Sotos-like features and dominant-negative effects of the truncated NFIX proteins cause Marshall–Smith syndrome.[6]

In this study, we screened for *NFIX* mutations in 48 Japanese patients who were suspected as Sotos syndrome, but showed neither deletions nor mutations in *NSD1*. Detailed genetic and clinical data are presented.

MATERIALS AND METHODS

Subjects

A total of 48 patients suspected as Sotos syndrome were analyzed for *NFIX* mutations. *NSD1* investigation by sequencing and fluorescent *in situ* hybridization analysis was negative in these patients. In this study, the patients presenting with cardinal features of Sotos syndrome (specific craniofacial features, intellectual disability and overgrowth to same extent) but showing no *NSD1* abnormalities are referred as those with 'Sotos-like features'. Experimental protocols were approved by the Committee for Ethical issues at Yokohama City University School of Medicine. All individuals were investigated in agreement with the requirements of Japanese regulations.

Mutation analysis

Genomic DNA was isolated from peripheral blood leukocytes according to standard methods. DNA for mutation screening was amplified by illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Buckinghamshire, UK). Sequencing of exon 1 and high-resolution melting curve (HRM) analysis of exon 2–9 covering the *NFIX* coding region (GenBank accession number NM_002501.2) were performed. For exon 1, the 12 μl PCR mixture contained 30 ng DNA, 0.3 μM each primer, 0.4 mM each dNTP, 1× PCR buffer for KOD FX and 0.3 U KOD FX polymerase (Toyobo, Osaka, Japan). For exons 2–9, real-time PCR and HRM analysis were serially performed in 12 μl mixture on Rotor-Gene Q (QIAGEN, Hilden, Germany). For exon 7, the PCR mixture contained 30 ng DNA, 0.3 μM each primer, 0.4 mM each dNTP, 0.36 μl SYTO9 (Invitrogen, Carlsbad, CA, USA), 0.4 mM each dNTP, 1× PCR buffer for KOD FX and 0.3 U KOD FX polymerase (Toyobo). For the remaining exons, the PCR mixture contained 30 ng DNA, 0.25 μM each primer, 0.36 μl SYTO9 (Invitrogen), 0.2 mM each dNTP, 1× ExTaq buffer and 0.375 U ExTaq HS (Takara, Otsu, Japan). Primers and conditions of PCR are shown in Supplementary Table 1. The PCR products showing an aberrant melting curve were sequenced. All the novel mutations in DNA amplified by GenomiPhi were verified by sequencing of PCR products using genomic DNA as a template. Mutations were checked in 250 Japanese normal controls (500 alleles) by HRM analysis.

Parentage testing

For the family showing *de novo* mutations, parentage was confirmed by microsatellite analysis as previously described.[8] Biological parentage was judged if more than four informative markers were compatible and other uninformative markers showed no discrepancies.

Prediction of functional effect

The effect of the mutations for protein features was predicted by following web-based prediction tools: SIFT (http://sift.jcvi.org/), PolyPhen (http://genetics.bwh.harvard.edu/pph/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), Mutation Taster (http://www.mutationtaster.org/) and Align GVGD (http://agvgd.iarc.fr/agvgd_input.php).

RESULTS

NFIX mutations

Two heterozygous missense mutations were identified. The c.179T>C (p.Leu60Pro) mutation in patient 1 were not found in her parents, indicating that the mutation occurred *de novo* (Figure 1a). Biological parentage was confirmed by several microsatellite markers (data not shown). The c.362G>C (p.Arg121Pro) mutation in patient 2 was found in his mother (Figure 1a). These two mutations occurred at evolutionary conserved amino acids (Figure 1b) and were absent in 250 Japanese normal controls. Interestingly, the missense changes were located in DNA-binding/dimerization domain of the NFIX protein (Figure 1c). Evaluation with web-based prediction tools strongly suggested that these substitutions are pathogenic (Supplementary Table 2).

Clinical information of the patients

Patient 1 is a product of unrelated healthy parents. The body weight at birth was 2816 g (−0.6 s.d.), height 48.8 cm (0 s.d.) and OFC 33.5 cm (+0.3 s.d.). Neonatal hypotonia was recognized. At 17 months of age, her weight was 9.24 kg (−0.5 s.d.), height 84.9 cm (+2 s.d.) and OFC 48 cm (+1.2 s.d.). The facial appearance showed long/narrow and triangular face, high forehead, midface hypoplasia, prominent ears, epicanthal folds, strabismus, down-slanting palpebral fissures, short nose with anteverted nares, prominent long philtrum, everted lower lip and narrow palate (Figure 1d). Large hands/feet, prominent fingertips, pectus excavatum were also noted. Her primary dentition started at 7 months of age and was completed by 17 months of age. Bone age was estimated as 3 years at 17 months of age and as 5 years at 3 years of age. Bullet-shaped phalanges, which are typical features of Marshall–Smith syndrome, were not observed. She was initially diagnosed as Sotos syndrome. She showed mental retardation and severe developmental delay with developmental quotients of 19. Scoliosis was noted at 18 months of age and surgically treated for several times. Complex partial seizures were noted at 4 years of age and were controlled with phenytoin and zonisamide. At present (17 years of age), prognathia was observed (Figure 1e). Her weight was 40 kg (−2 s.d.) and height 156.5 cm (−0.2 s.d.).

