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Research Article
Screening of *ACTN4* and *PLCE1* Genes Mutations in Saudi Children Patients with Steroid Resistant Nephrotic Syndrome

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Abstract

Background and Objective: Nephrotic syndrome (NS) is the most well-known glomerular kidney disease in children. Genetic risk is highly frequently detected amongst childhood steroid-resistant NS (SRNS) disease. Therefore, the aim of the present research was to evaluate *ACTN4* and *PLCE1* genes mutations in children with SRNS in western region of Saudi Arabia. **Materials and Methods:** Twenty SRNS patients were identified and screened for gene mutations within *ACTN4* (21 exons) and *PLCE1* (33 exons) genes by direct sequencing method to elucidate the correlation between these genes and SRNS. **Results:** Three novel heterozygous missense mutations were detected in *ACTN4* gene in 3 patients. The renal biopsy findings for the 3 patients were focal segmental glomerulosclerosis. The *in-silico* analysis for these mutations propose that they are deleterious. In case of *PLCE1* gene, 2 missense mutations were detected in exons 24 and 26. These mutations were found previously as non-disease-causing mutations. **Conclusion:** *ACTN4* is a significant causative gene mutation of FSGS in Saudi children patients with SRNS But causative mutations in *PLCE1* gene can not identified.

Key words: *In-silico* analysis, point mutations, renal biopsy, steroid resistant nephrotic syndrome, glomerulosclerosis

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nephrotic syndrome (NS) is the famous glomerular kidney disease in children that is characterize with proteinuria, hypoalbuminemia and edema[1]. Even though NS is accompanying with several forms of renal disease, the most frequent type (>90%) in pediatric is idiopathic NS. The worldwide incidence of NS in children is reported to be 2-16.9/100,000 children[2]. Until now, the treatment by corticosteroid remains the most typical treatment for NS patients. Therefore, the types of NS were classified, according to patient's reaction to steroid therapy as steroid-sensitive NS and steroid-resistant NS (SRNS)[3]. Several reports documented that about 50% of SRNS patients are developing to end-stage renal disease (ESRD) within 15 years[4-6]. In addition, only around 8-10% of inherited genetic NS is responsive to steroid therapy, which consequently means 90% of SRNS patients are presumed to be multidrug resistant[6,7]. As stated by the International Study of Kidney Disease in Children, from the pathological point of view, 75% of SRNS patients have focal segmental glomerulosclerosis (FSGS), whereas 20% exhibit minimal change nephrotic syndrome[8].

The main reason for the familiar phenotype of SRNS is genetic background, whether recessive or dominant genes[9]. Consequently, recent studies in SRNS patients discovered single rare mutations in podocyte genes that responsible for SRNS[10,11]. Sadowski *et al.*[12] reported in 2015 the rate of single-gene causation of SRNS was 29.5% of all tested cases. Recent advances in histology and molecular facilities extended the consideration to the detection of the causative, especially at the molecular level, of SRNS cases. Numerous contributing genes causing NS disease have been distinguished by either utilizing the direct DNA sequencing technique or next-generation sequencing innovation. For example, recessive mutation in *NPHS1*, *NPHS2*, *LAMB2*or *WT1* genes is responsible for about 85% of SRNS patients with onset by three months of age and 66% with onset by 1st year of age[13]. Additionally, single-gene mutation causing SRNS later in life is linked with the late onset of proteinuria and the disease progression to ESRD[14-16]. To date, with the recent advances of genome studies, there are over 30 single genes associated with SRNS in children[17]. Of these, the recognized podocyte genes clarify about 20-30% of hereditary NS and 10-20% of sporadic patients[18].

