SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 18: 5095-5100, 2018

Whole exome sequencing identification of a novel insertion mutation in the phospholipase C ε-1 gene in a family with steroid resistant inherited nephrotic syndrome

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Received November 5, 2017; Accepted March 27, 2018

DOI: 10.3892/mmr.2018.9528

Abstract. Nephrotic syndrome (NS) represents a heterogeneous group of kidney disorders characterized by excessive proteinuria, hypoalbuminemia and edema. Defects in the filtration barrier of the glomeruli results in the development of NS. The genetic cause of NS remains to be fully elucidated. However, previous studies based on positional cloning of genes mutated in NS have provided limited insight into the pathogenesis of this disease. Mutations in phospholipase C ε-1 (PLCE1) have been reported as a cause of early onset NS characterized by histology of diffuse mesangial sclerosis. In the present study, the underlying cause of NS in a consanguineous family was identified. Clinical and molecular aspects of a consanguineous Saudi family comprised of five individuals with steroid resistant NS were examined. Seven healthy individuals from the same family were also studied. Whole exome sequencing (WES) was performed to detect the genetic defect underlying NS. WES identified a homozygous novel insertion mutation (c.6272_6273insT) in the *PLCE1* gene. Pedigree and segregation analysis confirmed an autosomal recessive inheritance pattern. This mutation may result in a bi-allelic loss of the C-terminal Ras-associating domain in PLCE1 that results in NS. The present study expanded the mutational spectrum of PLCE1 in NS. In addition, the present study provided further evidence that supports the important involvement of PLCE1 in the physiological function of the glomerular filtration barrier.

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Key words: phospholipase C ε-1, mutations, congenital, nephrotic syndrome, autosomal recessive

Introduction

Nephrotic syndrome (NS) is a renal disorder characterized by a tetrad of clinical conditions, including proteinuria, hypoalbuminemia, hyperlipidemia and edema (1). It is one of the most prevalent defective states of the glomerular filtration barrier, whereby macromolecules, including albumin, are excreted in the urine. Worldwide prevalence is reported to be ~1-3 out of 100,000 children under the age of 16 (2,3). Congenital nephrotic syndrome (CNS) represents a dysfunctional glomerular state that is presented *in utero* or during the first three months of life; contrary to infantile nephrotic syndrome, in which disease symptoms appear at 4-12 months of age (4). Cases in which the symptoms of NS manifest in children aged between 2-5 years old are classified as childhood NS.

As patients with NS typically respond well to steroid therapy, NS is characterized as either steroid resistant nephrotic syndrome (SRNS), which occurs in ~20% of children with NS, or as steroid susceptible nephrotic syndrome, occurring in ~80% of patients (5,6). SRNS manifests as a glomerular disorder that may lead to end-stage renal disease (7-9). A great deal of pathogenic genetic defects for CNS have been reported since 1998 (10). Over the last 15 years, mutations in >39 dominant or recessive genes have been revealed to cause NS or SRNS, including KN motif and ankyrin repeat domains 1, 2 and 4; nephrin, podocin, nucleoporin 93, 107 and 205; and Wilms- tumor 1 (11-14).

Next generation sequencing (NGS) is a technique that can effectively identify genetic defects underlying inherited disorders with high precision and reliability, which consequently increases the amount of known genetic mutations. Whole exome sequencing (WES) in NGS is a powerful tool used to identify underlying genetic elements (15,16) without any prior knowledge of the gene or genes involved in the pathogenesis (17). The pathological genetic mutations in a variety of human genetic disorders have been identified by WES (18,19). However, there are a limited number of reports that have identified genetic mutations that result in NS in the

Saudi population, using microarray and direct sequencing techniques (20,21). The aim of the present study was to utilize WES in a Saudi consanguineous family suffering from SRNS to identify the underlying genetic defects, in order to broaden the disease understanding and improve disease management. To the best of our knowledge, this is the first report from Saudi Arabia in which WES was performed to identify novel mutations in the phospholipase C ε-1 (PLCE1) gene in a family with segregating SRNS.

Patients and methods

Ethical approval and patient recruitment. The Ethical Review Committee of Taibah University (Medina, Saudi Arabia) approved the protocol of the present study (approval no. TU-REC-2016018). Informed written consent was obtained from all participants prior to study commencement. Experiments were performed in the Centre for Genetics and Inherited Diseases, Taibah University (Medina, Saudi Arabia).