Patient 2 is a male at age of 20 years. The birth weight was 2938 g (−0.4 s.d.), height 51 cm (+0.8 s.d.) and OFC 35.5 cm (+1.4 s.d.). Respiratory insufficiency was noted, but no visceral malformations were pointed out. Bilateral tubing therapy was performed for recurrent bilateral exudative otitis media at 4 years of age. At 14 years of age, his weight was 58.1 kg (+0.6 s.d.) and height 185.7 cm (+3.5 s.d.). Mental retardation was evident as the IQ score (Tanaka–Binet intelligence test) was 59. Craniofacial features included high forehead, down-slanting palpebral fissures and prognathia. He was suspected as Sotos syndrome. His mother showed tall stature, suggesting that c.362G>C led to overgrowth in the mother. Unfortunately, further details of clinical features in the mother are unavailable. Clinical information of two patients is summarized in Table 1.

DISCUSSION

NFIX is a member of the nuclear factor I (NFI) family proteins, which are implicated as site-specific DNA-binding proteins known to function in viral DNA replication and gene expression regulation.[9] NFI proteins form homo- or heterodimers and bind to the palindromic DNA consensus sequence through its N-terminal DNA-binding/dimerization domain.[10] Point mutations in DNA-binding/dimerization domain of NFI protein have been shown to cause loss of dimerization, DNA-binding and replication activities,[11] highlighting the importance of structural integrity of DNA-binding/dimerization domain. It has been reported that the DNA binding domain of SMADs and NFI transcription factors shared considerable structural similarity, and the secondary structure of the DNA-binding domain of NFI was estimated based on that of SMADs.[12] In this study, we identified two heterozygous missense mutations, the c.179T>C (p.Leu60Pro) and the c.362G>C (p.Arg121Pro), in the DNA-binding/dimerization domain. Of note, two mutations are estimated to be localized within α-helical region of DNA-binding domain and at evolutionally conserved amino acids between SMADs and NFI.[12] In addition, two mutations cause substitutions to a proline residue,