The *ACTN4* gene was firstly mapped to chromosome 19q13 and encodes for α-actinin-4 protein, an actin bundling protein of the cytoskeleton that is highly expressed in podocytes[19]. *ACTN4* has a significant role for the cytoskeletal function of the podocyte, with several reports documenting that the knock-down or over expression of the transgenic mouse model with *ACTN4* is associated with proteinuria and podocyte alterations[20,21]. The mutations in *ACTN4* were recognized as autosomal dominant late onset causing FSGS[19]. The *PLCE1* gene is mapped to chromosome 10q23-q24 and encodes for the enzyme phospholipase C epsilon-1. The PLCE1 enzyme has a significant role with intracellular signal transduction, therefore, it is extensively expressed in numerous tissues including podocytes[22]. Several gene mutations in *PLCE1* were documented as a reason for autosomal recessive diffuse mesangial sclerosis (DMS)[22]. In addition, it was found that low protein expression of PLCE1 is correlated with development of podocyte foot process effacement and edematous outer appearance of the zebrafish, elucidating the precise function of the PLCE1 enzyme in the maintenance of the glomerular filtration barrier[22]. Moreover, *PLCE1* gene mutations were considered the major reason for isolated DMS in children, with a rate of 28.6%[23]. On other hand, Gilbert *et al.*[24] demonstrated that the homozygous mutation in *PLCE1* is not always enough to cause DMS. The percentage of consanguineous marriage in the Kingdom of Saudi Arabia (KSA) was reported to be around 56%[25,26]. Pediatric renal diseases are more likely to be predominant in countries with high percentages of consanguineous marriages, such as KSA[27]. Furthermore, congenital and infantile NS are documented with a higher incidence in KSA than in other countries[28].

Previously, same Saudi children patients with SRNS that are presented here were screened the for mutation analysis within the *NPHS2* gene and exons 8 and 9 of the *WT1* gene[29]. The gene mutations of *NPHS2* gene are present in 10% of the patients, while *WT1* gene mutation is found in 5%[29]. To further evaluate the existence of single gene mutation within the same cohort, mutational screening of all exons of *ACTN4* and *PLCE1* genes was achieved. Until now, this study is consider the first detection of *ACTN4* gene mutation in Saudi children with SRNS.

MATERIALS AND METHODS

Study area: This study was carried out at molecular genetics Lab, Deanship of Scientific Research, Taif University, KSA from January, 2018-April, 2019. The study was approved by the bioethics committee of Al-Hada Armed Forces Hospital (PTRC#15-05-227). Twenty children who entered Al-hada Armed Forces Hospital during the period of 2015-2017 with age between 1 and 16 years old and with a clinical finding of SRNS were enrolled in this study, participation involved informed consent. NS cases were characterized as proteinuria, hypoalbuminemia and generalized edema[30]. SRNS patients

were described as the inability to respond to daily treatment of prednisone dose (2 mg kg^{-1}) for a period of 4-6 weeks[30]. SRNS patients were described as the inability to response to daily treatment of prednisone dose (2 mg kg^{-1}) for a period of 4-6 weeks[30]. The inclusion criterion comprised all Saudi childhood SRNS patients. Exclusion criteria were steroid sensitive NS patients and/or patients with secondary reason (e.g., IgA nephropathy). The renal biopsy samples were achieved by renal pathologists. Several clinical analysis were recorded, such as: sex, family history, age of onset, hypertension, hematuria, failure to the response to steroid therapy, kidney biopsy and interval time of development to ESRD.

Genomic DNA extraction: Total DNA was extracted immediately from blood by Genomic DNA Purification Kit (Promega, USA) as described by the protocol of the manufacture. The purity and concentration of the genomic DNA was evaluated through agarose gel electrophoresis. All coding sequence and exon-intron borders of both genes, *ACTN4* (accession number NM_004924.5, 21 exons) and *PLCE1* (accession number NM_016341.3, 33 exons), were amplified by PCR using specific primers as previously reported by Dai *et al.*[31] and Boyer *et al.*[32]. PCR reaction mixture and conditions were performed as reported previously by Dai *et al.*[31] and Boyer *et al.*[32].

Detection mutations by PCR-restriction fragment length polymorphism (RFLP) analysis: The two mutations that were found in the *PLCE1* gene (c.5964C>T in exon 24, c.6414A>G in exon 26) were confirmed using the PCR/RFLP technique. PCR assay was conducted using specific primers for exon 24 and exon 26 as described previously by Dai *et al.*[31] and Boyer *et al.*[32]. PCR reaction mixture of the final volume of 50 µL included: 50 ng of genomic DNA, 0.2 µM of forward and reverse primer and 25 µL of GoTaq® green master mix, with the remaining volume comprising Nuclease-Free Water (Promega, USA). The RFLP assay was carried out with the BseYI enzyme (New England Biolabs, USA) in order to detect c.5964C>T mutation in exon 24 (544 bp), while the BstUI enzyme (New England Biolabs, USA) was used to detect c.6414A>G mutation in exon 26 (488 bp). Conditions of digestion with restriction enzymes were achieved as described by the manufacture's protocol.

