregulated if its mean reads were > 50 in the NS groups and it exhibited a \geq 5-fold change in the NS groups compared to the control groups (*P* <.05). Consequently, 30 miRNAs were upregulated in NS patients, and the top 15 miRNAs were selected for validation (Supplemental Table 2).

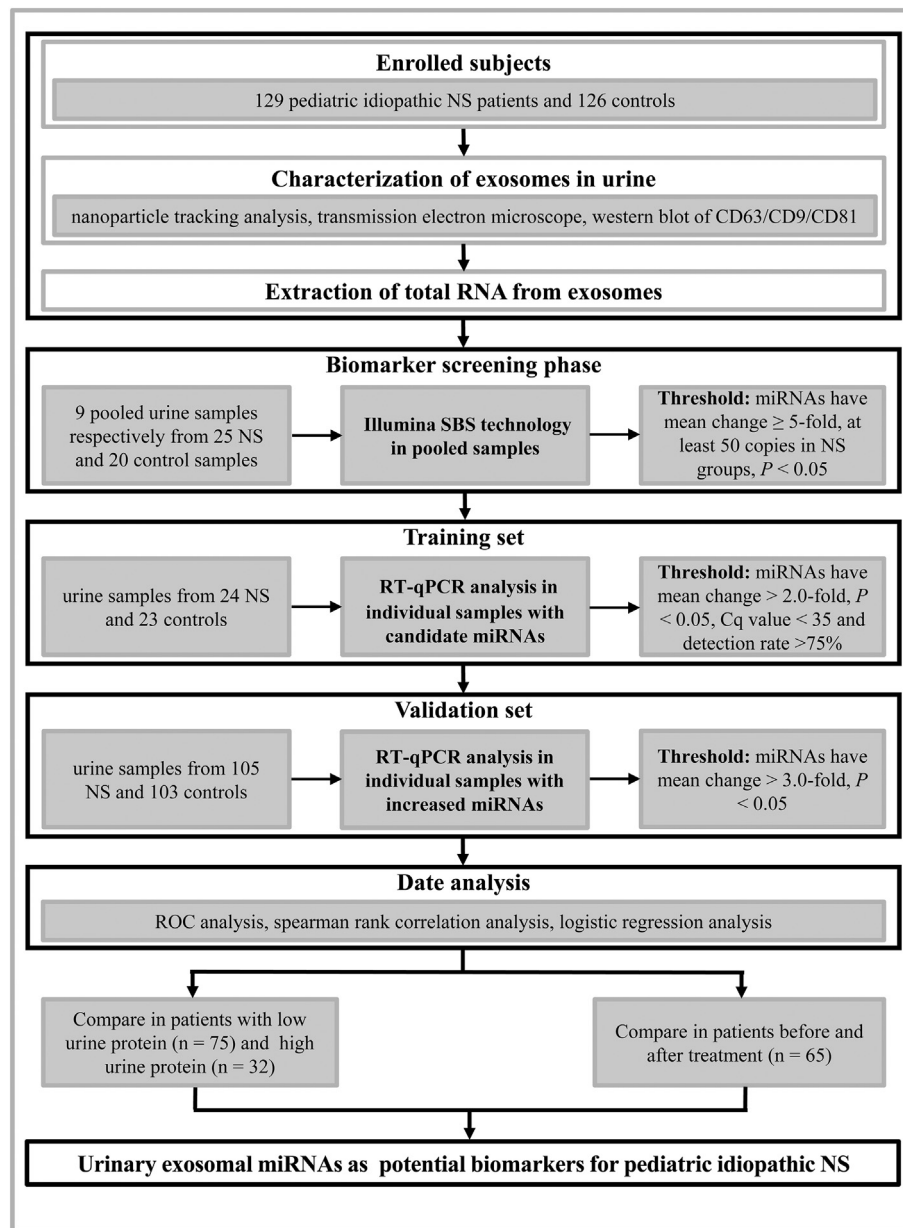


Fig. 1. An overview of the experimental design.

3.3. Validation results of urinary exosomal miRNAs by RT-qPCR

We then performed RT-qPCR assays to confirm the results of the Illumina SBS technology in two independent cohorts (129 NS and 126 controls). No significant distinction was present in the age and gender composition between the control group (male/female: 87/39; mean age (SD): 5.86 (3.00)) and the NS group (male/female: 89/40; mean age (SD): 6.58 (3.66)). The concentrations of 15 candidate urinary exosomal miRNAs were first measured in the training set, which consisted of 24 NS patients and 23 controls, including samples from the screening phase. Because of a shortage of the synthetic probe, miR-514a-3p was not measured. Instead, miR-30a-5p, which was found to be significantly elevated in the serum and urine samples of children with idiopathic NS in our previous study [7], was selected for examination by RT-qPCR assay. MiRNA was regarded as markedly increased if it met the following criteria: a mean > 2.0-fold increase, a *P*-value < .05 for comparison of the NS and controls, a quantification cycle (Cq) value < 35 and a detection rate > 75% in the cases. Five

miRNAs, including miR-194-5p, miR-146b-5p, miR-378a-3p, miR-23b-3p and miR-30a-5p, were significantly increased and chosen for further validation in an additional, larger cohort (validation set) involving 105 NS and 103 controls (Table 1). In accordance with the results of the training set, the concentrations of these 5 miRNAs were also significantly increased in NS cases compared with controls, with changes ranging from 3- to 13-fold (*P* < .0001) (Table 1). The significant differences in the expression of the 5 miRNAs between 129 NS and 126 controls are shown in Supplemental Fig. 4.

