Gene 628 (2017) 134–140

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

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

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ARTICLE INFO

Keywords:
Indian children
NPHS2 gene
Steroid-resistant nephrotic syndrome
Steroid-sensitive nephrotic syndrome

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 ultrafiltrate 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 weaken 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

Abbreviations: NS, nephrotic syndrome; SRNS, steroid resistance nephrotic syndrome; SSNS, steroid sensitive nephrotic syndrome; GFR, glomerular filtration rate; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; *NPHS2*, nephrosis-2-idopathic-steroid resistance; SNPs, single nucleotide polymorphisms; nsSNP, non-synonymous SNPs; NGS, next generation sequencing; qRT-PCR, quantitative real time-polymerase chain reaction; cDNA, complementary DNA; GATK, Genome Analysis Toolkit; SAS, Statistical Analysis System; LD, linkage disequilibrium

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http://dx.doi.org/10.1016/j.gene.2017.07.029
Received 23 February 2017; Received in revised form 2 June 2017; Accepted 10 July 2017
Available online 13 July 2017
0378-1119/

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 (Fotouhi 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 m^2. Other baseline criteria includes urinary protein excretion > 0.05 g/kg per 24 h with hypoalbuminemia < 25 g/l. SRNS patients were identified when they show lack of response to daily oral administration of prednisone treatment at 60 mg/m^2 for continuous four weeks. The clinical characteristic of SRNS and SSNS patients has been described in Table 1. Children diagnosed with other systemic disease based on clinical and laboratory examinations were not included in the current study. Human subjects research was approved by the Institutional Ethics Committee of Muljibhai Patel Urological Hospital (MPUH), Nadiad, Gujarat, India (EC/264/2014). The diagnosis of NS was made by pediatric nephrologists at authorized center MPUH. Further genetic studies were carried out after properly approaching parents of all the patients with informed consent form.

2.1.2. Mutation analysis

Genomic DNA was isolated from blood samples using the modified John's nonidet method (John et al., 1991). Mutational analysis of *NPHS2* gene by direct exon sequencing (i.e. all 8 exons) was performed using previously published primers (Boute et al., 2000). PCR conditions were modified as follows; 94 °C - 4 min initial denaturation, followed by 32 cycles of denaturation at 94 °C - 20 s, annealing temperature at 58 °C - 40 s for exon 3, 7 and 8 rest all were kept at 56 °C and terminated by 72 °C - 5 min final elongation. Sequencing analysis was performed using ion Torrent PGM platform sequencer (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 multilocus haplotype frequency in a set of genotypes.

Table 1
Clinical characteristics between SRNS and SSNS patient sample with their mean ± standard deviation values[a].

Parameters	SRNS	SSNS	*p*-Value
Urine protein creatinine ratio (UPCR)N:1	7.10 ± 1.53	9.06 ± 3.77	0.09
S/creatinine (mg dL^{-1})	0.32	0.43	0.223
Serum albumin (g dL^{-1})	2.05 ± 0.46	2.07 ± 0.52	0.87
S/sodium (meq dL^{-1})	134.2 ± 25.1	137.2 ± 21.57	0.336
S/potassium (meq dL^{-1})	3.315 ± 25.68	3.98 ± 13.33	0.458

[a] U, stands for urine; S stands for serum, here *p* value < 0.05 was considered significant.

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 DNAase (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 oligodT and 11.5 μl MilliQ) and reaction mix 6.5 μl (1 μl reverse transcriptase, 0.5 μl riboblock, 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 QuantiFluor™-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

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 two protein molecules, and the output 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%; however, 35.3% SNPs did not show any significant difference and were predicted in both the group of patients. The number and location of SNPs detected through sequencing data are summarized and shown in Table 2.

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

These 17 SNPs were present in both coding 7(41.17%) and non-coding 10(58.82%) regions of *NPHS2* gene. From coding region, 3 SNPs were missense 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.Gly34Gly) 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).

