Comprehensive analysis of microRNA expression and target prediction in children with Nephrotic syndrome

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Research Article

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Abstract

Background

Nephrotic syndrome is one of the common cause among the kidney disease in children worldwide. It is characterised by the edema proteinuria, hypoalbuminemia and hypocholesteremia. Recently many studies have emerged with the association of microRNAs playing an potential roles in many pathophysiological functions. MicroRNAs (miRNAs) and RNA binding proteins (RBPs) are found to be two most important needed transcriptional regulators of gene expression as well as for the aberrant expression that tend to contribute to the development of the disease. It can reduce translation neither by translation repression through or MicroRNA cleavage. In this present study we have checked for the expression pattern of the targeted microRNAs miR-17-5P, miR-155p, miR-424-5p, miR-1 and 215-5p in the Children among Steroid Sensitive Nephrotic syndrome (SSNS) Steroid Resistance Nephrotic Syndrome (SRNS) along with the healthy individuals. Total RNA was isolated from the urine samples among the three groups (SSNS = 100, SRNS = 100 and healthy individuals = 100). The expression pattern for theses microRNAs was carried out using RT-PCR. Bioinformatics tools such as miRWalk, miR-Tar link were used in predicting targets for the microRNAs an online data bases and g profiler software is used which was also helpful in evaluating the targets based on the biological functions, Molecular functions and the pathways related to the selected microRNAs, along with that ROC analysis was also performed which was widely helpful in selecting the microRNAs that could be used as a potential biomarker as well as a therapeutic target. Among the five microRNAs miR-1, miR-215, miR-17-5P, miR-155-5p & miR-424-5p, two microRNAs miR-424-5p & miR-155p is found to be up regulated in the SRNS group.

1. Introduction

Nephrotic syndrome a glomerular kidney disease mainly characterised by the recurrent edema which was found to be associated with the loss of protein in urine, along with the lower concentrations of albumin, gamma globulin along with the hyperlipemia. It was discovered that the renal glomerulus' enhanced porousness was caused by compromised basement membrane, which led to infections. For younger ages, the etiology of NS is more likely to be minimal change nephrotic syndrome (MCNS) and focal segmental Glomerulosclerosis (FSGS), while there are varieties of etiologies for older ages, including MCNS, FSGS, membranous glomerulonephropathy (MGN) and others.

According to the US report it was stated that the cause of incidence is about 7 per 1 million resulting in the primary glomerular disorder leading to the end stage renal diseases. Almost 80% of the children with idiopathic nephrotic syndrome who are showing absolution of proteinuria under the treatment of corticosteroids are known as Steroid sensitive and it was also reported that nearly 40–60% they may tend to experience for intermittent relapses. Steroid resistance denotes the deflection of remission although with 4 weeks of prednisone therapy. A definite disease phenotype liaison was observed in
patients with the Nephrotic syndrome requires the foot process eradicating or spreading of the podocyte foot process, but still the detailed view of association is found to be unclear\textsuperscript{14}.

MicroRNAs may have a role in any pathophysiological function, according to numerous studies that have recently come out. Both microRNAs (miRNAs) and RNA binding proteins (RBPs) are discovered to be two of the most crucial required transcriptional regulators of gene expression, as well as for the aberrant expression that tends to contribute to the development of the disease. These two factors also act to reduce translation by neither translation repression through nor MicroRNA cleavage\textsuperscript{5}. They are found to be a biomarkers not only in diseases such as viral infections, cardiovascular disorders, diabetes, cancer but also in kidney diseases\textsuperscript{15}. A study by (luo \textit{et al.}, 2017) states that certain microRNAs such as miR-30a-5p, miR-151-3p, miR-150-5p, miR-191 and also miR-19b in serum and miR-30a-5p in urine samples of NS patients have shown to have the increased levels\textsuperscript{16}.

Based on the data available in the literature studies for the role of actin cytoskeleton components in nephrotic syndrome, microRNAs miR-1 and 215-5p, miR-155p, miR-424-5p and miR-17-5p were selected. The present study has seen for the differential expression of miR-17-5p, miR-155p, miR-424-5p, miR-1 and 215-5p with miR-484-5p as endogenous control in urine samples from the 100 healthy volunteers and from two case groups (SSNS = 100 & SRNS = 100).

\section*{2. Methods}

\subsection*{2.1 Selection of participants:}

The study was conducted at Sri Ramachandra institute of Higher Education and Research form the Department of Nephrology SRIHER, Chennai, India. The children who have met the criteria's have been rolled for the study with the age group from 1–12 years. For this study the sample size was taken as 300 among which 200 cases (SSNS, SRNS) and 100 aged matched control groups.