The concentrations of these 5 miRNAs were also compared between patient groups with different histological subtypes, including MCD, MsPGN, GML and FSGS. However, there were no significant differences among the subtypes (Supplemental Table 8).

3.4. ROC curve analysis

Subsequently, ROC curve analysis was performed to investigate the diagnostic value of the 5 urinary exosomal miRNAs for NS. The areas

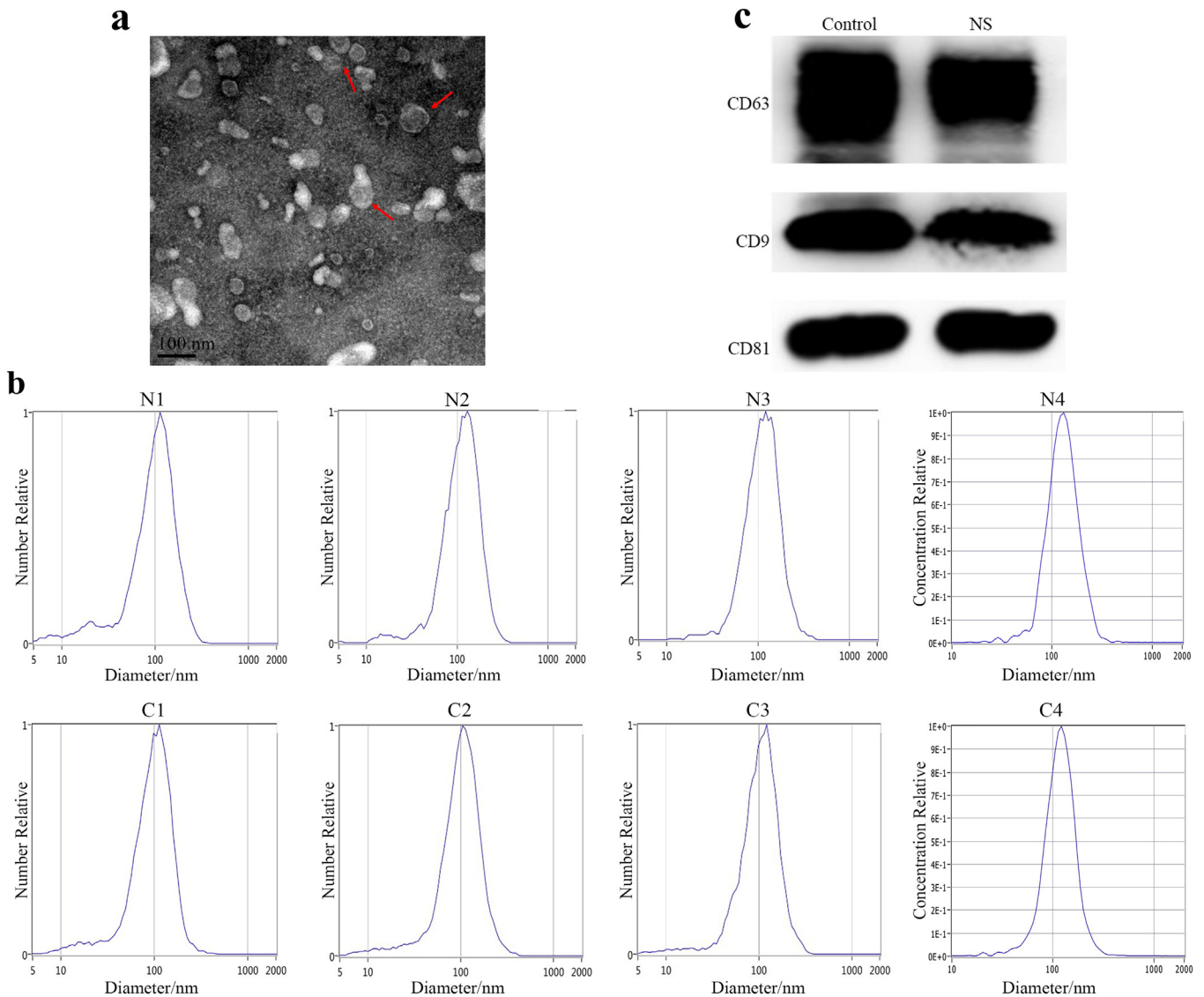


Fig. 2. Characterisation of exosomes derived from the urine of NS patients and controls. (a) The shape and structure of urinary exosomes isolated by ultracentrifugation under TEM. The red arrow represents exosomes with typical characteristics. (b) The size of exosomes derived from control groups and NS groups was analysed by NTA. N represents the control group ($n = 4$) and C represents the NS group ($n = 4$). N1–N3 and C1–C3 denotes ultracentrifugation; N4 and C4 denotes use of ExoQuick exosome precipitation solution. (c) Western blots of exosomal membrane markers, including CD63, CD9 and CD81.