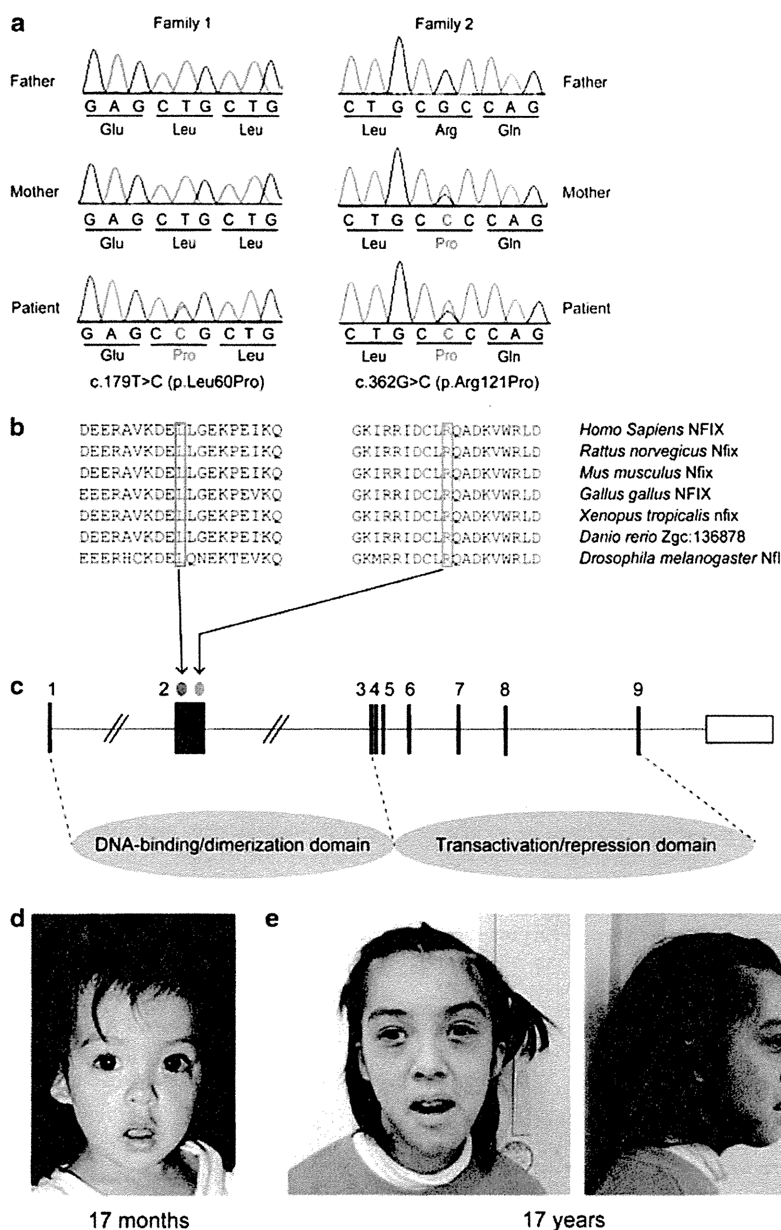

Figure 1 Missense mutations in *NFIX* in individuals with Sotos-like features. (**a**) Electropherogram of family 1 (left) and family 2 (right). The c.179T>C (p.Leu60Pro) mutation occured *de novo*. The c.362G>C (p.Arg121Pro) mutation was inherited from his mother. (**b**) An amino-acid sequence alignments of NFIX protein including amino-acid positions 60 and 121. Protein sequences were obtained through the NCBI protein database and multiple sequence alignment was performed by CLUSTALW web site (http://clustalw.ddbj.nig.ac.jp/). (**c**) Schematic representation of *NFIX* consisting of nine exons. UTR and coding exons are indicated by open and filled rectangles, respectively. The location of mutations is indicated by red (c.179T>C) and blue (c.362G>C) dots. At the bottom, C-terminal DNA-binding/dimerization domain and N-terminal transactivation/repression domain are depicted. Both the c.179T>C and c.362G>C mutations are located in exon 2 encoding a part of DNA-binding/dimerization domain. (**d**) Facial appearance of patient 1 at 17 months of age, showing long/narrow and triangular face, down slanting, short nose with anteverted nares and everted lower lip. (**e**) At 17 years of age, prognathia was noted in patient 1.

which is a unique amino acid characterized by imino radical. Proline has a pyrrolidine ring that restricts the available conformational space; therefore, it has effects on chain conformation and the process of protein folding.[13] Thus, it is very likely that two mutations could affect DNA-binding activity of NFIX protein through conformational changes of the DNA-binding domain.

Because *NFIX* mutations could cause both Marshall–Smith syndrome and Sotos-like features,[6] it is great concern to which of them two patients with missense mutations could be classified. Main clinical features of Sotos syndrome are childhood overgrowth including tall stature and/or macrocephaly, characteristic face and mental retardation. Other minor features are scoliosis, hypotonia in infancy, seizures,

Table 1 Clinical features of two patients with missense mutations in *NFIX*

				Reported by Malan et al.[6]		
		Patient 1	*Patient 2*	*Patient A*	*Patient B*	*Patient C*
Genetics	*NFIX deletion/mutation*	*c.179T>C*	*c.362G>C*	*del 19p13.3*	*del 19p13.3*	*c.568C>T*
Epidemiology	Age at last evaluation (years)	17	14	14	10	27
	Sex	F	M	M	M	F
	Mat/pat age	48/52	?/?	31/33	25/30	31/31
Prenatal growth	Birth weight (g)	2816 (−0.6 s.d.)	2938 (−0.4 s.d.)	4500 (>95)	3110 (10–50)	3600 (50–90)
	Birth height (cm)	48.8 (0 s.d.)	51 (+0.8 s.d.)	53 (95)	49 (50)	52 (95)
	OFC (cm)	33.5 (+0.3 s.d.)	35.5 (+1.4 s.d.)	38 (>95)	33.5 (10)	37.5 (>95)
Postnatal growth	Weight (kg)	9.24 (−0.5 s.d.)[a]	58.1 (+0.6 s.d.)[b]	>P98	>P98	>P98
	Height (cm)	84.9 (+2 s.d.)[a]	185.7 (+3.5 s.d.)[b]	>P98	>P98	>P98
Development						
SS	Autistic traits	−	−	+	+	+
	Behavioral anomalies	NA	−	+	+	+
	Motor retardation	+	+	+	−	−
	Hypotonia	+	+	+	+	−
Overlapped	Mental retardation	+	+	+	+	+
	Degree of delay	DQ19	IQ42	NA	NA	NA
	Speech delay	+	+	+	+	+
	First words (months)	24	18	NA	NA	NA
Craniofacial features						
SS	Long/narrow face	+	−	+	+	+
	Down-slanting palpebral fissures	+	+	+	−	+
	Small mouth	NA	−	+	−	+
	Prognathia	+	+	+	−	−
Overlapped	High forehead	+	+	+	+	+
MSS	Everted lower lip	+	−	+	−	+
	Underdeveloped midface	+	−	NA	NA	NA
	Proptosis	NA	−	NA	NA	NA
	Short nose	+	−	NA	NA	NA
	Prominent premaxilla	NA	−	NA	NA	NA
	Gum hypertrophy	+[c]	−	NA	NA	NA
	Retrognathia	−	−	NA	NA	NA
Eyes						
SS	Hypermotropia	−	−	+	+	−
	Strabismus	+	−	+	−	+
	Nystagmus	−	−	−	−	+
	Astigmatism	NA	NA	−	+	−
MSS	Myopia	NA	−	NA	NA	NA
	Blue sclerae	NA	−	NA	NA	NA
Musculo-skeletal abnormalities						
SS	Abdominal wall hypotonia	−	−	+	−	+
	Pectus excavatum	+	−	+	+	−
	Coxa valga	−	−	+	+	−
Overlapped	Scoliosis	+	−	+	−	+
	Advanced bone age	+	NA	+	+	+
MSS	Abnormal bone maturation	NA	NA	NA	NA	NA
	Bone fractures	−	−	NA	NA	NA
	Kyphosis	−	−	NA	NA	NA
	Umbilical hernia	−	−	NA	NA	NA

