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Analysis of microRNAs signatures in juvenile glomerular proteinuria

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ABSTRACT

Background : Urinary microRNAs (miRNAS) are found to be as non-invasive biomarkers in many diseases, including nephrotic syndrome (NS). NS is a common kidney disorder predominant in children and has also been reported in adults. Approximately 85 to 90% of patients with NS respond to steroid treatment with complete remission of proteinuria are known as steroid sensitive nephrotic syndrome (SSNS), while 10 to 15% have partial or even no response to steroid therapy termed as steroid resistant nephrotic syndrome (SRNS). The global urinary miRNA signature in paediatric NS patients and its clinical significance have not been explored. The present study was therefore attempted to analyse the miRNA profile in urinary samples by high-throughput Illumina sequencing via synthesis (SBS) technology in control and NS patients.

Methodology: MicroRNA isolation was carried out in urine samples collected from SSNS (n=10), SRNS (n=10), and healthy controls (n=10). Isolated RNA enriched for small RNA high throughput sequencing (HTS), and the sequence data were generated using Illumina HiSeq sequencing technology (Clevergene Biocorp Pvt. Ltd., Bengaluru, India). The expression profile of the differentially expressed miRNAs across the samples is presented in volcano plot and heatmap. miRNA sequencing revealed significant increase in 4 and 19 miRNAs in SSNS and SRNS samples respectively, compared to that of normal subjects.

Results: Among the 4 miRNAs of SSNS patients, 3 miRNAs were found to be significantly elevated, while only one was found to be downregulated as compared to control group. Among the 19 miRNAs of SRNS patients, 8 miRNAs were found to be significantly upregulated, while 11 were found to be downregulated when compared to control. The GO analysis showed that the target genes were mainly involved in biological processes, molecular function and cellular components. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed pathways in Ras signaling, MAPK signaling, calcium signaling etc. The miRNA sequencing was submitted in the SRA database in NCBI, Bio Project ID :PRJNA858929 (Accession Number for submission (SSNS: SAMN29759595 , SRNS: SAMN29759596, Control: SAMN29759597).

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Conclusion : Urinary miRNAs identified in this studys could be promising and non-invasive potential biomarker candidates for diagnosis and prognosis of pediatric NS.

Keywords: *microRNA profiling, nephrotic syndrome, next-generation sequencing, urine biomarkers*

INTRODUCTION

MicroRNAs (miRNAs) are small, single stranded, non-coding RNAs of 22–25 base length, regulating posttranscriptional gene expression primarily by promoting mRNA degradation or inhibiting translation[1,2] and thus have a globalized role in a wide variety of biological functions, such as cellular proliferation, apoptosis, tumorigenesis, and fibrosis in various target organs.[3] The advent of next-generation sequencing (NGS) technology has enabled the sequencing of the entire set of miRNAs present in an RNA sample. Expression profiling, identification of sequence isoforms, prediction of novel miRNAs, and prediction of possible mRNA target molecules can all be done with the data collected from such sequencing investigations to evaluate miRNA expression and function.[4] NGS involves simultaneous sequencing of many molecules at a time, as opposed to "first generation" sequencing, which requires sequencing of one molecule at a time.[5,6] This advancement is fast, reliable, and has lowered the time and cost per sequenced base, as well as increased the scope of sequencing applications.[7,8]

MiRNAs, in view of their stability, specificity, ease of detection and quantification are commonly reported as potential biomarkers for various diseases, including nephrotic syndrome (NS).[9] In children, responsiveness to glucocorticoid (GC) therapy has been shown to be the best indicator of prognosis. Based on the responsiveness to GC therapy, NS is classified as steroid-sensitive (SSNS) or steroid-resistant (SRNS). According to International Study of Kidney Disease in Children,[10] 80–90% of patients with childhood NS achieve complete remission (SSNS). In contrast, approximately 20% of children fail to enter complete remission after 8 weeks of GC therapy and are clinically steroid resistant (SRNS). Even though kidney biopsy is the benchmark process to differentiate these diseases, it is an invasive method with possible complications. Therefore, a non-invasive method is required to differentiate between different etiologies of NS. Although blood is the most common specimen used for studying biomarkers, using urine specimen has several advantages which include (i) ease and non-invasiveness of collection procedure, (ii) availability in large amounts, (iii) If well preserved, urine samples are stable for very long time periods, (iv) Multiple samples can be collected without causing discomfort to the patient.[11] Hence, in the present study, urine specimens are opted to identify potentially useful miRNAs as biomarkers by NGS as well as to focus on the data analysis aspect, and bioinformatics analysis of miRNA-sequencing data.