3.1.2. Prediction of clinically significant NPHS2 SNPs

Almost all the observed SNPs showed heterozygous state of condition in either SSNS or SRNS group of patients. While only 1 SNP, (c.275-56T > G) was showing homozygous state of condition. Fascinatingly, 4 of these intronic SNPs (i.e. c.954C > T, c.451 + 58A > T, c.378 + 42T > G and c.102A > G) were found in homozygous as well as heterozygous conditions. All these 17 SNPs were statistically examined for their level of significance using SAS software. 2 SNPs, (c.451 + 23C > T and c.451 + 58A > T) were showing a high level of clinical significance within genomic and allelic frequency along with haplotypes and linkage disequilibrium count, thus showing clear clustering difference among two groups of patients. For c.451 + 23C > T variant scores were: NegLog10_Prob allelic (2.623), genomic (3.019), haplotypes (4.637) and linkage disequilibrium score (3.511). Similarly for c.451 + 58A > T scores were: NegLog10_Prob allelic (1.751), genomic (1.210), haplotypes (4.210) and linkage disequilibrium score (3.321). Score above 1.3 (NegLog10_Prob > 1.3 is equal to $\alpha < 0.05$). Remaining all 15 SNPs were showing less level of significance having their NegLog10_Prob allelic, genomic, dominance-recessive, haplotypes and LD score below 1.3.

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

Barely detectable amplification product was observed in SRNS as

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

No.	Position	rs IDs	Effect	Exon/intron	Mutation status
1	c.1038A > G	rs3818587	Leu346Leu	Exon 8	Het.
2	c.954C > T	rs1410592	Ala318Ala	Exon 8	Both Het./Hom.[a]
3	c.873 + 11C >	rs372303141		Intron 7	Het.
4	c.873 + 7A > G	rs115778946	Splice region	Intron 7	Het.
5	c.535-2548G > A	rs61747728	Arg229Gln	Exon 5	Het.
6	c.452-21C > T	rs12401708		Intron 3	Het.
7	c.452-46C > T	rs12401711		Intron 3	Het.
8	c.451 + 58A > T	.		Intron 3	Both Het./Hom.
9	c.451 + 23C > T	.		Intron 3	Het.
10	c.378 + 42T > G	.		Intron 2	Both Het./Hom.
11	c.288C > T	rs3738423	Ser96Ser	Exon 2	Het.
12	c.275-56T > G	.		Intron 1	Hom.
13	c.104G > A	.	Gly35Asp	Exon 1	Het.
14	c.102A > G	rs1079292	Gly34Gly	Exon 1	Both Het./Hom.
15	c.59C > T	rs74315344	Pro20Leu	Exon 1	Het.
16	c. − 51G > T	rs12406197	5_prime_UTR_premature_start_codon_gain_variant	Intron 1	Het.
17	c. − 79G > A	.	5_prime_UTR_variant	Intron 1	Het.

[a] Het., heterozygous mutation; both Het./Hom., here some of the patients were showing heterozygous state while others were showing homozygous state.
[b] SRNS, steroid resistance nephrotic syndrome; both, here some of the patients were SRNS while others were SSNS condition.

Table 3
Fold expression changes at transcript level in targeted proteins.

Sample	Gene	ΔΔ CT mean	Fold change	Expression
Control	NPHS2	0		
Cases	NPHS2	0.1794	− 1.148	Down-regulation

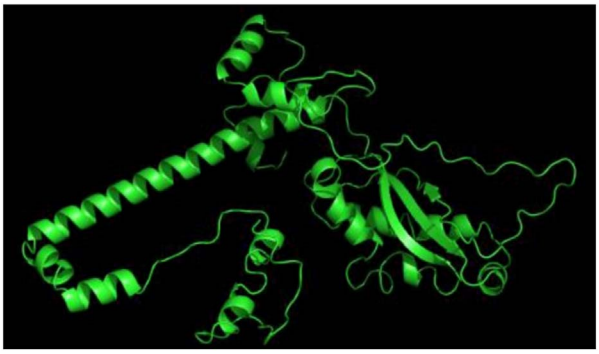

Fig. 1. 3D model of podocin protein constructed protein podocin protein using Raptor X.

compare to SSNS which suggests that the transcript of interest was expressed at a low level in SRNS patients (Table 3). Thus the qRT-PCR results showed a decrease in fold expression changes of podocin at transcript level expression in SRNS as compare to SSNS patients, which may be due to a presence of SNPs in the corresponding gene.