***In-silico* analysis of the mutated nucleotides:** Expectation of the functional influence of each pathogenic mutation found in *ACTN4* and *PLCE1* genes was achieved using 3 distinctive computational programs freely accessible online, (1) The PROVEAN program (http://provean.jcvi.org/seq_ submit.php) analyzes the single nucleotide polymorphisms and/or indels[33]. As described by PROVEAN, a value of -2.5 is assumed to be a default threshold. In this way, mutations with a score equivalent to or lower than -2.5 are considered deleterious. On other hand, mutations with a score higher than -2.5 are considered neutral, (2) The PolyPhen2 program (http://genetics.bwh.harvard.edu/pph2/) employs the sequence homology and information of the 3D structure of the protein to anticipate the likely influence of an amino acid substitution on the structure and function of a human protein. The results are categorized as benign, possibly damaging, probably damaging or unknown[34], (3) The SIFT program, (https://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html) assesses whether the replacement of an amino acid disturbs protein function[35]. To further explore the impact of all mutations found in this study on the 3D structure and folding of ACTN4 and PLCE1 proteins, the active domains of both proteins were modeled using Protein Fold Recognition Server (Phyre2)[36] and the structure of domains and mutation site were visualized using PyMol[37].

Statistical analysis: Sequencing of the purified specific fragments were performed as previously described[29]. The raw sequencing data were assembled by the Data collection software version 3.1 (Applied Biosystems). Sequence base-calling and point mutation analysis were investigated using Seqscape software version 2.7 (Applied Biosystems). Mutational records were named through the nomenclature of the Human Genome Variation Society[38].

RESULTS

Effect of *ACTN4* gene mutation on protein structure: The summary of clinical data of the individual cases that have the mutations of *ACTN4* and *PLCE1* genes is shown in Table 1. The reference sequences of the *ACTN4* gene are NG_007082.2, NM_004924.5 and NP_004915.2. Three novel mutations were detected in *ACTN4* gene in three SRNS patients as heterozygous state (Fig. 1). The first one found in exon 8 (c.870T>A, p.V247E). The second mutation (c.2136T>A, p.M669K) was in exon 16, while the third mutation (c.2464C>A, p.D778E) was in exon 18. The three mutations occurred in 3 distinct domains of the *ACTN4* gene (Fig. 1a, b). On the level of the 3D protein structure and function, the three mutations were found to affect the structure and polar charge of the amino acid residues that have a significance for