\subsection*{2.2 Inclusion and Exclusion criteria}

The inclusion criteria was involved as the histological confirmation with or without the familial history of NS along with the histological confirmation of MCNS or FSGS. Then the children who do not respond to prednisilone therapy which requires minimum period of 4 weeks has been included. Secondary NS and SRNS with the history other than FSGS or MCNS are taken as exclusion criteria. In the study proper inform consent from all the parents/guardian along with the asset form was obtained. Ethics committee approval has been obtained from the Institutional Ethics committee at Sri Ramachandra Institute of Higher Education and Research [IEC-NI/21/FEB/77/28]

\subsection*{2.3 Sample collection}
About 6 ml of urine that was taken in a sterile container from children aged 1 to 12 years. A total of 200 cases of SSNS and SRNS, along with 100 children who were age-matched controls. As soon as the samples were obtained, they were processed by centrifuging them for 20 minutes at 3,000 rpm, discarding the supernatant. 2.4 Isolation of RNA from the urine pellet.

The urinary pellet was lysed with RNA lysis buffer and it was stored in the deep freezer at 80°C for further purpose. After that MicroRNA isolation was carried out for both the cases SSNS, SRNS and for the control groups by using the MiRNeasy Mini Kit and the procedure was performed according to the manufactures protocol. Urine samples were mixed thoroughly with an equal volume of miRNeasy Serum/Plasma Spike-In Control, incubated for 2–3 minutes at room temperature, and then analysed. centrifuged at 12,000 g for 15 minutes at 4°C. After transferring the upper aqueous phase to a new collecting tube, 1.5 litres of 100% ethanol were added. A 2 ml collection tube containing 700 l of material was pipetted into an RNeasy MinElute spin column and spun at 8000 g for 15 s at room temperature. The supernatant was thrown away. The RNeasy MinElute spin column received about 500 l of elution buffer, and it was centrifuged at the same speed. The RNeasy MinElute spin column was filled with approximately 500 l of 80% ethanol and centrifuged. The concentrations and purity of the samples were calculated from the isolated nucleic acids. On the NanoDrop 1000, the concentration and quality of the isolated RNA were evaluated using spectrophotometry (Thermo Scientific, Waltham, MA). To determine how pure a nucleic acid is, utilise the A260/280 ratio, which is the absorbance at 260 and 280 nm. The lower pedestal of the nano drop was filled with around 2 l of the separated sample, and using the nano drop apparatus, the nucleic acid purity of each collected urine sample was determined. Most people considered a ratio of 1.7–2.0 to be of good quality 2.6 All the 300 samples were checked for quality and quantity are found to be good and with adequate proportion for expression study 40.

2.5 Gene Expression Analysis using quantitative real time PCR

The cDNA conversion was performed using the Taqman advanced cDNA synthesis kit and the converted cDNA templates were further diluted to proceed for microRNA expression analysis using Rotar Gene Q software for the selected miRNAs miR-17-5P, miR-155p, miR- 424 -5p , miR-1 and 215. The expression levels for all the microRNAs was normalized using miR- 484-5p as endogenous control.

2.7 Computational prediction for microRNAs using online data base

MicroRNA prediction tool such as miRWalk and miR Target link human (sticht C, De Torre C, Parveen A, Gretz et al 2018) which was useful to analyse and the select the specific target MicroRNAs with the database available for the study and experimentally validated predicted microRNAs were further analysed using the g profer software in order to identify and the select the specific function relate to this disease.

2.8 Statistical Analysis

Relative Quantification was carried out for all the microRNAs by calculating $2^{-\Delta\Delta CT}$ method and t-test was used to calculate the level of significance with the fold change with the p value less than 0.05 were
found to be statistically significant. Along with that Pearson coefficient correlation was also performed for the selected microRNAs.

3 Results

3.1 Target Prediction Analysis

The Target prediction software human miRtarlink has revealed for the selected microRNAs miR-1 and 215-5p, miR-155p, miR-424-5p and miR-17-5p are represented in Figure 1 and 2 and miRWalk is represented in Table 2. The targeted microRNAs has been further analyzed for the various pathways that are associated with the Nephrotic syndrome. A detailed analyses for involving various functions has been widely represented with their respective IDs in the Table 3.

