Retrospective mutational analysis of *NPHS1*, *NPHS2*, *WT1* and *LAMB2* in children with steroid-resistant focal segmental glomerulosclerosis – a single-centre experience

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Abstract

The aim of our study was to examine *NPHS1*, *NPHS2*, *WT1* and *LAMB2* mutations, previously reported in two thirds of patients with nephrotic syndrome with onset before the age of one year old. Genomic DNA samples from Polish children (n=33) with Steroid-Resistant Nephrotic Syndrome (SRNS) due to focal segmental glomerulosclerosis (FSGS), manifesting before the age of 13 years old, underwent retrospective analysis of *NPHS1*, *NPHS2*, *WT1* (exons 8, 9 and adjacent exon/intron boundaries) and *LAMB2*. No pathogenic *NPHS1* or *LAMB2* mutations were found in our FSGS cohort. SRNS-causing mutations of *NPHS2* and *WT1* were detected in 7 of 33 patients (21%), including those with nephrotic syndrome manifesting before one year old: five of seven patients. Four patients had homozygous c.413G>A (p.Arg138Gln) *NPHS2* mutations; one subject was homozygous for c.868G>A (p.Val290Met) *NPHS2*. A phenotypic female had C>T transition at position +4 of the *WT1* intron 9 (c.1432+4C>T) splice-donor site, and another phenotypic female was heterozygous for G>A transition at position +5 (c.1432+5G>A). Genotyping revealed a female genotypic gender (46, XX) for the first subject and male (46, XY) for the latter. In addition, one patient was heterozygous for c.104dup (p.Arg36Profs*34) *NPHS2*; two patients carried a c.686G>A (p.Arg229Gln) *NPHS2* non-neutral variant. Results indicate possible clustering of causative *NPHS2* mutations in FSGS-proven SRNS with onset before age one year old, and provide additional evidence that patients with childhood steroid-resistant nephrotic syndrome due to focal segmental glomerulosclerosis should first undergo analysis of *NPHS2* coding sequence and *WT1* exons 8 and 9 and surrounding exon/intron boundary sequences, followed by gender genotyping.

KEY WORDS: gene variant, *NPHS2*, *WT1*, children, steroid-resistant nephrotic syndrome, FSGS

INTRODUCTION

Genetically-heterogenous Steroid-Resistant Nephrotic Syndrome (SRNS) affects approximately 10% of children with idiopathic nephrotic syndrome, progresses toward End-Stage Renal Disease (ESRD), and typically shows Focal Segmental GlomeruloSclerosis (FSGS) in renal histology [1, 2]. To the best of our knowledge, mutations of at least 24 genes have been associated with hereditary SRNS [3]. In 1998, Kestila et al. [4] discovered that the *NPHS1* gene, encoding the podocytic protein nephrin, is mutated in the Finnish type of congenital nephrotic syndrome (CNS). This finding was the first proof of concept for the heredity of childhood nephrotic syndrome. In 2007, Hinkes et al. [5] found that mutations in *NPHS1*, *NPHS2* (encoding podocin), *WT1* (exons 8 and 9) and *LAMB2* (encoding laminin-β2) are causes of two thirds of cases of nephrotic syndrome with onset in the first year of life. Mutational analysis of seven podocyte genes (*NPHS1*, *NPHS2*, *WT1*, *CD2AP*, *ACTN4*, *TRPC6* and *PLCE1*) in 19 non-familial childhood-onset, steroid resistant, biopsy-proven FSGS patients revealed variants of *NPHS1*, *NPHS2*, *WT1* and *CD2AP* that could be the cause of the disease in four subjects (21%). In addition, these results have suggested the role of combinations of genetic variants (bigenic heterozygosity) in the pathogenesis of steroid-resistant FSGS [6]. Santin et al. [2] proved

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Submitted: 7 February 2014 / Accepted: 1 March 2014

the clinical utility of a genetic testing algorithm, for SRNS occurring before adolescence, based on an analysis of *NPHS1*, *NPHS2*, *WT1* and *PLCE1* genes. Recently, Rood et al. [7] suggested that there might be no need for *PLCE1* analysis, for steroid-resistant FSGS occurring before adolescence, in this genetic testing algorithm. On the other hand, Hasselbacher et al. [8] and Matejas et al. [9] indicated that analysis of the *LAMB2* gene, which is mutated in Pierson syndrome (OMIM #609049), could be included in the diagnostics of early onset nephrotic syndrome with absence of extrarenal abnormalities. Therefore, to further address this issue we decided to analyse retrospectively *NPHS1*, *NPHS2*, *WT1* (exons 8 and 9 and the adjacent exon/intron boundaries) and *LAMB2* genes in DNA samples from 33 patients with steroid-resistant focal segmental glomerulosclerosis manifesting before adolescence.