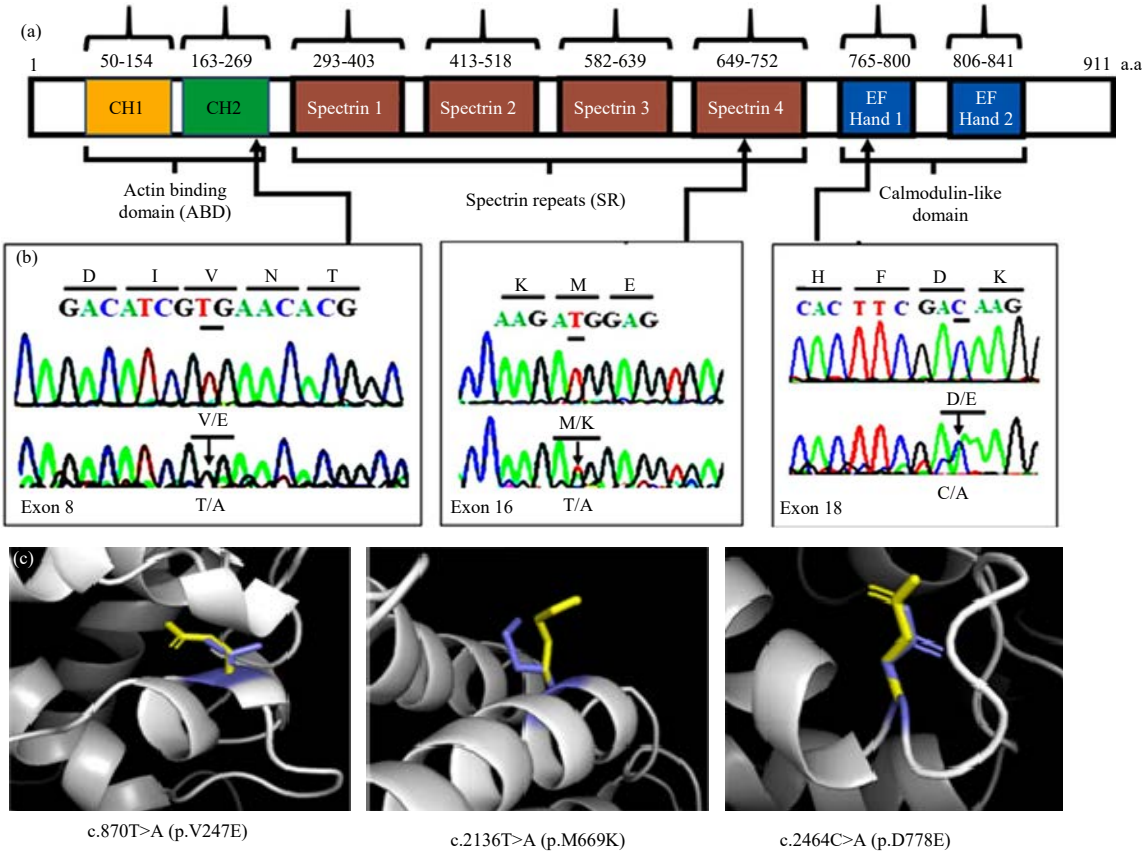

Fig. 1(a-c): DNA sequence analysis of *ACTN4* gene, (a) Functional different domains of the human *ACTN4* protein, (b) Direct sequencing of the PCR product amplified from exons 8, 16 and 18 of *ACTN4* gene. The affected individuals are heterozygous for the 3 mutations (arrow) and (c) Superimposed tertiary structure of *ACTN4* protein and the predictions of normal and mutated amino acid

Normal amino acid is showed in purple, while the mutated amino acid in the three mutations is illustrated in yellow

Table 1: Clinical data of the individual children patients with *ACTN4* and *PLCE1* gene mutations

Sex	Parental consanguinity	Gene mutation	Age of onset (Year)	Histology	Age at ESRD (Year)	HPT	Hematuria
Female	Yes	*ACTN4*	3	FSGS	5	No	No
Male	No	*ACTN4*	4	FSGS	6	No	No
Male	Yes	*ACTN4*	4	FSGS	7	Yes	Microscopic
Female	Yes	*PLCE1*	4	UA	8	No	No
Male	No	*PLCE1*	2	FSGS	10	Yes	No
Female	Yes	*PLCE1*	9	FSGS	12	Yes	Microscopic
Female	Yes	*PLCE1*	3	MPGN	12	Yes	No
Female	Yes	*PLCE1*	5	FSGS	7	Yes	Microscopic
Female	No	*PLCE1*	8	FSGS	13	Yes	No
Female	Yes	*PLCE1*	9	FSGS	13	Yes	No
Male	No	*PLCE1*	2	FSGS	6	No	Microscopic
Male	No	*PLCE1*	3	UA	4	Yes	No

FSGS: Focal segmental glomerulosclerosis, HPT: Hypertension, MPGN: Membranoproliferative glomerulonephritis, UA: Unavailable, ESRD: End stage renal disease

the coiled coil structure (Fig. 1c). The coiled coil structure has a significant function in key cellular interactions. The replacement of the amino acids valine and methionine by glutamic acid and lysine in p.V247E and p.M669K mutations, respectively, altered the surface charge of the amino acids in this area from non-polar to negative and positive charges, respectively. Consequently, the three mutations of *ACTN4* gene could be deleterious within the SRNS patients.

