3.0 Materials and Methodology

The aim of the present study is to perform identification of SNPs of targeted genes using \textit{in silico} and sequencing analysis (Fig.14). This investigation may reveal advantages over the experimental bases due to its convenience, reliability, speed, and cost-effective.

![Flow-chart](image)

**Fig.14** Flow-chart representing the overall work flow of the present study, it includes \textit{both in silico} analysis and case-control studies using NGS to study polymorphism of targeted genes and their association with NS in Indian children.
3.1 *In silico* analysis of targeted genes

To accomplish the first objective, *in silico* analysis was performed in the targeted podocyte gene (*TRPC6* and *NPHS2*) using various bioinformatics tools.

The steps for the *in silico* analysis are as follows: (1) Data mining of targeted gene SNPs from various public datasets, (2) Modeling 3D structure of protein, (3) Structural analysis of modeled protein, (4) Predicting effect on the energy minimizing values of native and mutant protein models, (5) Identification of ligand binding sites and post-translation modified sites in targeted protein models.

3.1.1 Retrieval of SNPs

SNPs data of human *TRPC6* and *NPHS2* genes, SNP ID and protein accession number of TRPC6 and podocin, were obtained from NCBI dbSNP for further *in silico* analysis.

3.1.2 Characterization of functional Non-synonymous SNPs

Detection of deleterious nsSNPs of targeted genes was performed using the four *in silico* tools, SIFT, PolyPhen2, SNP&GO and PROVEN. nsSNPs predicted to be probably deleterious by these algorithms were characterized to be high-risk nsSNPs and were selected for further structural studies.

**SIFT:** SIFT is a sequence homology based tool available at (http://sift.jcvi.org/). Queries are submitted in the form of SNP ids or as protein sequences. SIFT detects the changes in the conserved amino-acids sequences on the basis of multiple sequence alignment and further calculates the normalized probabilities scores for all possible substitutions at each position from the alignment. The cutoff value in SIFT program is tolerance index of 0.05, higher the
tolerance index, a particular amino acid substitution is likely to have less functional impact (Ng and Henikoff, 2003).

**PolyPhen2**: PolyPhen severe is a sequence homology based tool, available at (http://genetics.bwh.harvard.edu/pp2). The query input format is either protein sequence (fasta), SWALL database ID or accession number together with sequence position with two amino acid variants. PolyPhen calculates the tolerance index on the basis of position-specific independent counts (PSIC) scores for each of the two variants, and then computes the PSIC scores difference between them (Ramensky et al., 2002).

**SNP&GO**: SNP&GO is an algorithm available at (http://snps-and-go.biocomp.unibo.it/snps-and-go/). It explores protein sequence annotation and accurately predicts whether a variation is disease related or not. The information collected is in the form of protein sequence in fasta format, evolutionary information and function as encoded in the terms of gene ontology terms (Capriotti et al., 2013).

**PROVEN**: It is new prediction based software which works for genome wide analysis of both SNPs and InDels. PROVEN predicts whether the amino acid substitution have an effect on biological function of a protein. It works on the principle of pairwise alignment scores and available at (http://provean.jcvi.org/index.php)(Choi and Chan, 2015) (published by Joshi et al., 2015).

### 3.1.3 Phylogenic analysis of SNPs found in conserved region of targeted genes

ConSurf web-server available at (consurf.tau.ac.il/) was used to determine the evolutionary conserved regions of targeted protein. FASTA sequences of targeted proteins are submitted online where the calculation is based on phylogenic relations between homologous
sequences. The conserved regions were predicted via conservation scores, colouring scheme and further divided into distinct scales of nine grades (Ashkenazy et al., 2010a) (published by Joshi et al., 2015).

### 3.1.4 Developing 3D Structural in targeted proteins

The 3D protein structure of human TRPC6 and Podocin is not available in the Protein Data Bank. Hence SWISS-MODEL server (http://swissmodel.expasy.org/interactive) was used to generate 3D structural model for wild type protein (Pascal and Marco, 2011). SWISS-MODEL is a platform for automated protein structure prediction based on homology modeling.