MATERIAL AND METHODS

Patient recruitment and data collection

The study group consisted of 30 children with NS (10 SSNS and 10 SRNS) and 10 normal subjects without a family history of renal disease. They were recruited from the pediatric nephrology department of Sri Ramachandra Institute of Higher Education and Research (SRIHER) Chennai, India. The research protocol was approved by the Institutional Review Board/ethics committee of SRIHER (IEC-NI/21/FEB/77/27).Written informed consent was obtained from the parents of both patients and healthy controls. All cases fulfilled the criteria of the International Standard of Kidney Disease in Children[10] for the diagnosis of the NS. Children with NS aged between 16 months and 16 years were included in the study if they present clinical symptoms of proteinuria, hypoalbuminemia, and edema. The clinical response of each patient to GC (i.e., SRNS or SSNS) was assessed approximately 6 to 10 weeks after initial presentation. Cases with secondary NS and those from consanguineous families, urinary tract infection, other immune diseases, and age more than years old were excluded.

Sample collection and processing

About 5ml of urine samples were collected from 20 NS patients and 10 healthy control groups among which in NS, 10 subjects responded to steroid therapy (SSNS) and 10 subjects after 6 to 10 weeks of GC therapy showed steroid

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resistance (SRNS). The samples are processed immediately by centrifuging at 3000×g for about 20 min and at 13,000×g for 5 min at 4°C. The supernatant was discarded and the urinary cell pellet was stored for microRNA isolation.

MicroRNA isolation

MicroRNA isolation was carried out for SSNS (n=10), SRNS (n=10), and healthy controls (n=10), using MiRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. About 3.5 µl miRNeasy Serum/Plasma Spike-In Control was added to urine samples, mixed thoroughly with equal volume of chloroform with a vortex, stood at room temperature for 2–3 min and centrifuged at 12,000 x g at 4°C for 15 min. The upper aqueous phase was transferred to a sterile and RNase-free tube, and 1.5 volumes of 100% ethanol was added.

About 700 µl of sample was pipetted into an RNeasy Min Elute spin column in a 2 ml collection tube, centrifuged at ≥ 8000 x g for 15 seconds at room temperature. Supernatant was discarded. Around 500 µl of elution buffer was added to the RNeasy Min Elute spin column and centrifuged at the same rpm. About 500 µl of 80% ethanol was added to the RNeasy MinElute spin column and centrifuged for 2 min at ≥ 8000 x g. The flow through was discarded followed by dry spin. Finally, the RNeasy MinElute spin column was placed in a new 1.5 ml collection tube and 14 µl RNase-free water added directly to the center of the spin column followed by centrifugation for one min at full speed to elute the microRNA.

The isolated nucleic acids were quantified to determine the concentrations and purity of the samples. The concentration and quality of extracted RNA were assessed by spectrophotometry on the NanoDrop 1000 (Thermo Scientific, Waltham, MA). The ratio of the absorbance at 260 and 280 nm (A260/280) is used to assess the purity of nucleic acids. About 2 µl of isolated sample was added to the lower pedestal of the nano drop and the purity of nucleic acid was assessed for the entire collected urine sample using nano drop equipment. A ratio of 1.7-2.0 was generally accepted as a good quality. All 30 samples were subjected for both qualitative and quantitative analysis.

