Mutations in *NPHS2* (podocin) in Mexican children with nephrotic syndrome who respond to standard steroid treatment

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ABSTRACT. Human nephrotic syndrome has been related to mutations in glomerular proteins. Mutations in the *NPHS2* gene that encodes podocin have been described as responsible for steroid-resistant nephrotic syndrome. It has been advised to test for *NPHS2* mutations in parallel or before giving steroid treatment in nephrotic syndrome patients in order to avoid unnecessary therapy. We identified *NPHS2* mutations in Mexican children with nephrotic syndrome. The study included 13 children with nephrotic syndrome and 2 healthy control individuals; 8 patients were steroid-resistant and 5 were steroid-sensitive. We analyzed the 3rd exon of *NPHS2* by DNA sequencing. Podocin heterozygous missense mutations L139R and L142P were found; the former was found in both steroid-sensitive and steroid-

resistant children, while the latter was found in a steroid-resistant child. We conclude that *NPHS2* mutations should be investigated to help decide the course of treatment in nephrotic syndrome patients.

Key words: Podocin; Nephrotic syndrome; Mexican children; Mutation

INTRODUCTION

Nephrotic syndrome (NS) is defined as a combination of proteinuria, hypoalbuminemia, edema, and hyperlipidemia. Approximately 80% of all children with sporadic NS respond to steroid treatment. For decades, NS has been separated into 2 broad categories on the basis of the patient's response to standard steroid therapy, i.e., steroid-sensitive NS (SSNS) and steroid-resistant NS (SRNS) (Ruf et al., 2004). This syndrome is related to the *NPHS2* gene, which codes for an exclusive glomerular protein (podocin) that participates in blood filtration and cell signaling (Somlo and Mundel, 2000; Huber and Benzing, 2005). Podocin mutations are apparently restricted to SRNS (Niaudet, 2004a; Otukesh et al., 2009; Maddalena et al., 2011); they represent approximately 40% of familial SRNS patients and have also been found in 10-30% of sporadic SRNS cases in groups from European and Middle Eastern countries (Caridi et al., 2001; Karle et al., 2002; Ruf et al., 2004) and 20% of NS in the Japanese population (Maruyama et al., 2003). The identification of *NPHS2* mutations in NS patients is important for therapeutic decisions and genetic counseling, and according to several authors, patients should be tested for mutations in this gene before receiving immunosuppressive therapy (Karle et al., 2002; Niaudet, 2004a; Ruf et al., 2004; Caridi et al., 2005).

Inter-ethnic differences have also been suggested to play a role in the incidence of *NPHS2* mutations (Andreoli, 2004; Özçakar et al., 2006). Mutations in this gene in Mexican patients with NS remain largely unknown, since databases lack this information to date. Thus, we determined the presence of mutations in the most commonly tested exon of the *NPHS2* gene, which is involved in SRNS.

MATERIAL AND METHODS

Patients and controls

We examined 15 Mexican children, including 8 diagnosed with SRNS and 5 with SSNS and 2 healthy children. All patients presented normal renal function (data not shown). These cases were seen at State Children's Hospital in Hermosillo, Sonora, Mexico, under parents' consent and approved protocols of the Hospital Bioethics committee. NS patients were admitted to the hospital from May to September 2010. Parental consanguinity was not reported. Nephrotic syndrome was defined as proteinuria (>40 mg·m^{-2}·h^{-1}), hypoalbuminemia (<2.5 g/dL), hyperlipidemia (serum total cholesterol >200 mg/dL), and edema. Familial and sporadic NS types were also determined by familial history. SRNS was defined as the failure to respond to 6- to 8-week daily administration of 2 mg/kg prednisone from the onset of the disease.

DNA extraction and detection of *NPHS2* gene mutations

Genomic DNA was extracted and purified from peripheral whole blood samples using a QIAmp kit (Qiagen, Valencia, CA, USA). The primers used were designed based on GenBank NG_007535.1. DNA (100 ng) was amplified using 1 U Platinum Taq DNA polymerase (Invitrogen, Life Technologies, Mexico) in a 25-μL reaction mixture, which consisted of 2.5 μL 10X PCR buffer, 2.0 μL 10 mM dNTPs, 10 μM primers, and 1.5 μL 25 mM $MgCl_2$. The PCR conditions were as follows: 1 cycle at 95°C for 10 min, a second stage consisting of 29 cycles at 95° (1 min), 55° (1 min), and 72°C (1 min), and a third consisting of 1 cycle at 72°C for 7 min. Aliquots (7 μL) of the PCR product were electrophoresed on 2% agarose gels and visualized under UV illumination after staining with Sybr Safe (Invitrogen). The primers utilized for amplification and sequencing of the 3rd exon of the *NPHS2* gene were as follow: forward: 5'-GGGAAAGTTGGCCATG-3' and reverse: 5'-GGCTCTCTGTACCAATTCTCTC-3'. The PCR products were purified using a QIAquick PCR Purification kit (Qiagen). Sequencing of both strands of the 3rd exon of the *NPHS2* gene was performed at the University of Arizona Genetics Core sequencing laboratory (Tucson, AZ, USA). Sequences obtained were analyzed using the Blast algorithm from NCBI and compared against the amino acid sequence of *NPHS2* (GenBank NP_055440).