The template protein was searched through BlastP algorithm against PDB database (Ashkenazy et al., 2010b). Once the template sequence and target sequence were aligned, 3D model was constructed automatically using automodel class. Models were saved in .pdb format and visualized using PYMOL tool that works in Linux platform (Pymol: http://pymol.sourceforge.net/). Targeted protein mutant models were constructed and analyzed from their wild type using same PYMOL tool (published by Joshi et al., 2015).

### 3.1.5 Validation of modeled protein structure using PROCHECK-Ramchandran plot

Structure validation of targeted proteins was carried using Ramchandran plot (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/) (Tabassum et al., 2014). Ramchandran plot is a scattered two-dimensional (2D) plot of Φ and Ψ pairs, comparing them with a predicted distribution regions. This tool uses database statistics calculations for validation of modeled protein structure.
3.1.6 Evaluating protein structure using QMEAN and MUSTER scores

Evaluation of modeled protein structure stability was also carried out using QMEAN and MUSTER server. QMEAN server available at (http://swissmodel.expasy.org/qmean) (Benkert et al., 2008), provides access to both composite scoring function clustering method for protein structure validation (Pascal & Marco, 2011). MUSTER (http://zhanglab.ccmb.med.umich.edu/MUSTER/) is a Multi-score program, which is a linear combination of 5 terms: (1) primary and secondary structure derived from template sequence, (2) structured derivation profiles, (3) solvent accessibility, (4) torsion energy (Ψ and Φ angles) and (5) hydrophobic energy. Z-score provided by MUSTER includes complete full length models in support to MODELLER software (Wu, 2008) (published by Joshi et al., 2015).

3.1.7 Protein structure stability analysis using I-Mutant

I-Mutant version 2.0, an online tool accessible at (http://folding.biofold.org/i-mutant/) (Capriotti et al., 2005). It depends on the ProTherm database and is used to evaluate protein stability changes induced in presence of nsSNP. It estimates the Gibbs free energy change value (DDG) for the wild type protein, subtracting it from the mutant protein. It also predicts the sign (increase or decrease) of the free energy change value, along with a protein reliability index scores. A DDG< 0 corresponds to a decrease in protein stability, whereas a DDG>0 corresponds to an increase in protein stability. However, according to the ternary classification system, a large decrease in protein stability corresponds to a DDG < -0.5 and a large increase in protein stability corresponds to a DDG > 0.5 (Wu, 2008) (published by Joshi et al., 2015).
3.1.8 Post-translation modification sites present on targeted protein

Glycation sites of targeted protein were predicted using NetGlycate 1.0 server (http://www.cbs.dtu.dk/services/NetGlycate/) (Johansen et al., 2006). Residues showing score of >0.5 were considered glycated. NetPhos2.0 server (http://www.cbs.dtu.dk/services/NetPhos/) (Blom et al., 1999) was used to check phosphorylation sites of predicted protein Serine, threonine, and tyrosine residues with a score of >0.5 were considered phosphorylated. UbPerd server (www.ubpred.org) (Radivojac et al., 2010) was used to predict ubiquitylation sites in targeted protein. In UbPerd, residues with a score of ≥0.52 were considered ubiquitylated. Sumoylation sites were predicted using the GPS-SUMO tool (http://sumosp.biocuckoo.org/) (Zhao et al., 2014), residues having score of >0.5 were considered sumoylated (Jenna and Stephen, 2014) (Wu, 2008) (published by Joshi et al., 2015).

3.1.9 Identification of nsSNPs on Ligand Binding Sites using FTsite server

Prediction of ligand binding sites of targeted proteins was carried out using FTsite (http://ftsite.bu.edu) (Ngan et al., 2012). It accurately makes out ligand binding sites of targeted proteins depending upon structure-based prediction, functional relationships among proteins, protein engineering and drug design (Wu, 2008) (published by Joshi et al., 2015).

