2.0. Study population:

The study was approved by Institutional Ethics Committee of Sri Ramachandra University, Chennai, India (IEC-NI/10/JUNE/17/15). This is a case control study consisting of three groups - healthy controls (Group I); children with SSNS (Group II) and children with SRNS (Group III). The sample size in each group is 100. The diagnosis of cases and their response to therapy based on biopsy reports were confirmed by the physician (1). The mean age for cases was 12.0 ± 5.39 years and for controls, 12.5 ± 5.86 years. The male to female percentage was found to be 68% : 32% for cases and 58% : 42% for control subjects. Both the cases and controls were similar in ethnicity, mainly from South India. The study subjects were recruited from Sri Ramachandra Hospital and Mehta Children’s Hospital, Chennai after obtaining an informed consent from a parent or guardian.

2.1 The criteria for selection of the study subjects are enlisted below:

2.1.1 Selection criteria for cases (n= 200): The KDIGO recommendations was adopted to recruit the cases.

Inclusion:
- One to twelve years of age with/without family history of NS.
- Histological confirmation of MCNS (or) FSGS.
- Steroid resistance NS children are those who do not respond to prednisilone therapy given in adequate and appropriate dose for a minimum period of 4 weeks.

Exclusion:
- Secondary NS.
- SRNS with histology other than MCNS (or) FSGS.

2.1.2 Selection criteria for controls: (n= 100)

Age and gender matched healthy volunteers with no kinship to the cases were recruited from the same geographic area and time period. Subjects with a history of genetic diseases or other birth defects were excluded.
2.2 Materials:

2.2.1 Reagents for DNA isolation: QIAamp DNA Mini Kit (Cat. # 51104) consists the following reagents:

- Collection Tubes
- Buffer AL
- Buffer ATL
- Buffer AW1
- Buffer AW2
- Buffer AE
- QIAGEN® Protease
- Protease Solvent
- Proteinase K

2.2.2 Reagents for agarose gel electrophoresis

2.2.2.1 TAE buffer (Tris- acetate EDTA buffer) - 50X (pH 7.2)

- Tris base – 2M
- Glacial acetic acid - 1N
- Na$_2$ EDTA.2 H$_2$O - 0.05M

Tris base and disodium EDTA were dissolved in sterile double distilled water. Using glacial acetic acid, the pH was adjusted to 7.2. The final volume was made up to 1000 ml and sterilized by autoclaving. The solution was stored in a clean sterile reagent bottle at 25°C.

2.2.2.2 Tris HCl-Himedia (Cat. # TC073)

2.2.2.3 Ethidium bromide (EtBr): Sigma: (Cat. # 8751): To 1ml of sterile double distilled water, 10 mg of ethidium bromide was added and mixed well for the complete dissolution of the dye (10mg/ml). The stock solution was stored in aliquots in air tight containers wrapped with aluminum foil.
2.2.2.4 **Bromophenol blue**: (Himedia Cat. #MB123).

2.2.2.5 **Agarose low EEO**: Bangalore Genei (Cat. # 612600501001730).

2.2.2.6 **DNA sample loading dye**- (6X): Biolabs (Cat. #B7025S).

- Ficoll 400 - 6%
- Bromophenol blue - 0.12%
- Xylene cyanol FF - 0.12%
- Tris- HCl (pH 7.5) - 12mM
- Na₂ EDTA₂ H₂O - 120mM

All the components were dissolved in sterile double distilled water and stored at 25°C for further use.

2.2.3 **Reagents for polymerase chain reaction (PCR)**

- Taq DNA polymerase (3 units/µL): Bangalore Genei (Cat. # 610602500051730)
- 10 X Taq DNA polymerase buffer (10X) Bangalore Genei (Cat.#610602500051730)
- dNTP Mix Solution (10mM) Bangalore Genei (Cat. # 610651200011730)
- Dimethyl sulfoxide (DMSO) Sigma Aldrich (Cat. # D8418)

2.2.3.1 **Primers**: Shrimpex/Sigma

The lyophilized powder of different OD values was reconstituted with sterile water. The primer sequences for the selected genes *NP*HS₁, *NP*HS₂, *ACE* and *MDR1* are specified in Table 2.1.