Small RNA sequencing using NGS platform

Isolated miRNA from each subject was pooled before proceeding with small RNA high throughput sequencing (HTS), and the sequence data were generated using Illumina HiSeq NGS technology, (Clevergene Biocorp Pvt. Ltd., Bengaluru, India). The quality of the data was checked using FastQC[12] and MultiQC[13] software. The adapter sequences, low-quality bases, and the reads shorter than 14 bp were removed using the Trim Galore[14] and Cutadapt v3.4[15] program. The generated miRNA sequencing data were submitted in Sequence Read Archive (SRA) database in NCBI (Ref: Bio Project ID : PRJNA858929). Alignment and Identification of miRNA

The QC passed reads were mapped onto indexed Human reference genome (GRCh38.p7) using mapper.pl script of miRDeep2 v2.0.1.2 [5]. Reads mapped to the reference genome were used to identify miRNA with miRDeep2 using known and novel miRNA identification parameters. Human mature miRNAs from mirBase v22.1[16] were used for miRNA prediction. Total 212 novel and 284 known miRNAs were identified; miRNAs with miRDeep score less than 1 were excluded from further analysis resulting in 205 novel and 284 known miRNAs. Expression levels of miRNAs were estimated using miRDeep2 quatifiler.pl script.

Differential Expression

Differential expression analysis was carried out using the DESeq2[17] package. The read counts were normalized and differential expression was tested comparing the test group with control group. MiRNAs with absolute log2 fold change ≥ 1 and p-value ≤ 0.05 were considered significant. The expression profile of the differentially expressed miRNAs across the samples is presented heatmap and as volcano plot.

Statistical analysis

Expression similarity between biological replicates was checked by spearman correlation (data is in 03_Correlation_matrix.xlsx) and Principal Components Analysis.

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RESULTS

Small RNAs from Control, SSNS and SRNS

To identify miRNAs in control and NS patients, we generated total RNAs from urine specimens for high-throughput sequencing, yielding 18,447,872, 15,342,660 and 16,075,142 raw read totals from the normal, SSNS and SRNS patients, respectively (Table 1).

Following filtration and trimming, 4,185,183, 5,732,351 and 7,104,038 clean reads were obtained and mapped onto the NS reference genome, respectively (mature miRNAs from mirBase v22.1 and were used for miRNA prediction). The length distribution analysis of raw and filtered reads showed that the small RNA length from control and NS patients varied from 17 to 50 nt in normal subjects, SSNS and SRNS.

TABLE 1: Filtering raw data using Trim Galore and Cutadapt tools to remove unwanted reads

Raw Reads		
Sample Name	**Number of reads**	**Read length**
US- CONTROL	18447872	50
US-SSNS	15342660	50
SUS-SRNS	16075142	50
Reads after Qc filtering and trimming		
Sample Name	**Number of reads**	**Read Length**
CONTROL	4185183	17-50
SSNS	5732351	17-50
SRNS	710438	17-50

miRNA identification from control, SSNS and SRNS patients

The processing of filtered reads from high-throughput sequencing was carried out according to miRDeep-P workflow, and the expression levels of miRNAs were estimated using miRDeep2 quatifiler pl script with the NS genome as the reference sequence(Table 2). For the control samples, a total of 322 miRNAs were identified, out of which 293 miRNAs were expressed, resulting in 180 known and 113 novel miRNAs. In the SSNS patients, 281 expressed miRNAs including 149 known and 132 novel miRNAs were identified, and in the SRNS RNA samples, out of 230 expressed miRNAs, 79 known and 151 novel miRNAs were identified (Table 3).

TABLE 2: Identification of known and novel miRNA using miRDeep2 tool

Sample Name	Total miRNAs (Known Novel)	Expressed miRNAs	Known miRNAs	Novel miRNAs
CONTROL	322	293	180	113
SSNS	322	281	149	132
SRNS	322	230	79	151

TABLE 3: Known and Novel miRNAs predicted using miRDeep2 after filtering

	Total miRNA	**Filtered miRNA**	**miRNA Predicted miRDeep2**
Known miRNA	284	284	190
Novel miRNA	212	205	132

