Characterization of NPHS2 gene polymorphisms associated to steroid resistance nephrotic syndrome in Indian children

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ABSTRACT

Nephrotic syndrome (NS) is the common glomerular disease in children. These children are treated with steroids, depending upon their behavior. They are either steroid sensitive (SSNS) or steroid resistant (SRNS). NPHS2 gene mutants are linked to the risk of autosomal recessive SRNS and in some cases to SSNS. The present study has been performed to screen single nucleotide polymorphisms (SNPs) of the NPHS2 gene in a group of 90 Indian children suffering with NS (30 SSNS, 30 SRNS and 30 Controls) by PCR method followed by direct exon sequencing. Effect of SNPs on fold expression changes at transcript level of podocin was checked using quantitative real time PCR (qRT-PCR). SNPs identified through sequencing helps to carry out in-silico analysis. Overall 17 SNPs were identified in NPHS2 gene where 6 were found novel. Three missense SNPs p.R299Q, p.P20L and p.G35D were also identified in this population where SNP, p.G35D was found novel. In addition to sequencing analysis, results of in silico analysis shows that a mutant with these three missense SNPs has least ligand binding efficiency compared to native model. Moreover the significant observation of this study included two intronic SNPs c.451 + 23C > T and c.451 + 58A > T present in SRNS group of patients. These SNPs has shown high level of clinical significance within genomic and allelic frequency along with haplotypes and linkage disequilibrium count. The qRT-PCR analysis shows, down expression of podocin protein at transcript level in SRNS patients compared to SSNS patients. All these results support the fact that SNPs present in this population could affect the protein structural stability. Thus it is concluded that the polymorphisms predicted in this study might be disease causing in the NPHS2 gene and may have influence on the therapeutic response of NS patients.

1. Introduction

Kidneys execute numerous central physiological functions including filtration of metabolic waste products from blood (Niaudet, 1991). Glomerular ultrafilterate is generally free from large plasma proteins and cellular elements. On the other side, if the integrity of the filtration machinery is disturbed, the glomerular per-selectivity gets weakened which permits higher amount of plasma proteins, specifically albumin to bypass into the urinary space, so end with proteinuria and Nephrotic Syndrome (NS) (Spitzer, 1985). A good number of NS patients act in response to steroid therapy; however, 15–20% of them fail to respond to steroid treatment. It may direct to end-stage kidney disease (ESRD) which requires dialysis or transplantation (Ruf et al., 2004). Even though it is a common medical finding, the etiology and pathogenesis of NS are still poorly understood.

The exact factors to determine steroid-resistant form have not been fully identified yet. In many cases circulating agents produced by immune cells showed their involvement while in other patients genetic factors were involved (Yu et al., 2005). In recent years, advance researches in molecular genetics permitted some researchers to suggest that a subset of individuals with steroid resistance nephrotic syndrome (SRNS) have a prime imperfection of podocyte proteins such as podocin, nephrin and a-actinin 4 in the glomerular barrier (Papez and Smoyer, 2004). Podocin protein encoded by NPHS2 gene, is an essential...
integral membrane protein that very precisely organizes and regulates structure of glomerular membrane by interacting with Nephrin, CD2AP, TRPC-6 and various other genes via its C-terminus region (Reiterova et al., 2012). Various studies have been confirmed that NPHS2 gene mutation is a common source of sporadic SRNS, found in 10%–30% of children (Caridi et al., 2001). SNP, p.R229Q is one of most frequently reported polymorphism among European population with frequency of 5% in SRNS as compared to healthy individuals (Futouhi et al., 2013).

Literature shows 1–2 folds increase in the occurrence of NS in Asian population, with high risk of NS in black persons (50%) than that of white (35%) (D’Agati, 2008). In 2005, Zihua Yu et al. established the role of NPHS2 gene SNPs in among Chinese children. Though NPHS2 gene polymorphisms are clearly established as causative agents for NS among European as well as Asian countries, but still there are very few studies showing the effect of NPHS2 gene SNPs in Indian children (Yu et al., 2005; Jaffer et al., 2014; Vasudevan et al., 2012). Therefore this study is aimed to perform mutational analysis of NPHS2 gene using next generation sequencing (NGS) and computational analysis to validate the role of SNPs in Indian children.