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,

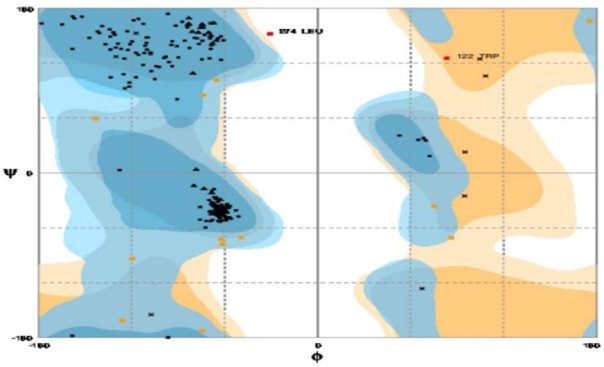

Fig. 2. Ramachandran plot of modeled using Rampage.

Table 4
QMEAN results.

Parameters	Score
C_beta interaction energy	− 66.55 (Z-score: − 0.28)
All-atom pairwise energy	− 3396.87 (Z-score: − 1.23)
Salvation energy	− 11.34 (Z-score: − 2.05)
Torsion energy	3.53 (Z-score: − 4.62)
Secondary structure arrangement	64.1% (Z-score: − 3.47)
Solvation accessibility agreement	59.4% (Z-score: − 4.07)
Total QMEAN-score	59.4% (Z-score: − 5.89)

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 (Bouchireb 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).