Sequencing and differential expression analysis revealed significant differences in the 4 miRNAs expressed (1 upregulated, and 3 downregulated) in the SSNS versus control samples. When SRNS samples were compared with control samples, significant difference was observed in the 19 miRNAs expressed (8 upregulated and 11 downregulated). The down regulated genes include the following : hsa-mir-129-5p, hsa-mir-199a-5p, hsa-mir-199a-5p, hsa-mir-129-5p,

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hsa-mir-320a-5p, hsa-mir-141-5p, hsa-mir-200c-5p, hsa-mir-192-5p, hsa-mir-1307-5p, hsa-mir-200a-5p, hsa-mir-29c-5p (Tables 4). We constructed a network on the significantly upregulated novel miRNA based on TargetScan (miRNA target prediction tool) consisting of 134 miRNA targeted genes with 139 nodes and 138 edges, The score 170 was adopted as the cut-off value, the more positive the score, the greater the repression. Volcano plot and heatmap represented the expression profile of the differentially expressed miRNAs across the samples (Figure 1-4 respectively). The miRNA sequencing was submitted in the SRA database in NCBI, Bio Project ID :PRJNA858929(Accession Number for submission (SSNS: SAMN29759595 , SRNS: SAMN29759596, Control: SAMN29759597)

TABLE 4: Number of differentially expressed miRNA comparing test with control groups

Condition	Tested miRNAs (Known +Novel)	Significantly Expressed miRNAs	Upregulated miRNAs	Down regulated miRNAs
Control vs SSNS	320	4	1	3
Control vs SRNS	320	19	8	11

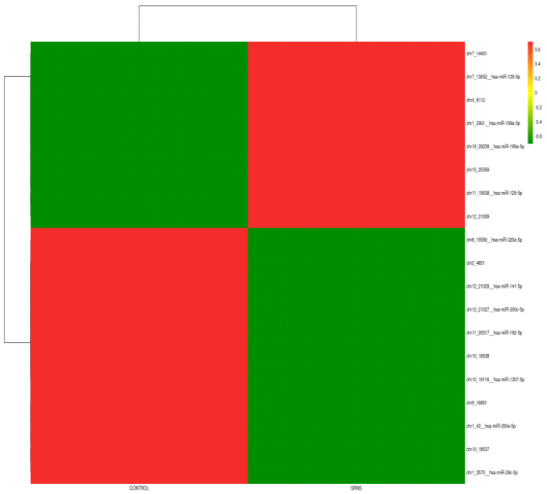

FIGURE 1: Heat map representing significantly expressed miRNAs Control vs SRNS group

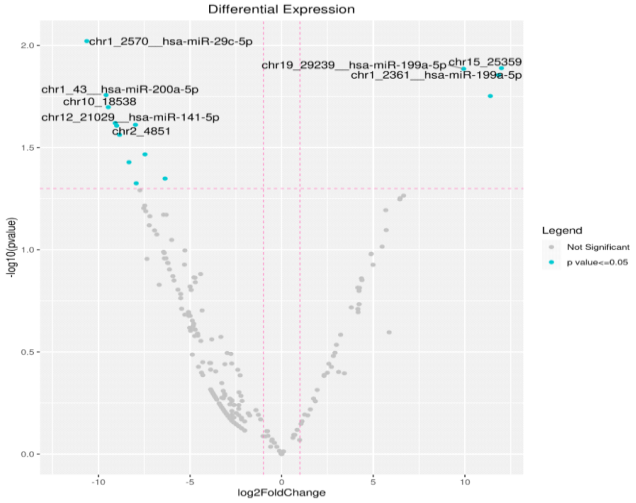

FIGURE 2: Volcano plot showing differential expression level of miRNAs Control vs SRNS

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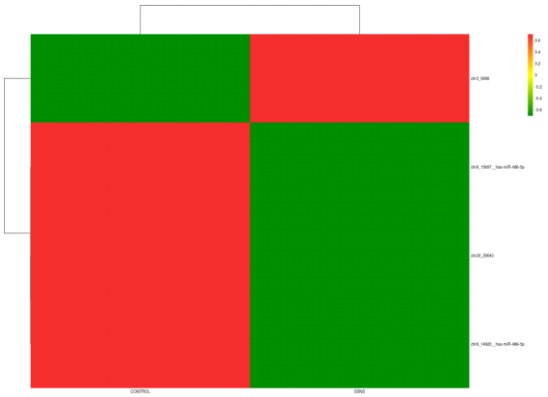