3.1.10 Docking Analysis by PatchDoc

Molecular docking studies, were carried out to find protein-protein interactions (PPI) among targeted proteins using Patchdock (Schneidman-Duhovny et al., 2005). The input is two protein molecules; TRPC6 and podocin, and the output will be feasible complex structure
organized by complementary criteria. This provides the valuable information about the differences between the native and mutant protein models of targeted protein (Wu, 2008) (published by Joshi et al., 2016).

3.2 Molecular genomic analysis of nephrotic syndrome using NGS technology

To accomplish the second objective, molecular genomic analysis was performed using NGS technology. On the basis of the literature survey (TRPC6 and NPHS2) gene were selected for the mutational analysis via case-control studies (Obeidová et al., 2012; Reiterova et al., 2012). The work includes: (1) Screening of SNPs in targeted genes using NGS technique, (2) Evaluation of SNP data obtained through sequencing (3) Statistical analysis of probably significant SNPs.

3.2.1 Blood and biopsy sample collection

A total 90 Indian children diagnosed with primary NS and histological proven FSGS/MCD were selected in the present study. Children diagnosed with other systemic disease based on clinical and laboratory examinations were excluded from current study. The blood samples were characterized in two different groups: Group I: Normal healthy children (n=30) and Group II: Children having nephrotic syndrome (NS) (n=60). Blood samples were collected in EDTA coated vacuainter tubes by vein puncture. Further genetic study was carried out only after properly approaching parents of all the children with informed consent form. The present study was approved by the Ethics Committee, Govindbhai Jorabhai Patel Ayurved College and Research Center, Anand, Gujarat, India (Approval no. - IEC-3/GJPIASR/2015-16/E/C) and Muljibhai Urological Hospital, Nadiad, Gujarat, India (Approval no. -
EC/264/2014). Fresh biopsy samples were collected from ten control and ten cases to perform change in fold expression of targeted proteins at transcript level using quantitative real time PCR (RT-qPCR). Here biopsy sample collection of control children was a major concern, so biopsy samples from kidney transplant patients were collected as controls.

### 3.2.2 Isolation of genomic DNA from blood sample

Isolation of genomic DNA from peripheral lymphocytes was carried out using modified John’s nonidet method (John et al., 1991). The detailed modified procedure is described below:

- In 1ml of blood sample, equal amount of RNA lysis buffer and 120 µl of non-i-det P₄₀ were added and tubes were mixed properly for 10 minutes by inverting them several times. Tubes and centrifuged at 2000 rpm for 15 minutes.
- Supernatant was poured off gently without disturbing the pellets. Pellets were resuspended in 800 µl of WBC lysis buffer and 600 µl of phenol solution.
- Tubes were centrifuged at 11000 rpm for 9 minutes at 4 °C. Upper aqueous phase
- Plasma proteins were removed by the adding of equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to supernatant. Tubes were centrifuged at 11000 rpm for 9 minutes at 4 °C.
- The upper phase was taken again and chloroform:isoamyl alcohol (24:1). Tubes were centrifuged at 11000 rpm for 9 minutes at 4 °C.
- DNA was precipitated by adding equal volume of chilled ethanol and 1/10th volume of 3M Sodium acetate. The tubes were centrifuged at 11000 rpm, 9 minutes, 4°C, the pellets were washed with 0.5ml of 70% ethanol.
The pellets were suspended in 70 l in TE (Tris EDTA) buffer and stored at -20°C for further analysis.

DNA concentration and purity was checked by nanodrop (Thermoscientific, NANODROP, and U.S.A).