3.2 MicroRNA expression analysis

The normalization of expression for the all the microRNAs were performed using miR-484-5P as endogenous control. The results were analyzed for the expression and it was found that miR 215-5p, miR-424 and miR-155-5P showed 2 fold increase in expression where as miR1 and miR-17-5p showed 1 fold increase in expression when compared to the control group. miR-1, miR-17-5P, miR-155p, miR-215 were found to up regulated in both SSNS and SRNS group where as miR-424-5p is found to be down regulated in SSNS group and shown up regulation for the SRNS group with the p values as mentioned in the table(1). Though miR-17-5p showed up regulation in cases it did not show significant for the SRNS was calculated by the t-test.

3.3 ROC Analysis

The expression of different genes were analysed to test their ability as a potential biomarker to differentiate SSNS and SRNS using Receiver-operating characteristic (ROC) analysis. ROC analysis is a useful graphical representation, commonly used in medical decision making, for evaluating the performance of diagnostic tests and more generally for evaluating the accuracy of a statistical model. ROC curve was plotted using the sensitivity and specificity of the biomarker assessed at various thresholds, represented as area under the curve (AUC). So, the sensitivity and specificity vary across the different threshold and the sensitivity is inversely related with specificity. Then, the plot of sensitivity versus 1-specificity is called receiver operating characteristic (ROC) curve and the area under the curve (AUC), as an effective measure of accuracy has been considered with a meaningful interpretations. Any biomarker with an AUC 0.7–0.8 was considered as fair, 0.8–0.9 as good and >0.9 as excellent model to differentiate between the two groups (Trifonova et al., 2013). In this study the individual ROC analysis showed miR-155 AUC-0.40 SSNS & SRNS AUC-0.58 and for miR-424 SSNS AUC-0.35 & SRNS AUC-0.46 and the combined analysis shows AUC-0.68 for SSNS & AUC-0.70 for SRNS as represented in Figure (6&7). According to the literatures proves 0.7 can be considered to be a fair biomarker in the diseased condition.
4. Discussion

MicroRNAs are small non-coding RNA of about 22 nucleotides involved in regulating post transcriptional modulators that tend to regulate gene expression\(^{19}\). Many Studies have reported the importance of microRNAs and its potential roles in apoptosis, cell differentiation, cell proliferation with the inclusion of the kidney disease. Differential expression miRNAs studies has been helpful in genome-wide profiling studies\(^{20,21}\). Among them, RNA has emerged as the most easily accessible molecule for the identification of prospective biomarkers in the near future.\(^{37}\). Among the kidney injury miR-21 and miR-155 are found to shown decreased level of expression in blood and urine samples. Luo et al studies states that miRNAs mir30a05p ,mir-151-3p ,miR- 191 are found to shown high level of expression in urine and serum samples in NS children when compared to the healthy individuals\(^{22}\). Similarly another study by Zhang et al 2018 have stated that mir-17-5p are found to significantly increased in Childhood Nephrotic syndrome when compared with the healthy subjects\(^{23}\). miR-192,miR-194,miR-215 and miR-216 are found to be highly expressed in human Kidneys according to Sun et al group\(^{24}\). In Renal childhood neoplasm mir215 are down regulated\(^{25}\) in turns can tend activate transforming growth factor-β (TGF-β) pathway and it can promote tumor formation since they have target activin A receptor in common\(^{26}\). White et al studies shows mir215-5p is found to be over expressed in cellular migration and also in invasion, moreover in gene expression profiling studies they identified mir215 have tend to contribute to tumor metastasis\(^{27}\) and also found to have low level of expression in certain types of tumor, including colon cancer, renal carcinoma and ovarian cancer\(^{28}\). Study by Deng et al., 2019 shows the role of mir1 which is cardiac specific miRNA and its main target in calcium channel in heart\(^{29}\). According to the evidence reported mir1 was the miRNA to be indicted in heart development and also found to be expressed in skeletal muscles and cardiac precursors\(^{30,31}\). Not only in cardiac and skeletal muscles mir1 is found to significantly reduced expression in lung cancer tissues as well as cancer cell lines\(^{32,33}\).Similarly mir-17 plays as a key regulator in G1/S phase cell cycle transition\(^{34}\). In acute kidney injury the urinary miRNAs are tend to have important clinical aspect which can be used as in early diagnosis in an non-invasive biomarker\(^{35}\). Many miRNAs are not only tend to be useful as diagnostic biomarkers but also used as therapeutic targets.\(^{36}\)

As the micro RNAs miR-1 and 215-5p ,miR-155p ,miR- 424 -5p and miR-17-5P which are selected for having an important role in Nephrotic syndrome our study has searched for the expression levels of these microRNAs among the SSNS and SRNS children. miR- 1,miR-17-5P,miR-215 were found to down and SRNS group where as miR-424-5p& miR-155 -5p is found to be up regulated in SRNS group. Computational prediction for the selected microRNAs reveled a common target that has various functions involving molecular function, cellular components and Biological Functions. Subsequently based on the combined ROC examination performed miR-424-5pand miR-155-5p has appeared a esteem of 0.7 which is considered to be reasonable biomarker that can be utilized as potential Biomarker for the Childhood Nephrotic Disorder.
5. Conclusion