FIGURE 3: Heat map representing significantly expressed miRNAs among Control vs SSNS

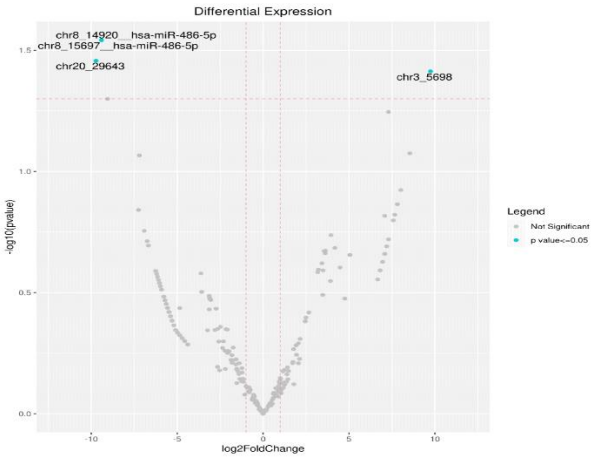

FIGURE 4: Volcano plot showing differential expression level of miRNAs among Control vs SSNS

Target Prediction

The cumulative weighted context++ score <-0.4 was adopted as the cut-off value. The cumulative weighted context++ score is the sum of the contribution of 14 features for each of the four site types, the more negative the score, the greater the repression. The target prediction results are provided for known and novel miRNAs in (Figure 5-6) respectively.

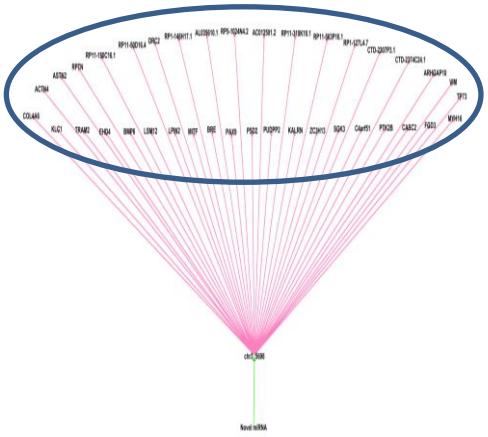

FIGURE 5: Upregulated novel-miRNAs among Control vs SRNS

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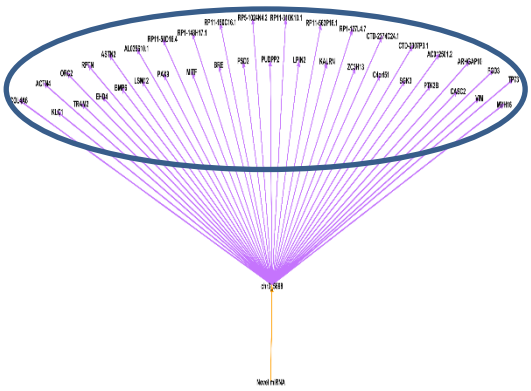

FIGURE 6: Upregulated novel-miRNA among Control vs SSNS

GO and Pathway enrichment analysis of target genes

Gene Ontology and pathways were enriched for target genes using the ClusterProfiler[19] R Bioconductor package. After identifying the differentially expressed genes (DEGs) in NS patients and control group, we employed the Database for Annotation, Visualization and Integration Discovery (DAVID) to identify the functions of the identified DEGs and performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg), pathway analyses. A total of 23 GO terms were significantly enriched among DEGs in samples obtained from SRNS children. To investigate the biological classification of DEGs, the overall genes in three ontologies were identified using DAVID. P-values < 0.05 after adjustment by the Benjamini-Hochberg method were regarded as statistically significant. The novel microRNAs targeted genes and their chromosome location along with the functions including the disease,[45-52] denoted in **(Figure7).**From the total data provided ,top 20 significantly enriched gene ontology terms and KEGG pathways are presented in **(Figure8).**Enrichment analysis also showed that the glomerular and tubulointerstitial compartments underwent a wide range of unique pathological changes during chronic injury. As revealed by the GO enrichment analysis, shared DEGs in glomeruli were significantly enriched in exosomes. Notably, other diseases such as Staphylococcus aureus infection and autoimmune thyroid disease were also listed as enriched terms, indicating that these disorders may share similar pathophysiological pathways with CKD.

