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ORIGINAL ARTICLE

Analysis of the genes responsible for steroid-resistant nephrotic syndrome and/or focal segmental glomerulosclerosis in Japanese patients by whole-exome sequencing analysis

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Steroid-resistant nephrotic syndrome (SRNS) represents glomerular disease resulting from a number of different etiologies leading to focal segmental glomerulosclerosis (FSGS). Recently, many genes causing SRNS/FSGS have been identified. These genes encode the proteins associated with the formation and/or maintenance of glomerular filtration barrier. Next-generation sequencing is used to analyze large numbers of genes at lower costs. To identify the genetic background of Japanese patients, we studied 26 disease-causing genes using whole-exome sequencing analysis in 24 patients with SRNS and/or FSGS from 22 different Japanese families. We finally found eight causative gene mutations, four recessive and four dominant gene mutations, including three novel mutations, in six patients from five different families, and one novel predisposing mutation in two patients from two different families. Causative gene mutations have only been identified in ~20% of families and further analysis is necessary to identify the unknown disease-causing gene. Identification of the disease-causing gene would support clinical practices, including the diagnosis, understanding of pathogenesis and treatment.
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INTRODUCTION

Idiopathic nephrotic syndrome (NS) is a kidney disorder characterized by high excretion of protein in the urine, hypoalbuminemia and edema. In Europe and the United States, 2 in 100 000 children will develop idiopathic NS in a single year.[1,2] Approximately 80% of patients are steroid responsive and have a favorable prognosis, and the other 20% of children with NS are resistant to steroid therapy and have an unfavorable prognosis.[2,3] Steroid-resistant NS (SRNS) represents glomerular disease resulting from a number of different etiologies leading to focal segmental glomerulosclerosis (FSGS).[4]

Recently, many genes causing SRNS/FSGS have been identified. These genes encode the proteins associated with formation and/or maintenance of the glomerular filtration barrier.[5–10] Genetic backgrounds of SRNS/FSGS may be different in each ethnic group. There are currently no detailed reports of genotype–phenotype correlations, including the responsibility to the ordinal treatment, complication, prognosis and post-transplant recurrence of SRNS/FSGS. These data will provide useful information for clinical practice and genetic counseling.[11] Next-generation sequencing is used to analyze a large number of genes associated with SRNS/FSGS and can be carried out at lower costs compared with the individual sequence analysis of many genes.[12] To identify the genetic background of Japanese patients, we studied 26 disease-causing genes using whole-exome sequencing analysis in 24 patients with SRNS and/or FSGS from 22 different families. We finally identified eight causative gene mutations in six patients from five different families and one predisposing gene mutation in two patients from two different families.

MATERIALS AND METHODS

Subjects

We studied 24 patients with SRNS and/or FSGS (male-to-female ratio, 12:12; ages from 2 to 42 years; median age, 12 years) from 22 different Japanese families (Supplementary Table 1). The institutional review board of the Yamagata University School of Medicine study approved this study, and written informed consent was obtained from the patients or parents of all children. The definitions and criteria for NS and steroid resistance were those used by the International Study of Kidney Disease in Children.[13,14] NS was defined as heavy proteinuria (urinary protein excretion $\geqslant 40\,mg\,m^{-2}\,h^{-1}$) with hypoalbuminemia $\leqslant 25\,g\,l^{-1}$. Steroid resistance was defined as a failure to achieve a response despite 4 weeks of prednisolone therapy ($2\,mg\,kg^{-1}$ per day prednisolone).

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Molecular analysis

We extracted genomic DNA from peripheral blood using a standard method.