2.2.4 **Reagents for DNA sequencing and purification**

2.2.4.1 **Big Dye Terminator V.3.1 Cycle sequencing kit**: Applied Biosystems (Cat. #4337455)

- The kit contains 200 μL of Big Dye Terminator V.3.1 ready reaction mix, PGEM control DNA, M13 (-21) primer, sequencing buffer (5x).

2.2.4.2 **Hi-Di Formamide**: Applied Biosystems (Cat. #4311320).
2.2.4.3 **Ethylene diamine tetra acetic acid (disodium salt: EDTA):** Merck (Cat. # 324503)

2.2.4.4 **Absolute ethanol:** Hayman (Cat. #F203640)

2.2.4.5 **70% ethanol:** 7ml of absolute ethanol was diluted with 3ml of sterile double distilled water.

2.3 Methodology:

2.3.1 **Sample collection:**

About 2-3ml of peripheral blood was collected in EDTA vacutainer from all the three groups after obtaining a signed informed consent/assent from the parents/guardians (A sample copy of IC is enclosed in annexure-VI).

2.3.2 **DNA isolation from blood:**

Genomic DNA from the blood samples was isolated and purified using QIAamp Blood Mini kit following the manufacturer’s instructions and stored until further process. About 200μl of peripheral blood was added into 20μl QIAGEN protease to the microcentrifuge tube. Then 200μl buffer AL was added, mixed by pulse-vortexing for 15 seconds and incubated at 56°C for 10 min. The contents were briefly centrifuged. Added 200 μl of 100% ethanol to the sample, and mixed again by pulse-vortexing for 15 seconds. The contents were added into QIAamp mini spin column (in a 2 ml collection tube) and centrifuged at 8000 rpm for 1 min.

The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. About 500 μl of buffer AW1 was added and centrifuged at 8000 rpm for 1 min. Then 500 μl buffer AW2 was added, centrifuged at 14,000 rpm for 3 min. Finally, 200 μl buffer AE or distilled water was added, incubated at room temperature (15–25°C) for 1 min, and then centrifuge at 8000 rpm for 1 min (2).

2.3.3 **Qualitative analysis of DNA:**

The quality of the DNA samples was checked in a 0.8% agarose gel. About 0.8g of agarose was dissolved in 100ml of 1X TAE buffer, boiled and allowed to become
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lukewarm followed by which Etbr (0.1mg/ml) was added. The gel was then poured on a gel-casting tray and allowed to solidify by placing the comb. The DNA samples were mixed with bromophenol blue dye and loaded on the gel. The gel was electrophoresed at 2 volts/cm and visualized under the UV trans-illuminator and photographed using gel documentation system (Biorad). Presences of sharp band as shown in Figure 2.1 indicate that the isolated DNA is in good quality.

**Figure 2.1: Agarose gel shows the quality of DNA**

![](image)

**Lane:** 1 to 8 represents sharp DNA bands; the band intensity depends on the DNA concentration

2.3.4 Quantitative analysis of DNA:

The quantity of DNA samples were measured using the nanodrop based on the ratio of absorbance at 260/280nm wavelength. About 2µl of DNA was added to the lower pedestal of the nanodrop and the purity of DNA was assessed for all 300 samples. A ratio of 1.8 was generally accepted as a good quality DNA (Figure 2.2).
2.3.5 Polymerase chain reaction (PCR):

Using gene specific primers (NPHS1, NPHS2, ACE and MDR1), PCR amplification was carried out and the subsequent amplified products was sequenced by the conventional DNA Sanger sequencing method (Table 2.1) followed by data analysis to assess mutations and/or polymorphisms in genes. (3-6). Amplification of the gene of interest was performed using specific primers under appropriate cycling conditions of denaturation, annealing and extension in a Thermal cycler (Master cycler gradient-Eppendorf). A master mix comprising of all the reagents outlined in Table 2.2 was prepared and added to each template DNA sample tube with the separate reactions yielding a final volume of 20 μl. The tubes were placed in the thermal cycler and subjected to the standardized PCR conditions.