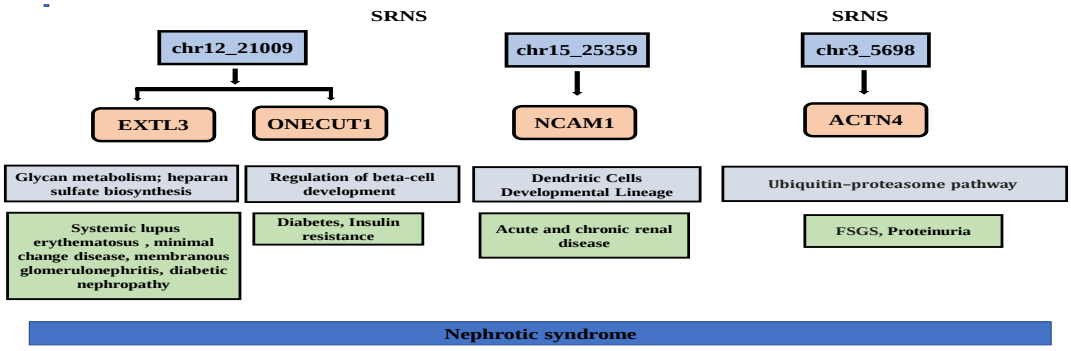

FIGURE 7: Novel microRNAs and their chromosome location along with the functions and disease involved

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Gene ontology

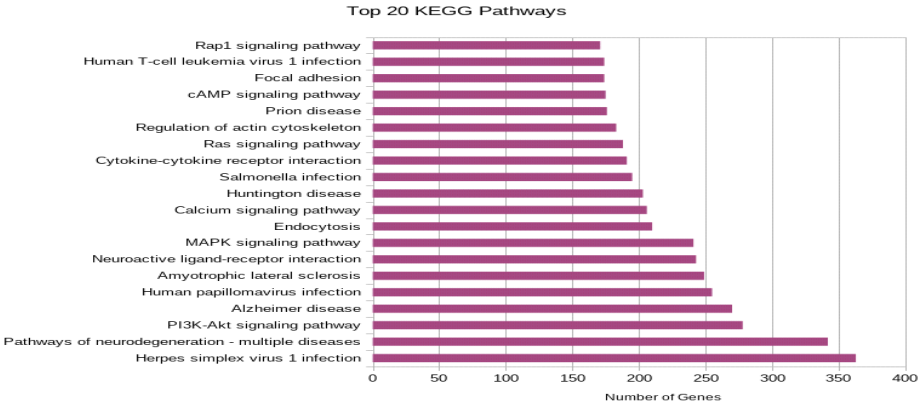

FIG 8: Kegg Pathway analysis

DISCUSSION

MicroRNAs have been widely reported as promising biomarkers for many diseases, including NS as they not only help in the diagnosis of specific diseases, but also for understanding the disease pathogenesis and even for establishing novel therapeutics.[9] The present study was attempted to investigate the urinary miRNA profile in a cohort of children with NS using Illumina SBS technology and bioinformatics.

The present study revealed that the signature of urinary miRNAs from paediatric NS was markedly different from that of healthy children, with 4 and 19 miRNAs being significantly increased in SSNS and SRNS samples, respectively, compared to that of normal subjects. Among the 4 miRNAs of SSNS patients, 3 miRNAs were found to be significantly elevated, while only one was found to be low as compared control. Among the 19 miRNAs of SRNS children, 8 miRNAs were found to be significantly upregulated, while 11 were found to be low as compared control. The down regulated genes include the following : hsa-mir-129-5p, hsa-mir-199a-5p, hsa-mir-199a-5p, hsa-mir-129-5p, hsa-mir-320a-5p, hsa-mir-141-5p, hsa-mir-200c-5p, hsa-mir-192-5p, hsa-mir-1307-5p, hsa-mir-200a-5p, hsa-mir-29c-5p .