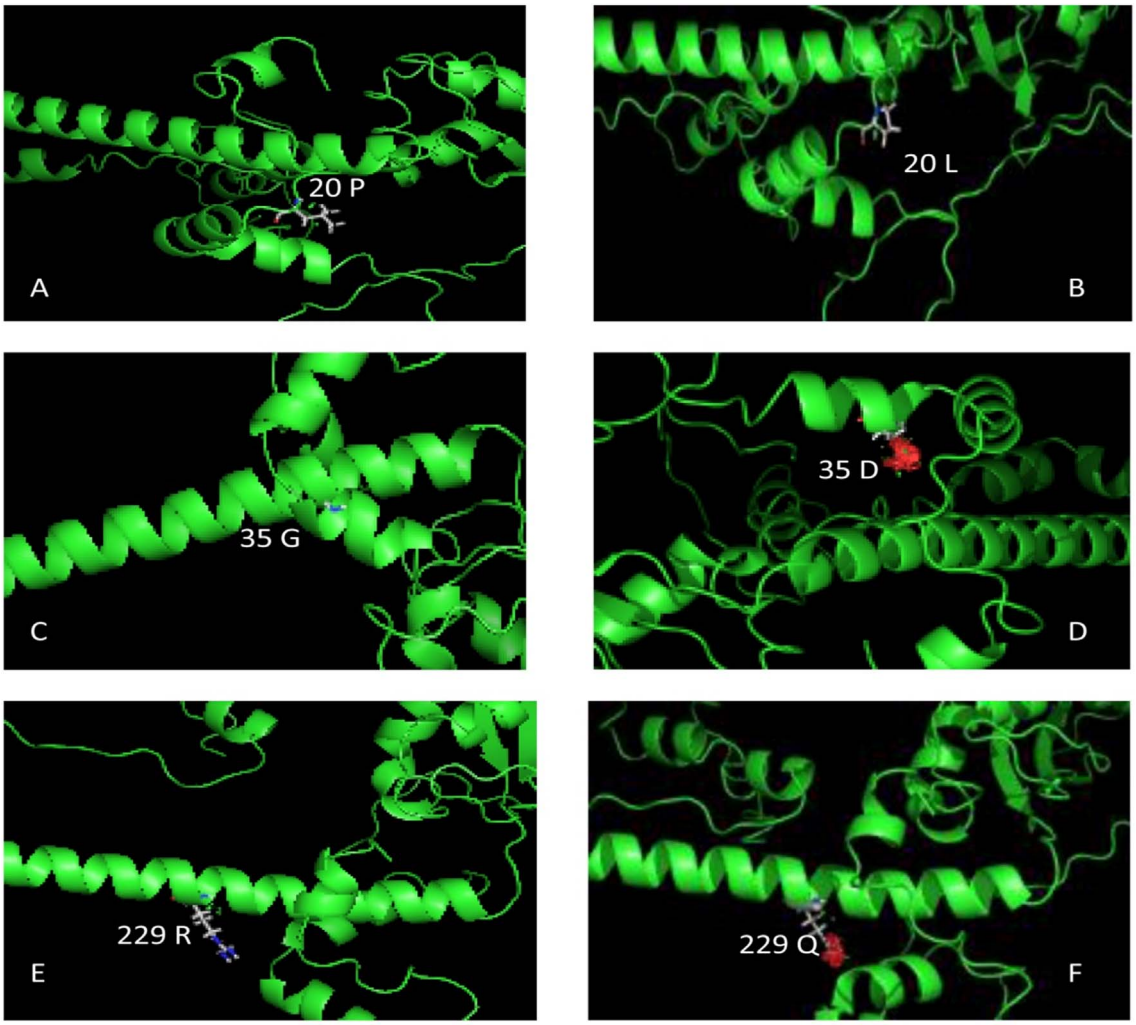

Fig. 3. (a) Wild type wild Proline at 20 position (b) Mutant Leucine at 20 position (c) Wild type Wild Glycine at 35 position (d) Mutant Aspartic acid 35 position (E) Wild type wild Arginine at 229 position (F) Mutant Glutamine at 229 position`.

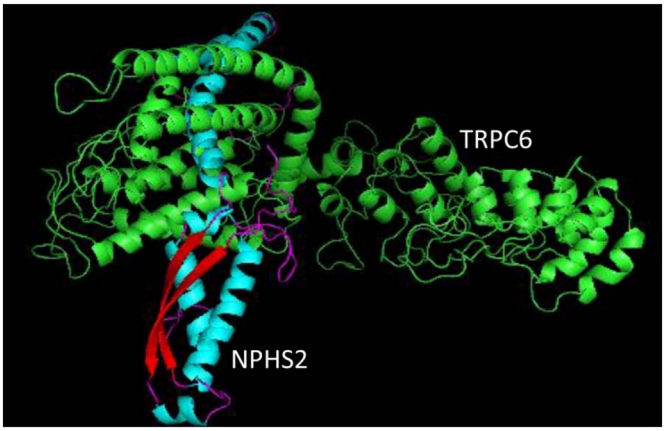

Fig. 4. Results of docking analysis using PatchDock, to study protein-protein interaction (ppi) among podocin protein as a receptor molecule and TRPC6 protein as a ligand molecule.

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

Table 5
Effect of *NPHS2* gene missense SNPs on the protein structure stability and ligand binding affinity checked via I-Mutant and PatchDock[a].

Substitution	I-Mutant			PatchDock		
	DGG	RI	Effect on protein stability	Molecule	Score	ACE[b]
R229Q	− 0.97	8	Decrease	Native	25,358	− 333.09
P20L	0.79	1	Increase	P20L	21,190	− 312.46
G35D	− 0.15	1	Decrease	R229Q	20,592	− 249.74
				G35D	24,318	− 328.13

[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.

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.,

2000; Fuchshuber et al., 1995).

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 at., 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 sample size are amid the chief essential sources of this discrepancy (Otukesh et al., 2009). We therefore examined genotypic and allelic frequencies with LD and haplotypes values of all 17 predicted SNPs of NPHS2 gene for their *p*-value (< 0.05) significance. Two intronic region SNPs, (c.451 + 23C > T and c.451 + 58A > T) were showing high level of significance, while in remaining 15 SNPs, less significant differences were found. These observations suggest that there is an association between the SNPs of *NPHS2* and SRNS. In the present study 29.41% of SNP's were predicted to be present in SSNS patients. Though *NPHS2* gene SNPs have been remarkably reported for SRNS, there are studies pointing the existence of *NPHS2* gene SNPs in few SSNS patients (Mishra et al., 2014).