3.2.3 Evaluation of isolated genomic DNA

The quantitative and qualitative estimation of all isolated DNA was done using NanoDrop (ND-100) and agarose gel electrophoresis respectively. In agarose gel electrophoresis 0.8% agarose was dissolved in 1X TBE buffer and melted until it gets completely saturated in buffer solution. After cooling, ethidium bromide (EtBr) was added at rate of 10ul /100ml in gel solution. Melted agarose was poured in sealed gel casting tray with the appropriately positioned comb. Comb was removed carefully after the gel got solidified at room temperature. Solidified gel was placed in electrophoresis unit filled with 1X TBE buffer. 3ul of isolated DNA samples along with 3ul of gel loading dye were loaded in the wells and electrophoresis was carried out at 80 to 100v until tracking dye reached 70% of gel. On the completion of electrophoresis, gel was visualized under UV transilluminator, here purified DNA appeared as orange fluorescent bands.

3.2.4 PCR amplification of targeted genes for detection SNP detection

The purified high quality RNA-free DNA stock concentration was calculated and samples were diluted to 30ng/µl for further PCR amplification. All exonic regions of targeted genes (i.e. TRPC6 and NPHS2) were amplified using polymerase chain reaction (PCR) (Corbett research – Master cycler gradient, Hamburg, Germany). For 25 1 reaction mixture included
100 ng template DNA, 10pM each primer, 200 nM dNTPs, 10 mM Tris HCl (pH 9.0), 50 mM KCl, and 0.5 U of Taq polymerase (EmeraldAmp GT PCR Master Mix, Takara, Clontech Laboratories, Inc., USA). Primers used here were prior crosschecked using in silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr) (Kuhn et al., 2013).

3.2.4.1 Thermal conditions for NPHS2 gene

Mutational analysis of NPHS2 gene by direct exon sequencing (i.e. all 8 exons) was performed using previously published primers (Boute et al., 2000) (Table 2). The modified conditions were: 95°C- 5 minutes of initial denaturation, followed by the 30 cycles of denaturation 95°C- 30 sec, annealing temperature at 58°C- 40sec for exon 3, 7 and 8 rest all were kept at 56°C and extension by 72 °C -15sec each, with final elongation 72°C -10 minutes.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primers Forward (5’-3’) / Reverse (5’-3’)</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F: GCAGCGACTCCACAGGGACT R: TCAGTGTTGCTCGTGAGGAT</td>
<td>362</td>
</tr>
<tr>
<td>2</td>
<td>F: AGGCAGTGAATACAGTGGAAG R: GGCCTCAGGAAATTACCTA</td>
<td>244</td>
</tr>
<tr>
<td>3</td>
<td>F: TTCTGGGAGTGATTTGAAAG R: GAAGAAATTGGAAGTCAG</td>
<td>213</td>
</tr>
<tr>
<td>4</td>
<td>F: AAGGTGAAACCCCAAACAGC R: GGTAGGTAGACCATGGGAAC</td>
<td>233</td>
</tr>
<tr>
<td>5</td>
<td>F: CATAGGAAAGAGCGCAAGGACCAAGAG R: TTTCCAGCATATGGCAGGAGATTA</td>
<td>344</td>
</tr>
<tr>
<td>6</td>
<td>F: CTCCCCACTGACATCTGA R: AAATTTAAAAATGAAACCAGAA</td>
<td>196</td>
</tr>
<tr>
<td>7</td>
<td>F: CTAATCTGAGCTGCAACCACTG R: CTTCCATAAGGCGAGTGCTGG</td>
<td>219</td>
</tr>
<tr>
<td>8</td>
<td>F: GGTTGAAGCCTTCAGGGGAATG R: TTCTATGGCAGGCCCTTTA</td>
<td>419</td>
</tr>
</tbody>
</table>
3.2.4.2 Thermal conditions for TRPC6 gene