2. Materials and methodology

2.1. DNA sequencing analysis using by NGS

2.1.1. Patient and data recruitment

In this study total 90 Indian children (30 SRNS, 30 SSNS and 30 Control), histologically proven with focal segmental glomerulosclerosis (FSGS)/minimal change disease (MCD) for NS were selected and grouped on the basis of their drug response (Age 2–14 years). Inclusion criteria is based on renal insufficiency with a glomerular filtration rate (GFR) < 80 ml/min per 1.73 m2. Other baseline criteria includes urinary protein creatinine ratio < 25 g/l. SRNS patients were identified based on daily oral administration of prednisone treatment at 60 mg/

2.2. Expression analysis by qRT-PCR

Total RNA was isolated from human fetal kidney biopsy from the same group of patients (SRNS and SSNS) described above. Fold expression at transcript level of podocin protein was checked in SRNS using SSNS as controls. Total RNA extraction includes various steps such as cell lysis using trizol solution (Invitrogen), followed by incubation with DNase (Qiagen) for 15 min. and final extraction using RNA Extraction Kit method (Qiagen). First and second strand cDNA was synthesized using the cDNA synthesis system (Roche Diagnostics) following manufacturer’s instructions. cDNA synthesis was performed using sample mix 13.5 μl (1 μl template RNA, 1 μl oligo dT and 11.5 μl MillQ) and reaction mix 6.5 μl (1 μl reverse transcriptase, 0.5 μl ribo-block, 1 μl dNTPs, 4 μl RT buffer) according to standard procedures.

Further, its quality and quantity was confirmed using High Sensitivity DNA Chip Kit, Agilent technologies on Bioanalyzer 2100 and Fluorometer QuantFlour™-ST (Promega).

Transcript fold expression analysis was performed with the help of quantitative real-time polymerase chain reaction (qRT-PCR) (Applied biosystem 7500HP). The qRT-PCR method used in the present study is based on the relative quantification approach, changes in fold expression of podocin at transcript level was checked in SRNS patients relative to SSNS patients using previously published primers (Boute et al., 2000). The specificity of amplification was confirmed by running PCR product on agarose gel electrophoresis and melt curve analysis. For RT-qPCR, all reactions were run in triplicate and cycle threshold (Ct) values for target genes were normalized with the GAPDH as an endogenous reference gene.

2.3. Computational analysis of NPHS2 gene

2.3.1. Developing and validating 3D structural of podocin protein

The 3D structure of human Podocin protein is not available in the Protein Data Bank. Hence RaptorX was used to obtain 3D structural model for wild type protein model (Pascal and Marco, 2011). Developed podocin models were visualized using PYMOL tool (Pymol: http://pymol.sourceforge.net/). Structure validation of podocin protein was performed using RAMPAGE: Ramachandran Plot Assessment and QMEAN server (Benkert et al., 2008).

2.3.2. Predicting effects of mutation on protein stability by I-Mutant server

I-Mutant 2.0 server was used to study the effects of mutation on protein stability (http://gpcr2.biocomp.unibo.it/~emidio/I-Mutant/I-Mutant_help.html). I-Mutant calculates free energy change and terminated by 72 °C - 5 min final elongation. Sequencing analysis was performed using ion Torrent PGM platform sequence (Life Technologies). Low quality reads were filtered based on quality score (< 20) and length (< 60 bp) using FastQC and PRINSEQ (Schmieder and Edwards, 2011; Leggett et al., 2013). We used Genome Analysis Toolkit (GATK) software (version 2.8; http://www.broadinstitute.org) to carry out multi-sample SNP-calling (McKenna et al., 2010). The published reference sequence of human (GRCh38.p5) was used as the relevant wild-type sequence. NPHS2 gene was explored for its possible association patterns (P-value (< 0.05) significance) in the SRNS and SSNS patients using Statistical Analysis System (SAS) software (version 9.2; SAS Institute Inc., Nashville, TN, USA). SAS is widely known potential, mainframe-based statistical software with a package application containing several computer languages within it. Analysis of SNP data through SAS basically include two procedures; (1) Calculation of descriptive statistics such as allelic and genomic frequency using Hardy-Weinberg equilibrium (HWE) and (2) Calculation of degree of pairwise linkage disequilibrium (LD) and haplotypes frequencies. Here, LD values reflect susceptibility of alleles to co-segregate at particular loci. Measurement of haplotypes is used to estimate the most likely multi-locus haplotype frequency in a set of genotypes.

Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SRNS</th>
<th>SSNS</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine protein creatinine ratio (UPCR)</td>
<td>7.10 ± 1.53</td>
<td>9.06 ± 3.77</td>
<td>0.09</td>
</tr>
<tr>
<td>S/creatinine (mg dl⁻¹)</td>
<td>0.32</td>
<td>0.43</td>
<td>0.223</td>
</tr>
<tr>
<td>Serum albumin (μg dl⁻¹)</td>
<td>2.05 ± 0.46</td>
<td>2.07 ± 0.52</td>
<td>0.87</td>
</tr>
<tr>
<td>S/sodium (meq dl⁻¹)</td>
<td>134.2 ± 25.1</td>
<td>137.2 ± 21.57</td>
<td>0.336</td>
</tr>
<tr>
<td>S/potassium (meq dl⁻¹)</td>
<td>3.315 ± 25.68</td>
<td>3.98 ± 13.33</td>
<td>0.458</td>
</tr>
</tbody>
</table>

* U, stands for urine; S stands for serum, here p value < 0.05 was considered significant.
estimates whether the protein mutation stabilize or destabilize the protein structure (Abagyan and Totrov, 1994; Schymkowitz et al., 2005).

2.3.3. Molecular docking by PatchDock

We used PatchDock server (Schneidman-Duhovny et al., 2005) for our molecular docking study. The input is list of possible complexes organized by structure complementarily criteria. Docking was carried to check protein-protein interaction (PPI) among podocin protein and TRPC6. This provides valuable insight into the difference between the wild type and mutant proteins models of podocin with ligand TRPC6. The structures of ligand TRPC6 was already generated and published in our previous article (Joshi et al., 2015) along with energy minimization values.

3. Results

Molecular genetics-characterization of NPHS2 gene was carried out in Indian children to identify the role of functional SNPs in NS. Clinically SRNS and SSNS patients were not showing any significant association but, both showed strong association through case-control studies using NGS. Moreover, the qRT-PCR analysis data also supported the role of SNPs in regulating the expression of podocin at transcript level.

3.1. NPHS2 gene analysis

Results of SNP calling predict total 17 SNPs in NPHS2 gene, none of these SNPs were showing association with healthy control group suggesting their disease causing nature. These SNPs were further investigated against SRNS and SSNS, which is the main objective of this study. Out of 17 SNPs, 11(64.7%) SNPs were already reported and the rest 6(35.29%) were novel. Novel SNPs were submitted and accepted by ClinVar-NCBI for their new identity numbers (i.e. ClinVar accession numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS, which is the main objective of this study. Out of 17 SNPs, 11(64.7%) SNPs were already reported and the rest 6(35.29%) were novel. Novel SNPs were submitted and accepted by ClinVar-NCBI for their new identity numbers (i.e. ClinVar accession numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449). The occurrence of SNPs among SRNS and SSNS was found to be 35.3% and 29.41%; numbers are from SCV000266417 to SCV000266449).

These 17 SNPs were present in both coding and non-coding regions of NPHS2 gene. From coding region, 3 SNPs were nonsense and balance 4 was synonymous. Missense SNP (p.G35D) was found novel in this study. Remaining 2 SNPs, (p.R229Q) and (p.P20L) were considerably found in SRNS patients. 2 out of 4 synonymous (p.Leu346Leu and p.Ser96Ser) were noted only among SSNS patients, while the other 2 synonymous (p.Ala318Ala and p.Gly343Gly) were present in both the groups of patients.

From non-coding region SNPs, 5(50%) were novel SNPs while the remaining 5(50%) were known SNPs with their reported rs IDs in NCBI. Majority of non-coding SNPs were present in intronic region (i.e. 7 SNPs of NPHS2 gene). 5 novel non-coding SNPs included, 4; intronic SNPs (c.451 + 58A > T, c.451 + 23C > T, c.378 + 42T > G and c.275-56T > G) and 1; 5’UTR SNP (c. −79G > A). Here SNPs c.378 + 42T > G and c.275-56T > G were found among both the group of patients, while SNPs c.451 + 58A > T, c.451 + 23C > T and (c. −79G > A) were substantially predicted among SRNS patients. 5 reported non-coding SNPs includes 3 intronic (c.873 + 11C > T, c.452-21C > T and c.452-46C > T), 1; 5’UTR premature start codon gain variant (c. −51G > T) and 1; splice region (c.873 + 7A > G).

3.1.1. SNPs identified in coding region and non-coding regions of NPHS2 gene

Table 2

Table 2: Total 17 SNPs found among steroid sensitive nephrotic syndrome and steroid resistance nephrotic syndrome patients and their effect in NPHS2 gene.