under the curve (AUCs) of these miRNAs ranged from 0.682 to 0.800 (Supplemental Table 4). Among them, miR-194-5p presented the largest AUC (0.800; 95% CI, 0.746–0.853), followed by miR-23b-3p (0.772; 95% CI, 0.716–0.829). The sensitivity and specificity of miR-194-5p were 68.2% and 75.4%, respectively. For miR-23b-3p, the sensitivity and specificity were 82.2% and 62.7%. In addition, we evaluated the diagnostic values of the different combinations of these miRNAs [24]. However, the AUCs for all the combinations were lower than miR-194-5p and miR-23b-3p alone (Supplemental Table 4). These results suggest that urinary exosomal miR-194-5p and miR-23b-3p have relatively high diagnostic accuracy for NS.

3.5. Binary logistic regression analysis for the risk score in NS patients and controls

To further explore the risk prediction effect of 5 candidate urinary exosomal miRNAs for NS patients, a univariate logistic regression analysis was conducted with SPSS software. Based on risk score analysis, we defined NS status as the dependent variable and the risk score as the covariate. The regression coefficients of these 5 miRNAs were larger

than 0 (ranging from 1.093 to 3.247), and the odds ratios were >1 (ranging from 2.983 to 25.707) (Supplemental Table 5), suggesting that these miRNAs are potential risk factors for childhood NS.

3.6. The concentrations of 5 candidate miRNAs in NS children with varying degrees of urine protein

Additionally, to observe whether concentrations of these miRNAs are related to the content of protein in the urine, we divided the 107 NS patients with 24-h urinary protein analysis into two groups, a high urine protein group and a low urine protein group, according to the average value of urinary protein (4.69 g/24 h). The concentrations of all 5 miRNAs were significantly increased in the low urine protein group compared with the normal control group, and the concentrations were higher in the high urine protein group than in the low urine protein group. However, only the concentrations of miR-194-5p and miR-23b-3p had a statistically significant difference between the high urine protein group and the low urine protein group ($P = .0005$ and $P = .0098$, respectively) (Fig. 4a and Supplemental Table 6). These results suggest that miR-194-5p and miR-23b-3p are interrelated with urine

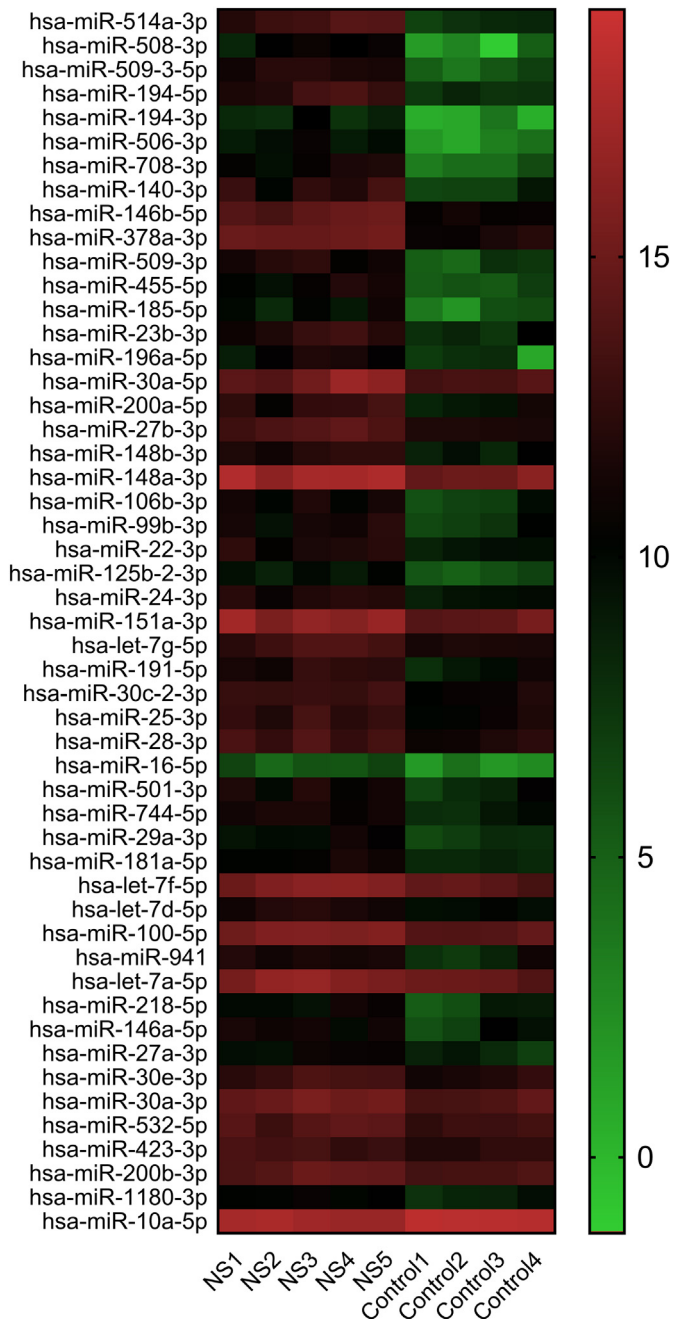


Fig. 3. Heat map describing the markedly upregulated exosomal miRNAs from Illumina SBS technology. Exosomes were derived from 5 pooled urine samples of NS patients and 4 pooled urine samples of controls.

protein content, and therefore can reflect the severity of the paediatric NS disease.