TRPC6 gene was screened for mutations using previously reported primers (Obeidová et al., 2012) (Table 3). PCR conditions were modified as follows; 95°C -5 minutes of initial denaturation, followed by the 30 cycles of denaturation 95°C- 30 sec, annealing temperature 56°C- 40sec for exon 3 and 4, rest all exon were kept at 60 °C and extension by 72°C- 15 sec each, with final elongation 72 °C -10 minutes.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primers</th>
<th>Product size</th>
</tr>
</thead>
</table>
| 1    | F: TGGAACTGCCCACCTCGGCTC  
R: TCCTGCAGCCGCAAAACC | 298 |
| 2A   | F: GGCAAGTCATTGCGACACT  
R: CCACATGATGGCATTCTGG | 316 |
| 2B   | F: CAGTGGTGGGAAGATGTTA  
R: TGGAGTCAAATGACTGGAGA | 332 |
| 2C   | F: TTTGCTGAAAGCGAGGAGTT  
R: AGCCGTCAATGACTGGATCTT | 336 |
| 2D   | F: TGACTCGTTTAGCCACTCCA  
R: GGTAGCGATCACAATTTTCG | 328 |
| 3    | F: TCTGAAGCATAAGTAAAACGTGGT  
R: CCCCTTTATCCCTTATTAGCACCACAA | 313 |
| 4    | F: TCGTTTATGCTGAACTTTCTT  
R: ACCCAACTGTGATTCCCTGA | 326 |
| 5    | F: GGAGATCATGGGAATGTCGAG  
R: CCAACTGCTAAGACTGCAACA | 365 |
| 6    | F: CAGCTAAAGGCTGAATGCGAT  
R: TGAGAATTGTGCAGTAAACGA | 377 |
| 7    | F: CGCAGAAAAAGAAGTTACTAAA  
R: CCCATGGACTTACATAAACGC | 420 |
| 8    | F: TTTGCAAGCATAAAACTCAATCA  
R: AGCAGTCATAAGACTACAAACA | 353 |
| 9    | F: TGCACTTTCTTGTCAAAGTCTG  
R: AAAGGGATGTGGCATAGTGG | 316 |
| 10   | F: AGCATTGGCAAAGGGAAAGGA  
R: AGATAAGGCCGTGACAGTG | 271 |
| 11   | F: GACAACCTCTAAACCAACAGCCA  
R: AAGAATCATCAGTTTCAAGACCTAAA | 369 |
| 12   | F: TCGCCTGCATCCTGGCTGAATC  
R: AGCTCTCCAGGCACCTCTGCG | 305 |
3.2.5 Visualization of amplified products and amplicon preparation of targeted genes

The PCR products in both the genes were checked via gel electrophoresis at 80-100 volts in a 2% agarose gel stained with EtBr. The amplified products observed under UV-transilluminator (300nm) to verify amplification and approximate quantity of DNA based on band intensities. The desired amplified products sizes were confirmed using a low range molecular weight marker (Invitrogen, USA) at the time of agarose gel electrophoresis. Concentration of all the amplicons was checked via nanodrop, these amplicons were then pooled in sample wise style by maintaining their equimolar concentrations. Polled amplicons were then purified by gel extraction method using gel extraction kit. (Qiagen QIAquick Gel Extraction Kit). Amplicons of each group were then subjected for sequencing using Ion Torrent PGM™ System sequencing.

3.2.6 Library preparation, bead enrichment and sequencing using Ion Torrent PGM system sequencing

Purified \textit{TRPC6} and \textit{NPHS2} amplicons were pulled according to their equimolar concentration and then subjected to ligation and nick repair in a 0.2 ml PCR tube, combining the reagents (Table-4).

| Table-4: Reaction setup for barcoded libraries in Ion Torrent PGM |
|-------------------|-------------------|-------------------|
| **Component**     | **Volume by input DNA (50-100ng)** |
| DNA               | 25 ng             |
| 10X Ligase Buffer | 10 ng             |
| Ion P1Adapter     | 2 ng              |
Materials and methodology

