Original Article

Detection of Novel Candidate Mutations as A Cause of Steroid-resistant Nephrotic Syndrome in Children using Next-generation Sequencing Techniques

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ABSTRACT

Background and Objectives: Steroid-resistant nephrotic syndrome (SRNS) is a serious chronic ailment that affects children and causes blood coagulation issues as well as an increased vulnerability to infections. Only around 10% of inherited genetic nephrotic syndrome cases are responding to steroid therapy, and, accordingly, 90% of SRNS patients have multidrug resistance. This study was done to detect novel candidate mutations as a factor for causing SRNS in children using the sequencing technique. **Materials and Methods:** This study included nine children ranging in age from one to sixteen years old who had a clinical diagnosis of SRNS. Phenotype-genotype correlations in these Saudi children were explored using next-generation sequencing techniques to assess the correlation and/or effect of mutations in multiple genes on phenotype variability. The enrichment analysis was carried out to identify genes. **Results:** Five genes were potentially new causative agents for SRNS. The enrichment analysis helped us identify nine causal genes, not previously reported, in six out of nine individuals (66%). These genes are phospholipase D family member 3, mitogen-activated protein kinase binding protein 1, solute carrier family 12 members 3, ezrin, and pancreatic lipase related protein. The other four nominee genes were wilms tumor 1, diacylglycerol kinase iota, coenzyme Q8B, and CASC3. **Conclusion:** The outcome of the study indicated that there is a new mutation as we had four replicates for each sample run on a different sequencing lane. The histopathological findings of these mutated patients were focal segmental glomerulosclerosis.

Keywords: Next-generation sequencing, Steroid-resistant nephrotic syndrome, Focal segmental glomerulosclerosis, Gene mutation, Pediatric.

INTRODUCTION

Nephrotic syndrome (NS) is a dangerous chronic condition that affects children and is categorized by a minimal change of the disease in the majority of individuals affected. Heavy proteinuria and hypoalbuminemia, often coupled with edema and widespread hyperlipidemia, are clinical hallmarks of NS caused by glomerular capillary wall changes.[1] The global incidence rate of NS is estimated to be 2-16.9 children per 100,000.[2] Geographic location and ethnic origin have an impact on the incidence and histologic pattern of NS.[3] Males outnumber females 2:1 in young children; however, by adolescence, this gender imbalance has vanished, and the prevalence of both males

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and females in adolescents and adults is equal.[4] The types of NS are categorized as steroid-sensitive NS (SSNS) or steroid-resistant NS (SRNS) based on the patient's response to steroid medication.[5] Severe proteinuria, edema, low blood protein levels, high blood fat levels, a proclivity for increased blood clotting, and an increased vulnerability to infection are all symptoms of SRNS and are all linked to increased morbidity and mortality. The condition can strike anyone at any age, but it is more prevalent in children. According to several studies, 50% of SRNS patients develop end-stage renal disease (ESRD) within 15 years after diagnosis.[6-8] Furthermore, only 8-10% of inherited genetic NS is susceptible to steroid therapy, resulting in multidrug resistance in 90% of SRNS patients.[8,9] NS is further divided as minimal-change nephrotic syndrome (MCNS), focal segmental glomerulosclerosis (FSGS), mesangioproliferative glomerulonephritis (MPGN), and membranous glomerulonephritis (MGN) based on renal biopsy or histopathological.[10] According to the International Study of Kidney Disease in Children, from a pathological standpoint, 75% of SRNS patients have focal FSGS, while 20% have minimal-change nephrotic syndrome.[11] T lymphocyte dysregulation and vascular permeability variables, which may affect podocyte function and perm selectivity, have been highlighted in research on the pathophysiology of SRNS. Using direct DNA sequencing or next-generation sequencing technologies, a number of contributory genes that cause SRNS disease have been identified.[12,13] Recessive mutations in *NPHS1*, *NPHS2*, *LAMB2*, *WT1, CD2AP, MYO1E,* and *PLCE1* induce severe clinical symptoms of early-onset SRNS and develop to ESRD throughout childhood or infancy.[14-16] On other hand, dominant mutations related to *ACTN4*, *TRPC6*, and *INF2* have been associated to late-onset proteinuria and the development of ESRD in the third and fourth decades of life.[17-19] To date, over 45 genes have been related to SRNS in humans.[15-21]