3.7. Comparison of candidate exosomal miRNAs before and after treatment

To investigate whether these urinary exosomal miRNAs can monitor the remission of NS in children, we collected paired urine samples from 65 of 129 patients before and after their regular daily therapy, including prednisone at $1.5\text{--}2\text{ mg/kg day}^{-1}$ ($\leq 60\text{ mg/day}$) and other adjuvant treatment. After 1 to 4 weeks of treatment, these patients tested negative for the contents of protein in the morning urine samples. We examined the 5 exosomal miRNAs by RT-qPCR in these paired urine samples and found that the concentrations of all 5 miRNAs were reduced significantly after treatment compared with the concentrations before

treatment ($P < .001$) and almost returned to the normal concentrations ($P > .05$, compared with healthy controls), with the exception of miR-194-5p (Fig. 4b and Supplemental Table 7). Among them, miR-23b-3p had the highest AUC (0.771; 95% CI, 0.691–0.851), followed by miR-194-5p (Supplemental Table 4). These results indicate that these urinary exosomal miRNAs, especially miR-23b-3p and miR-194-5p, can serve as additional indicators for monitoring NS.

3.8. Correlations of candidate urinary exosomal miRNAs with clinical parameters associated with renal function in NS children

To further investigate the association of these urinary exosomal miRNAs with NS, Spearman's rank correlation test was performed to assess whether the concentrations of these miRNAs were related to renal function parameters in patients. As shown in Table 2, the concentrations of all 5 miRNAs were significantly positively related to the levels of triacylglycerols, cholesterol, uric acid, urea and creatinine in the serum, while they were inversely related to the contents of albumin and total protein in the serum. Furthermore, miR-194-5p and miR-23b-3p were also significantly positively associated with 24-h urine protein content ($r = 0.295$, $P = .002$; $r = 0.200$, $P = .039$, respectively). These results demonstrate that the 5 urinary exosomal miRNAs, especially miR-194-5p and miR-23b-3p, are closely associated with kidney function, and may be used as auxiliary indicators for the diagnosis and condition judgment of childhood NS.

4. Discussion

Urine is collected non-invasively and is easily accessible and is fashionable for translation in clinical practice compared with blood and other clinical samples, making it an optimal source of biomarkers for kidney diseases [25]. Exosomes secreted by all nephron segments exist in human urine [18], and exosomes isolated from human urine have an RNA complete profile similar to that in renal tissue [26]. Moreover, urine-derived exosomes have significant enrichment and integrality of miRNAs compared with cell-free urine; exosomes with a membrane structure have a protective effect for miRNA, as they protect from hydrolysis by nuclease [27]. Thus, urinary exosomal miRNAs should be potentially useful as biomarkers for renal diseases. Several studies have indicated that an aberrant production of some miRNAs occurred in urinary exosomes in various kidney diseases. For instance, the urinary exosomal miR-181a [17] and miR-29c [28] were dramatically decreased in adults with CKD, compared with healthy individuals and miR-29c was related to glomerular filtration rate (GFR) and renal fibrosis. Recently, miR-193a contained in urinary exosomes was also observed to be significantly increased in children with FSGS ($n = 8$) compared to those with MCD ($n = 5$) [9]. However, the sample sizes enrolled in the above study was small, and the signature of exosomal miRNAs in urine for paediatric idiopathic NS has not been sufficiently examined.

In the present study, we comprehensively investigated the urinary exosomal miRNA profile in a large cohort of paediatric idiopathic NS with Illumina SBS technology combined with RT-qPCR assays. We observed that the signature of miRNAs in urinary exosomes from NS children was markedly different from that of healthy children, with 30 miRNAs being significantly increased in NS samples compared to controls. By RT-qPCR assay validation arranged in two independent cohorts, 5 urinary exosomal miRNAs, including miR-194-5p, miR-146b-5p, miR-387a-3p, miR-23b-3p and miR-30a-5p, were demonstrated to be significantly and steadily elevated and markedly decreased with remission after treatment in NS children. Among the 5 miRNAs, miR-194-5p and miR-23b-3p exhibited relatively high AUCs for NS diagnosis. Moreover, the concentrations of these two miRNAs were higher in the high urine protein group than in the low urine protein group and were significantly related with all renal function indexes, including 24-h urine protein content. Therefore, urinary exosomal miR-194-5p and miR-23b-3p