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>rs IDs</th>
<th>Effect</th>
<th>Exon/intron</th>
<th>Mutation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c.1038A &gt; G</td>
<td>rs3818587</td>
<td>Leu346Leu</td>
<td>Exon 8</td>
<td>Het.</td>
</tr>
<tr>
<td>2</td>
<td>c.954C &gt; T</td>
<td>rs1410592</td>
<td>Ala318Ala</td>
<td>Exon 8</td>
<td>Both Het./Hom.</td>
</tr>
<tr>
<td>3</td>
<td>c.873 + 11C &gt;</td>
<td>rs372303141</td>
<td>Intron 7</td>
<td>Het.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>c.873 + 7A &gt; G</td>
<td>rs115778966</td>
<td>Intron 7</td>
<td>Het.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>c.535-258G &gt; A</td>
<td>rs61747728</td>
<td>Arg229Gln</td>
<td>Exon 5</td>
<td>Het.</td>
</tr>
<tr>
<td>6</td>
<td>c.452-21C &gt; T</td>
<td>rs12401708</td>
<td>Intron 3</td>
<td>Het.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>c.452-46C &gt; T</td>
<td>rs12401711</td>
<td>Intron 3</td>
<td>Het.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>c.451 + 58A &gt; T</td>
<td>.</td>
<td>Intron 3</td>
<td>Both Het./Hom.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>c.451 + 23C &gt; T</td>
<td>.</td>
<td>Intron 3</td>
<td>Het.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>c.378 + 42T &gt; G</td>
<td>.</td>
<td>Intron 2</td>
<td>Both Het./Hom.</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>c.288C &gt; T</td>
<td>rs3738423</td>
<td>Ser96Ser</td>
<td>Exon 2</td>
<td>Het.</td>
</tr>
<tr>
<td>12</td>
<td>c.275-56T &gt; G</td>
<td>.</td>
<td>Intron 1</td>
<td>Hom.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>c.104G &gt; A</td>
<td>.</td>
<td>Gly35Asp</td>
<td>Exon 1</td>
<td>Het.</td>
</tr>
<tr>
<td>14</td>
<td>c.102A &gt; G</td>
<td>rs1079292</td>
<td>Gly34Gly</td>
<td>Exon 1</td>
<td>Both Het./Hom.</td>
</tr>
<tr>
<td>15</td>
<td>c.59C &gt; T</td>
<td>rs743153644</td>
<td>Pro20Leu</td>
<td>Exon 1</td>
<td>Het.</td>
</tr>
<tr>
<td>16</td>
<td>c.−51G &gt; T</td>
<td>rs12406197</td>
<td>5’_prime_UTR_premature_start_codon_gain_variant</td>
<td>Intron 1</td>
<td>Het.</td>
</tr>
<tr>
<td>17</td>
<td>c.−79G &gt; A</td>
<td>.</td>
<td>5’_prime_UTR_variants</td>
<td>Intron 1</td>
<td>Het.</td>
</tr>
</tbody>
</table>

3.2. Role of NPHS2 SNPs at fold expression changes at transcript level

Barely detectable amplification product was observed in SRNS as...
Table 3
Fold expression changes at transcript level in targeted proteins.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gene</th>
<th>ΔΔ CT mean</th>
<th>Fold change</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>NPHS2</td>
<td>0</td>
<td>-1.148</td>
<td>Down-regulation</td>
</tr>
<tr>
<td>Cases</td>
<td>NPHS2</td>
<td>0.1794</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

3.3. Computational prediction of functional SNPs in support to sequencing analysis

3D model generated through Raptor X covered total nos. of 315 residues out of total 383 amino acid residues of podocin protein (Fig. 1). Ramachandran plot was used to validate protein model obtained from RaptorX web server. Out of all the amino acids, 301(93.9%) were in most the favored region, 12(5.2%) in additional allowable regions and 2(0.9%) in disallowable regions. Thus, overall structure of podocin protein obtained from Ramachandran plot can be considered as appropriate one (Fig. 2). The QMEAN score includes combination of 6 different terms (Table 4). The total QMEAN-score which is 0.257 along with its Z-score = 5.89, is coming under estimated model reliability value between 0–1.