We analyzed all the exons of 26 genes: *NPHS1*, *NPHS2*, *NPHS3 (PLCE1)*, *CD2AP*, *TRPC6*, *ACTN4*, *MYH9*, *ARHGAP24*, *MYO1E*, *IFN2*, *WT1*, *LMX1B*, *SMARCAL1*, *LAMB2*, *MT-TL1*, *COQ6*, *COQ2*, *PDSS2*, *SCARB2*, *ZMPSTE24*, *PMM2*, *ALG1*, *PTPRO*, *GPC5*, *APOL1* and *ITGB4*,[9–11,15] using a next-generation sequencer. Exon capture was performed with the SureSelect Human All Exon Kit v.5 (Agilent Technologies, Santa Clara, CA, USA). Exon libraries were sequenced with the HiSeq 2000 platform (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Paired 100-base pair reads were aligned to the reference human genome (UCSC hg19) using the Burrows–Wheeler Aligner (version 0.7.3a).[16] Probably, PCR duplicates were removed with the Picard 1.88 (http://picard.sourceforge.net/). We picked up the sequences of 26 genes previously identified as the responsible genes for SRSN. Single-nucleotide variants and indels were identified using the Unified Genotyper module of the Genome Analysis Tool Kit (GATK) 2.5. 2 (Broad Institute of MIT and Harvard, Cambridge, MA, USA),[17] with the Best Practice Variant Detection with the GATK v.3 (https://www.broadinstitute.org/gatk/index.php). Single-nucleotide variants and indels were annotated against the set of RefSeq database, NHLBI-ESP 6500 exomes and dbSNP135 with the ANNOVAR (8 May 2013).[18] We predicted the functional effects of mutations using two PolyPhen scores (HumDiv and HumVar models) provided by PolyPhen-2 version 2.2.2,[19] Grantham,[20] PhastCons[21] and GERP[22] scores provided by SeattleSeq SNP annotation 137 (http://snp.gs.washington.edu/SeattleSeqAnnotation137/). We performed direct sequencing to confirm the mutations identified by exome sequencing in the patients and some of family members and to find additional gene mutations detected in cases 10, 11, 12 and 15. PCR was performed with the primers designed based on the genome database. The sequence reactions were analyzed on an ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

RESULTS

We studied 26 disease-causing genes using whole-exome sequencing analysis in 24 patients with SRNS and/or FSGS from 22 different families and found 10 nucleotide changes in eight genes (Supplementary Table 1 and Table 1). Based on the database and functional prediction analyses, we finally identified eight causative gene mutations in six patients from five different families and one predisposing gene mutation in two patients from two different families (Table 2).

For the genes of recessive types, we detected a heterozygous *NPHS2* p.R168H mutation[23] in Case 15 and a heterozygous *LAMB2* c.1405 +1g>a mutation (splicing mutation)[24] in Case 12 (Table 2). We also detected three polymorphic nucleotide changes as follows: *PTPRO* p. S338F (rs.200478856), *NPHS1* p.A219V (rs.757417823) and *NPHS2* p. Q287R (rs.200042397). Direct sequencing revealed that Cases 15 and 12 were a compound heterozygote of *NPHS2* p.[R138X];[R168H] mutation[25,26] and a compound heterozygote of *LAMB2* p.[abnormal splicing];[G699R] mutation, respectively. Case 15 presented as congenital NS, and steadily progressed to end-stage renal failure (ESRF) at age 11 years. Case 12 manifested the symptoms in infantile period and progressed rapidly to ESRF at age 2 years, but he did not have any ocular symptoms. Cases 15 and 12 had no relapse of FSGS after renal transplantation.

In the analysis of the genes of dominant types, we found five gene mutations in six cases from five families. Cases 10 and 11 carrying a novel *CD2AP* p.R74M mutation were unrelated and presented different clinical features. Case 10 partially responded to steroid and cyclosporin A therapy; however, he progressed to ESRF. He received cadaveric renal transplantation, but had a recurrence of FSGS. Case 11 frequently relapsed, but he was responsive to therapy and had a complete remission. We performed direct sequencing of all coding regions of *CD2AP* in Cases 10 and 11, but we did not find any additional mutations.