The Kingdom of Saudi Arabia (KSA) has a uniquely high first and second cousin consanguinity rate of 56 percent.[22,23] In nations with a high frequency of consanguineous marriages, such as Kingdom of Saudi Arabia (KSA),[24] pediatric renal disorders are more likely to be prevalent. Additionally, a higher frequency of congenital and infantile NS has been recorded in KSA than in other countries.[4,25] Kari[26] investigated the trend of histopathologic subgroups in idiopathic nephrotic disease in Saudi Arabia's western region. The findings revealed a trend toward an increase in the prevalence of FSGS and MGN in the KSA's western region. Kari *et al.*[27] also looked at the pattern of histopathology in children with SRNS who lived in Saudi Arabia. According to the findings, FSGS was the most common causal histopathology followed by IgM nephropathy, MGN and MPGN. Hereditary SRNS, like most genetic illnesses, has ethnic and regional variations. In KSA, however, no large-scale genetic research on pediatric patients with SRNS/FSGS have been conducted. Here, using next-generation sequencing techniques, phenotype-genotype correlations in Saudi children with SRNS/FSGS were investigated to determine the correlation and/or effect of mutations in numerous genes on phenotype variability.

MATERIALS AND METHODS

Study Area

The study was carried out from January to May 2022 in the laboratories of Taif University, Taif, Saudi Arabia.

Patients

The bioethics committees of Taif University (40-31-0176) and King Faisal Specialist Hospital and Research Centre-Jeddah (RC-J/234/41) gave their approval to this project. This study included nine children ranging in age from one to sixteen years old who had a clinical diagnosis of SRNS. Proteinuria, hypoalbuminemia, and widespread edema were all symptoms of NS (28). Patients with SRNS have been designated as being unable to respond to a daily prednisone dose of 2 mg/kg over a period of four to six weeks.[28] Every Saudi childhood SRNS case met the inclusion criteria. Patients with steroid-sensitive NS and/or patients with secondary causes were excluded. Renal pathologists took the biopsy samples from the kidneys. Gender, consanguinity, failure to react to steroid therapy, renal biopsy, the interval time of development to ESRD, and other clinical analysis were among the clinical findings.

Library preparation and sequencing

Total DNA was isolated from whole blood as soon as possible using a Wizard® Genomic DNA Purification Kit (Promega; USA). The purity and concentration of genomic DNA were examined using a Maestro-Nanodrop UV-Vis (Maestrogen Inc., Taiwan) at 260 and 280 nm and a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY, USA). For library preparation and exome enrichment, the Nextra Rapid Capture Exome kit (Illumina, Inc., San Diego, CA, USA) was utilized. As previously described,[29,30] the Illumina NextSeq500 instrument (Illumina, Inc.) was used to create clusters and DNA sequence reads. The BCL2FASTQ utility (Illumina, Inc.) was used to convert bcl files generated by the Illumina NextSeq500 instrument into fastq files.

Mutational and enrichment analysis

All sample raw reads were aligned using BWA mem 0.7.17. Gatk 4.1.3.0 was used for variant calling, variants aggregation, joint genotyping, and variants selection, as well as quality-based filtration. We used Haplotype Caller to call single nucleotide variants (SNVs) and short insertion/deletion (INDELs). Genomics DBImport and Genotype GVCFs were used to merge GVCFs of all samples and estimate genotype likelihoods, respectively. Select Variants were applied to select only SNVs and INDELs. Variant Filtration filtered the selected variants based on the quality criteria described in Table 1.

Filtered VCFs were annotated by ANNOVAR, and only exonic variants present in all sequenced samples per case were chosen. Further, non-synonymous, nonsense, stop-loss, and frameshift INDELS with MAF of 1% (gnomAD) and CADD scaled score of greater than 30 variants were selected as candidate causative mutations. We used STRING (https://string-db.org/) homo sapien data for enrichment analysis of the candidate genes. Human phenotype ontology (https://hpo.jax.org/), Orphadata (http://www.orphadata.org/cgi-bin/index.php), and DisGeNET (https://www.disgenet.org/) were downloaded (December 2021). Finally, all candidate genes and their interactive partners were queried against the phenotype databases and NS/SRNS genes (Figure 1).

Table 1: Variant filtration criteria.

Filter	Threshold	Selected variants class
QD	< 2.0	SNV, INDEL
QUAL	< 30.0	SNV, INDEL
SOR	> 3.0	SNV
FS	> 60.0, > 200.0	SNV, INDEL
MQ	< 40.0	SNV
MQRankSum	< -12.5	SNV
ReadPosRankSum	< -8.0, < -20.0	SNV, INDEL

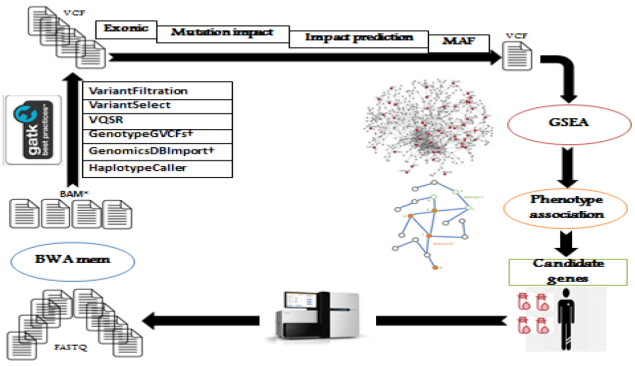

Figure 1: Sample process and analysis workflow.

* BAM files were sorted and indexed. † All study samples were used to calculate the genotype likelihoods.

RESULTS AND DISCUSSION

During the period of 2019 to 2020, nine Saudi pediatric patients with a clinical finding of SRNS admitted to several hospitals of the western region of Saudi Arabia were recruited for this study. The clinical information of each separate child patient included in this study was shown in Table 2. Five patients (55%) were females, while four (45%) were males. Three children (33%) had a family history of NS. The shared clinical symptom was periorbital edema, lower limb edema, and ascites. Hypertensive was found in six (66%) patients, while hematuria existed in five children (55%) (Table 2).

The sequence mutation and variations were named in agreement with the nomenclature of the Human Genome Variation Society. In the list of the nine identified causative genes, 44% were previously reported with NS, FSGS, or ESRD. The enrichment analysis helped us identify nine causal genes in six out of nine individuals (66%), and five of them were potentially new causative genes for SRNS. A novel genetic variant (NM_024426.6:c.1316G>A,R439H) in the Wilms Tumor 1 (*WT1*) gene was found in one 3-month-old patient (Table 3). *WT1* is estimated to be mutated in 4.4-4.8% in patients with early-onset SRNS. *WT1* has a relatively lower mutation rate in Asian populations than Western populations.[31] Generally, mutations in mitogen-activated protein kinase binding protein 1 (*MAPKBP1*) affect the encoded protein's cellular location, resulting in Nephronophthisis, an autosomal-recessive kidney condition.[32] In one patient, a novel variant was found (NM_001128608.2:c.2248C>T,R 750C) in the *MAPKBP1* gene (Table 2). Interestingly, the same patient has "healthy" parents and one affected sister with NS. Surprisingly, *MAPKBP1* is most possibly the first member of a non-ciliary gene family for Nephronophthisis, which could explain non-syndromic variants of Nephronophthisis for which causative mutant genes are still mostly unknown.[32] FSGS is a feature that can cause steroid-resistant type NS in some cases. The causality of the primary coenzyme Q10 (*CoQ10*) deficiency and FSGS is well established.[33,34] Recently, a study recommended measuring urinary *CoQ10* in patients with isolated proteinuria of unknown cause, as it might give diagnostic evidence of mitochondrial nephropathy.[35] The coenzyme is elementary for mitochondrial ATP generation and the respiratory chain. *CoQ8B* is a primary factor in *CoQ10* deficiency cases. We found a "novel" homozygous genetic variant (NM_024876.4:c.532C > T, R178W) in the *CoQ8B* gene (Table 3). This mutation was also recently reported in several patients with FSGS.[36,37]

Table 2: Clinical information of eac(h patient enrolled in the present study.

Case	Ns	Htn	Hematuria	Serum Creatinine	Serum Albumin	Histopathology	Family History	Onset Age	Age at Esrd	Gene
1	SRNS	Yes	Yes (Microscopic)	High	Low	not done	no record	3 M	3 M	*WT1*
2	SRNS	Yes	No	High	Low	FSGS	sister with NS	2.5 Y	14 Y	*MAPKBP1*
3	SRNS	No	Yes (Microscopic)	Normal	Low	FSGS	no record	1 Y	7 Y	*PNLIPRP1, CASC3*
4	SRNS	Yes	No	Normal	Low	not done	no record	3 Y	3 Y	DGKI
5	SRNS	Yes	Yes (Microscopic)	Normal	Low	not done	cousin on dialysis	4 Y	9 Y	no gene found
6	SRNS	Yes	Yes (Microscopic)	High	Low	FSGS	sister with NS	8 Y	12 Y	*PLD3, COQ8B*
7	SRNS	No	No	Normal	Normal	not done	no record	6 Y	10 Y	*EZR, SLC12A3*
8	SRNS	Yes	Yes (Microscopic)	High	Low	FSGS	no record	2 Y	13 Y	no gene found
9	SRNS	No	No	Normal	Low	MPGN	no record	4 Y	12 Y	no gene found

Htn: Hybertension; M: month; Y: year.

Table 3: Genotypes, protein partner, and interaction type of each mutation of SRNS patients considered in this study.

Cases	Gene	Variant	cDNA	Amino Acid	Zygosity	Partner(S)	Interaction Type	Interaction Score
1	WT1	11-32414250-C-T	NM_024426.6:c.1316G>A	R439H	heterozygous	na	na	na
2	MAPKBP1	15-42111094-C-T	NM_001128608.2:c.2248C>T	R750C	heterozygous	na	na	na
3	PNLIPRP1	10-118364985-G-A	NM_006229.4:c.1260G>A	W420*	heterozygous	DGKE	Binding	656
3	CASC3	17-38320381-C-T	NM_007359.5:c.1433C>T	P478L	homozygous	NUP85, NUP133, NUP205, NUP93, NUP160	Reaction	900, 902
4	DGKI	7-137255971-G-A	NM_004717.3:c.1897C>T	R633C	heterozygous	PLCE1	Binding	712
6	PLD3	19-40880407-G-A	NM_012268.4:c.899G>A	C300Y	homozygous	DGKE	Binding	650
6	COQ8B	19-41211045-G-A	NM_024876.4:c.532C > T	R178W	homozygous	na	na	na
7	EZR	6-159188098-C-A	NM_003379.5:c.1609G>T	E537*	heterozygous	ACTN4	Binding	900
7	SLC12A3	16-56913524-C-T	NM_000339.3:c.1406C>T	A469V	heterozygous	na	na	na

Interestingly, we found another novel homozygous variant (NM_012268.4:c.899G>A,C300Y) in the phospholipase D family member 3 (*PLD3*) gene, known for Alzheimer's mode of action (Table 3). The *PLD3* gene is known for its involvement in the glycerophospholipid metabolism pathway and binding to the *DGKE* gene. Mutations in *DGKE* are a genetic cause of glomerular microangiopathy, as the phosphatidylinositol cycle (which requires *DGKE*) is important for normal functioning of podocytes.[38] Moreover, the patient has a sister also diagnosed with NS. More verification is needed to confirm the causality of the *PLD3* gene in NS patients. Studying podocyte gene associations in hereditary, familial, and early onset of NS has revealed a high penetrance rate of those genes.[30] For example, they explained ~ 57-100% of familial and early-onset cases.[30] Multiple genes involved in actin dynamic regulation have been associated with NS. In one of our cases, we found a novel heterozygous mutation (NM_003379.5:c.1609G>T,E537*) in the ezrin (*EZR*) gene (Table 3).

Ezrin belongs to the *ERM* family, which is primarily expressed in epithelial cells. The *EZR* gene encodes ezrin protein, which connects the plasma membrane and the actin cytoskeleton, transmitting signals correspond to external inputs. The plasma membrane's integral

membrane proteins interact with the N domain, while the actin cytoskeleton interacts with the C domain. Ezrin controls signaling pathways involving PKA, PKC, Rho, PI3K, AKT, MAPK, and RTKs like EGFR and MET.[39,40] Therefore, it is a high possibility that ezrin has a high binding affinity to ACTN4, and both genes share acting maintenance molecular functions (e.g., actin cytoskeleton and actin filament bundle assembly) [Table 3]. Thus, we speculate that the *EZR* truncating mutation renders the ACTN4 interaction disrupted. The ACTN4 function disruption is documented to cause renal disorders, including FSGS and ESRD.[41] The same patient also exhibited an uncommon variant (NM_000339.3:c.1406C>T, A469V) in the solute carrier family 12 member 3 (*SLC12A3*), which was reported previously in cases of glomerulosclerosis (Table 3). *SLC12A3* gene encodes a thiazide-sensitive sodium-chloride cotransporter in the kidney, which is critical for electrolyte balance[42] and intercedes sodium and chloride reabsorption in the distal convoluted tubule. Gitelman syndrome may be caused by a homozygous mutation in the *SLC12A3* gene.[43] At the same time, these findings may pique interest in examining the link between various *SLC12A3* gene variants and renal disease.

Diacylglycerol kinase iota (*DGKI*) gene regulates the level of bioactive lipids diacylglycerol/DAG in phosphatidic acid/phosphatidate/PA by acting as a central converter.[44] *DGKI* showed a new missense heterozygous genetic variant (NM_004717.3:c.1897C>T,R633C) predicted to interact with phospholipase PLCE1 with good confidence in the phosphatidylinositol signaling pathway. The *PLCE1* gene is associated with nephrotic syndrome (type 3) in an AR hereditary mode.[31] CASC3 (Caspase 3) is a protein-coding gene that plays a critical role in cell apoptosis execution. Diseases linked with CASC3 contain Immunodeficiency-18.[45] *CASC3* exhibited a novel homozygous missense genetic variant (NM_007359.5:c.1433C>T,P478L) in a patient. Various podocytes' nuclear-altered proteins have been documented in patients with NS in both an autosomal dominant and recessive manner,[46] in particular, the nucleoporin 93kD and 107kD (NUP93, NUP107). The former gene is highly intractable with *CASC3* as structural constituents for nuclear pores. Furthermore, another new heterozygous nonsense mutation (NM_006229.4:c.1260G>A,W420*) in the pancreatic lipase-related protein 1 (*PNLIPRP1*) gene in the same patient was also found. The PNLIPRP1 lipase-related protein is likely to bind to the glycerolipid metabolism of the *DGKE* gene. We confidently report the new mutation, as we had four replicates for each sample run on a different sequencing lane. However, further functional evidence is required to verify our findings.

CONCLUSION

The current study used next-generation sequencing to investigate the phenotype–genotype correlations in Saudi pediatric patients with SRNS to determine the correlation and/or effect of mutations in various genes on phenotype variability. Nine causative genes in children aged 1 to 16 years with a clinical diagnosis of Steroid-resistant nephrotic syndrome were found in this study and histologically they were found with focal segmental glomerulosclerosis.

Significance Statement

The goal of this study was to identify any potential gene abnormalities that might contribute to children with steroid-resistant nephrotic syndrome (SRNS) developing vulnerability to infection and coagulation issues. This is one of the initial discoveries made utilising next-generation sequencing techniques on nine paediatric Saudi Arabian patients. The findings of this study indicate that nine causative genes are present in six of the nine SNRS patients. We identified patients with glomerulosclerosis histologically. Consanguineous marriages are common in Saudi Arabia, hence the likelihood of this kind of genetic mutation may be very high. In light of this, our study emphasises the significance of developing methods to lower this kind of mutation in future generations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

Author's Contribution

Walaa F. Alsanie, Abdulhakeem S. Alamri, Turki M. Sobahy, and Syed Mohammed Basheeruddin Asdaq conceptualized, reviewed, and edited the manuscript.

Samar A. Zailaie, Majid Alhomrani, and Moamen S. Refat carried out the experimental project. The formal analysis was done by Hamza Habeeballah, Syed Mohammed Basheeruddin Asdaq, and Ahmed Gaber. The original draft was written by Abdulhakeem Alamri, and Walaa F. Alsanie. Project administration was taken care of by Majid Alhomrani.

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