Since microRNA exerts its biological effects through suppression of target genes, it is necessary to identify microRNA–target pairs to understand the biological significance of specific microRNAs. miRNA-129-5p is one of the miRNAS identified in the present study. miRNA-129-5p is believed to be a potent downstream inhibitor of TGF-β1 in renal fibrosis.[20] According to them, decreased expression of miR-129-5p was accompanied with alterations in EMT-related genes and the expression of respective proteins in vivo. In addition, in vitro studies revealed reduced expression of E-cadherin and claudin-1 with increased cell migration in HMrSV5, a human peritoneal mesothelial cell line (HPMC), treated with TGF-β1 and increased expression of vimentin, fibronectin and transcription factors, Smad-interacting protein 1 (SIP-1) and SOX4.[20] Furthermore, alteration in EMT-related genes and proteins were reversed by overexpression of miR-129-5p. In systemic sclerosis—a disease characterized by widespread fibrosis—expression of the mmu-miR-29 family is decreased.[21] mmu-miR-29a-5p suppresses the expression of collagen type I and III.[21] Furthermore, TGF-β suppressed miR-29a-5p which resulted in feedforward upregulation of TGF-β.[21]

Another miR-192 was shown to be highly expressed in the kidney, as compared with other organs.[22,23] In the context of renal fibrosis, expression levels of miR-192 increased significantly in glomeruli isolated from diabetic mice.[24] Evidence shows that the levels of transforming growth factor β1 (TGF-β1), a major mediator of renal fibrosis are increased under diabetic conditions in renal cells, including mesangial cells (MC), can up-regulate ECM proteins such as collagens, and also can promote MC survival and oxidant stress.[25] to impair normal kidney function. A number of studies have shown that TGF-β regulates expression of

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several microRNAs, such as miR-21, miR-192, miR-200, miR-433, and miR-29. MiR-21, and miR-433 which are positively induced by TGF-β signaling play a pathological role in kidney diseases. In contrast, members in both miR-29 and miR-200 families which are inhibited by TGF-β signaling protect kidneys from renal fibrosis by suppressing the deposition of ECM and preventing epithelial-to-mesenchymal transition, respectively.[26] miR-192 was proved to repress zinc finger E-box binding homeobox 1/2 (Zeb1/2) and mediates TGF-β–induced collagen expression in MCs in vitro.[24,27] Further supportive evidence can be drawn from the findings in miR-192 KO mice.[28] Deletion of miR-192 gene in type I diabetic mice reduced proteinuria, albuminuria, renal fibrosis, and hypertrophy as compared to diabetic wild-type mice.[28] Taken together, these studies demonstrate a pro-fibrotic role of miR-192 in TGF-β-dependent renal fibrosis observed in animal models of diabetic and obstructive nephropathy.[24,27] In contrast, the reverse is true in human nephropathy. Krupa et al.[29] have shown that TGF-β1 suppresses miR-192 expression in human tubular epithelial cells (TEC) and loss of miR-192 promotes fibrogenesis in diabetic nephropathy. Therefore, in the present study, the differential expression of miR-192, miR-129-5p, miR-29a-5p, miR-200a, miR-200b, miR-200c and miR-141observed may be due to the participation of these miRNAs in the pathogenesis of SRNS and was a clear manifestation of renal fibrosis. Supportive evidence can be drawn from studies showing significant tubulointerstitial fibrosis and/or glomerulosclerosis in the biopsy of SRNS children.[30,31]