Abbreviations: F, female; M, male; Mat/pat, maternal/paternal; MSS, Marshall–Smith syndrome; NA, not ascertained; OFC, Occipitofrontal circumference; SS, Sotot's syndrome.
Growth of patients 1 and 2 is indicated with s.d. and that of patients in the report of Malan *et al.*[6] is indicated with percentile.
[a]At 17 months.
[b]At 14 years.
[c]Suggested the possibility of the adverse drug reaction.

cardiac defect and genitourinary anomalies.[5] On the other hand, main clinical features of Marshall–Smith syndrome are moderate to severe developmental delay with absent or limited speech, unusual behavior, disharmonic bone maturation, respiratory compromise secondary to upper airway obstruction, short stature and kyphoscoliosis.[14] One of remarkable differences between Sotos syndrome and Marshall–Smith syndrome is facial appearances. Although both syndromes has high forehead, Sotos syndrome has a long/narrow face, triangular shaped face with a prominent chin, down-slanting of the palpebral fissures,[1,4–5] whereas Marshall–Smith syndrome has proptosis, under-developed midface and prominent premaxilla.[7,14] In patient 1, although some characteristic features of Marshall–Smith syndrome such as everted lower lip, short nose and midface hypoplasia were observed, overall facial appearance, overgrowth features at 17 month of age, scoliosis, hypotonia and seizures were consistent with Sotos syndrome. Similarly, in patient 2, the facial appearance, tall stature and macrocephaly were consistent with Sotos syndrome. In both patients, their body weights were relatively low in comparison with their heights. This is consistent with the fact that, throughout childhood and early adolescence, the height was usually more significantly increased than weight in Sotos patients.[15] In addition, our patients did not show respiratory difficulties, one of specific features in Marshall–Smith syndrome, which cause early death in the neonatal period or early infancy.[7] Thus missense mutations in the DNA-binding/dimerization domain, which may lead to loss of transcriptional regulation by NFIX protein, could cause Sotos-like syndrome in two patients.

Many clinical features including tall statue, mental retardation, speech delay and high forehead are shared between our patients and three patients reported by Malan *et al.*[6] with *NFIX* abnormalities. The recognizable difference is autistic traits. Autistic traits are not observed in our patients but all of Malan *et al.*'s[6] patients. Thus there is a possibility that autistic traits are caused by haploinsufficiency of *NFIX* in Malan *et al.*'s[6] patients, but not by misssense mutations in the DNA-binding/dimerization domain. However, identification of a greater number of cases with *NFIX* mutations is required to confirm this hypothesis.

In conclusion, our report provides further evidences that *NFIX* is a causative gene for Sotos-like features. Abnormalities of *NSD1* are found in majority of Sotos syndrome cases and aberration of other genes including *NFIX* may be found in the minority of Sotos syndrome/Sotos-like features. Genetic testing of *NFIX* should be considered in such patients if no *NSD1* abnormalities were identified.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SHORT COMMUNICATION

Mutations in *PRRT2* responsible for paroxysmal kinesigenic dyskinesias also cause benign familial infantile convulsions

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Paroxysmal kinesigenic dyskinesia (PKD (MIM128000)) is a neurological disorder characterized by recurrent attacks of involuntary movements. Benign familial infantile convulsion (BFIC) is also one of a neurological disorder characterized by clusters of epileptic seizures. The BFIC1 (MIM601764), BFIC2 (MIM605751) and BFIC4 (MIM612627) loci have been mapped to chromosome 19q, 16p and 1p, respectively, while BFIC3 (MIM607745) is caused by mutations in *SCN2A* on chromosome 2q24. Furthermore, patients with BFIC have been observed in a family concurrently with PKD. Both PKD and BFIC2 are heritable paroxysmal disorders and map to the same region on chromosome 16. Recently, the causative gene of PKD, the protein-rich transmembrane protein 2 (*PRRT2*), has been detected using whole-exome sequencing. We performed mutation analysis of *PRRT2* by direct sequencing in 81 members of 17 families containing 15 PKD families and two BFIC families. Direct sequencing revealed that two mutations, c.649dupC and c.748C>T, were detected in all members of the PKD and BFIC families. Our results suggest that BFIC2 is caused by a truncated mutation that also causes PKD. Thus, PKD and BFIC2 are genetically identical and may cause convulsions and involuntary movements via a similar mechanism.
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Keywords: benign familial infantile convulsion; mutation analysis; paroxysmal kinesigenic dyskinesia; *PRRT2*