Results from expression analysis suggest that the fold change at transcript level in SRNS patients was significantly lower than SSNS patients. Analogous to our results Boute et al., reported down-regulation of podocin transcript due to the presence of SNPs in the *NPHS2* gene among SRNS patients (Boute et al., 2000). Moreover, the results from in silico analysis extensively supported the qRT-PCR analysis. The docking score of mutant and native molecule showed the momentous differences that lead to disruption of protein structure and PPI stability. Hiroyasu et al., reported pathogenic effect of p.R229Q, it causes decrease in binding efficiency of podocin to other membrane proteins such as nephrin and TRPC6 (Hiroyasu et al., 2002). Podocin is an integral structural protein of the podocyte, thus a disrupted protein will apparently effect in a glomerular filtration function, ultimately leading to NS (Nikou et al., 2013). Thus sequencing and in silico analysis results supported the role of *NPHS2* gene SNPs in NS, in harmony with assumption which we had postulate for this study.

5. Conclusion

Identification of total 17 novel and known SNPs, dispersed along with the entire coding and non-coding region of the *NPHS2* gene are reported in this study. This study demonstrates that *NPHS2* gene SNPs might be the major cause of NS in Indian children. The energy minimization values and docking score of mutant and native molecule confirmed the pathogenic effect of mutant molecule in compare to wild type, where the c.535-2548G > A (p.R229Q) showed more prominent effect in regulating protein structural and functional stability. This research supports the requirement of genetic assessment for *NPHS2* gene SNPs in Indian children with NS. Detected SNPs may serve as biomarkers and prevent patients from use of unnecessary renal biopsy and steroid treatment.

Acknowledgement

Authors are grateful to Charutar Vidya Mandal (CVM) and Anand Agriculture University Vallabh Vidya Nagar, Gujarat for providing platform for this research work. We are also thankful to Dr. Nilanjan Roy Director of Ashok and Rita Patel Institute of Integrated Study & Research in Biotechnology and Allied Sciences (ARIBAS) New Vallabh Vidya Nagar, for providing the facilities and his valuable suggestions during our research work.