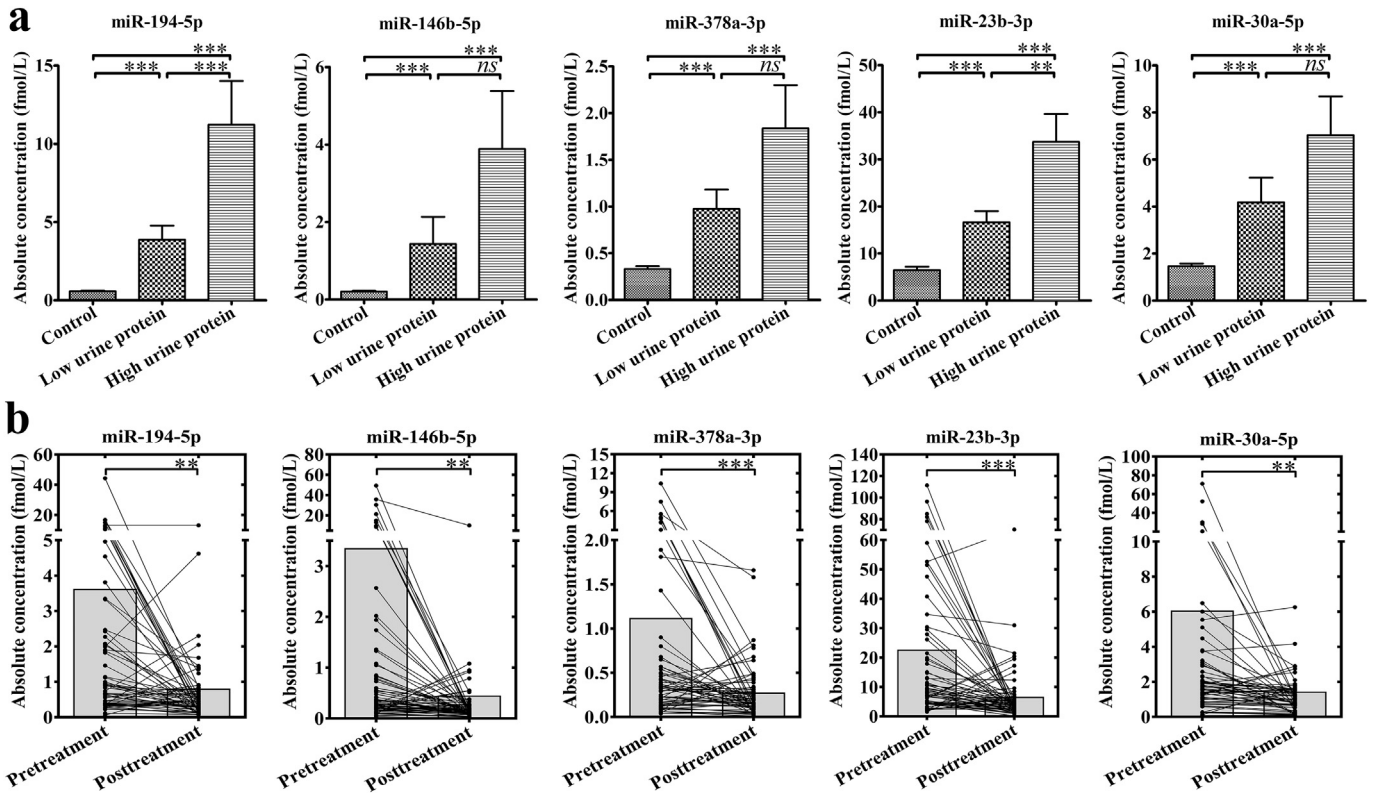


Fig. 4. The changes in concentrations of urinary exosomal miR-194-5p, miR-146b-5p, miR-378a-3p, miR-23b-3p and miR-30a-5p with the content of urine protein. (a) The concentrations of 5 candidate urinary exosomal miRNAs in the control group ($n = 102$), the low urine protein group ($n = 75$) and the high urine protein group ($n = 32$). Cq values were converted to absolute values based on the standard curve. Each P -value was calculated with a nonparametric Mann-Whitney test. $**P < .01$; $***P < .001$. (b) The concentrations of 5 miRNAs in urinary exosomes from NS patients before and after treatment ($n = 65$). Cq values were converted to absolute values based on the standard curve. Each P -value was calculated with a Wilcoxon matched pairs test. $**P < .01$; $***P < .001$. Each point represents the mean of triplicate samples.

may have potential as promising diagnostic indicators for childhood NS. Consistent with expectations, the alteration of miR-30a-5p in urinary exosomes in paediatric NS was similar to that in serum and urine, as we had previously observed [7]. It must be noted that we did not observe a significant difference in the concentrations of detected miRNAs among NS children with different pathological types. Considering that the sample size of each type was small in our study, more specimens will be needed to clarify whether these urinary exosomal miRNAs have the ability to distinguish different pathological types.