INTRODUCTION

Paroxysmal kinesigenic dyskinesia (PKD (MIM128200)) is a heritable paroxysmal movement disorder characterized by recurrent and brief attacks of involuntary movements triggered by sudden voluntary movements. PKD attacks consist of any combination of dystonic, choreoathetotic and ballistic components, often occur daily and frequently more than once a day, and usually last from a few seconds to 1–2 min.[1–3] Age of onset is usually during early adolescence. Most of PKD cases are usually inherited as an autosomal dominant trait, but many sporadic cases have also been reported.[2–4] In our previous study, we performed a genome-wide linkage and haplotype analysis and defined disease locus within the pericentromeric region of chromosome 16.[1,5] Subsequently, we performed mutation analysis on 229 genes between D16S3131 and D16S503; however, we failed to identify the causative gene.[5,6] Recently, Chen *et al.*[7] identified truncating mutations within protein-rich transmembrane protein 2 (*PRRT2*) in eight Han-Chinese families with histories of PKD using whole-exome sequencing.

Benign familial infantile convulsion (BFIC) is a clustered epileptic syndrome occurring from 3 to 24 months.[3] Seizures usually occur in clusters over a day, and four associated loci have been identified. BFIC1 (MIM601764), BFIC2 (MIM605751) and BFIC4 (MIM612627) have been mapped to chromosome 19q, 16p and 1p, respectively,[2,3] while BFIC3 is caused by the mutations in *SCN2A* (182390) on chromosome 2q24.[3] Patients with BFIC2 have also been observed within a family that concurrently had PKD.[3] Caraballo *et al.*[8] suggested that BFIC2, PKD, and infantile convulsion and paroxysmal choreoathetosis (ICCA; MIM 602066) might be allelic disorders because of mapping evidence and that these three conditions have

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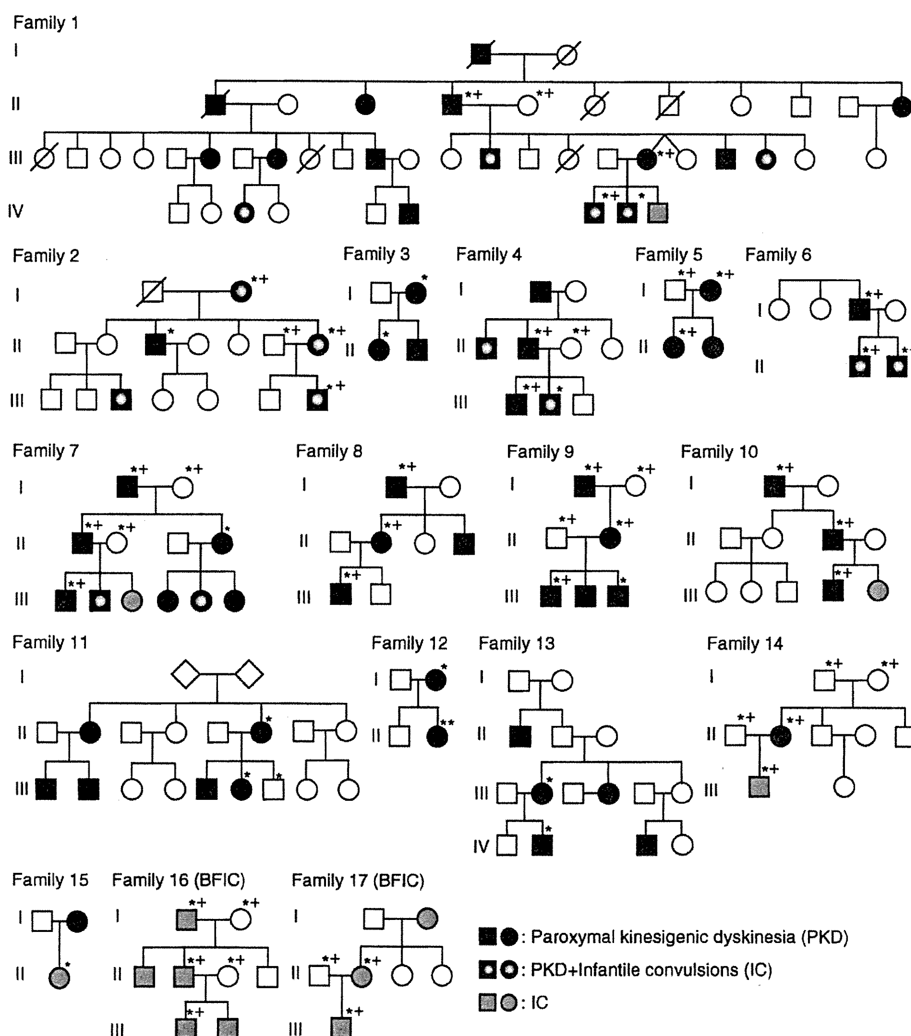

Figure 1 Pedigrees of the 17 families with paroxysmal kinesigenic dyskinesia (PKD) and/or benign familial infantile convulsion (BFIC). Filled-in symbols indicate individuals with PKD or PKD with infantile convulsions or BFIC. Empty symbols indicate unaffected individuals. * indicates individuals whose DNA was used in the mutation analysis. + indicates individuals whose DNA was used in the haplotype analysis.

been found in one family. However, the genetic background and clinical features of BFIC remain unclear.