Case 16 carrying a novel heterozygous *TRPC6* p.E875V mutation presented FSGS at age 12 years, did not respond to steroid and cyclosporin A and progressed to ESRF. Her grandfather died of renal disease at age 30 years and her father presented proteinuria.

Case 19 was a daughter of Case 20, and Cases 19 and 20 presented the symptoms at school age and progressed to ESRF. They were complicated with bicornuate uterus and had a novel heterozygous *WT1* p.[K141Q; P249T] mutation.

Table 1 Nucleotide changes detected in 24 patients with FSGS and/or SRNS from 22 different families

No.	Gene	Mutation		Status	PolyPhen-2 (HumDiv)	PolyPhen-2 (HumVar)	Grantham score	PhastCons score	GERP score	References and information
1	*PTPRO*	c.1013c>t	p.S338F	Heterozygote	1	0.998	155	0.996	5.23	rs.200478856
6	*NPHS1*	c.656c>t	p.A219V	Heterozygote	0.692	0.148	64	0.334	−0.75	Sibling of case 7 rs.757417823
7	*NPHS1*	c.656c>t	p.A219V	Heterozygote	0.692	0.148	64	0.334	−0.75	Sibling of case 6 rs.757417823
15	*NPHS2*	c.412c>t	p.R138X	Heterozygote	NA	NA	NA	1	4.91	Grantham[20]
		c.503g>a	p.R168H	Heterozygote	1	0.999	29	1	4.52	
22	*NPHS2*	c.860a>g	p.Q287R	Heterozygote	0.989	0.979	43	1	5.34	rs.200042397
12	*LAMB2*	c.1405+1g>a	ab. spl.	Heterozygote	0.996	0.755	43	0.719	3.44	Siepel *et al.*[21]
		c.2095g>c	p.G699R	Heterozygote	0.986	0.593	125	0.992	5.49	rs.28364667
10	*CD2AP*	c.221g>t	p.R74M[a]	Heterozygote	0.966	0.641	91	1	4.65	
11	*CD2AP*	c.221g>t	p.R74M[a]	Heterozygote	0.966	0.641	91	1	4.65	
16	*TRPC6*	c.2624a>t	p.E875V[a]	Heterozygote	1	0.982	121	1	5.89	
19	*WT1*	c.421a>c	p.K141Q[a]	Heterozygote	0.960	0.545	53	1	2.58	Daughter of case 20
		c.745c>a	p.P249T[a]		0.035	0.027	38	1	5.62	
20	*WT1*	c.421a>c	p.K141Q[a]	Heterozygote	0.960	0.545	53	1	2.58	Mother of case 19
		c.745c>a	p.P249T[a]		0.035	0.027	38	1	5.62	
24	*IFN2*	c.550g>a	p.E184K	Heterozygote	1	0.999	56	0.709	4.48	Cooper *et al.* [22]

Abbreviations: Ab. spl., abnormal splicing; FSGS, focal glomerular sclerosis; GERP, Genomic Evolutionary Rate Profiling; NA, not analyzed; SRNS, steroid-resistant nephrotic syndrome.
[a]Novel mutations.