A Saudi family was recruited in September 2015. In total, the family included five members (IV:1, IV:2, IV:3, IV:5 and IV:7) with SRNS. All individuals with SRNS as well as seven healthy individuals (III:1, III:2, III:3, III:4, IV:4, IV:6, IV:8) belonging to the family were clinically examined by a nephrologist in the Department of Nephrology, Madinah Maternity and Children Hospital (Medina, Kingdom of Saudi Arabia). Clinical reports of three affected individuals (IV:1, IV:3, IV:7) were obtained; however, DNA samples were not available, as these individuals succumbed to mortality prior to the initiation of the genetic studies. The family pedigree was drawn upon querying the elders of the family. The following parameters were used to diagnose patients: Proteinuria or spot urine protein:creatinine ratio, serum albumin, peripheral edema on clinical examination and hyperlipidemia (total blood cholesterol).

Genomic DNA extraction. Blood samples were obtained for genetic analysis from the affected individuals (IV:2, IV:5), asymptomatic siblings (IV:4, IV:6) and the parents (III:1, III:2). Furthermore, blood samples (3 ml) were collected from 30 healthy individuals (aged 5-27 years old; 15 males and 15 females) in the Centre for Genetics and Inherited Diseases, Taibah University (Medina, Saudi Arabia). These individuals were not related to the family; however, they were from the same population. Blood vacutainers containing EDTA were used to collect the blood specimens. QIAquick DNA extraction kits (Qiagen, Inc., Valencia, CA, USA) were used to extract genomic DNA from the whole blood samples. DNA quantification was performed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Qubit fluorometer (Thermo Fisher Scientific, Inc.). DNA integrity was resolved through 1% agarose gel electrophoresis.

Whole exome sequencing (WES). WES was performed using DNA from the two affected individuals (IV:2 and IV:5) of the family. Library preparation and exome enrichment was performed using the Nextra Rapid Capture Exome kit (Illumina, Inc., San Diego, CA, USA), which captured 214,405 exons and splice sites with 98.3% RefSeq coverage. Subsequently, the Illumina NextSeq500 instrument (Illumina, Inc.) was used to produce clusters and DNA sequence reads, as described previously (22).

The Illumina NextSeq500 instrument generated bcl files that were converted to fastq files using the BCL2FASTQ tool (Illumina, Inc.). BaseSpace (Illumina, Inc.; basespace.illumina.com/home/index) was used to align fastq files to the reference genome using the Burrows-Wheeler Aligner-MEM algorithm (23). Variant calling was performed with the genome analysis toolkit (24). Finally, VariantStudio software (version 3.0; Illumina, Inc.) was used for the annotation of the variants obtained. Variant filtration and prioritization was performed as described previously (18).

Sanger sequencing. Genetic variants that were exposed by WES were further confirmed by Sanger sequencing as described by Basit *et al* (25). The genomic sequence of human PLCE1 gene (12,024 bp; ENST00000371380.7) was downloaded from the Ensembl genome browser (asia.ensembl.org/index.html). In order to amplify and validate the targeted sequence variant with their flanking sites, primers (forward, 5-GAATAAGTT GTGCCGTTGCC-3- and reverse, 5-TTCAGGGAGGTTGTG AGTGG-3') were designed using Primer3 software (version 0.4.0; frodo.wi.mit.edu/primer3/). PCR master mix (Promega Corporation, Madison, WI, USA) was used to amplify the target region. Following this, amplified regions were resolved on a 2% agarose gel and visualized using a Bio-Rad gel documentation system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The amplified sequences were analyzed using BIOEDIT sequence alignment editor (version 6.0.7; Ibis Biosciences, Inc., Carlsbad, CA, USA). Thermocycling conditions used were as follows: 95˚C for 1 min; followed by 30 cycles of 95˚C for 35 sec, 60˚C for 35 sec and 70˚C for 3.5 min; followed by a single incubation at 70˚C for 10 min.

Mutation prediction analysis. The Simple Modular Architecture Research Tool (SMART) (26) was used to identify the predicted effect of the identified single base pair insertion (c.6272_6273insT) on the product of PLCE1 enzyme.

Results

Clinical evaluation of patients. The family pedigree is presented in Fig. 1. The affected individuals were clinically examined by a pediatric nephrologist in Madinah Maternity and Children Hospital (Medina, Saudi Arabia). Blood and urine investigations were used to diagnose patients: Proteinuria (>3-3.5 g/24 h) or spot urine protein:creatinine ratio (>300-350 mg/mmol); serum albumin (<25 g/l); and peripheral edema on clinical examination and hyperlipidemia (total blood cholesterol >10 mmol/l).

The affected son (IV:2) was evaluated at the age of 11 months and was determined to have severe pulmonary edema, sarcoidosis, oliguria and severe hypertension. A kidney biopsy revealed vasculitis. The overall presentation of this case was diffuse mesangial sclerosis (DMS). Renal transplantation was eventually performed for this patient, who is 21 years old at present. The subject is living a normal life without disease recurrence.