In the present study the results were analyzed for the expression and it was found that miR 215-5p ,miR-424 and miR-155-5P showed 2 fold increase in expression where as miR1 and miR- 17-5p showed 1fold increase in expression when compared to the control group. miR- 1,miR-17-5P,miR-215 were found to up regulated in both SSNS and SRNS group where as miR-424-5p & miR-155p is found to up regulated in SRNS group and shown up regulation for the SSNS group with the p values as mentioned in the table(2). Further the combined analysis of gene expression along with the targeted microRNAs can give better understanding in bringing out the Knowledge in the Nephrotic syndrome in future studies.

Declarations

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors does not have any conflict of interest.

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23. Paul vesley at al., miR-192, miR-194, miR-215, miR-200c and miR-141 are downregulated and their common target ACVR2B is strongly expressed in renal childhood neoplasms.


39. Mohanapriya Chinambedu Dandapani1 • Vettriselvi Venkatesan2 • Pricilla Charmine1 • Sangeetha Geminiganesan3 • Sudha Ekambaram. Differential urinary microRNA expression analysis of miR-1, miR-215, miR-335, let-7a in childhood nephrotic syndrome.

Tables

Table: 1 Baseline Characteristics of the Nephrotic Syndrome Children
<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Steroid Sensitive Mean(SD)</th>
<th>Steroid Resistant Mean(SD)</th>
<th>Control Mean(SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=100)</td>
<td>(n=100)</td>
<td>(n=50)</td>
<td>T test</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>71</td>
<td>68</td>
<td>r</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>29</td>
<td>32</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-3</td>
<td>2.2±0.74 (n=36)</td>
<td>1.75±0.79 (n=38)</td>
<td>2.54±0.52 (n=11)</td>
<td>.644979</td>
</tr>
<tr>
<td>Age(yr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-6</td>
<td>5.7±5.18 (n=32)</td>
<td>4.84±0.67 (n=26)</td>
<td>4.6±0.97 (n=18)</td>
<td>.000894</td>
</tr>
<tr>
<td>7-9</td>
<td>8.2±0.77 (n=20)</td>
<td>8.25±0.71 (n=22)</td>
<td>8.2±0.56 (n=17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.5±0.54 (n=12)</td>
<td>10.2±0.57 (n=4)</td>
<td>10.57±0.53 (n=14)</td>
<td></td>
</tr>
</tbody>
</table>

The p-value is .644979. Not significant at p < .05.

The p-value is .000894. The result is significant at p < .05.
<table>
<thead>
<tr>
<th>Laboratory Factors</th>
<th>0.26±0.82 g/dl</th>
<th>0.23±0.01 g/dl</th>
<th>0.4±0.7 g/dl</th>
<th>The p-value is .00012. The result is significant at p &lt; .05.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum albumin, g/dl</td>
<td>1.3±0.14 mg/dl</td>
<td>5.4±0.17 mg/dl</td>
<td>&lt; 20–30 mg/g</td>
<td>The p-value is &lt; .00001. The result is significant at p &lt; .05.</td>
</tr>
</tbody>
</table>

Table:2 Relative expression of microRNAs as fold change
<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-424</td>
<td>SSNS = 0.12</td>
<td>SSNS = &lt; .0001</td>
</tr>
<tr>
<td></td>
<td>SRNS = 2.80</td>
<td>SRNS = .00056</td>
</tr>
<tr>
<td>miR-17</td>
<td>SSNS = 2.80</td>
<td>SSNS = .001779</td>
</tr>
<tr>
<td></td>
<td>SRNS = 1.72</td>
<td>SRNS = .329064</td>
</tr>
<tr>
<td>miR-155</td>
<td>SSNS = 1.33</td>
<td>SSNS = .179375</td>
</tr>
<tr>
<td></td>
<td>SRNS = 2.95</td>
<td>SRNS = .0009</td>
</tr>
<tr>
<td>miR-215</td>
<td>SSNS = 3.94</td>
<td>SSNS = .00001</td>
</tr>
<tr>
<td></td>
<td>SRNS = 1.06</td>
<td>SRNS = .00001</td>
</tr>
<tr>
<td>miR-1</td>
<td>SSNS = 4.04</td>
<td>SSNS = .00031</td>
</tr>
<tr>
<td></td>
<td>SRNS = 1.98</td>
<td>SRNS = .23442</td>
</tr>
</tbody>
</table>