RESULTS

The 13 patients with confirmed NS had normal renal function, and the median age of onset was 3.5 years. Eight patients showed primary steroid-resistance and 5 patients were initially steroid-sensitive. Since none of the patients had a family history of NS, we identified these cases as sporadic NS. Patients had cholesterol levels above 200 mg/dL, ranging from 218 to 1060 mg/dL. Three of the 13 NS patients (23%) showed normal triglycerides levels under 200 mg/dL with no distinctions between steroid-resistant and steroid-sensitive patients (Table 1).

Table 1. Clinical data and podocin mutations in 13 children with nephrotic syndrome and 2 healthy controls.

Individuals	Drug response	Mutation	Cholesterol (mg/dL)	Triglycerides (mg/dL)
1	S	L139R	349	288
2	S	No	533	157
3	S	No	350	261
4	S	L139R	245	117
5[HD]	R	L139R	575	463
6	R	No	438	752
7	R	No	265	411
8	R	No	218	91
9	R	No	682	311
10	R	No	376	796
11	R	L142P	1060	1993
12	R	No	358	611
13	S	No	401	517
14*	NT	No	154	154
15*	NT	No	130	123

*Healthy controls; S = steroid-sensitive; R = steroid-resistant; NT = no treatment; [HD]hepatic damage.

Mutational analysis was carried out in 15 children, where 2 of them were healthy. We analyzed the sequence of the *NPHS2* gene exon 3, and 2 novel missense mutations were found in 4 of the patients (26.6%) (Table 1).

In steroid-resistant patients, we observed 6 of 8 individuals with no mutations in *NPHS2* gene exon 3, with cholesterol levels ranging from 218 to 682 mg/dL and triglycerides from 91 to 796 mg/dL. However, 1 individual with a heterozygous missense mutation L142P had a cholesterol level of 1060 mg/dL and triglycerides of 1993 mg/dL.

Three patients showed the heterozygous missense mutation L139R, where 2 of them were identified as steroid-sensitive patients. The corticoid-resistant patient with this missense mutation displayed hepatic damage and higher serum lipid levels compared to the 2 others. Cholesterol ranged from 245 to 575 mg/dL and triglycerides ranged from 117 to 463 mg/dL in patients with mutation L139R. The rest of the patients and controls had no mutation in the *NPHS2* gene exon 3.

DISCUSSION

Previous studies on the frequency of *NPHS2* mutations in different populations showed that ethnicity plays an important role; however, there is no consensus on which exon is more critical for predicting NS and which mutations are most deleterious for the patient (Otukesh et al., 2009). However, exon 3 is the most recurrent source of mutations in the *NPHS2* gene. Ruf et al. (2004) found that almost 50% of the missense mutations reported are concentrated in this region. One of the most common mutations, R138Q, is localized in exon 3 and it is mainly reported in the European population, including countries such as Italy, France, and Germany (Niaudet, 2004b; Löwik et al., 2009). However, it has also been found in the Greek population (Megremis et al., 2008), and we suggest that it would be worthwhile searching for this mutation or any variation through this exon in the Mexican population.

We found 2 novel missense mutations in the *NPHS2* gene exon 3 and no incidence of R138Q in Mexican pediatric NS patients. One of them appeared in a corticoid-resistant NS patient with exacerbated serum lipid levels (L142P). Another mutation (L139R) appeared in 2 of 5 corticoid-sensitive and 1 of 8 corticoid-resistant patients, where this last one developed hepatic damage and markedly higher lipid levels than corticoid-sensitive patients with the same mutation. The hepatic damage and altered lipid levels could be factors that contribute to resistance to corticoid treatment in the patient according to Ueda et al. (2011). Both missense mutations affect amino acids that are highly conserved across many species (Figure 1), which denotes their importance in the protein's function and its possible role in the cause of NS.