<table>
<thead>
<tr>
<th>Ion Xpress™ Barcode X*</th>
<th>2 l</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTP Mix</td>
<td>2 l</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>49 l</td>
</tr>
<tr>
<td>DNA Ligase</td>
<td>2 l</td>
</tr>
<tr>
<td>Nick Repair Polymerase</td>
<td>8 l</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>100 l</td>
</tr>
</tbody>
</table>

X* - All samples were ligated with different barcodes

- PCR tubes were then kept in PCR for 25°C for 15 minutes, 72°C for 5 minutes and 4°C for hold.
- Whole mixture was then transferred to 1.5 ml Eppendorf LoBind® tube for next cleanup step.
- Nick translated amplicon purification for adapter-ligation and was carried out using AMpure® XP Agencourt® reagent (1.4X sample volume) according to manufacturer’s protocol.
- Qualitative and Quantitative measurement of pooled barcoded libraries was performed using Bioanalyser® instrument. Diagrammatic presentation of barcoaded amplicons is shown in figure 15.

![Diagramatic presentation of barcoaded amplicons library](image)

**Fig.15** Diagrammatic presentation of barcoaded amplicons library
Determination of library dilution required for template preparation: The template preparation using an appropriate Ion template preparation kit was carried out determining the library dilution that gave $210 \times 10^6$ molecules of template per 20 µl; $210 \times 10^6$ was the recommended library input for whole exonic libraries. A conversion factor was used of $8.3 \text{ nM} = 5 \times 10^9 \text{ molecules/ l}$, and the following formula

$$\text{Template dilution factor} = \frac{(\text{Library concentration in nM}) \times \left[\frac{(5 \times 10^9 \text{ molecules/ l})}{(8.3 \text{ nM})}\right] \times \left[\frac{\text{(volume per template preparation reaction in µl)}}{(210 \times 10^6 \text{ molecules})}\right]}$$

Amplicon libraries were diluted serially (1:100) until library concentration was ≤20 nM.

Template preparation and emulsion based clonal amplification: In this procedure, each library template was clonally amplified on Ion Sphere™ particles for sequencing on the Ion PGM System.

Emulsion was carried out using Ion OneTouch™ 200 Template Kit v2 DL with the Ion OneTouch™ System. In 1.5 ml Eppendorf LoBind® tubes following components were mixed at 15-30°C (Table 5).

**Table 5** Components of amplification solution used for emulsion based clonal amplification in Ion Torrent PGM sequencing system.

<table>
<thead>
<tr>
<th>Order</th>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ion PGM™ Template OT2 400 Reagent Mix</td>
<td>500 µl</td>
</tr>
<tr>
<td>2</td>
<td>Ion PGM™ Template OT2 400 PCR Reagent B</td>
<td>285 µl</td>
</tr>
<tr>
<td>3</td>
<td>Ion PGM™ Template OT2 400 Enzyme Mix</td>
<td>50 µl</td>
</tr>
<tr>
<td>4</td>
<td>Ion PGM™ Template OT2 400 Reagent X</td>
<td>40 µl</td>
</tr>
<tr>
<td>5</td>
<td>Diluted library</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

Ion PGM™ template OT2 400 Ion Sphere Particles (ISPs) were vortex for 1 minute, centrifuge at 2 sec and then add in to amplification solution. 900 µl of Amplification solution was mixed with 100 µl of ISPs. The complete amplification solution was vortex
again at maximum speed for 5 seconds.

- 1000 µl of Ion One Touch™ reaction oil (27ml size) was pipet through the sample port. Invert and then install the filled Ion PGM™ one touch plus reaction filter assembly was first inverted and then installed into the three holes on the top stage of the Ion One Touch™ instrument.

- Ion One Touch™ instrument was run and template positive Ion PGM™ template OT2 400 ISPs was recovered. Enrichment of the template positive ISP particles was performed in Ion OntTouch™ ES system as per manufacturer’s instructions.