The affected daughter (IV:5) was admitted to the hospital at the age of 2 years with a history of persistent vomiting for two

days and a low-grade fever without convulsion. Furthermore, the patient had been unwell for the last two years with a history of abnormal movements during sleep with hyperpigmentation on the back. Laboratory investigations revealed high blood pressure reaching 140/90 mm/Hg with anemia, and highly elevated urea and creatinine levels in blood with imbalanced electrolytes. The patient remained admitted to the hospital due to chronic kidney disease and renal failure. Ultrasound scan of the kidneys revealed an echogenic cortex with poor cortico-medullary differentiation and no back pressure. A renal biopsy and transplant was also performed for IV:5 and the patient is 16 years old at present. Histology revealed mesangial matrix expansion, hypertrophic podocytes and thickened basement membranes (data not shown; Madinah Maternity and Children Hospital).

Three affected individuals (IV:1, IV:3 and IV:7) had previously succumbed to mortality. Clinical reports show similar phenotypic manifestations as found in IV:2 and IV:5. Interviews with the elder members of the family confirmed that the deceased individuals had similar clinical manifestations to the affected living individuals (IV:2 and IV:5). Renal transplantation was not performed for these deceased individuals. Blood and urine analysis of the heterozygous carriers (parents) revealed a normal proteinuria and protein:creatinine ratio range. As symptoms were present from birth, patients under investigation were most likely suffering from congenital NS.

Novel variant identification by WES analysis. The resulting variant call format files obtained from the fastq files contained ~80,000 variants. Different filters were applied to these genomic variants, whilst considering any previous links with the disease phenotype, potential effects on the protein, genome frequency, quality and pathogenicity. Rare and potentially harmful variants present in the homozygous state were of primary interest given the reported positive consanguinity in this family. Searching for rare homo/hemizygous variants within the protein coding regions of all genes that have previously been associated with NS yielded a reasonable candidate variant. A novel homozygous frameshift variant (c.6272_6273insT; p.2090Met_2091GlnPheSer) in exon 29 of the PLCE1 gene was detected in the two affected individuals (Fig. 2). Recessive mutations in this gene cause early-onset nephrotic syndrome (13,27). Taken together, this variant is likely to contribute to the phenotype of these patients based on its novelty, predicted deleterious effect and good phenotype match.

c.6272_6273insT is inherited in an autosomal recessive manner. The identified variant (c.6272_6273insT) in *PLCE1* gene was further validated through Sanger sequencing. All members of the family were screened and it was determined that the variant segregated in the family in an autosomal recessive manner (Fig. 3). Furthermore, 30 unrelated, normal control individuals were screened from the same population. Variant (c.6272_6273insT) was not found in the DNA isolated from any of the control individuals.

Mutant PCLE1 lacks a complete Ras-association (RA) domain. SMART was used to predict the effect of the identified single

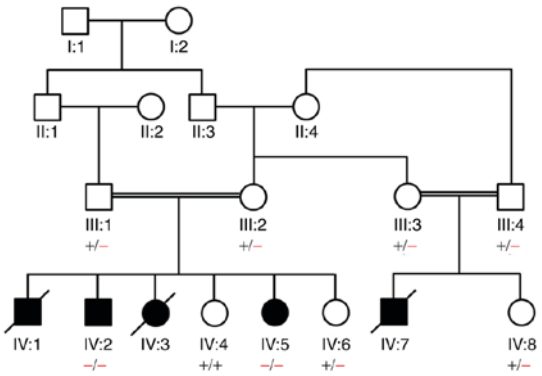

Figure 1. Extended pedigree of a Saudi family with congenital nephrotic syndrome. Filled symbols represent affected individuals; symbols with diagonal lines represent individuals who have succumbed; and squares indicate males while circles indicate females. Double lines of the pedigree represent the consanguineous relationships. -/-, +/+ and -/+ indicate homozygous mutants, wild type phenotypes and heterozygous mutants, respectively.