Podocin mutants were modeled from their wild type by PYMOL server (Fig. 3). The total energy after minimization and electrostatic constant of native protein were −10,786.841 kJ/mol, −8633.33 where as that of p.P20L were −10,818.770 kJ/mol, −8635.47, p.G35D was −9994.296 kJ/mol, −8562.60 and p.R229Q was −10,689.00 kJ/mol, −8532.38. The energy minimization and electrostatic constant values of the mutant models presented thermodynamically favorable changes when compared with wild-type model, but these values showed more difference in mutant p.G35D and p.R229Q when judge against p.P20L. These genomic mutants are more stable and possibly add to the stability of podocin protein.

3.3.1. I-Mutant score

The three missense SNPs, p.R229Q, p.P20L and p.G35D of NPHS2 gene screened through sequencing analysis have been selected and submitted to I-Mutant web server to predict the DDG stability and reliability index (RI) upon mutation; results are mentioned in (Table 4). If DDG value is < 0, protein stability decreases and when DDG value is > 0 protein stability increases. There are more chance that protein stability might get affected in mutation at position p.R229Q and p.P20L (DDG score = −0.97 and 0.79) as compared to mutation at position p.G35D (DDG score = −0.15).

3.3.2. Docking analysis by PatchDock

Results of docking analysis predicted the effect of mutation on PPI of podocin protein with TRPC6 as a ligand molecule (Fig. 4). Here 4 complexes namely; wild type podocin, p.R229Q, p.P20L and p.G35D were used in the study. Docking scores and atomic contact energy (ACE) of the wild type and mutant complexes are elucidated in Table 5. Acquired results verify that wild type complex exhibited the highest docking score of 25,358 and ACE value of −333.09 when compared to the other three mutant complexes. This result signifies better conjugation of native podocin in the binding pocket of the TRPC6. Mutant p.R229Q complex exhibited the least binding affinity towards TRPC6, which was confirmed by the docking score of 20,592 and ACE value of −249.74.

4. Discussion

This is the first study that correlates sequencing and expression analysis with in silico analysis to understand the role of NPHS2 gene SNPs in Indian children with NS. NPHS2 gene SNPs are identified in a subset of childhood SRNS and SSNS with FSGS and MCD. Any apparent difference between SRNS and SSNS patients could not be detected from clinical data, but in these patients significant genetic variation has been identified through NGS and in-silico analysis which is the key finding of this study. This gene is chosen as a candidate gene, as the literature indicate common presence of homozygous or compound heterozygous SNPs in patients with NS leading towards ESRD within age of 6–14 years. In some cases there are chances of recurrence even after renal transplantation (Bouchireh et al., 2014). Moreover the comparative studies reported high occurrence rate (i.e. around 20%) of NPHS2 gene SNPs in Germany, Italy, Israel and the USA population. Mayumi et al., describes the NPHS2 gene SNPs to be present in Italy, French, German, and Israeli-Arab origin patients, but were absent in Israeli-Jewish and Japanese children, the rate of occurrence of these SNPs may be due to the interethnic variations (Sako et al., 2005). Reports showed 10–30% of European children with SRNS have SNPs in the NPHS2 gene (Karle et al., 2002). Jaffer A et al., in 2014 predicted 4 different SNPs (G92C, P118L, R138Q and D160G) of NPHS2 gene in SRNS patients. This findings help in establishing the role of NPHS2 gene SNPs in the Indian population (Jaffer et al., 2014).
Mutation analysis of the NPHS2 gene was executed in all 8 exons using the direct DNA sequencing method. 6 novel SNPs were identified in addition to the 11 already known SNPs. The SNPs were distributed in coding and non-coding region and lead to all kinds of alterations such as 3 missense, 4 synonymous, 7 intronic, 1 splicing, and 2 in the 5′UTR region of NPHS2 gene. Similar to this study, Boute et al., described NPHS2 as the causative gene for autosomal-recessive SRNS among 16 different families emerged from Europe, Egypt, North Africa, Saudi Arabia and Turkey (Boute et al., 2000). Our results indicate the presence of heterozygous as well as homozygous SNPs in both the group of patients, but the heterozygous form were more prevalent. Various studies indicated the probability of single heterozygous NPHS2 SNPs as a foremost cause of disease (Weber et al., 2004; Karle et al., 2002; Ruf et al., 2004). There are some cases where the patients with entire range of MCD, diffuse mesangial proliferation, and FSGS having homozygous mutation do not respond to immunosuppressive agents (Boute et al.,...