The PCR conditions were standardized for each gene by gradient PCR. The PCR program was outlined with specific annealing temperature and time for each gene; the remaining PCR conditions were constant and are given in Table 2.3. PCR done using specific primer for genotyping of ACE (intron 16) described by Rigat B et al (3), and MDR1 (C3435T, C1236T G2677T/A) described by Jafar T et al (4). Similarly, of the mutation frequency was studied in NPHS1 and NPHS2 (Table 2.1) with gene primers.
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described earlier (5, 6). Upon completion, the products were stored at 4° C until further use.

<p>| Table 2.1. Primer sequences adopted to screen mutations in NPHS1, NPHS2 and SNP in ACE and MDR1 |</p>
<table>
<thead>
<tr>
<th>Exon (n)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size (bp)</th>
<th>Annealing temperature (°C) and Duration (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NPHS1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1&amp;2</td>
<td>GAGAAAGCCAGA CAGACGCAG</td>
<td>AGCTTCGCTGGTG GGCT</td>
<td>491</td>
<td>61.0/45</td>
</tr>
<tr>
<td>E3&amp;4</td>
<td>AGCCACCAGCGG AAGCT</td>
<td>CTCCCCTCACCACCT CCAGAGG</td>
<td>530</td>
<td>57.5/45</td>
</tr>
<tr>
<td>E5</td>
<td>CAGAATCTATCTTT GGGGGAG</td>
<td>CATGGGAAAAT TAGGGGTAAG</td>
<td>187</td>
<td>60.0/45</td>
</tr>
<tr>
<td>E6&amp;7</td>
<td>TCTCCTGACCTCC CCAATTC</td>
<td>CTCAGGACTGGCT CCCAGAC</td>
<td>544</td>
<td>61.0/45</td>
</tr>
<tr>
<td>E8&amp;9</td>
<td>GACAGTGGGGTCT GGGAGCCAGTCCT GAG</td>
<td>GAGTCATGCCCCTC AGCCCC</td>
<td>623</td>
<td>61.0/45</td>
</tr>
<tr>
<td>E10&amp;11</td>
<td>CACGATGAGTGA GGGTGCTG</td>
<td>CCTGGTCCTCACCACATT</td>
<td>465</td>
<td>61.0/45</td>
</tr>
<tr>
<td>E12&amp;13</td>
<td>AACCAGTTGGGC AGGATAGGGG</td>
<td>GACATCGTGGA GGGGGCGA</td>
<td>668</td>
<td>58.0/60</td>
</tr>
<tr>
<td>E14</td>
<td>CCTAGTGCCTTC CAGCC</td>
<td>GAGTAGTCTAGGG TCAAGAAGG</td>
<td>288</td>
<td>59.2/60</td>
</tr>
<tr>
<td>E15&amp;16</td>
<td>CCTGATCTCCAAAT CTGTCCTTGG</td>
<td>CCACAATGGGCA AGGGGCACCCAGTTTGG</td>
<td>484</td>
<td>60.0/60</td>
</tr>
<tr>
<td>E17</td>
<td>CACCCAGACCTGT CGGCC</td>
<td>GTCGCCACTCAGCA AGGAACCT</td>
<td>257</td>
<td>60.0/45</td>
</tr>
<tr>
<td>E18&amp;19</td>
<td>GAGGCTACAGAA GGGGAAATTG</td>
<td>GCTGGGGTACCA GACCTGGG</td>
<td>561</td>
<td>60.0/45</td>
</tr>
<tr>
<td>E20</td>
<td>GGATGGATGCTA GATGCCATCC</td>
<td>CAATCGTTGATGT GGGAATG</td>
<td>297</td>
<td>60.0/60</td>
</tr>
<tr>
<td>E21&amp;22</td>
<td>CCTGACAGAATG TTCTGGAAATT</td>
<td>CCTACACATCTC TGAGGAATAC</td>
<td>487</td>