Here, we describe the results of *PRRT2* mutation analyses in 15 PKD and 2 BFIC2 Japanese families.

MATERIALS AND METHODS

Subjects

We recruited 17 Japanese families for our study, comprised 15 PKD families and two BFIC families with only infantile convulsion patients (Figure 1). Among all these families, 68 patients were diagnosed with PKD and 13 patients were diagnosed with BFIC or infantile convulsion. Out of all 68 PKD patients, 16 patients were diagnosed with infantile convulsion as infants. Detailed information of all families is shown in Figure 1 and Table 1. Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp DNA Mini kit (QIAGEN, Hilden, Germany). Experimental procedures were approved by the Committee for Ethical Issues on Human Genome and Gene Analysis at Nagasaki University.

Mutation analysis

We carried out mutation analysis of *PRRT2* by direct sequencing after PCR amplification (primer sequences are available on request). Amplified fragments were directly sequenced using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and were run on an ABI PRISM 3130×l Genetic Analyzer (Applied Biosystems).

Haplotype analysis

Phased haplotypes were constructed with the Merlin software[9] using rs648559, rs7190132, rs183529, rs402720, rs235659, rs10204, rs1057451, rs9938630, rs4788186, rs1291771, rs12919612, rs1129700 and rs7201384. These single nucleotide polymorphisms spanned ~80 kb of the telomeric and ~100 kb of the centromeric sides flanking *PRRT2*. The rs11645263, which is located 53 kb on the telomeric side from (3′ end) of *PRRT2*, was a repeat length polymorphic marker instead of a single nucleotide polymorphism. Therefore, we genotyped the sequences using the GeneMapper (Applied Biosystems) after being run on an ABI PRISM 3130xl Genetic Analyzer.

RESULTS

The c.649dupC mutation, generating a truncated protein with only 223 amino acids, was found in 16 Japanese families with PKD and BFIC2 out of 17 families (Table 1). This mutation in Family 14 was confirmed as *de novo* (Figure 2a). Interestingly, the c.649dupC mutation found in PKD families was also detected in Family 16 and 17 with BFIC2 only. The c.748C>T (p.Q250X) mutation, which substitutes the codon for Q250 with a stop codon, was found in Family 15 (Figure 2b). Furthermore, these two mutations were not found in 288 ethnically matched normal controls.

Haplotype analyses indicated that 11/12 families have the common single nucleotide polymorphism haplotype that carried the mutation flanking *PRRT2*. This haplotype pattern consisted of all major alleles. However, these 11 families could be divided into six groups based on repeat length markers (Supplementary Figure).

DISCUSSION

We report three novel findings in this study. First, the c.649dupC mutation was found not only in PKD families but also in two BFIC2 families that lacked PKD patients. This mutation has been previously reported as the causative gene for PKD by two studies.[7,10] Several reports also indicate that PKD and BFIC2 may be allelic disorders because these two diseases occurred in the same families and map to the same region on chromosome 16.[11] To date, it has been recognized that these two diseases are closely related, yet non-identical, because the main symptom of BFIC2 is convulsion and that of PKD is involuntary movement.[3] Results of our study strongly suggest that PKD and BFIC2 are genetically caused by identical mutations. The difference between these two diseases may simply be due to phenotype differences caused by fluctuating PRRT2 mRNA level during aging. Another possible cause is the *cis*-acting polymorphisms or base changes around the mutation, because BFIC families without PKD (for example Family 16 and Family 17) are obviously observed. Additional mutation searches in many PKD and BFIC families and sporadic cases, along with further analysis of base alterations distinguishing PKD and BFIC, are needed to confirm these assumptions. Because the same mutation can cause the PKD or BFIC, we speculate that convulsions and involuntary movements might be caused by a similar mechanism, and thereby may help in the treatment of involuntary movements.