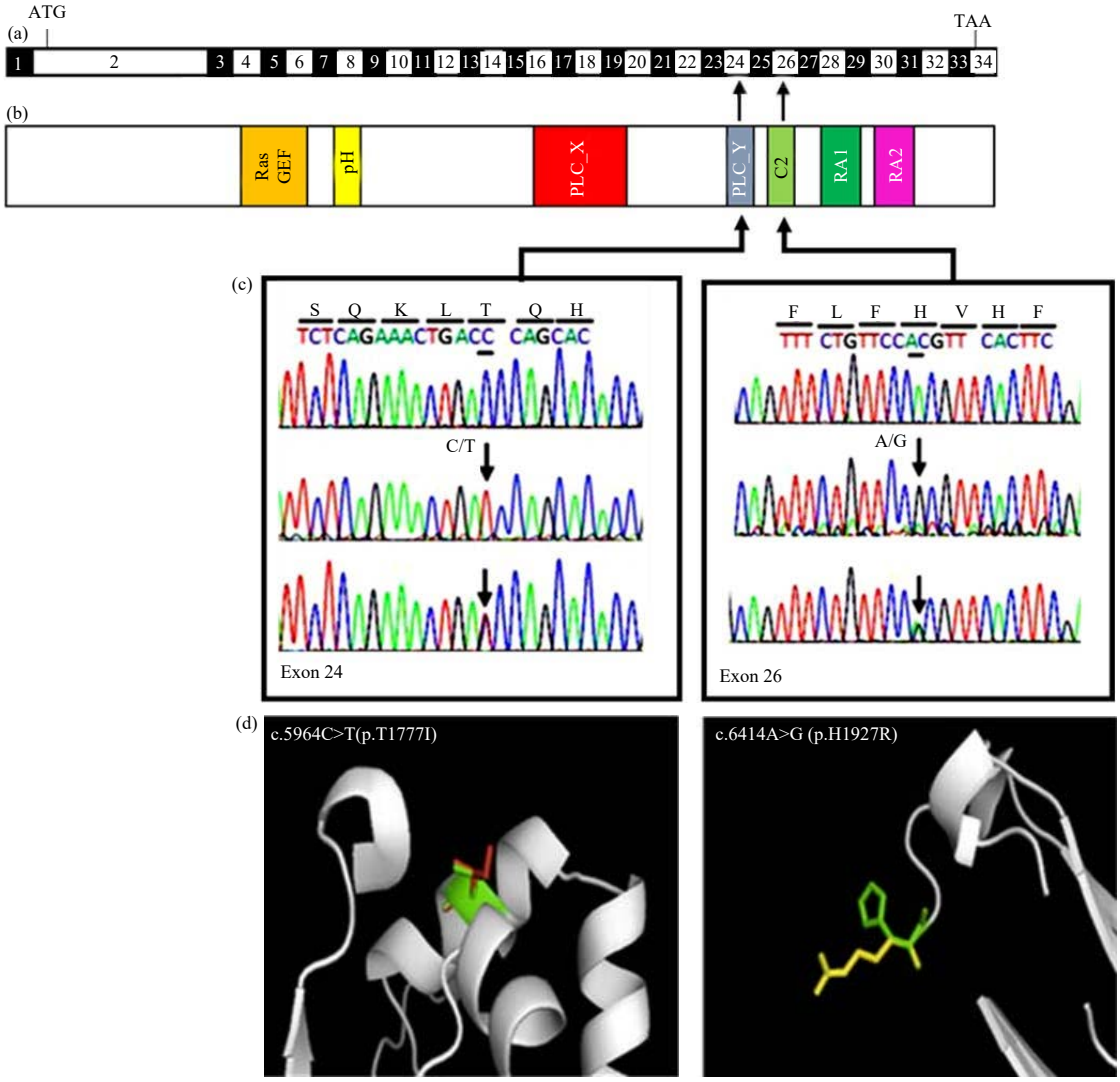

Fig. 2(a-d): DNA sequence analysis of *PLCE1* gene, (a) Exon structure of human *PLCE1* gene-viewing positions of start and stop codons, (b) Locations of the protein domains regarding the encoding exon site, (c) Direct sequencing of the PCR product amplified from exons 24 and 26 *PLCE1* gene. Arrows indicate relative positions of mutations and (d) Superimposed tertiary structure of *PLCE1* protein and the predictions of normal and mutated amino acid
Normal amino acid is showed in green, while the mutated amino acid in c.5964C>T mutation is illustrated in red and in c.6414A>G is showed in yellow