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Apis florea|ref|XP_003694878      48 VIFRLGRLLSGGAKGPGIFFILPCVDNYARVDLRTRTYDVPPQEVLTKDS 98
Apis mellifera|ref|XP_001123020   48 VIFRLGRLLSGGAKGPGIFFILPCVDNYARVDLRTRTYDVPPQEVLTKDS 98
Homo sapiens|ref|NP_055440.1     134 IIFRLGHLLPGRAKGPGLFFFLPCLDTYHKVDLRLQTLEIPFHEIVTKDM 184
Macaca mulatta|ref|XP_002802082  134 IIFRLGHLLPGRAKGPGLFFFLPCLDTYHKVDLRLQTLEIPFHEIVTKDM 184
Equus caballus|ref|XP_001487958  134 IIFRLGHLLPGRAKGPGLFFFLPCLDTYHKVDLRLQTLEIPFHEIVTKDM 184
Mus musculus|emb|CAC44636.1      136 IIFRLGHLLPGRAKGPGLFFFLPCLDTYHKVDLRLQTLEIPFHEVVTKDM 186
Canis lupus|ref|XP_547443        148 IIFRLGHLLPGRAKGPGLFFFFPCLDTYHKVDLRLQTLEIPFHEVVTKDM 198
Gallus gallus|ref|XP_422265.2    133 IVFRLGHLLPGRARGPGLFFFLPCLDTYHKVDLRLKTLEIPFHQVVTKDM 183
Danio rerio|ref|NP_001018155     145 VKFRLGHLLQKRPRGPGLMFYLPFLDVCHIVDIRLQILKIPPHMVVTKDL 195
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Figure 1. Alignment of podocin amino acids of different species. Red box corresponds to invariant leucine residue (human L139) and blue box corresponds to invariant human L142. GenBank accession numbers are included after the scientific name of the organism.

Due to the position and change in the polarity of the amino acid in the mutation L139R, we suggest that this mutant podocin is not able to associate with lipid rafts in the plasma membrane and may not be able to recruit nephrin to the lipid rafts, losing its ability to

enhance nephrin signaling, as in a patient carrying the R138Q mutation (Huber et al., 2003), resulting in NS. However, according to observed *in silico* secondary structure prediction (Garnier et al., 1996) (Table 2), we believe that in the mutation L139R the conformational change in the protein may not be drastic enough to impair the molecular mechanism of action of corticoids, and that the patients can respond to treatment. Steroid resistance in the patient with the same mutation could be due to various factors that can affect response to treatment such as changes in corticoid receptors, low levels of corticoid transporter proteins due to hepatic damage, or the high persistent hyperlipidemia (Ueda et al., 2011). Therefore, it is suggested that the mutation L139R is not related to treatment resistance because it is present in corticoid-sensitive patients as well as in a corticoid-resistant patient (Table 1).

Table 2. Prediction of secondary structure composition of human podocin.

Structure	Normal podocin	Mutant L139R	Mutant L142P
Alpha helix	39.43%	39.43%	37.60%
Extended strand	12.27%	12.27%	13.84%
Random coil	48.30%	48.30%	48.56%

These results can be supported by sporadic cases where the heterozygous missense mutations such as R138Q (Ruf et al., 2004), which has been proven to cause SN (Huber et al., 2003), homozygous L169P (Caridi et al., 2001), or S211T (Caridi et al., 2005), all excluded as polymorphism, are present in SSNS patients. The specific molecular mechanism of action for immunosuppressive therapy in SN patients has not been well elucidated.

Some authors argue that mutations in podocin are an important cause of SRNS. Ruf et al. (2004) argue that homozygous or compound heterozygous are directly related to steroid-resistant patients. Karle et al. (2002) found 4 German families with SRNS and heterozygous mutations. Yu et al. (2005) reported on 1 of 23 SRNS patients, who showed a single heterozygous missense mutation L361P in podocin and no mutation in control individuals. In this study, we found a heterozygous leucine-to-proline change (L142P) that could be related to resistance to steroid treatment. It is well known that proline residues disrupt secondary structure, especially alpha helices; in this case, the mutation L142P could drastically disrupt secondary structure conformation and possibly affect the function of the protein (Table 2). Furthermore, a significant change in the conformation of the protein can affect the mechanism of action of corticoids in treatment, leading to a resistance condition. However, further studies are required to elucidate the specific mechanism. This heterozygous mutation was present in 1 SRNS patient in this study.

The mutations reported here were searched as single nucleotide polymorphisms in *NPHS2* for the Mexican population and none was found in available databases (Silva-Zolezzi et al., 2009).

On the basis of our results, we support a model where ethnicity plays an important role in specific *NPHS2* mutations, since 2 novel mutations were found in this study. However, a larger study is needed. The relationship between *NPHS2* mutations and resistance to steroid treatment in NS patients is controversial, because steroid treatment is recommended only in patients who have a non-mutant *NPHS2* genotype (Karle et al., 2002; Niaudet, 2004a; Ruf et al., 2004; Caridi et al., 2005). Further study is recommended and it is underway to generate a predictive model to understand in a more thorough way the molecular etiology of NS.

ACKNOWLEDGMENTS

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