**Table 5**

Effect of NPHS2 gene missense SNPs on the protein structure stability and ligand binding affinity checked via I-Mutant and PatchDock.

<table>
<thead>
<tr>
<th>Substitution</th>
<th>I-Mutant</th>
<th>PatchDock</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substitution</td>
<td>Score</td>
</tr>
<tr>
<td></td>
<td>RI</td>
<td>Effect on protein stability</td>
</tr>
<tr>
<td>R229Q</td>
<td>−0.97</td>
<td>Decrease</td>
</tr>
<tr>
<td>P20L</td>
<td>0.79</td>
<td>Increase</td>
</tr>
<tr>
<td>G35D</td>
<td>−0.15</td>
<td>Decrease</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a WT: aminoacid in wild-type protein, RI: reliability index, NEW: new aminoacid after mutation, DDG: DG(NewProtein) DG(WildType) in Kcal/mol.
b ACE: stands for atomic contact energy.

**Fig. 3.** (a) Wild type wild Proline at 20 position (b) Mutant Leucine at 20 position (c) Wild type Wild Glycine at 35 position (d) Mutant Aspartic acid 35 position (e) Wild type wild Arginine at 229 position (f) Mutant Glutamine at 229 position.

**Fig. 4.** Results of docking analysis using PatchDock, to study protein-protein interaction (ppi) among podocin protein as a receptor molecule and TRPC6 protein as a ligand molecule.

**NPHS2** as the causative gene for autosomal-recessive SRNS among 16 different families emerged from Europe, Egypt, North Africa, Saudi Arabia and Turkey (Boute et al., 2000). Our results indicate the presence of heterozygous as well as homozygous SNPs in both the group of patients, but the heterozygous form were more prevalent. Various studies indicated the probability of single heterozygous NPHS2 SNPs as a foremost cause of disease (Weber et al., 2004; Karle et al., 2002; Ruf et al., 2004). There are some cases where the patients with entire range of MCD, diffuse mesangial proliferation, and FSGS having homozygous mutation do not respond to immunosuppressive agents (Boute et al.,...
A novel heterozygous SNP, p.G35D was identified in exon 1 of the NPHS2 gene, the total energy minimization and electrostatic constant values of p.G35D were showing thermodynamic difference compared to native molecule. Additionally the docking score and ACE values proved that p.G35D could affect the structure stability of podocin protein. All these facts mentioned above strongly suggest that the mutation p.G35D of NPHS2 gene is pathogenic. 2 more known SNPs; p.R229Q and p.P20L, were also identified in the present study. Comparative results of in silico analysis predicted p.R229Q with highest the possible damaging effect among p.G35D and p.P20L. There are few studies where p.R229Q and p.P20L are predicted to have disease causing effect in European, North American Caucasian and South American populations (Tory et al., 2014; Machuca et al., 2009). In 2003, Caridi predicted p.P20L to cause non-conservative substitutions (Caridi et al., 2001). The highest frequency of p.R229Q has been reported in the Czech population (i.e. 12%) (Reiterova et al., 2012). Patients with p.R229Q, tend to have later-onset disease (i.e. typically FSGS) with an increased (2–3 folds) risk of microalbuminuria and progresses slowly to ESRD in the third and fourth decades of life (Tryggvason et al., 2006). Yu et al., predicted 2 NPHS2 gene SNPs, a heterozygous 1082T > C and a homozygous 954T > C, through which they established the fact that the single heterozygous mutation (in this case, p.R229Q) could not by itself be acknowledged as a causative mutation (Yu et al., 2005).

In this study some synonymous SNPs and non-coding region SNPs (intronic and splice region) were also found. Though these SNPs do not affect amino acid substitution, they may cause phenotypic change by affecting the structural folds of the mRNA or inactivating genes by activating cryptic splicing sites. Therefore there are some possibilities, where non-coding SNPs can reduce or remove protein function by causing premature termination of the protein and it cannot be ignored. Also introns towards the 5’ UTR region of a gene contain some regulatory elements whose mutation might impact on the rate of transcription (Dusel et al., 2005). As podocin is expressed entirely in glomerular podocytes, it is not easy to evaluate the consequence of intronic and splice region SNPs in affected cells.

Various reports have verified that gene SNPs may affect the disease vulnerability. The difference in the frequency of NPHS2 gene SNPs illustrates the importance of ethnicity in different populations and countries. On the other hand, the numeral of estimated exons and the lustrates the importance of ethnicity in di...