MATERIALS AND METHODS

Samples

Genomic DNA samples (n=33) for analysis were chosen from the DNA Repository for Childhood Nephrotic Syndrome (NS) at the Department of Clinical and Molecular Biochemistry, Pomeranian Medical University, Szczecin, Poland. The DNA samples were selected from children of Central European descent (Polish origin) who met the following criteria for inclusion: age of NS onset before adolescence (before the age of 13 years), biopsy-proven FSGS, primary steroid resistance of nephrotic syndrome defined as the persistence of heavy proteinuria (> 50 mg/kg/day) after eight weeks of prednisone treatment (60 mg/m²/day) and no significant extrarenal manifestations. The study was approved by the ethics committees of the Pomeranian Medical University, Szczecin, Poland and the Children`s Memorial Health Institute, Warsaw, Poland, and conforms to the ethical standards laid down in the 1964 Declaration of Helsinki; parents gave informed consent.

Procedures

Genomic DNA from peripheral blood leucocytes was extracted using a commercially available DNA isolation kit (QIAamp Blood DNA Mini Kit, QIAGEN, Hilden, Germany). Amplification of *NPHS1* (NCBI Reference Sequence NG_013356), *NPHS2* (NCBI Reference Sequence NG_007535), *WT1* (NCBI Reference Sequence NG_009272) and *LAMB2* (NCBI Reference Sequence NG_008094) were performed by PCR. Primer data are available on request [binia@pum.edu.pl]. Subsequently, PCR amplification products were purified using a GenElute PCR Clean-Up Kit (Sigma-Aldrich, Poznan, Poland). Sequencing of the products used BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Life Technologies Polska, Warsaw, Poland). Electrophoresis and analysis were performed using an ABI PRISM 3100-Avant machine (Data Collection Software v2.0, Sequencing Analysis Software v5.1; Applied Biosystems).

RESULTS

The *NPHS1*, *NPHS2*, *WT1* and *LAMB2* genes were analyzed in genomic DNA samples of 33 Polish children with primary SRNS due to FSGS. The study cohort (15 phenotypic males and 18 phenotypic females) consisted of seven children with SRNS with onset in the first year of life, 15 subjects with early childhood-onset SRNS (13 to 60 months) and 11 subjects with late childhood-onset SRNS (61 to 132 months) (Table 1). The median age of NS onset was 36 months (range: 1 to 132 months); for males: 45 months (range: 3 to 126 months); for females: 27 months (range: 1 to 132 months). No pathogenic *NPHS1* or *LAMB2* variants were found in our FSGS cohort. The SRNS-causing mutations of *NPHS2* and *WT1* were detected in 7 of 33 patients (21%), and in those with nephrotic syndrome manifesting within the first year of life: five out of seven patients. Five patients had homozygous missense *NPHS2* mutations; four of these with c.413G>A (p.Arg138Gln) mutations, and the fifth with a c.868G>A (p.Val290Met) mutation. DNA sequencing revealed a C>T transition at position +4 of the splice-donor site in *WT1* intron 9 (c.1432+4C>T, previous nomenclature: IVS9ds+4C>T) from a phenotypic female (subject P20) and a G>A transition at position +5 of the splice-donor site in *WT1* intron 9 (c.1432+5G>A, previous nomenclature: IVS9ds+5G>A) from another phenotypic female (subject P6) (Table 1). No parental DNA samples were available for analysis. Genotyping using the AmpFℓSTR NGM PCR Amplification Kit and AmpFℓSTR Yfiler PCR Amplification Kit revealed a female origin (46, XX) for the DNA sample from subject P20 and a male origin (46, XY) for the DNA sample from subject P6. In addition, the P7 subject was heterozygous for c.104dup (p.Arg36Profs*34) (previous nomenclature: c.104_105insG) without any additional pathogenic *NPHS2* variant and two patients (P8 and P20 subjects) were heterozygous for the c.686G>A (p.Arg229Gln) *NPHS2* non-neutral polymorphism.

DISCUSSION

Results of our study revealed that one fifth of cases of steroid-resistant FSGS which occurred before adolescence were caused by mutations in either the *NPHS2* or *WT1* gene. Neither *NPHS1* nor *LAMB2* mutations were found in our FSGS cohort. It has also not escaped of our notice that 5 out of 7 from the *NPHS2* or *WT1* disease-causing mutations were detected in a small subgroup of children with SRNS (n=7) manifesting in the first year of life. Recent results, from similarly-designed studies with SRNS

TABLE 1. Background characteristics and genetic variants in children with steroid-resistant FSGS (patients listed in order of age of NS onset).