<td>61.0/45</td>
</tr>
<tr>
<td>E23</td>
<td>GAGGCTGAGAAA TATTTAAGCCTTA GAG</td>
<td>GAGACCAGGAGG TTCCATTCT</td>
<td>188</td>
<td>60.0/45</td>
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</tbody>
</table>
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**Identification of novel gene biomarkers associated with steroid responsiveness in children with Nephrotic syndrome**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Probes</th>
<th>Primer Sequences</th>
<th>Length</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NPHS2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>GCAGCGACTCCAC AGGGACT</td>
<td>TCCACCTTATCTG ACGCCCC</td>
<td>420</td>
<td>60.0/30</td>
</tr>
<tr>
<td>E2</td>
<td>AGGCAGTGAATA CAGTGAAG</td>
<td>GCCCTCAGGAAA TTACCTA</td>
<td>203</td>
<td>55.0/30</td>
</tr>
<tr>
<td>E3</td>
<td>TTCTGGGGATGT TTGAAAG</td>
<td>TGAAGAAATGG CAAGTCAG</td>
<td>168</td>
<td>55.0/45</td>
</tr>
<tr>
<td>E4</td>
<td>AAGGTGAAACC CAAACAGC</td>
<td>CGGTAGGTAAGC CATGAAA</td>
<td>204</td>
<td>55.0/60</td>
</tr>
<tr>
<td>E5</td>
<td>CATAGGAAAAGGA GCCCAAGA</td>
<td>TTTCAGCATATTG GCCATTA</td>
<td>293</td>
<td>55.0/60</td>
</tr>
<tr>
<td>E6</td>
<td>CTCCCACTGACAT CTGA</td>
<td>AATTTAAAAATGAA ACCAGAA</td>
<td>155</td>
<td>50.0/30</td>
</tr>
<tr>
<td>E7</td>
<td>CTAAATCATGGCT GCACACC</td>
<td>CTTCCTAAAGGGC AGTCTGG</td>
<td>167</td>
<td>60.0/45</td>
</tr>
<tr>
<td>E8</td>
<td>GGTAAGGCTTCA GGGATG</td>
<td>TTCTATGGCAGGC CCCCTTA</td>
<td>380</td>
<td>60.0/30</td>
</tr>
<tr>
<td><strong>ACE</strong></td>
<td><strong>Pro_5’ UTR</strong></td>
<td>GAAAGTTGGGG A</td>
<td>CAATCAAAGCTTC GGGCGGCT</td>
<td>639</td>
</tr>
<tr>
<td><strong>MDRI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3435T</td>
<td>TCTTTTCAGTGGC TTGATGG</td>
<td>AAGGCATCTATGT TGGCCTC</td>
<td>197</td>
<td>61.0/30</td>
</tr>
<tr>
<td>G2677T/A</td>
<td>TGCAGGGCTATAGG TCCAGG</td>
<td>TTTAGTTTGACTC ACCTTCCCG</td>
<td>224</td>
<td>64.3/30</td>
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<tr>
<td>C1236T</td>
<td>TATCCTGTGTCTC TGAATTGCC</td>
<td>CCTGACTCCACC ACAATG</td>
<td>366</td>
<td>63.7/45</td>
</tr>
</tbody>
</table>
Identification of novel gene biomarkers associated with steroid responsiveness in children with Nephrotic syndrome

Table 2.3 Standard PCR program adopted to amplify the target sequences

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>45 seconds</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>45sec-1 minute</td>
</tr>
<tr>
<td>Repeated for 30-35 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>Hold</td>
</tr>
</tbody>
</table>