Table 1 Mutations within *PRRT2* in patients with paroxysmal dyskinesia

Family	*Number of affected individuals (detailed information)*	*Nucleotide mutation*	*Protein alteration*
1	15 (PKD: 9, PKD+IC: 5, IC: 1)	c.649dupC	p.P217fsX7
2	5 (PKD: 1, PKD+IC: 4)	c.649dupC	p.P217fsX7
3	3 (PKD: 3)	c.649dupC	p.P217fsX7
4	5 (PKD: 3, PKD+IC: 2)	c.649dupC	p.P217fsX7
5	3 (PKD: 3)	c.649dupC	p.P217fsX7
6	3 (PKD: 1, PKD+IC: 2)	c.649dupC	p.P217fsX7
7	9 (PKD: 6, PKD+IC: 2, IC: 1)	c.649dupC	p.P217fsX7
8	4 (PKD: 4)	c.649dupC	p.P217fsX7
9	5 (PKD: 5)	c.649dupC	p.P217fsX7
10	4 (PKD: 3, PKD+IC: 1)	c.649dupC	p.P217fsX7
11	6 (PKD: 6)	c.649dupC	p.P217fsX7
12	2 (PKD: 2)	c.649dupC	p.P217fsX7
13	5 (PKD: 5)	c.649dupC	p.P217fsX7
14	2 (PKD: 1, IC: 1)	c.649dupC	p.P217fsX7
15	2 (PKD: 1, IC: 1)	c.748C>T	p.Q250X
16 (BFIC)	5 (IC: 5)	c.649dupC	p.P217fsX7
17 (BFIC)	3 (IC: 3)	c.649dupC	p.P217fsX7

Abbreviations: BFIC, benign familial infantile convulsion; IC, infantile convulsion; *PRRT2*, protein-rich transmembrane protein 2; PKD, paroxysmal kinesigenic dyskinesia.

Second, the c.748C>T (p.Q250X) mutation, which was found in Family 15, is a new detected mutation in *PRRT2*. This mutation is located within the N-terminal extracellular domain, thereby generating a truncated protein, which is similar to the mutant protein generated by c.649dupC mutation in that it also lacks a transmembrane domain.[7,10] Chen *et al.* and Wang *et al.*[7,10] suggested that the truncated proteins caused by mutations located in the second transmembrane motif influence the function of ion channels and might thereby cause PKD.[7,10] Our results are congruent with this suggestion, given that all patients in our study had truncated *PRRT2* mutations.

Finally, the c.649dupC mutation observed in Family 14 was found to be a *de novo* mutation. In our study, the mutation was also observed in 16/17 families. This observation suggests that the mutation observed in our study is potentially a founder mutation. The results of haplotype analysis using repeat length markers, along with the observed *de novo* mutation in Family 14, led us to suggest that the mutation c.649dupC arose independently in most cases. This mutation is the most commonly found in patients with family based PKD, as supported by two previous reports.[7,10] Our results also suggest that the associated sequence, which has nine consecutive cytosines, is a mutational hotspot that has expanded one base to

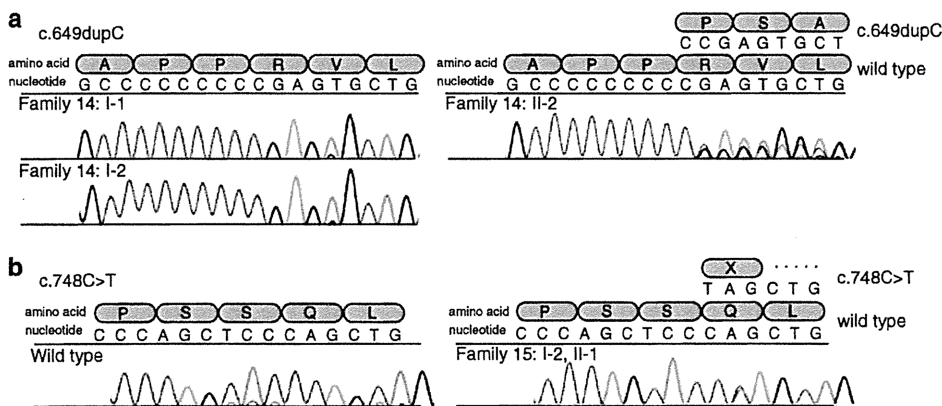

Figure 2 Base substitutions detected in individuals with PKD or infantile convulsion in Family 14 and 15. (**a**) The c.649dupC mutation was detected as a *de novo* mutation in Family 14. (**b**) The c.748C>T mutation was detected in Family 15 and generated a truncated protein with only 249 amino acids. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

result in frameshift. The observed poly C tract in the exon could explain the existence of many sporadic PKD cases. In accordance with this discussion, we clearly identified *de novo* c.649dupC mutation in Family 14. To date, we have analyzed familial PKD, BFIC or ICCR, however, we further need to perform mutation screening in such sporadic cases. Increased attention should be given to the homopolymer within the exon such as *PRRT2*, especially when searching for mutations using the PCR-direct sequencing method. This is because it is potentially difficult to analyze >9 or 10 homopolymers within the elctropherogram by the PCR-direct sequencing method due to slippage causing mixed signals during the PCR amplification, and that homopolymer sites could be mutation hot spots for the development of *de novo* mutations as observed in *PPRT2*.