- Five sequencing runs covering all 100 barcoded libraries were performed on Ion PGM system using Ion 318™ chip following manufacturer’s instructions.

- After sequencing of all 100 samples (50 each of HC and HN) on Ion Personal Genome Machine® (PGM™) system, data were generated in the form of electrical pulses. The series of electrical pulses transmitted from chip to computer were translated into DNA sequence reads.

### 3.2.7 Evaluation of sequencing data using GATK tool

The, low quality reads per length were filtered and pre sequence quality score along with GC scores was checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and PRINSEQ (http://sourceforge.net/projects/prinseq/files/) java based software (Schmieder and Edwards, 2011; Leggett et al., 2013). SNP calling was performed with the aim to determine the number and positions of SNPs in compare to a reference sequence.

Genome Analysis Tool Kit (GATK) on Google Genomics software was used to carry out aligned reads in multi-sample SNP-calling (McKenna et al., 2010). The GATK is
premeditated to run on Linux and other POSIX-compatible platforms with a Cloud Platform project to store and process genomic data. This α release tool supports whole human genome sequencing and human exome sequencing; here it uses human_g1k_v37_decoy as the reference genome. Further the pipeline, BWA MEM aligns the reads against the reference genome, GATK then merges alignments from the same sample, calculate genotype likelihoods with HaplotypeCaller, and finally combines call variants. The overall work flow of GATK pipeline is presented in figure 16.

![Fig. 16 The overall workflow of GATK tool (https://software.broadinstitute.org/gatk/)](image-url)
3.2.8 Statistical analysis of significant SNPs

Targeted gene variants were explored for their possible association patterns in the case-control study using SAS software. SAS (http://www.sas.com/en_us/insights/analytics/statistical-analysis.html) is widely known potential, mainframe-based statistical software with a package application containing several computer languages within it. SNPs were tested to show their p-value (<0.05) significance along with their genotype and allelic frequency calculations. The pairwise linkage disequilibrium (LD) statistic and their haplotypes were estimated and assessed for association studies.

3.3 Analysis of fold expression at transcript level using RT-qPCR

Change in fold expression at transcript level was performed in targeted proteins (TRPC6 and podocin) using RT-qPCR. We isolated total RNA from kidney samples. Fresh biopsy samples were collected from ten control and ten cases and transferred immediately in RNAlater® (Sigma) to avoid RNA degradation. The samples were stored immediately in liquid nitrogen.

3.3.1 mRNA isolation and cDNA synthesis

Total RNA was isolated from the case-control tissue sample using TRIzol (Invitrogen) following manufacturer's instructions, and impurities of genomic DNA was removed by DNaseI enzyme (Fermentas) treatment at 37°C incubation for 1h. mRNA was isolated from total RNA using the mRNA isolation Kit (Roche Diagnostics) following the manufacturer's instructions. Total RNA quality and quantity was verified on Bioanalyzer (Agilent Technologies-2100).
cDNA synthesis was performed using sample mix 13.5 µl (1 µl template RNA, 1 µl oligo dT 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. Change in fold expression at transcript level was performed with the help of RT-qPCR (Applied biosystem 7500HP). Amplification of podocin transcript was performed using previously published primers (Boute et al., 2000), where as primers for TRPC6 protein were designed using Primer 3 software v.0.4 (Table 6) (biinfo.ut.ee/primer3-0.4.0/) (Untergasser et al., 2012) and crosschecked by Primer-Blast program through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) (Ye et al., 2012).

**Table 6** List of Podocin and TRPC6 primers for expression analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPC6</td>
<td>F: GCAACGAGAGCCAGGACTA</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>R: TGTCAGATCCCCCGGAAGCAG</td>
<td></td>
</tr>
<tr>
<td>Podocin</td>
<td>F: AGGTGGTTGGCGCTTGGAG</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>R: GAAGCAGATGTCCCAGTGCGGAATAT</td>
<td></td>
</tr>
</tbody>
</table>