Mutation screening of *PLCE1* gene: The reference symbols of the *PLCE1* gene and protein are NG_015799.1, NM_016341.3 and NP_057425.3. Two missense mutations were detected in *PLCE1* gene in 2 different domains (Fig. 2a, b). First one at the position c.5964C>T (p.T1777I) in exon 24 and the other at the position c.6414A>G (p.H1927R) in exon 26 (Fig. 2c). These two variants were found as homozygous, heterozygous and compound mutation states in 9 out of 20 (45%) SRNS patients. Unfortunately, parent's DNA of the compound mutations were not analyzed to investigate if these variants are located on one chromosome and whether, therefore, the compound mutations would be characterized as a haplotype heterozygous mutation or if they are located on two separate chromosomes and they would be compound heterozygous mutations. The amino acid Isoleucine and Arginine that replace Tyrosine and Histidine in the two mutations have a different polar structure (Fig. 2d).

PCR-RFLP analysis of *PLCE1* gene mutations: By using BseYI restricted enzyme, The c.5964T>C mutation in exon 24 display one band at 544 base-pair in homozygous carrier patient, while 3 bands were observed in the patients with

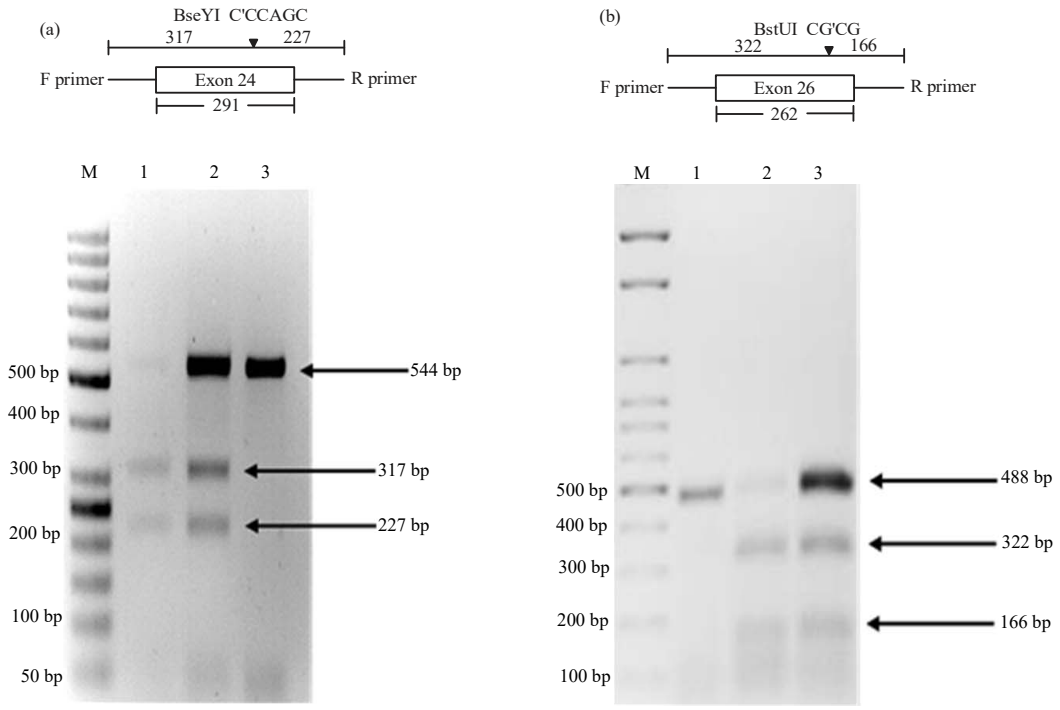

Fig. 3(a-b): PCR/RFLP analysis confirmation of PLCE1 gene mutations, (a) Schematic diagram and gel electrophoresis of c.5964T>C mutation after cutting exon 24 with BseYI enzyme and (b) Schematic diagram and gel electrophoresis of c.6414A>G mutation after cutting exon 26 with BstUI enzyme

a: M: 50 bp marker, 1: Wild type exon, 2: Heterozygous mutated exon, 3: Homozygous mutated exon, b: M: 100 bp marker, 1: Wild type exon, 2: Heterozygous mutated exon, 3: Homozygous mutated exon