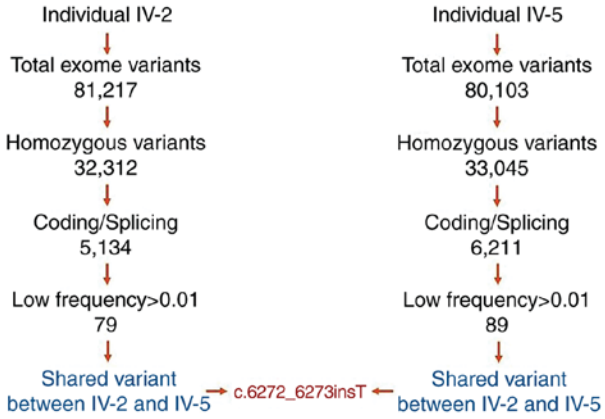

Figure 2. Variant calling and variant filtration of the exome data.

base pair insertion (c.6272_6273insT) on the PLCE1 enzyme product. The insertion was determined to result in a frameshift of the protein from position 2090Met_2091GlnPheSer, just prior to the RA domain. The RA domain consists of 104 amino acids and the mutant protein lacked the complete RA domain (Fig. 4).

Discussion

A novel homozygous insertion mutation was identified in exon 29 of the PLCE1 gene in two siblings with SRNS using the WES method. The resultant genetic variation results in a frameshift of the genetic code from position 2090Met_2091GlnPheSer. PLCE1 is one of the genes implicated in early onset NS.

The frameshift mutation identified in the present study was predicted to cause inactivation of the RA domain that subsequently leads to PLCE1 protein loss of function, resulting in the aforementioned patient phenotype. Ras proteins function as molecular switches, transmitting a signal in the active guanosine 5'-triphosphate (GTP)-bound state and reverting to an inactive state when the bound GTP is hydrolyzed to guanosine 5'-diphosphate. Ras interacts with the RA domain of PLCE1 to initiate downstream signaling pathways. The