References

Abagyan, R., Totrov, M., 1994. Biased probability Monte Carlo conformational searches and electrostatic calculations for peptides and proteins. J. Mol. Biol. 235, 983–1002.
Benkert, P., Tosatto, S.C., Schomburg, D., 2008. QMEAN: a comprehensive scoring function for model quality assessment. Proteins 71, 261–277.
Bouchireb, K., Boyer, O., Gribouval, O., Nevo, F., Huynh-Cong, E., Moriniere, V., Campait, R., Ars, E., Brackman, D., Dantal, J., Eckart, P., Gigante, M., Lipska, B.S., Liutkus, A., Megarbane, A., Mohsin, N., Ozaltin, F., Saleem, M.A., Schaefer, F., Soulami, K., Torra, R., Garcelon, N., Mollet, G., Dahan, K., Antignac, C., 2014. NPHS2 mutations in steroid-resistant nephrotic syndrome: a mutation update and the associated phenotypic spectrum. Hum. Mutat. 35, 178–186.
Boute, N., Gribouval, O., Roselli, S., Benessy, F., Lee, H., Fuchshuber, A., Dahan, K., Gubler, M.C., Niaudet, P., Antignac, C., 2000. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. Nat. Genet. 24, 349–354.
Caridi, G., Bertelli, R., Carrea, A., Di Duca, M., Catarsi, P., Artero, M., Carraro, M., Zennaro, C., Candiano, G., Musante, L., Seri, M., Ginevri, F., Perfumo, F., Ghiggeri, G.M., 2001. Prevalence, genetics, and clinical features of patients carrying podocin mutations in steroid-resistant nonfamilial focal segmental glomerulosclerosis. J. Am. Soc. Nephrol. 12, 2742–2746.
D'Agati, V.D., 2008. The spectrum of focal segmental glomerulosclerosis: new insights. Curr. Opin. Nephrol. Hypertens. 17, 271–281.
Dusel, J.A., Burdon, K.P., Hicks, P.J., Hawkins, G.A., Bowden, D.W., Freedman, B.I., 2005. Identification of podocin (NPHS2) gene mutations in African Americans with non-diabetic end-stage renal disease. Kidney Int. 68, 256–262.
Fotouhi, N., Ardalan, M., Jabbarpour Bonyadi, M., Abdolmohammadi, R., Kamalifar, A., Nasri, H., Einollahi, B., 2013. R229Q polymorphism of NPHS2 gene in patients with late-onset steroid-resistance nephrotic syndrome: a preliminary study. Iran J. Kidney Dis. 7, 399–403.
Fuchshuber, A., Jean, G., Gribouval, O., Gubler, M., Broyer, M., Beckmann, J., Niaudet, P., Antignac, C., 1995. Mapping a gene (SRN1) to chromosome 1q25-q31 in idiopathic nephritic syndrome confirms a distinct entity of autosomal recessive nephrosis. Hum. Mol. Genet. 4, 2155–2158.
Hiroyasu, T., Akulapalli, S., Tu, C., Trang, N., Jun, Y., Joshua, A., Asher, D., Esteban, P., Patricia, F., Gerald, B., Aparecido, P., Raghu, K., Martin, P., 2002. NPHS2 mutations in late-onset focal segmental glomerulosclerosis: R229Q is a common disease-associated allele. J. Clin. Invest. 110, 1659–1666.
Jaffer, A., Unnisa, W., Raju, D.S., Jahan, P., 2014. NPHS2 mutation analysis and primary nephrotic syndrome in southern Indians. Nephrology (Carlton) 19, 398–403.
John, S.W., Weitzner, G., Rozen, R., Scriver, C.R., 1991. A rapid procedure for extracting genomic DNA from leukocytes. Nucleic Acids Res. 19, 408.
Joshi, B.B., Koringa, P.G., Mistry, K.N., Patel, A.K., Gang, S., Joshi, C.G., 2015. In silico analysis of functional nsSNPs in human TRPC6 gene associated with steroid resistant nephrotic syndrome. Gene 572, 8–16.
Karle, S., Uetz, B., Ronner, V., Glaeser, L., Hildebrandt, F., Fuchshuber, A., 2002. Novel mutations in NPHS2 detected in both familial and sporadic steroid-resistant nephrotic syndrome. J. Am. Soc. Nephrol. 13, 388–393.