Table 2: *In-silico* analysis of the missense mutations identified in *ACTN4* and *PLCE1* genes

				Provean		PolyPhen 2		SIFT	
Genes	Nucleotide change	Amino acid change	Exon number	Prediction	Score	Prediction	Score	Prediction	Score
ACTN4	c.870T>A	p.V247E	8	Deleterious	-4.309	Possibly damaging	0.540	Affect protein function	0.01
	c.2136T>A	p.M669K	16	Deleterious	-3.454	Benign	0.004	Affect protein function	0.02
	c.2464C>A	p.D778E	18	Deleterious	-3.582	Probably damaging	1.000	Tolerated	0.20
PLCE1	c.5964T>C	p.T1777I	24	Neutral	3.826	Benign	0.000	Tolerated	0.94
	c.6414A>G	p.H1927R	26	Neutral	2.511	Benign	0.000	Tolerated	0.99

heterozygous state for the mutation (Fig. 3a). In case of c.6414A>G mutation in exon 26, the BstUI restriction enzyme was used in order to detect the carrier patients with mutations. Thus, the samples from patients with homozygous state generated 2 bands, whereas, three bands were detected in all patients that have the heterozygous state (Fig. 3b).

***In-silico* evaluation of *ACTN4* and *PLCE1* genes mutations:** The mutation of p.V247E in exon 8 was predicted to be possibly damaging and consequently affect the function of the ACTN4 protein (Table 2). Thus, the amino acid valine at the position 247 may be functionally significant and the mutation may lead to damaging the interference with conformation and function of the *ACTN4* protein. Additionally, the 2 mutations p.M669K and p.D778E in *ACTN4* gene were found to be possibly deleterious by PROVEAN software (Table 2). The variation p.D778E was predicted to be probably damaging and tolerated by the PolyPhen2 and SIFT programs, respectively. In case of *PLCE1* gene, the two mutations were predicted to be benign by the three web-based programs (Table 2).

DISCUSSION

NS is one of the most widely recognized reasons for kidney disease in children[1]. To date, numerous genes have been associated with NS in children, which is indicative of the wide

genetic cause of this syndrome[17]. Mutation screening in SRNA patients would be beneficial for clinicians to minimize the risk of immunosuppressive drugs, since gene mutations are involved with a poor response to immunosuppression[39,40].

The current study presents the first molecular screening analysis for mutations of the *ACTN4* gene in a cohort of Saudi child patients with SRNS. The mean age of the present patients with SRNS at the onset of diagnosis was 4.5 years. Similar results were published recently of childhood NS in tropical Africa with a mean age of 4.8 years[41] and 4.0 years in Brazilian children[42]. On other hand, the mean age at the onset of diagnosis was 5.4 years in Indian children[43] and 2.3 years in African American child patients[44].

There is a paucity in the molecular research for screening mutations of genes that are related to SRNS, especially in Saudi society. In a recent study, mutation screening for the complete 8 exons of the *NPHS2* gene and exons 8 and 9 of the *WT1* gene were performed using the same number of child patients that are represented here[29]. Additionally, Al-Hamed *et al.*[45] detected mutations in *NPHS1, NPHS2, PLCE1* and *MYO1E* genes in a group of 49 Saudi families having congenital NS, infantile NS and childhood SRNS. Furthermore, Kari *et al.*[46] reported a lower frequency rate for the mutations in *NPHS2* and *NPHS1* genes in Saudi child patients with SRNS. More recently, Hashmi *et al.*[47] detected the cause of SRNS in a consanguineous Saudi family by detecting a homozygous novel insertion mutation (c.6272_6273insT) in the *PLCE1* gene using whole exome sequencing. Therefore, this study presented the first mutation screen analysis of the *ACTN4* gene for Saudi children with SRNS. Molecular screening analysis revealed three mutations in the *ACTN4* gene that were found in three patients with a frequency of 15% (3/20) in the heterozygous state. These mutations are not recognized in the db SNP, 1000G and ExAC databases and are anticipated to be deleterious and disease-causing by PROVEAN, Polyphen2 and SIFT programs (Table 2). The first mutation p.V247E is located in the actin-binding domain (Fig. 1). To date, all mutations that were identified in the *ACTN4* gene have been found in the actin-binding domain of the encoded protein, resulting in irregular actin rich cellular aggregates[48]. The recognizable proof of *ACTN4* mutations as a reason for human kidney disease proves a significant cellular pathway of the *ACTN4* mutations and podocyte dysfunction[49].