Patient	Age of NS onset (months)	Time to ESRD (months)	Gender phenotype	*NPHS2* variants* [allele1];[allele2]	*WT1* variants* [allele1];[allele2]
P1	1	160	female	[c.413G>A];[c.413G>A]	
P2	3	3	male		
P3	5	39	male	[c.413G>A];[c.413G>A]	
P4	5	59	female	[c.868G>A];[c.868G>A]	
P5	8	135	female	[c.413G>A];[c.413G>A]	
P6	8	n.a.	female		[c.1432+4C>T];[=]
P7	9	63	male	[c.104dup];[=]	
P8	14	28	male	[c.686G>A];[=]	
P9	16	46	male		
P10	17	11	male		
P11	19	70	female		
P12	20	64	female		
P13	20	64	female		
P14	21	102	female		
P15	26	n.a.	female		
P16	27	13	female		
P17	36	0	female		
P18	41	6	male		
P19	45	21	male		
P20	47	n.a.	female	[c.686G>A];[=]	[c.1432+5G>A];[=]
P21	50	55	male		
P22	51	8	male		
P23	61	n.a.	female		
P24	72	36	female		
P25	72	66	male		
P26	76	0	male		
P27	78	44	female		
P28	80	n.a.	male		
P29	95	66	female		
P30	99	0	male	[c.413G>A];[c.413G>A]	
P31	103	26	female		
P32	126	18	male		
P33	132	38	female		

ESRD = End Stage Renal Disease:
n.a. = not available;
[=] = reference sequence;
*Blank spaces indicate that reference sequences were detected for the genes analysed.

children which focused on searching for mutations in panels of genes, were published by Al-Hamed et al. [10] and McCarthy et al. [3]. Al-Hamed et al. detected disease-causing *NPHS1* and *NPHS2* variants in 12% (6 of 49) and 22% (11 of 49) of Saudi Arabian families with childhood nephrotic syndrome, respectively. The *NPHS1* and *NPHS2* mutations were exclusively detected in children of consanguineous parents [10]. McCarthy et al. sequenced 24 hereditary SRNS-associated genes, including the *NPHS1*, *NPHS2*, *WT1* and *LAMB2* genes, in 36 SRNS patients from the UK Renal Registry. These patients were ethnically-heterogeneous and between 1 month and 16 years of age at onset of disease. Molecular analysis revealed causative *NPHS1* and *NPHS2* mutations in 14% (5 of 36) and 8% (3 of 36) of the patients, respectively [3]. The *NPHS1* mutations identified by Al-Hamed et al. [10] and McCarthy et al. [3] in congenital nephrotic syndrome have also been detected in early childhood-onset SRNS [10] and late childhood-onset SRNS [3]. In addition, the *NPHS1* mutations were also identified in adult-onset SRNS [2], including SRNS due to FSGS [11]. Therefore, the lack of *NPHS1* mutations in our group is somewhat surprising. However, the homogeneity of ethnic origin (of Central European/Polish descent) and renal histology (FSGS), as well as the absence of children from consanguineous marriages, should be taken into consideration in relation to this lack of *NPHS1* mutation. In addition, Hinkes et al. reported that *NPHS1* mutations in patients of Central European descent were solely found in nephrotic syndrome with congenital onset (0–3 months)[5], which would only apply to two patients in our cohort (Table 1). Note also that, in one of these two patients, the homozygous *NPHS2* mutation found confirms previous reports that CNS may be caused by mutations in genes other than *NPHS1* [12-17]. Benoit et al. [18] has even recommended that patients with nephrotic syndrome occurring later than the congenital period (and with FSGS in renal histology) should first undergo screening for *NPHS2* mutations followed by *NPHS1* analysis. Lipska et al. reported recessive pathogenic *NPHS2* mutations in 20 of 141 (14%) Polish children with steroid-resistant nephrotic syndrome [19]. The detection rate of *NPHS2* mutations was very similar to that revealed in our study (15%). If from the study of Lipska et al. [19] the c.1032delT *NPHS2* variant is excluded, as it is found solely in Kashubian patients who were not enrolled in our study, then the c.413G>A (p.Arg138Gln) *NPHS2* mutation was the most frequently detected genetic variant both by us and by Lipska et al. [19], and is the most common in populations of European descent. Lipska et al. [19] also reported that *NPHS2* mutations were to be found in the highest prevalence (26 %, 9/35) in patients with SRNS diagnosed within the first year of life, and it is of some interest that in our study 5 out of 7 patients with FSGS-proven SRNS caused by *NPHS2* mutations were found with age of onset within the first year of life (Table 1). Both McCarthy et al. [3] and Al-Hamed et al. [10] found no *LAMB2* or *WT1* mutations in their studied cohorts.