Table 3: Pearson r correlation analysis of the microRNAs (miR-1, miR-215, miR-424, miR-155 and miR-17 among cases (SSNS and SRNS) and control group
### Control vs SSNS

<table>
<thead>
<tr>
<th>miRNA</th>
<th>r value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-215</td>
<td>.230**</td>
<td>.001</td>
</tr>
<tr>
<td>miR-1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>miR-424</td>
<td>.244**</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>miR-17</td>
<td>.247**</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>miR-155</td>
<td>.243**</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

### Control vs SRNS

<table>
<thead>
<tr>
<th>miRNA</th>
<th>r value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-215</td>
<td>.043</td>
<td>.544</td>
</tr>
<tr>
<td>miR-1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>miR-424</td>
<td>.353**</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>miR-17</td>
<td>0.36</td>
<td>0.612</td>
</tr>
<tr>
<td>miR-155</td>
<td>.411**</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Table: 4 Targets for microRNAs miR-1, miR-215, miR-17, miR-424 and miR-155 predicted using miTarbase and mirWalk databases

<table>
<thead>
<tr>
<th>microRNAs</th>
<th>Predicted Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>has-miR-1</td>
<td>CD2AP, COQ6, ACTN4, GATA3</td>
</tr>
<tr>
<td>Has-miR-215-5p</td>
<td>PLCE1</td>
</tr>
<tr>
<td>has-miR-424-5p</td>
<td>VEGFA</td>
</tr>
<tr>
<td>has-miR-17-5p</td>
<td>IL8</td>
</tr>
<tr>
<td>has-miR-155-5p</td>
<td>IL8, AGTR1, TRPS1, NR3C1</td>
</tr>
</tbody>
</table>

**Correlation is significant at the 0.01 level (2-tailed)**

**TABLE:** 5 Significantly enriched BioGRID interactions of the predicted target genes indicating the biological process, cellular component, cellular component, molecular Functions, Wikipathway, HP)
<table>
<thead>
<tr>
<th>Description</th>
<th>Term Id</th>
<th>Corrected P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cytokine receptor binding</td>
<td>GO:0005126</td>
<td>3.952×10⁻⁴</td>
</tr>
<tr>
<td>signaling receptor binding</td>
<td>GO:0005102</td>
<td>1.025×10⁻³</td>
</tr>
<tr>
<td>vascular endothelial growth factor receptor binding</td>
<td>GO:0005172</td>
<td>1.902×10⁻³</td>
</tr>
</tbody>
</table>
Biological Process

- cell migration  
  - GO:0016477  
  - 1.620×10^{-2}
- localization of cell  
  - GO:0051674  
  - 2.953×10^{-2}
- cell motility  
  - GO:0048870  
  - 2.953×10^{-2}

Cellular Components

- platelet alpha granule lumen  
  - GO:0031093  
  - 3.905×10^{-2}

Reactome Pathway

- Nephrin family interactions  
  - REAC:R-HSA-37375  
  - 2.911×10^{-2}

Wiki pathway

- Nephrotic syndrome  
  - WP:WP4758  
  - 9.984×10^{-1}

HP

- Abnormal nephron morphology  
  - HP:0012575  
  - 1.165×10^{-3}
- Focal segmental Glomerulosclerosis  
  - 1.165×10^{-3}
- Abnormal renal glomerulus morphology  
  - HP:0000097  
  - 1.715×10^{-2}
- Abnormal renal glomerulus morphology  
  - HP:0000095  
  - 1.942×10^{-2}

Proteinuria
Glomerular sclerosis

Abnormal urine protein level

Abnormal renal cortex morphology

Abnormal urine protein level

Abnormal renal cortex morphology

Figures
Figure 1

Target prediction for the microRNA miR-424-5P using miRtarget link human 2.0
Figure 2

Target prediction for the gene INF2 using miRartlink
Figure 3

miRNA expression of urinary miR-1 and miR-215 in children with SSNS and SRNS
Figure 4

miRNA expression of urinary miR-424, miR-17 & miR-155 in children with SSNS and SRNS
Figure 5

Bar diagrams showing fold changes in the expression levels of miR-1 (Fig. 3a), miR-215 (Fig. 3b), miR-424 (Fig. 3c), miR-17 (Fig. 3d) and miR-155 (Fig. 3e)
Figure 6

Showing the Individual ROC Analysis for the upregulated microRNAs miR-155-5P & miR-424-5p
Figure 7

Showing the combined ROC Analysis for the upregulated microRNAs mir-155-5p & miR-424-5p