*Annealing temperature varies for each exonic region mentioned in the table 2.1

2.3.6 Confirmation of PCR amplification:

The presence of amplicons was confirmed by running the amplified product into a 2% agarose gel electrophoresis. A 100bp DNA molecular weight marker was used to confirm the amplicon size. Two grams (2%) of agarose powder was dissolved in 100 ml TAE buffer (1 X) and kept in microwave oven for 2 min. The solution was cooled at
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Room temperature and ethidium bromide was added to a final concentration of 0.1mg/ml and mixed thoroughly. One µl Bromophenol blue and 5 µl of PCR product was mixed and loaded in the wells. Electrophoresis was carried out at 4V/cm at 100 volts for 15min. The amplified PCR products in the gel were visualized under the UV trans-illuminator and photographed using gel documentation system (Biorad) (Figure 2.3). The quality of the amplified products was assessed based on the bands intensity. For example presences of band at 155bp confirm the amplification of Exon 6 of NPHS2.

**Figure 2.3: Confirmation of amplicons in agarose gel**

![Image of agarose gel with bands](image)

**MW:** Molecular weight marker (100bp DNA ladder)

**Lane:** 1, 2, 3, 4 & 5 represents product size 155 bp

2.3.7 Sequence analysis:

The amplified products of NPHS1, NPHS2 and MDRI were preceded for Cycle sequencing in a MicroAmp 96 well plate with one of the respective forward or reverse primers (7). A master mix prepared with the reagents supplied from Applied Biosystems and cycle sequencing reaction were performed in a GenAmp PCR systems 9700 thermal cycler with the program represented in Table 2.4 and Table 2.5.
Identification of novel gene biomarkers associated with steroid responsiveness in children with Nephrotic syndrome

2.3.8 Purification of the amplicons:

The product obtained was subjected to purification after the completion of the program. Two microliter of 125 mM EDTA and 30µl of 100% ethanol was added to each well and incubated at room temperature use as 26°C for 10 min. The plate was centrifuged at 3500 rpm for 30 min at 25°C. The plate was kept inverted and spin at 200 rpm for 10 seconds to remove the unwanted contents (extra primers). About 30µl of 70% ethanol was added and centrifuged at 3500 rpm for 5 min maintaining at 25°C. The plate inverted again and centrifuged at 200 rpm to wash and get the purified product and kept

<table>
<thead>
<tr>
<th>S.No</th>
<th>Contents/Concentration</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ready reaction mix (2.5X)</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>Dilution buffer (5X)</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>Template (PCR product -25ng)</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>Forward / Reverse Primer (5pmol):1:1 dilution</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>Sterile water</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>96</td>
<td>60</td>
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<tr>
<td>Denaturation</td>
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<td>Extension</td>
<td>60</td>
<td>240</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.4: Composition of cycle sequencing reaction mixture

Table 2.5: PCR program for cycle sequencing
for drying at room temperature. Ten μl of the denaturing reagent HIDI formamide was added to all the wells and plate was linked for sequencing to the equipment 3730 Genetic analyser from ABI (6). The sequences obtained were then analyzed using Chromas and SeqScape analysis software V2.5 further confirmed by mutation taster for mutation analysis (Figure 2.4).

**Figure 2.4: Mutation analysis using SeqScape software**
2.4 Statistical analysis:

The genotype and allele frequencies for the observed polymorphisms were calculated for the cases and controls. These frequencies were tested for Hardy-Weinberg equilibrium using Chi square method. Association of the genotypes among the steroid sensitive and steroid resistant groups was determined by calculating the odds ratio with 95% confidence interval using multiple logistic regression analysis using SPSS software version 16. A difference was considered to be statistically significant when \( p-values \) were <0.05. Haplotype and pair wise linkage disequilibrium analysis was performed to estimate the frequency of haplotypes between cases and controls and linkage between the SNPs using SNPStats tool.
References:


