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ORIGINAL ARTICLE

Genetic mutation in Egyptian children with steroid-resistant nephrotic syndrome

Manal Micheal Thomas ^{a,*}, Mohamed S. Abdel-Hamid ^b,
Nermine Nabil Mahfouz ^c, Emad Emil Ghobrial ^d

^a Human Genetics and Genome Research Division, Clinical Genetics Department, National Research Centre, Cairo, Egypt

^b Medical Molecular Genetics Department and Genome Research Division, National Research Centre, Cairo, Egypt

^c Child Health Department, National Research Centre, Cairo, Egypt

^d Kasr Al Ainy School of Medicine, Cairo University, Cairo, Egypt

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Abstract *Background/Purpose:* Nephrotic syndrome is the commonest etiology of proteinuria in children. Steroid-resistant nephrotic syndrome (SRNS) is defined by resistance to standard steroid therapy, and it continues to be one of the most intractable etiologies of renal failure. Molecular studies discovered specialized molecules in podocytes that play a role in proteinuria. Mutations in *NPHS2* that encodes for podocin constitute a frequent cause of SRNS worldwide. This study aimed to screen for podocin mutations in SRNS Egyptian children and their parents.

Methods: Our study included patients from 10 unrelated Egyptian families diagnosed with SRNS. Mutational analysis of the *NPHS2* gene was performed by polymerase chain reaction amplification of the whole coding region of the gene and direct sequencing.

Results: Positive consanguinity was detected in five cases, and four of them had a positive family history of SRNS in a family member. Mutational analysis of *NPHS2* revealed pathogenic mutations in four cases (40%) including a novel missense in one patient (c.1A>T; p.M1L).

Conclusion: Our study concludes that mutations of *NPHS2* gene are common among Egyptian children with SRNS. We support a model where ethnicity plays an important role in specific *NPHS2* mutations, since a novel mutation was found in one patient in this study. Future study on a large number of Egyptian patients with SRNS is warranted to identify the actual genetic

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* Corresponding author.

E-mail address: nula_m@hotmail.com (M.M. Thomas).

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contribution of this gene in the development of SRNS in our population, which might help in patients' prognosis and management.

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Introduction

Nephrotic syndrome (NS) is one of the commonest primary kidney diseases, and its progressive forms can end up in chronic kidney disease.¹

NS is the result of an injury to the glomerular filtration barrier and presents clinically with heavy proteinuria, hypoalbuminemia, edema, and hyperlipidemia. Most patients with NS show a good response to steroid therapy and have a good prognosis. On the contrary, approximately 10% of children and 40% of adults are steroid resistant [steroid-resistant nephrotic syndrome (SRNS)], showing no response to steroid therapy and having a poor prognosis.²

The progressive fate of SRNS to end-stage renal disease (ESRD) is seen in 50–70% of patients.³ Inherited structural defects of the glomerular filtration barrier have been detected in isolated as well as familial cases of SRNS.⁴

The pathological picture of focal segmental glomerulosclerosis (FSGS) is revealed in approximately 63–73% of patients with childhood-onset SRNS.⁵

Recent molecular studies involving children with sporadic primary SRNS have described mutations in many genes encoding proteins responsible for the integrity of the glomerular filtration barrier.⁶ These genes include nephrin (*NPHS1*), podocin (*NPHS2*), alpha-actinin 4 (*ACTN4*), CD2-associated protein (*CD2AP*), Wilms' tumor 1 gene (*WT1*), transient receptor potential cation channel 6 (*TRPC6*), and Laminin-beta-2 (*LAMB2*). Proteins encoded by these genes (nephrin, podocin, alpha-actinin-4, an adapter protein anchoring CD2, and others) alter the function of the podocytes.⁷

Mutations of *NPHS1*, *NPHS2*, or *WT1* may be the cause of severe forms of NS in children, progressing to ESRD. Of them, *NPHS2* mutations are considered the most common and are observed in 10–30% of sporadic cases of SRNS with FSGS.⁸ The clinical scope of *NPHS2* mutations has widened, with the proof that mutations in the corresponding gene podocin may lead to NS at birth, in childhood, or in adulthood.^{8,9}

It is recommended to check for *NPHS2* mutations in parallel or prior to starting steroid therapy in NS patients to judge treatment benefits.¹⁰ *NPHS2* mutations were first identified in children with SRNS diagnosed before the age of 6 years who reached ESRD during the first decade of life.¹¹

This study aims to screen for podocin mutations in Egyptian patients with SRNS and compare it with other published series.

Patients and Methods

This study was approved by the Ethical Scientific Committee in the Cairo University Hospital, Giza, Egypt and was conducted in accordance with the university bylaws for

human research. It conforms to the provisions of the Declaration of Helsinki in 2000. All caretakers have given their informed consent.

This study was conducted in the Pediatric Nephrology Clinic, Cairo University Children's Hospital and Genetics Department, National Research Centre. Ten Egyptian children diagnosed with SRNS were included in the study. This study was approved by the Research Ethics Committee of Cairo University Hospital according to the "World Medical Association Declaration of Helsinki", and written informed consent was obtained from the guardians of all patients.

Primary resistance to steroid treatment was defined as the absence of remission to less than a trace of proteinuria on dipstick analysis or <4 mg/m² per hour within the initial 6 weeks of standard steroid therapy.¹²

Patients were included in the study if they fulfilled the following criteria: age group of the patients between 6 years and 16 years, laboratory investigations consistent with SRNS, and patients regularly attending periodic visits and taking prescribed medications. Patients with congenital NS (NS occurring before 3 months of age), cases with secondary NS, and patients with any other medical illness were excluded from the study.

Patients were subjected to detailed history (including demographic data, age at the onset, detailed pedigree construction and analysis with special emphasis on parental consanguinity, similar disease in the family, treatment modalities and response to them, and progression to chronic kidney disease) and careful clinical evaluation (including anthropometric measurements, vital signs, presence of edema or hypertension, and presence of complications).

Information regarding biochemical investigations and, if present, histological diagnosis of NS (renal biopsy) was recorded from the files.

Methods

Genomic DNA was extracted from peripheral blood lymphocytes of the patients and their parents using a standard extraction procedure. The coding region of the *NPHS2* gene (8 exons) were amplified using eight pairs of primers, and the sequence of primers is available upon request from the corresponding author. The primers were designed using PRIMER 3 INPUT SOFTWARE version 0.4.0 (Boston, USA). The coding regions and their exon/intron boundaries of approximately 50 bp sequence were investigated to identify any splice site variation as well. Our standard polymerase chain reaction cycling conditions were as follows: initial denaturation at 96°C for 5 minutes, 30 cycles of denaturation at 96°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 minutes, and an additional extension at 72°C for 5 minutes. The polymerase chain reaction

products were purified by QIAquick PCR purification kit (Qiagen, Redwood City, Germany), directly sequenced in both directions using the Big Dye Termination kit (Applied Biosystems, Foster City, CA, USA), and analyzed on the ABI Prism 3500 Genetic Analyzer (Applied Biosystems) according to manufacturer's instructions. The sequences of chromatograms were aligned and compared with the reference sequence (NPHS2, NM_001297575.1). Pathogenicity of the mutation was checked using various bioinformatic tools and the novel mutation identified was further screened in 100 normal control individuals of Egyptian origin.

Statistical analysis

Data analysis was performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). The descriptive analysis was performed by calculating the means and standard deviations for continuous variables or number and percentages for categorical variables. All the data were normally distributed and tested using sample K-S test. A comparative analysis of the two groups, one positive and the other negative for the gene, was performed, using two-sample *t* test for comparing the means and Chi-square test for comparing categorical variables. In all statistical tests, a *p* value < 0.05 was considered significant.

Results

Clinical data

Our 10 SRNS patients had a full-blown clinical picture of NS; five patients were male and five female, with a male to female ratio of 1:1. Five cases had edema at the time of this study, denoting severe relapse. The age of our cases, duration of the disease, age of the first attack, and number of relapses are shown in Table 1.

The initial full dose of steroid treatment was attempted in all patients. All cases were steroid nonresponders to a dose of 2 mg/kg/d of prednisolone in three divided doses for 6 weeks. The steroid dose at the time of the study ranged from 5 mg every other day to 40 mg every other day, with mean \pm standard deviation of 18.33 ± 11.99 mg every other day. Only one female patient stopped steroids completely as her creatinine rose.

Positive consanguinity was detected in five cases, and four of them had a positive family history of SRNS in a family member. Two cases have one affected sibling and two have an affected cousin. The remaining six were the first encounter in their respective families.

Table 1 Age, age of onset, duration of illness, and number of relapses among patients.

	Minimum	Maximum	Mean \pm SD
Age (y)	3.75	16	9.27 \pm 4.86
No. of relapses	1	14	5.5 \pm 4.58
Duration of disease (y)	1.5	13	6.4 \pm 4.6
Age of first attack (y)	1.25	5.5	2.9 \pm 1.4

SD = standard deviation.

Table 2 Laboratory investigations in SRNS patients.

	Minimum	Maximum	Mean \pm SD
24 h urinary proteins (g/d)	0.24	70	12.92 \pm 21.6
BUN (mg/dL)	5	48	16.1 \pm 13.07
Creatinine (mg/dL)	0.2	3.0	0.8 \pm 0.86
Cholesterol (mg/dL)	157	650	393.8 \pm 172.62
Total proteins (g/dL)	4	7	5.19 \pm 1.03
Albumin (g/dL)	1	4	2.24 \pm 0.94
Calcium (mg/dL)	7	10	8.18 \pm 1.11
Corrected calcium (mg/dL)	8.5	10.7	9.58 \pm 0.67

BUN = blood urea nitrogen; SD = standard deviation; SRNS = steroid-resistant nephrotic syndrome.

The weight of two patients (20%) was below the third percentile. Only one patient was hypertensive, who was controlled on amlodipine and captopril. All patients (hypertensive or nonhypertensive) used angiotensin-converting enzyme inhibitor (captopril) as antiproteinuric and renoprotective medication. Six patients used diuretics (spironolactone or furosemide) during their course of disease.

All cases used at least another immunosuppressant in the form of levamisole, cyclophosphamide, azathioprine, cyclosporine, chlorambucil, or mycophenolate mofetil.

Table 2 shows laboratory investigations in the studied SRNS patients. Seven patients (70%) had corrected calcium level below 8.5 mg/dL. Only one patient had renal impairment. She is a female offspring of positive consanguineous parents with a positive family history of NS (cousin).

Complications were encountered in six cases, mostly in the form of infection (peritonitis, pneumonia, urinary tract infection, cellulitis, and chicken pox) in five cases and psychosis in one case, as shown in Figure 1.

Renal biopsy, which was obtained from eight cases, showed FSGS in four patients (50%), minimal-change NS in one patient (12.5%), membranoproliferative glomerulonephritis in two patients (25%), and membranous glomerulopathy in one patient (12.5%).

Table 3 shows a comparison between cases with positive NPHS2 gene and those with negative gene as regards the age of the patients, age of the first attack, duration of illness, number of relapses, steroid dose, and laboratory investigations.

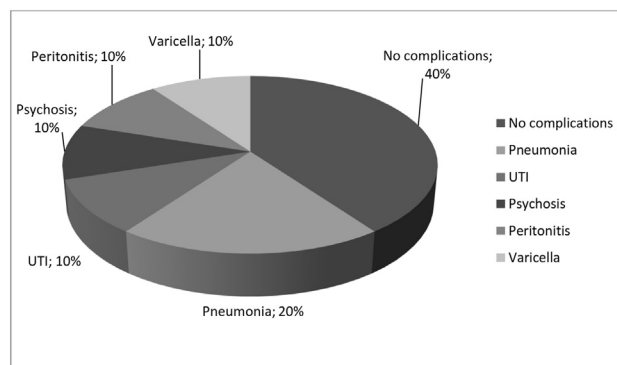


Figure 1 Complications among patients. UTI = urinary tract infection.

Table 3 Comparison between cases with positive *NPHS2* gene and cases with negative gene.

	Positive gene (n = 4)	Negative gene (n = 6)	p
Age (y)	10.10 ± 5.75	8.71 ± 4.65	0.683
Duration of illness (y)	6.68 ± 5.52	6.20 ± 4.45	0.886
Age of 1 st attack (y)	3.43 ± 1.87	2.54 ± 1.03	0.358
No. of relapses	7.50 ± 6.40	4.17 ± 2.79	0.284
Steroid dose (mg)	13.75 ± 13.77	18.33 ± 12.91	0.6068
24 h urinary proteins (g/d)	24.25 ± 31.36	5.36 ± 8.84	0.190
BUN (mg/dL)	20.75 ± 18.48	13.00 ± 8.58	0.389
Creatinine (mg/dL)	1.13 ± 1.27	0.57 ± 0.45	0.348
Total proteins (g/dL)	5.38 ± 0.28	5.07 ± 1.06	0.696
S. albumin (g/dL)	2.05 ± 0.6	2.37 ± 0.9	0.629
Cholesterol (mg/dL)	387 ± 114	398.33 ± 166.44	0.926
Calcium (mg/dL)	8.28 ± 0.83	8.17 ± 0.96	0.840
Corrected calcium (mg/dL)	9.85 ± 0.43	9.4 ± 0.57	0.326

BUN = blood urea nitrogen; S. albumin = serum albumin.

Molecular data

Molecular results revealed *NPHS2* mutations in four cases (frequency = 40%) as shown in (Table 4). By contrast, no pathogenic mutations were identified in the remaining six patients. Four distinct mutations were identified including a novel missense mutation, c.1A>T, p.M1L. The three other mutations were two missense (c.503G>A; p.R168H and c.709G>C; p.E237Q) and one frameshift (c.419delG; p.G140Dfs*41). The E237Q and p.R168H were found in the heterozygous form in Patients 1 and 2, whereas p.G140Dfs*41 and p.M1L were detected in the homozygous state in Patients 3 and 4, respectively.

Discussion

Detection of *NPHS2* mutations in children with SRNS is crucial, as children carrying homozygous or compound heterozygous mutations in this gene have a severe form of

SRNS characterized by primary resistance to steroid treatment and rapid deterioration toward ESRD, in contrast to those without.¹³ Thus, finding a mutation in an SRNS patient has clinical influences: it obviates the nonbenefit of further immunosuppression and points to the unlikely recurrence of NS in a renal graft.¹⁴ Rood et al⁵ proposed mutation screening in familial and sporadic SRNS cases to spare the patient an unnecessary long-term therapy with corticosteroids or cyclophosphamide.

Our study evaluated the frequency of *NPHS2* mutations in a cohort of Egyptian patients with nonsyndromic SRNS. Four patients (40%) were found to carry pathogenic *NPHS2* mutations. A total of four *NPHS2* mutations were detected each in one patient, suggesting a possible genetic heterogeneity of *NPHS2* mutations in our population. A novel mutation affecting the first initiation codon was identified—p.M1L. This mutation was found in the homozygous form in Patient 4, and both parents were heterozygous for the mutation. To our knowledge, this mutation is not reported in the dbSNP, 1000G, and ExAC databases, and is predicted to be

Table 4 Characteristics of patients with positive *NPHS2* gene.

	Case 1	Case 2	Case 3	Case 4
Age (y)	16	14	4.67	6
Sex	Male	Male	Female	Female
Consanguinity	+ve	−ve	+ve	+ve
FH of NS	+ve	−ve	+ve	−ve
Age of onset (y)	5.5	1.5	2	4.5
No. of relapses	14	12	2	2
Kidney functions	Normal	Normal	CKD	Normal
Steroid dose	30 mg every other day	5 mg every other day	Stopped	20 mg every other day
No. of immunosuppressants	3	5	2	2
Biopsy result	Parents refused	Membranous GN	FSGS	FSGS
Genotype	Heterozygous mutation in exon 5, c.709G>C (p.E237Q)	Heterozygous mutation in exon 4, c.503G>A (p.R168H)	Homozygous pathogenic mutation in exon 3, c.419delG (p.G140Dfs*41)	Homozygous pathogenic mutation in exon 1, c.1A>T, (p.M1L)

CKD = chronic kidney disease; FH = family history; FSGS = focal segmental glomerulosclerosis; GN = glomerulonephritis; NS = nephrotic syndrome.

deleterious and disease causing by Polyphen2, SIFT, and mutation taster. Furthermore, it was not found in 200 normal chromosomes of Egyptian origin excluding the possibility of being a rare variant in our population.

Three other *NPHS2* mutations were identified in the current study, two missense and a frame shift mutation. The p.R168H and p.E237Q mutations were previously reported.¹⁵ On the contrary, the p.G140Dfs*41 seems to be a relatively common *NPHS2* gene as it was found in several patients from various ethnic groups.^{4,11,15,16} Previously, Bakr et al¹⁷ reported *NPHS2* gene mutations and identified four novel mutations—p.R238fs, p.P45fs, p.I136L, and p.F216Y.

Santín et al³ found pathogenic *NPHS2* mutations in 10% (6 of 61) of cases with early childhood-onset SRNS, and always in homozygous or compound heterozygous state.

In a more recent study, mutations in the *NPHS2* gene were involved in the pathogenesis of 18.5% of steroid-resistant patients.¹

The mean age of the first attack of our study patients was 2.9 ± 1.4 years (range, 1.25–5.5 years). A slightly higher age of first attack was reported by Cho et al⁶ (median, 4.7 years; range, 4 months–13 years). Chernin et al¹⁸ reported an older age of presentation (median age, 7.2 years).

In our study, eight patients underwent renal biopsy; in four of them (50%), biopsy revealed FSGS (2 patients with gene mutation and 2 without mutation). We concluded that the histologic findings are not enough to recognize those with or without mutation. Basiratnia et al¹⁹ found that most cases (88%) corresponded to FSGS, and emphasized the fact that a greater number of cases are recommended to clarify the relationship between genotype and histological classification.

In our study, there were no statistically significant differences between patients with or without mutation as regards the demographic data and laboratory investigations. Similarly, Basiratnia et al¹⁹ demonstrated that patients with and without podocin mutation were comparable with one another regarding the severity of proteinuria, age, sex, affection of glomerular filtration rate at onset, and hypertension. This is also in accordance with the study of Caridi et al,¹⁶ which demonstrated no genotype–phenotype correlation between children with *NPHS2* mutation and idiopathic FSGS.

Our study supports the suggestion that ethnicity plays an important role in specific *NPHS2* mutations, since a rare mutation was found in one patient in this study.

Özçakar et al²⁰ suggested that interethnic differences have an influence on the incidence of *NPHS2* mutations. In addition, Cho et al⁶ stated that although several genetic causes of SRNS have been defined, still race has an effect on the appearance of these genetic abnormalities.

Chernin et al¹⁸ observed that knowledge of mutation rate of *NPHS2* in different populations of SRNS patients serves as guidance for the physician in choosing the appropriate genetic screening plan.

Similar to our results, the prevalence of mutations in *NPHS2* is higher in Europe and North America, affecting between 10.5% and 28% of the sporadic SRNS children.^{19,21} Tsukaguchi et al²² reported *NPHS2* variants in 23% of late-onset familial cases and 2% of sporadic ones. Lipska et al²³ showed a 14% detection rate of *NPHS2* mutations in Polish patients.

In contrast to our results, the prevalence of *NPHS2* mutations observed in a cohort of Indian children with sporadic

SRNS is low (4%),²⁴ and is similar to that in Chinese (3%),²¹ Korean (0%),⁶ and Japanese (0%)²⁷ populations. Chernin et al¹⁸ also found that no homozygous or compound heterozygous mutations were detected in *NPHS2* in their study on African-American children with SRNS.

Moreover, in 2009, Otukesh et al²⁵ did not detect *NPHS2* mutations in exons 5 and 7 in 20 Iranian children with SRNS. Therefore, they did not recommend *NPHS2* mutation screening in Iranian children with SRNS.

Abid et al²⁶ found out a low frequency of mutation of this gene in children with NS in Pakistan. Similarly, Vasudevan et al²⁷ who screened 25 Indian children with sporadic SRNS for *NPHS2* mutations found only 4% pathogenic mutation.

In our study, positive consanguinity was detected in five cases and four of them had a positive family history of SRNS in a family member (40%). Two cases have one affected sibling and two cases an affected cousin. The remaining six (60%) were sporadic (the 1st encounter in their families).

Ruf et al¹³ reported *NPHS2* mutations in as many as 26% of families with familial SRNS, and Caridi et al¹⁶ found that 12–19% of cases of sporadic pediatric SRNS were noted in children of European descent.

In a Turkish study, the incidence of *NPHS2* mutations was 60% in cases of familial SRNS and 4% in children with sporadic SRNS.²⁰

Benoit et al²⁴ found the mutations in *NPHS2* in 40% of familial SRNS cases (where it follows an autosomal recessive pattern of inheritance) as well as in 6–17% of sporadic SRNS cases.

The current study had some limitations; it was not a prospective longitudinal trial and had a small sample size.

Conclusion

Our study concludes that mutations of *NPHS2* gene are common among Egyptian children with SRNS. On the basis of our results, we support a model where ethnicity plays an important role in specific *NPHS2* mutations, since a rare mutation was found in one patient in this study. The mutations in *NPHS2* gene should be searched for in every child with SRNS. More studies are recommended to find out the precise frequency of *NPHS2* mutations in the Egyptian population, and such an analysis may help predict disease course better, eliminate prolonged unnecessary immunosuppressive therapy, and develop mutation-specific therapeutic interventions in future.

Acknowledgments

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