MiR-194-5p is enriched in kidney tissue and has been demonstrated to play a critical role in the pathogenesis of diabetic kidney disease [29]. Furthermore, miR-194-5p was found to be highly abundant in urinary exosomes [27] and was significantly increased in the urine extracellular vesicles from diabetic nephropathy patients with microalbuminuria compared with the normoalbuminuric and control subjects [30]. MiR-23b-3p and miR-30a-5p are abundantly expressed in podocytes, and significantly reduced in the podocytes of Dicer-knockout mice, resulting

in multiple lesions, including foot process effacement, damage to the glomerular basement membrane, podocyte apoptosis and depletion [31,32]. Both miR-23b-3p and miR-30a-5p are exosome-specific and exosome-enriched miRNAs in human urine [27,33]. Furthermore, it was reported that miR-23b downregulated the Hairy/enhancer of split protein (Hes1), which is expressed in specific nephron segments during development [32]. In addition, miR-30a may guard podocytes by suppressing *Notch1* and *P53*, and its loss promotes podocyte injury [34]. Clinically, miR-30a expression was decreased in the podocytes from patients with FSGS and elevated in urinary exosomes from patients with diabetic kidney disease [26,34]. The overexpression of miR-378a-3p causes oedema, proteinuria and podocyte effacement, and it is more concentrated in the renal tissue of patients with FSGS [35]. The above results suggest that these miRNAs can regulate multiple cellular processes in podocytes, which is critical to maintaining the glomerular filtration barrier. MiR-146b-5p is an immune and inflammatory related miRNA, and its overexpression can reduce the levels of IL-6 and IL-8

Table 1

The concentrations of miR-194-5p, miR-146b-5p, miR-378a-3p, miR-23b-3p and miR-30a-5p confirmed with RT-qPCR in urinary exosomes from controls and NS children in the training set and the validation set.^a

miRNAs	Training set				Validation set			
	Controls ($n = 23$)	NS ($n = 24$)	Fold change NS vs Controls	P	Controls ($n = 103$)	NS ($n = 105$)	Fold change NS vs Controls	P
miR-194-5p	0.99 (0.12)	12.26 (3.24)	12.38	< 0.0001	0.49 (0.04)	4.79 (0.99)	9.78	< 0.0001
miR-146b-5p	0.23 (0.02)	1.07 (0.31)	4.65	< 0.0001	0.18 (0.03)	2.22 (0.68)	12.33	< 0.0001
miR-378a-3p	0.46 (0.05)	1.52 (0.34)	3.30	0.0008	0.29 (0.03)	1.11 (0.20)	3.83	< 0.0001
miR-23b-3p	4.27 (0.59)	20.15 (3.30)	4.72	< 0.0001	6.86 (0.78)	21.80 (2.62)	3.18	< 0.0001
miR-30a-5p	1.24 (0.08)	4.10 (0.99)	3.31	0.0015	1.56 (0.15)	4.93 (0.91)	3.16	< 0.0001

^a miRNA data are expressed as mean, fmol/L (SE).

Table 2
The results of Spearman's rank correlations between 5 candidate urinary exosomal miRNAs and other variables in NS children (r/P).

miRNAs	Serum albumin (n = 127)	Serum total protein (n = 127)	Serum total cholesterol (n = 127)	Serum triacylglycerols (n = 126)	Serum urea (n = 127)	Serum uric acid (n = 127)	Serum creatinine (n = 127)	24-h urine protein (n = 107)	Sex (n = 129)	Age (n = 129)
miR-194-5p	−0.361** <i>P</i> < .0001	−0.345** <i>P</i> < .0001	0.303** <i>P</i> = .0010	0.318** <i>P</i> < .0001	0.263** <i>P</i> = .0030	0.262** <i>P</i> = .0030	0.231** <i>P</i> = .0090	0.295** <i>P</i> = .0020	−0.115 <i>P</i> = .1940	0.025 <i>P</i> = .7820
miR-146b-5p	−0.193* <i>P</i> = .0300	−0.271** <i>P</i> = .0020	0.200* <i>P</i> = .0240	0.262** <i>P</i> = .0030	0.315** <i>P</i> < .0001	0.259** <i>P</i> = .0030	0.347** <i>P</i> < .0001	0.057 <i>P</i> = .5580	−0.079 <i>P</i> = .3710	0.140 <i>P</i> = .1140
miR-378a-3p	−0.297** <i>P</i> = .0010	−0.333** <i>P</i> < .0001	0.270** <i>P</i> = .0020	0.247** <i>P</i> = .0050	0.302** <i>P</i> = .0010	0.295** <i>P</i> = .0010	0.308** <i>P</i> < .0001	0.152 <i>P</i> = .1180	−0.056 <i>P</i> = .5270	0.122 <i>P</i> = .1680
miR-23b-3p	−0.354** <i>P</i> < .0001	−0.404** <i>P</i> < .0001	0.321** <i>P</i> < .0001	0.365** <i>P</i> < .0001	0.274** <i>P</i> = .0020	0.299** <i>P</i> = .0010	0.263** <i>P</i> = .0030	0.200* <i>P</i> = .0390	−0.095 <i>P</i> = .2870	0.052 <i>P</i> = .5610
miR-30a-5p	−0.288** <i>P</i> = .0010	−0.310** <i>P</i> < .0001	0.276** <i>P</i> = .0020	0.304** <i>P</i> = .0010	0.350** <i>P</i> < .0001	0.262** <i>P</i> = .0030	0.352** <i>P</i> < .0001	0.094 <i>P</i> = .3350	−0.069 <i>P</i> = .4370	0.122 <i>P</i> = .1690

and inhibit glomerular mesangial cell proliferation to some degree in lupus nephritis [36]. Moreover, miR-146b-5p was found to be enriched in established mouse models of acute kidney injury and fibrosis [37]. Because the damage to the podocyte is recognized as the major pathological change for NS, and the dysfunction of the immune system has also been demonstrated to be involved in idiopathic NS [38], these miRNAs identified in our study likely play important roles in paediatric NS.