Table 2 Gene mutations and clinical features

No.	*Sex*	*Age at onset*	*Response for treatment*	*Pathology*	*Prognosis*	*Age at ESRF*	*Gene*	*Causative or predisposing mutation*	*Clinical features*
15	M	At birth	No trials	FSGS	ESRF	11y3m	*NPHS2*	c.[412c>t];[503g>a]	p.[R138X]; [R168H]	No relapse after renal transplantation at age 11y4m Sister with FSGS died of renal failure at age 5y
12	M	1y9m	Resistant	FSGS	ESRF	2y2m	*LAMB2*	c.[1405+1g>a]; [2095g>c]	p.[ab. spl.]; [G699R]	No relapse after renal transplantation at age 7y2m No ocular symptoms No affected family members
10	M	5y4m	Initially responsive	FSGS	ESRF	7y6m	*CD2AP*	c.221g>t	p.R74M[a]	FSGS relapsed after renal transplantation at age 8y1m No affected family members
11	M	5y2m	Responsive Frequently relapse	FSGS	Complete remission	—	*CD2AP*	c.221g>t	p.R74M[a]	No affected family members
16	F	12y5m	Resistant	FSGS	ESRF	23y11m	*TRPC6*	c.2624a>t	p.E875V[a]	Hemodialysis at age 23y Father had proteinuria; grandfather died of renal disease at age 30y
19	F	6y7m	No trials	FSGS	ESRF	16y6m	*WT1*	c. [421a>c;745c>a]	p.[K141Q[a]; P249T[a]]	Daughter of case 20; Bicornuate uterus Grandfather died of renal disease at age 42y
20	F	10y	Unknown	NA	ESRF	42y	*WT1*	c. [421a>c;745c>a]	p.[K141Q[a]; P249T[a]]	Mother of case 19; Bicornuate uterus Father died of renal disease at age 42y
24	M	10y	Resistant	FSGS	ESRF	16y11m	*IFN2*	c.550g>a	p.E184K	No relapse after renal transplantation at age 20y No affected family members

Abbreviations: ab. spl., abnormal splicing; ESRF, end-stage renal failure; FSGS, focal glomerular sclerosis; m, months; y, years.
[a]Novel mutations.

Case 24 carrying a heterozygous *IFN2* p.E184K mutation presented FSGS at age 10 years, did not respond to steroid, cyclosporin A and plasma exchange and progressed to ESRF at age 16 years.[27] He did not have any signs of Charcot-Marie-Tooth disease (CMT).

The patients carrying those dominant gene mutations manifested the symptoms in childhood period; they were older than the patients carrying the genes of recessive types. The patients carrying the mutations except *CD2AP* mutation did not respond to therapy and did not relapse FSGS after renal transplantation (Supplementary Table 1 and Table 2).

DISCUSSION

We studied 26 disease-causing genes using whole-exome sequencing analysis in 24 patients with SRNS and/or FSGS from 22 different families and finally found eight causative gene mutations, four recessive and four dominant gene mutations, in six patients from five different families, and one predisposing mutation in two patients from two different families (Table 2). Recently, Sadowski *et al.* studied 1783 families with SRNS that manifested before 25 years of age and detected a single-gene cause in 526 families (29.5%).[10] Our study was very small, but detected gene mutations in 22.7% of families.

Cases 15 and 12 carrying the recessive gene mutations manifested the symptoms during infantile period. Case 15 was a compound heterozygote of *NPHS2* p.[R138X];[R168H] mutation[25,26] (Table 2). Podocin encoded by *NPHS2* is expressed mainly in the podocytes and localized to the intercellular junction of the podocytes foot processes. Podocin is a lipid raft component of the slit diaphragm that is essential for the localization of nephrin and other components. Most missense mutations including the p.R168H mutation alter the trafficking to the plasma membrane[25] and the p.R138X mutation was demonstrated to be present at the plasma membrane but fail to recruit nephrin in lipid rafts.[26] Case 15 was resistant to therapy, progressed to ESRF and had no recurrence of FSGS after renal transplantation as reported previously.[28] Case 12 was a compound heterozygote of *LAMB2* c.[1405+1g>a];[2095g>c]; that is, p.[abnormal splicing];[G699R] mutation[24] (Table 2). Laminins are the major noncollagenous constituent of basement membranes and laminin β-chain encoded by *LAMB2*, a component of laminins, is highly expressed in the basement membrane of muscles at the neuromuscular junctions, kidney glomerulus and vascular smooth muscle. Mutations with complete loss of function are associated with the features of Pierson syndrome and missense mutations cause mild and variable phenotypes.[29] The homozygous c.[1405+1g>a] mutation was reported in the patient showing Pierson syndrome.[24] The p.G699R is registered as a rare variation (frequency is 0.00274), but is predicted to have a deleterious functional effect using several prediction programs, PolyPhen-2 (HumDiv), MutationTaster score (data not shown) and MutationAssessor score (data not shown). These suggest that a compound heterozygous *LAMB2* p.[abnormal splicing];[G699R] mutation is a causative mutation of FSGS.