Leggett, R.M., Ramirez-Gonzalez, R.H., Clavijo, B.J., Waite, D., Davey, R.P., 2013. Sequencing quality assessment tools to enable data-driven informatics for high throughput genomics. Front. Genet. 4, 288.
Machuca, E., Hummel, A., Nevo, F., Dantal, J., Martinez, F., Al-Sabban, E., Baudouin, V., Abel, L., Grunfeld, J.P., Antignac, C., 2009. Clinical and epidemiological assessment of steroid-resistant nephrotic syndrome associated with the NPHS2 R229Q variant. Kidney Int. 75, 727–735.
McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., DePristo, M.A., 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 20, 1297–1303.
Mishra, P., Kakani, N., Singh, K., Narayan, G., Abhinay, A., Prasad, R., Batra, V., 2014. NPHS2 R229Q Polymorphism in Steroid Resistant Nephrotic Syndrome: is it Responsive to Immunosuppressive Therapy? 60. pp. 231–237.
Niaudet, P., 1991. Steroid-resistant idiopathic nephrotic syndrome and ciclosporin. Fr. Club Pediatr. Nephrol. Nephron 57, 481.
Nikou, F., Mohammadreza, A., Mortaza, B., Reza, A., Amir, K., Hamid, N., Behzad, E., 2013. R229Q polymorphism of NPHS2 gene in patients with late-onset steroid-resistance nephrotic syndrome a preliminary study. Iran. J. Kidney Dis. 7, 399–403.
Otukesh, H., Ghazanfari, B., Fereshtehnejad, S.M., Bakhshayesh, M., Hashemi, M., Hoseini, R., Chalian, M., Salami, A., Mehdipor, L., Rahiminia, A., 2009. NPHS2 mutations in children with steroid-resistant nephrotic syndrome. Iran. J. Kidney Dis. 3, 99–102.
Papez, K.E., Smoyer, W.E., 2004. Recent advances in congenital nephrotic syndrome. Curr. Opin. Pediatr. 16, 165–170.
Pascal, B., Marco, B.T., 2011. Toward the estimation of the absolute quality of individual protein structure models. Bioinformatics 27, 343–350.
Reiterova, J., Safrankova, H., Obeidova, L., Stekrova, J., Maixnerova, D., Merta, M., Tesar, V., 2012. Mutational analysis of the NPHS2 gene in Czech patients with idiopathic nephrotic syndrome. Folia Biol. (Praha) 58, 64–68.
Ruf, R.G., Lichtenberger, A., Karle, S.M., Haas, J.P., Anacleto, F.E., Schultheiss, M., Zalewski, I., Imm, A., Ruf, E.M., Mucha, B., Bagga, A., Neuhaus, T., Fuchshuber, A., Bakkaloglu, A., Hildebrandt, F., Arbeitsgemeinschaft Fur Padiatrische Nephrologie Study, G, 2004. Patients with mutations in NPHS2 (podocin) do not respond to standard steroid treatment of nephrotic syndrome. J. Am. Soc. Nephrol. 15, 722–732.
Sako, M., Nakanishi, K., Obana, M., Yata, N., Hoshii, S., Takahashi, S., Wada, N., Takahashi, Y., Kaku, Y., Satomura, K., Ikeda, M., Honda, M., Iijima, K., Yoshikawa, N., 2005. Analysis of NPHS1, NPHS2, ACTN4, and WT1 in Japanese patients with congenital nephrotic syndrome. Kidney Int. 67, 1248–1255.
Schmieder, R., Edwards, R., 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27, 863–864.
Schneidman-Duhovny, D., Inbar, Y., Nussinov, R., Wolfson, H.J., 2005. PatchDock and SymmDock: servers for rigid and symmetric docking. Nucleic Acids Res. 33, W363–7.
Schymkowitz, J., Borg, J., Stricher, F., Nys, R., Rousseau, F., Serrano, L., 2005. The FoldX web server: an online force field. Nucleic Acids Res. 33, 382–388.
Spitzer, A., 1985. The developing kidney and the process of growth. In: Seldin, D.W., Giebisch, G. (Eds.), The Kidney: Physiology and Pathophysiology. Raven Press, pp. 1979–2015.
Tory, K., Menyhard, D.K., Woerner, S., Nevo, F., Gribouval, O., Kerti, A., Straner, P., Arrondel, C., Huynh Cong, E., Tulassay, T., Mollet, G., Perczel, A., Antignac, C., 2014. Mutation-dependent recessive inheritance of NPHS2-associated steroid-resistant nephrotic syndrome. Nat. Genet. 46, 299–304.
Tryggvason, K., Patrakka, J., Wartiovaara, J., 2006. Hereditary proteinuria syndromes and mechanisms of proteinuria. N. Engl. J. Med. 354, 1387–1401.
Vasudevan, A., Siji, A., Raghavendra, A., Sridhar, T.S., Phadke, K.D., 2012. NPHS2 mutations in Indian children with sporadic early steroid resistant nephrotic syndrome. Indian Pediatr. 49, 231–233.
Weber, S., Gribouval, O., Esquivel, L., Morinière, V., Tete, J., Legendre, C., Niaudet, P., Antignac, C., 2004. NPHS2 mutation analysis shows genetic heterogeneity of steroid-resistant nephrotic syndrome and low post-transplant recurrence. Kidney Int. 66, 571–579.
Yu, Z., Ding, J., Huang, J., Yao, Y., Xiao, H., Zhang, J., Liu, J., Yang, J., 2005. Mutations in NPHS2 in sporadic steroid-resistant nephrotic syndrome in Chinese children. Nephrol. Dial. Transplant. 20, 902–908.