The lack of *LAMB2* mutations in our FSGS cohort, in patients from the UK Renal Registry [3] and in Saudi Arabian SRNS children [10] fully justifies removal of laminin-β2 genetic analysis from the algorithm introduced by Santin et al. [2] for molecular genetic diagnostics of non-syndromic childhood-onset primary steroid-resistant nephrotic syndrome. Despite a suggestion to consider *PLCE1* testing only in children with diffuse mesangial sclerosis [7], Al-Hamed et al. [10] showed the clinical utility of *PLCE1* analysis in childhood-onset steroid resistant FSGS, and this could be further considered (but was not analysed in our study). As well as *NPHS1*, *NPHS2* and *PLCE1* sequencing, Santin et al. also proposed the analysis of *WT1* exons 8 and 9 in molecular genetic diagnostics of childhood onset SRNS [2]. To the best of our knowledge, Wasilewska et al. in 2009 [20] identified the first Polish patient with Frasier syndrome caused by a *WT1* splice-site mutation (c.1432+5G>A), and Lipska et al. (in 2013) [21], in a SRNS cohort from the international PodoNet registry (including 22 patients from Poland), found one Polish patient with classical Frasier syndrome caused by a de novo c.1432+5G>A *WT1* mutation. The prevalence of *WT1* splice-site mutations with sporadic steroid-resistant FSGS in our study (6%) was similar to results published previously by other authors [6, 17, 21-23]. Both c.1432+5G>A or c.1432+4C>T mutations affect *WT1* alternative splicing, leading to an imbalance between two *WT1* isoforms with or without (+KTS or –KTS, respectively) three amino acids lysine (K)-threonine (T)-serine (S) between zinc fingers 3 and 4 [24]. According to Klamt et al. [24] the pathology of Frasier syndrome suggests that gonadal development may be especially sensitive to under-representation of the *WT1* +KTS isoforms. Patients with c.1432+5G>A or c.1432+4C>T *WT1* and FSGS present either an XY disorder of sex differentiation or no gonadal dysgenesis in XX subjects [6, 15-17, 20-23, 25, 26], and we can assume that this also applied to the *WT1* splice-site mutations identified in our FSGS cohort. Note, however, that although Lipska et al. [27] recently reported *WT1* mutations in 6% (46/746) of sporadic SRNS cases in the PodoNet registry, only one third of these (15 out of 17 with FSGS) were due to intronic KTS splice-site variants. The exonic *WT1* mutations were associated with an early age of SRNS onset: mean 1.1 years, whereas KTS intronic splice site variants were not associated: mean age of onset 4.5 years. Recently, Lipska et al. [27] reported *NPHS2* mutations in 6% (11 of 179; [21]) and *WT1* mutations in 6% (46 of 746;) of patients with adolescent-onset sporadic SRNS from the international PodoNet registry, and recommended routine screening of *WT1*, in addition to *NPHS2*, in children with isolated, sporadic SRNS because of the high prevalence of "truly or apparently isolated SRNS". Lowik et al. indicated the cost-effectiveness and the clinical value of genetic screening based on sequencing of *NPHS2* and *WT1* exons 8 and 9, with the adjacent exon/intron boundaries, also for childhood-onset steroid-resistant FSGS [6]. In addition, although the series of FSGS patients in their study was rather small (n=19), the results strongly suggest that both recessive homozygous *CD2AP* mutations or combined haploinsufficiency in *NPHS2* and *CD2AP* might cause primary steroid-resistant FSGS in children [6]. Therefore, it seems to reasonable to consider the screening of *PLCE1*, as discussed earlier, and *CD2AP* as second-choice targets in a genetic testing approach for the diagnosis of primary steroid-resistant FSGS occurring before adolescence [2, 6, 10].

CONCLUSION

In conclusion, our results indicate possible clustering of causative *NPHS2* mutations in FSGS-proven SRNS with onset in the first year of life, and provide additional evidence that children with steroid-resistant nephrotic syndrome due to focal segmental glomerulosclerosis, in whom NS occurs before the age of 13 years, should first undergo analysis of the *NPHS2* coding sequence and the *WT1* gene, especially focused on exons 8 and 9 and the surrounding exon/intron boundary DNA sequences, followed by, in cases with *WT1* KTS intronic splice site variants, genotyping for gender.

DECLARATION OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by Research Grant No. N402 420 438 from the Polish Ministry of Science and Higher Education. We are grateful to Dr. Andrzej Ossowski for the gender genotyping of *WT1* patients and to the anonymous native speaker of English for help with preparation of the manuscript.

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