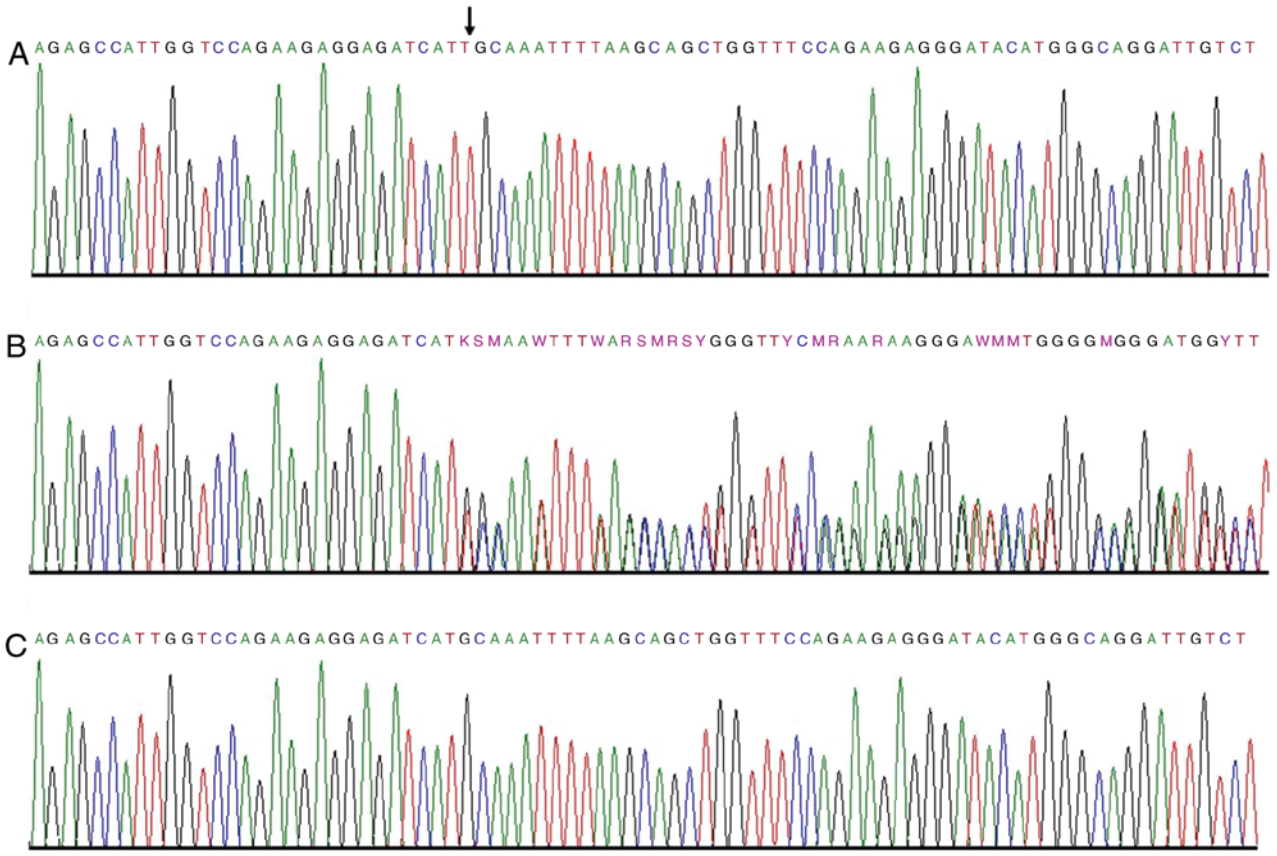

Figure 3. Electropherograms generated by Sanger sequencing confirmed the identified homozygous novel insertion mutation. Partial sequence chromatogram of phospholipase C ε-1 gene from individuals IV:2 and IV:5 exhibiting the homozygous insertion of (A) nucleotide T, (B) the parents III:1, III:2, III:3 and III:4, and heterozygous siblings IV:6 and IV:8, and (C) 30 control individuals. The arrow head indicates the point of insertion. Pink letters indicate disrupted sequence due to heterozygous insertion.

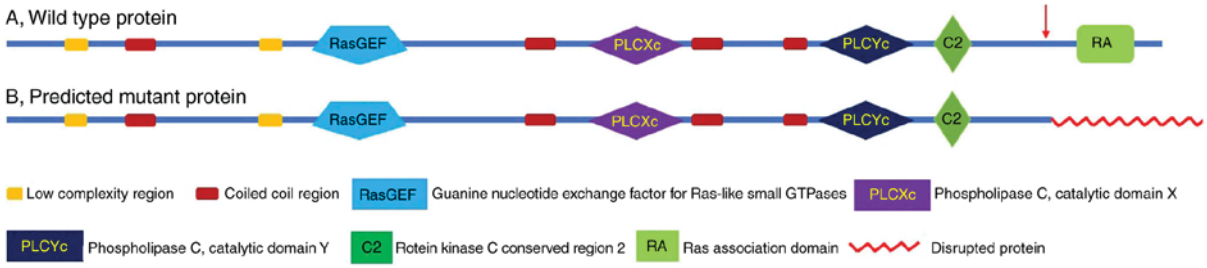

Figure 4. Schematic diagram of phospholipase C e-1 (A) wild type protein and (B) the predicted effect of the mutation on the protein. The red arrow indicates the position of insertion.

congenital NS, in the present case, was steroid resistant, as the patients did not respond to the standardized steroid therapy.

NS is a heterogeneous genetic disorder. To date, >39 different disease-causing genes have been identified in individuals with syndromic and non-syndromic forms of NS (11-14,28). When mutated, these genes lead to dysfunctional glomeruli and subsequently, focal segmental glomerulosclerosis (29). The proteins encoded by these genes are involved in either the development and/or function of the glomerular filtration barrier.

NGS has provided a cost-effective platform for the accurate analysis of a large number of genes. WES has broadened the genetic heterogeneity and mutation continuum of the disease by identifying novel candidate genes and novel mutations responsible for NS (30).

The PLCE1 gene spans over 334.4 kb on chr10q23.33 and has 34 exons. The protein encoded by PLCE1 gene belongs to the phospholipase family of proteins. In 2006, positional cloning was used to identify mutations in PLCE1 responsible for a novel cause of recessive NS type 3 (31). Mutations in PLCE1 are associated with early onset NS (32). At present, 17 different mutations have been identified in PLCE1.

PLCE1 protein is essential for the functional development of the glomeruli at the capillary loop stage. The identification of a novel pathogenic mutation in PLCE1 gene using WES in a Saudi family with congenital nephrotic syndrome expands the existing mutation spectrum of PLCE1 gene within the Saudi population and worldwide. Besides its potential role in diagnosis and disease pathogenicity, this discovery also

highlighted the importance of WES as a tool for the molecular diagnosis of NS.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Strategic Technology Program of the National Plan for Science, Technology and Innovation (Riyadh, Saudi Arabia; grant no. 13-MED2088-05).

Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JAH performed variant validation using the Sanger approach, and wrote the initial draft of the manuscript. RAS recruited the family included in the present study and performed clinical diagnosis. SA performed polymerase chain reactions using the control samples. FA designed the primer sequences. AMA extracted and quantified the genomic DNA, and performed exome sequencing. ZI performed experiments investigating variant identification. SB designed the study and analyzed exome data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in the present study were approved by The Ethical Review Committee of Taibah University (Medina, Saudi Arabia; approval no. TU-REC-2016018) and were also in accordance with the Declaration of Helsinki. Written informed consent was obtained from all individual participants or the guardians of underage participants included in the study.

Patient consent for publication

All patients and unaffected participants have provided consent for publication of genetic data.

Competing interests

The authors declare that they have no competing interests.

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