TGF-β and miR-192 increased the expression of miR-200 family members (miR-200a, miR-200b, miR-200c and miR-141) which can also target Zeb1/2.[24] The levels of many miRNAs, such as miR-200a, miR-200c, miR-141, miR-429 and miR-192, are lower in patients with active lupus nephritis than those in healthy controls.[32] Studies of the intra-renal expression of miRNAs in IgAN patients revealed that miR-200c was down-regulated while miR-141, miR-192 and miR-205 were up-regulated.[33] These authors have also reported that the downregulation of intrarenal miR-200c is inversely correlated with proteinuria. Another study reported that patients with minimal change disease (MCD) or focal glomerulosclerosis (FSGS) had higher levels of urinary miR-200c than those with other causes of NS.[34] Cai et al.[35] reported that serum miR-192 and miR-205 levels were higher in FSGS patients compared to MCNS patients. Levels of serum miR192 and miR-205 were correlated with proteinuria in FSGS patients while miR-192 levels, not miR-205 levels, were correlated with proteinuria in MCNS. In addition, serum miR-192 levels were correlated with interstitial fibrosis in FSGS patients. miR-320 was also reported to be higher in FSGS patients relative to control and patients with FSGS remission.[36] miR-320 expression level was also significantly elevated in patients with acute kidney disease.[34]

MicroRNA-141-5p, an important member of the miR-200 family, has been reported to be involved in cellular proliferation, migration, invasion, and drug resistance in different kinds of human malignant tumors. However, the role and function of miR-141-5p in SSNS or SRNS are not known. Bioinformatics analysis predicted that RAB32 was the potential target of miR-141-5p. miR-141 plays a dual role of an oncogene or tumor suppressor by regulating target genes.[37] miR-199a was shown to be involved in the renal development and disease. MiR-199a expression is downregulated in human renal cell carcinoma.[38] and deregulated in rodent genetic models of polycystic kidney disease,[39] in lupus nephritis,[40] and in ischemia/reperfusion injury.[41]

Bioinformatics has emerged as a powerful tool for providing comprehensive insights into the molecular mechanisms of disease and identifying potential biomarkers and therapeutic targets.[42.43] The GO function annotation is divided into three functional groups, biological process (BP), molecular function (MF), and cell component (CC), [Fig. 2; biological process (BP), n = 401, molecular function (MF), n = 89; cellular component (CC), n = 66] as determined by adjusted p-values (Benjamini-Hochberg).

The top 20 enriched pathway terms are shown in Fig. 4 and 5. For BP, several signaling pathways (e.g., membrane potential, SMAD1-mediated signal transduction, cell-cell adhesion via plasma membrane adhesion molecule, neuron projection and development, sodium ion transmembrane transport, axonogenesis etc) were found to be dysregulated. Molecular functions include calcium ion transmembrane transporter activity, ligand gated ion channel activity, microtubule binding, actin binding, phosphatase binding, protein serine/threonine kinase activity. Cellular

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components include microtubule, neuronal cell body, focal adhesion, glutaminergic synapse, synaptic membrane, cell-cell communication, KEGG includes Rap1 signaling pathway, cAMP signaling pathway, Ras signaling pathway, calcium signaling pathway, MAPK pathway, PI3K-AKT signaling pathway. Notably, other diseases such as Prion disease, Huntington disease, Amyotrophic lateral sclerosis, Alzhemier disease, Human T cell Leukemia virus1 infection and Salmonella infection, were also listed as enriched terms, indicating that these disorders may share similar pathophysiological pathways with NS.

To sum up, the present study explored the role of urinary miRNAs as non-invasive biomarkers for the diagnosis of children with NS using high-throughput sequencing technology. The present study is unique in reporting few novel miRNAs associated with steroid resistance of nephrotic children. Bioinformatics analysis further shows target genes of these miRNAs were associated with neuronal and renal pathological changes. Although studies have shown that the characteristic expression changes in miR-29a, miR-192 and miR-200c, can be used as markers for disease diagnosis and severity evaluation in the urine of adult patients with NS[44], studies on the direct role of miRNAs in children with NS are limited. Therefore, inference can be drawn based on the previous studies on the expression profiles of miRNAs and their targets in various kidney diseases.

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Compliance with Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all the individual participant included in the study.

Statement and Declarations

Conflict of interest

The authors report no conflicts of interest and they are responsible for the content and writing of this article.

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