For the dominant type, Cases 10 and 11 were heterozygous for *CD2AP* p.R74M mutation. CD2AP is an adapter protein with an SH3 domain, is expressed in lymphoid cells, and interacts with the T-cell adhesion protein CD2.[30] CD2AP is also expressed in the slit diaphragm of the podocytes and interacts with podocin and nephrin to form a signaling complex.[31,32] Mice with null mutation die of massive proteinuria shortly after birth and mice with heterozygous null mutation developed glomerular change similar to FSGS at 9 months of age.[30] For the human, only one case carrying homozygous p.R612X mutation was clearly demonstrated in association with renal disease.[33] Several patients with heterozygous *CD2AP* mutations have

been reported in association with adult onset FSGS.[30,34] However, segregation data was not available in all mutations and the heterozygous mutations were also detected even in unaffected individuals. Case 10 presented FSGS and relapsed FSGS after renal transplantation. In contrast, Case 11 frequently relapsed, but he was responsive to therapy and had a complete remission. In addition to their clinical difference, Cases 10 and 11 had no affected family members. These suggested that a heterozygous *CD2AP* p.R74M mutation is not a disease-causing mutation, but is a predisposing factor towards glomerular disease.

Case 16 had a novel heterozygous *TRPC6* p.E875V mutation. TRPC6 is a cation channel and is widely distributed in the body including the podocytes cell bodies and the slit diaphragms. The majority of mutations associated with FSGS are gain-of-function mutations, which can increase intracellular calcium influx and lead to the apoptosis of the podocyte.[35] Case 16 had similarly affected family members, but we could not confirm the linkage because their specimens were not available for analysis.

Cases 19 and 20 had heterozygous *WT1* p.[K141Q; P249T] mutation. These mutations were located to exons 1 and 2. Nearly all mutations of WT1 associated with FSGS are located in exons 8 and 9. However, SNPs located in *WT1* (promoter to exon 5) and *WITI* (gene immediately 5′ to *WT1*) were significantly associated with FSGS.[36] and Gebeshuber *et al.*[37] reported that transgenic expression of the microRNA miR-193a in mice rapidly induced FSGS via downregulation of WT1. Those reports suggest that a *WT1* mutation located in other than exons 8 and 9 will cause FSGS. Cases 19 and 20 had bicornuate uterus as genitourinary malformations, which may be associated with the sexuality and/or the type of the *WT1* mutation.[38]

Case 24 had a heterozygous *IFN2* p.E184K mutation and was not complicated with CMT as reported previously.[27] IFN2 is a member of the formin family and has a domain structure similar to the diaphanous formins: a diaphanous-inhibitory domain (DID), formin homology 1 and 2 domains and a diaphanous autoregulatory domain. IFN2 is strongly expressed in podocytes and Schwann cells. *IFN2* mutations were detected in the patients with isolated FSGS and the patients with FSGS associated with CMT. All mutations are distributed in different parts of the DID; however, the mutations detected in patients with FSGS and CMT are located in the inner face of the central core of the DID.[39,40] The p.E184K mutation is located in the DID, but not in the inner face of the central core of the DID. The clinical phenotype of the IFN2 mutation may depend on the site and/or type of the mutation.

Detection of an SRSN-causing mutation will support clinical practices, including the diagnosis, understanding of pathogenesis and treatment. It will also avoid an unnecessary steroid therapy, introduce supplement therapy for the patients with a defect in CoQ biosynthesis[41,42] and provide a prenatal diagnosis in severe cases. However, causative gene mutations have been identified only in ~ 20% of families and further analysis is necessary to identify the unknown disease-causing gene.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)