The renal biopsy pattern for the three patients having *ACTN4* gene mutations is FSGS that progressed to ESRD after 2-3 years from the onset of the diagnosis (Table 1). These results agree with a clinical study reported by Choi *et al.*[50], in which they found the mutation of *ACTN4* gene at position S262F as a heterozygous form was connected with FSGS at age 3 to 4 years old and directly progressed to ESRD. Additionally, three different mutations occurring in *ACTN4* in three different families were the cause of FSGS[20]. The incidence of FSGS has increased remarkably in the past 20 years and accounted for 50-60% of the total kidney biopsies in childhood SRNS[51]. There is a close relationship between the single gene mutations of the podocyte proteins, such as NPHS1, NPHS2, ACTN4, LAMB2, INF2, CD2AP or TRPC6 and the FSGS pattern[52]. By immunofluorescence staining, it was documented that the ACTN4 protein is dispersed in podocytes, with a little spreading in another vasculature in the renal cortex. These results confirmed the reports of the high expression of ACTN4 protein in the kidney[53].

It is difficult to estimate whether the novel mutations of *ACTN4* gene are the reason for the SRNS in the three patients without examining the family pedigree and segregation. However, *in-silico* programs have been used to identify the pathogenicity of these novel mutations (Table 2). Several reports used the same *in-silico* programs to anticipate the effect of gene mutations within SRNS patients[45,54,55]. Furthermore, the effect of the three mutations on the 3D structure of the ACTN4 protein indicated that the three mutations disturb the coiled coil alpha helix in three domains of the ACTN4 protein (Fig. 1c). These three domains are important for the function of ACTN4. In addition, the sequence alignment of the ACTN4 protein with other proteins from different organisms showed that about 99% of all amino acids in theses domains are conserved (data not shown). Consequently, a mutation in these domains allowed to change the protein structure has to affect the original function of this protein.

According to the literature, the 2 mutations (p.T1777I and p.H1927R) found in the *PLCE1* gene were non-pathogenic variants and considered as single nucleotide polymorphisms[39]. Interestingly, in several reports, same variants were found to associate with the risk of cancer. Malik *et al.*[56] documented that these mutations were significantly linked to the elevated risk of esophageal cancer in Kashmir Valley. Moreover, Wang *et al.*[57] confirmed that the variant p.H1927R might play an essential role in esophageal carcinogenesis in the Chinese population by changing the protein structure and enzyme activity and thus expanding the inflammatory process in esophageal epithelium. Consequently, Wang *et al.*[57] conclude that the p.H1927R variant may establish a promising biomarker for esophageal cell carcinoma risk stratification, early detection and progression prediction. Moreover, Yuan *et al.*[58] found that p.H1927R was remarkably linked with the increased risk of head and neck cancer in Chinese populations. All these results

suggest the association of the 2 variants of the *PLCE1* gene with different types of cancer. Therefore, the 2 variants of the *PLCE1* gene might be used as a biomarker for childhood SRNS.

The present study has some limitations. The number of patients is small, the inability to identify the compound mutations and the inability to include a control group. However, a relationship between single gene mutation in *ACTN4* gene and Saudi child patients with SRNS might be established.

CONCLUSION

The present study investigates causative mutations linked with SRNS by screening 2 well-known genes (*ACTN4* and *PLCE1*). Among 20 children patients who enrolled for PCR sequencing for all exons of *ACTN4* and *PLCE1* genes, 3 patients carried three novel heterozygous mutations causing disease in *ACTN4* gene. Furthermore, two known variants were detected in *PLCE1* gene in nine patients. As indicated by kidney biopsies, there is a high frequency of FSGS among children patients. Identification of mutations causing SRNS is of importance, for helping in therapeutic considerations as well as for genetic advising.

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SIGNIFICANT STATEMENT

The present study elucidated for the first time the existence of the *ACTN4* gene mutations in Saudi children patients with steroid resistant nephrotic syndrome. These results are helpful to understand the role of such gene in the development and pathogenesis of SRNS among Saudi children.

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