At present, the cellular source of urinary exosomal miRNAs remains unclear, but there is some evidence that they may be primarily derived from kidney-related cell types [17,18]. We speculate that the upregulation of miR-23b-3p in urine exosomes may be caused by the increased secretion of miR-23b-3p from damaged glomeruli and podocytes of NS patients. As for miR-194-5p, its exact pathophysiological function in the nephropathy hasn't been reported. However, because this miRNA may be involved in inflammatory process [39,40], we suppose that the upregulated miR-194-5p in urinary exosomes may result from the elevated inflammation in glomeruli and podocytes of NS children. These increased urinary exosomal miRNAs may provide valuable insights into renal pathophysiology. A study using bioinformatics target prediction followed by in vitro renal epithelial cell assay confirmation has provided initial evidence that the exosome-derived miRNAs in urine are biologically active and capable of modulating key kidney tubule functions by paracrine [33]. However, the origin, pathophysiological role and mechanism of the urinary exosomal miRNAs still need to be further addressed.

In summary, we have performed a global and detailed analysis of the urinary exosomal miRNAs profile in NS children and identified changes in the concentrations of miR-194-5p, miR-23b-3p, miR-146b-5p, miR-378a-3p and miR-30a-5p with disease progression and treatment with prednisone together with adjuvant treatment. Specifically, miR-194-5p and miR-23b-3p are demonstrated to be promising biomarkers for predicting and monitoring children with serious complications. These results suggest that these urinary exosomal miRNAs might play critical roles in the pathogenesis and development of paediatric NS and warrant further study.

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Conflicts of interest

The authors have no competing interests to declare.

Author contributions

C.Z., C.W. and C.-Y.Z. designed the study. T.C., H.Y., X.L. and C.Z. collected the data. T.C., C.W. and C.Z. did the data analysis and interpretation. T.C., C.Z. and C.W. wrote the manuscript. All authors reviewed the report and approved the final version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.11.018>.

References

- [1] Eddy AA, Symons JM. Nephrotic syndrome in childhood. *Lancet* 2003;36:629–39.
- [2] Noone DG, Iijima K, Parekh R. Idiopathic nephrotic syndrome in children. *Lancet* 2018;392:61–74.
- [3] Abeyagunawardena AS. Treatment of steroid sensitive nephrotic syndrome. *Indian J Pediatr* 2005;72:763–9.
- [4] Szeto CC. Urine miRNA in nephrotic syndrome. *Clin Chim Acta* 2014;436:308–13.
- [5] Li HQ, Wu J, Niu DM, Shi YH, Zhang CN, Wang JJ. The level of native and oxidized lipoprotein(a) in children with nephrotic syndrome. *Clin Biochem* 2012;45:101.
- [6] Tarshish P, Tobin JN, Bernstein J, Jr EC. Prognostic significance of the early course of minimal change nephrotic syndrome: report of the International Study of Kidney Disease in Children. *J Am Soc Nephrol* 1997;8:769–76.
- [7] Luo Y, Wang C, Chen X, et al. Increased serum and urinary microRNAs in children with idiopathic nephrotic syndrome. *Clin Chem* 2013;59:658–66.
- [8] Lorenzen JM, Thum T. MicroRNAs in idiopathic childhood nephrotic syndrome. *Clin Chem* 2013;59:595–7.
- [9] Huang Z, Zhang Y, Zhou J, Zhang Y. Urinary exosomal miR-193a can be a potential biomarker for the diagnosis of primary focal segmental glomerulosclerosis in children. *Biomed Res Int* 2017;2017 7298160.
- [10] Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 2008;105:10513–8.
- [11] Lu M, Wang C, Yuan Y, et al. Differentially expressed microRNAs in kidney biopsies from various subtypes of nephrotic children. *Exp Mol Pathol* 2015;99:590–5.
- [12] Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;9:654–9.
- [13] Vallabhajosyula P, Korutla L, Habberthuer A, et al. Tissue-specific exosome biomarkers for noninvasively monitoring immunologic rejection of transplanted tissue. *J Clin Invest* 2017;127:1375–91.
- [14] Habberthuer A, Korutla L, Rostami S, et al. Donor tissue-specific exosome profiling enables noninvasive monitoring of acute rejection in mouse allogeneic heart transplantation. *J Thorac Cardiovasc Surg* 2018;155:2479–89.
- [15] Min QH, Chen XM, Zou YQ, et al. Differential expression of urinary exosomal microRNAs in IgA nephropathy. *J Clin Lab Anal* 2017;32.
- [16] Perezhernandez J, Forner MJ, Pinto C, Chaves FJ, Cortes R, Redon J. Increased urinary exosomal microRNAs in patients with systemic lupus erythematosus. *Plos One* 2015;10:e0138618.
- [17] Khurana R, Ranches G, Schafferer S, et al. Identification of urinary exosomal non-coding RNAs as novel biomarkers in chronic kidney disease. *RNA* 2016;23:142–52.

- [18] Barutta F, Tricarico M, Corbelli A, et al. Urinary exosomal microRNAs in incipient diabetic nephropathy. *PLoS One* 2013;8:e73798.
- [19] Butz H, Nofechmzoes R, Ding Q, et al. Exosomal microRNAs are diagnostic biomarkers and can mediate cell-cell communication in renal cell carcinoma. *Eur Urol Focus* 2016;2:210–8.
- [20] Listed N. Primary nephrotic syndrome in children: clinical significance of histopathologic variants of minimal change and of diffuse mesangial hypercellularity. A report of the International Study of Kidney Disease in Children *Kidney Int* 1981;20:765–71.
- [21] Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* 2006;3 Unit [3.22].
- [22] Ding M, Wang C, Lu X, et al. Comparison of commercial exosome isolation kits for circulating exosomal microRNA profiling. *Anal Bioanal Chem* 2018;410:3805–14.
- [23] Wang C, Yang C, Chen X, et al. Altered profile of seminal plasma microRNAs in the molecular diagnosis of male infertility. *Clin Chem* 2011;57:1722–31.
- [24] Zhang C, Wang C, Chen X, et al. Expression profile of microRNAs in serum: a fingerprint for esophageal squamous cell carcinoma. *Clin Chem* 2010;56:1871–9.
- [25] Wang G, Szeto CC. Quantification of gene expression in urinary sediment for the study of renal diseases. *Nephrology (Carlton)* 2007;12:494–9.
- [26] Xu WC, Qian G, Liu AQ, Li YQ, Zou HQ. Urinary extracellular vesicle: a potential source of early diagnostic and therapeutic biomarker in diabetic kidney disease. *Chin Med J (Engl)* 2018;131:1357–64.
- [27] Cheng L, Sun X, Scicluna BJ, Coleman BM, Hill AF. Characterization and deep sequencing analysis of exosomal and non-exosomal miRNA in human urine. *Kidney Int* 2014;86:433–44.
- [28] Lv LL, Cao YH, Ni HF, et al. MicroRNA-29c in urinary exosome/microvesicle as a biomarker of renal fibrosis. *Am J Physiol Renal Physiol* 2013;305:F1220–7.
- [29] Sun Y, Koo S, White N, et al. Development of a micro-array to detect human and mouse microRNAs and characterization of expression in human organs. *Nucleic Acids Res* 2004;32:e188.
- [30] Jia Y, Guan M, Zheng Z, et al. MiRNAs in urine extracellular vesicles as predictors of early-stage diabetic nephropathy. *J Diabetes Res* 2016;2016:7932765.
- [31] Shi S, Yu L, Chiu C, et al. Podocyte-selective deletion of *dicer* induces proteinuria and glomerulosclerosis. *J Am Soc Nephrol* 2008;19:2159–69.
- [32] Ho J, Ng KH, Rosen S, Dostal A, Gregory RI, Kreidberg JA. Podocyte-specific loss of functional microRNAs leads to rapid glomerular and tubular injury. *J Am Soc Nephrol* 2008;19:2069–75.
- [33] Gracia T, Wang X, Su Y, et al. Urinary exosomes contain microRNAs capable of paracrine modulation of tubular transporters in kidney. *Sci Rep* 2017;7:40601.
- [34] Wu J, Zheng C, Fan Y, et al. Downregulation of microRNA-30 facilitates podocyte injury and is prevented by glucocorticoids. *J Am Soc Nephrol* 2014;25:92–104.
- [35] Muller-Deile J, Dannenberg J, Schroder P, et al. Podocytes regulate the glomerular basement membrane protein nephrin by means of miR-378a-3p in glomerular diseases. *Kidney Int* 2017;92:836–49.
- [36] Sheng ZX, Yao H, Cai ZY. The role of miR-146b-5p in TLR4 pathway of glomerular mesangial cells with lupus nephritis. *Eur Rev Med Pharmacol Sci* 2018;22:1737–43.
- [37] Pellegrini KL, Gerlach CV, Craciun FL, et al. Application of small RNA sequencing to identify microRNAs in acute kidney injury and fibrosis. *Toxicol Appl Pharmacol* 2016;312:42–52.
- [38] Jg VDB, Weening JJ. Role of the immune system in the pathogenesis of idiopathic nephrotic syndrome. *Clin Sci (Lond)* 2004;107:125–36.
- [39] Meng J, Zhang D, Pan N, et al. Identification of miR-194-5p as a potential biomarker for postmenopausal osteoporosis. *PeerJ* 2015;3:e971.
- [40] Li G, Tang X, Chen H, Sun W, Yuan F. miR-148a inhibits pro-inflammatory cytokines released by intervertebral disc cells by regulating the p38/MAPK pathway. *Exp Ther Med* 2018;16:2665–9.