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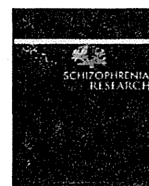

Letter to the Editor

Expression analysis of a novel mRNA variant of the schizophrenia risk gene *ZNF804A*

Dear Editors,

Schizophrenia is a severe psychiatric disease characterized by delusions, hallucinations, impairment of cognitive function and incoherent behavior. It affects approximately 1% of the general population worldwide. Schizophrenia is a "common disease" and is predicted to be associated with many susceptibility genes that each play minor roles. Candidate gene association studies have implicated many susceptibility genes for schizophrenia. However, these studies are hampered by their focus on a single gene and reliance on incomplete biological information. The genome-wide association study (GWAS) is currently the most powerful, systematic and unbiased genetic approach to study the common disease/common variant hypothesis of complex disorders, such as schizophrenia. A GWAS study by O'Donovan et al. (2008) first identified an association between schizophrenia and the risk SNP, rs1344706 encoding zinc-finger protein 804A (*ZNF804A*) gene. Subsequent GWAS and meta-analysis studies have replicated these results and highlighted *ZNF804A* as a robust genome-wide supported susceptibility gene for schizophrenia (Steinberg et al., 2011). Although the risk SNP, rs1344706 of *ZNF804A* has been implicated in brain connectivity, brain volume, cognitive function, and personality trait (Esslinger et al., 2009; Hashimoto et al., 2010; Donohoe et al., 2011; Rasetti et al., 2011; Yasuda et al., 2011), its specific biological functions have not been thoroughly investigated and the regulation of its transcription remains uncharacterized. The risk SNP of *ZNF804A*, rs1344706 is located in intron 2, 47 kb from the 3′-end of exon 2 and 20 kb from the 5′-end of exon 3. This SNP is not within the splicing site or the splicing branch point. Thus, it has been hypothesized that this SNP might be a *cis*-acting transcriptional regulator of the gene. Recently, an immunoprecipitation (IP) experiment performed using a synthetic oligonucleotide containing the rs1344706 region revealed the existence of allele specific binding proteins (Hill and Bray 2011). These findings support the *cis*-element hypothesis. In this study, we searched for a *ZNF804A* transcriptional variant and tested whether its expression is altered in patients with schizophrenia.

To search for a novel *ZNF804A* transcriptional variant, the genomic, mRNA, and expressed sequence tag (EST) NCBI databases and UCSC Genome Browser were used. ESTs that included conserved sequences were chosen as candidate novel exons. We performed exploratory RT-PCR using lymphoblastoid cell lines (LCLs) and amplicons were observed for seven primer pairs, five of which are shown in the supplementary Fig. 1. These amplicons correspond to the products from four recognized exons and one novel candidate exon referred to as exon 2.2 in intron 2. Postmortem brain samples (occipital lobe) from five schizophrenic patients were obtained from the Postmortem Brain Bank of Fukushima for Psychiatric Research. The expression of the variant was also observed in postmortem brain samples from schizophrenic patients (Supplementary Fig. 1). This variant is predicted to generate an immature 88 amino acid protein which has N-terminal zinc finger motif only by making stop codon immediately after the end of exon 2. The Expression levels of the newly identified *ZNF804A* variant and major recognized transcript were much higher in LCLs than in postmortem brain samples and further expression analyses was performed using LCLs.

The expression levels of the newly identified *ZNF804A* variant and major recognized transcript in the LCLs from forty-five schizophrenic patients and forty-five controls (Supplementary Table 1) were measured by quantitative real-time PCR assay as previously described (Hashimoto et al., 2004). The subjects included in this study were the same subjects as previously described (Yamamori et al., 2011). Because the expression level of the newly identified variant transcript in LCLs was low for the quantitative assay, semi-quantitative expression analysis was performed. We divided schizophrenic patients and controls into two categories, based on the presence (detected) or absence (not-detected) of expression. In the detected samples, amplification was observed before thirty-five cycles, and in the not-detected samples, amplification was observed after thirty-five cycles or not observed until forty cycles. Significantly fewer schizophrenic patients than controls expressed the variant transcript (Table 1, chi-square test; $\chi^2 = 7.90$, $p = 0.0049$). There was no significant difference between the forty-five schizophrenic patients and the forty-five controls with respect to the expression of the major recognized *ZNF804A* transcript (all p values>0.1).

In conclusion, we have identified a novel transcriptional variant of *ZNF804A* in human LCLs and postmortem brain samples. We found that significantly fewer schizophrenic patients than controls expressed the variant, as demonstrated in cultured LCLs. Because this variant is more expressed in peripheral blood than in brain, this variant might be used as a peripheral disease marker of schizophrenia. The identification of this *ZNF804A* transcriptional variant might give better characterization of this gene in the pathophysiology of schizophrenia. The regulation of the splicing of this variant could represent a new therapeutic target.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.schres.2012.08.015.

Table 1
Semi-quantitative analysis of the exon2.2 containing variant expression.

Diagnosis	Detected	Not-detected	Chi-square	*p*-value
Control	34	11	7.90	<u>0.0049</u> *
Schizophrenic patients	21	24		

* Significant *p*-value (<0.05) is underlined.

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Evaluation of *CNTNAP2* gene polymorphisms for exfoliation syndrome in Japanese

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Purpose: To investigate the contactin-associated protein-like 2 (*CNTNAP2*) gene for single-nucleotide polymorphisms (SNPs) in Japanese patients with the exfoliation syndrome (XFS).
Methods: One hundred and eight unrelated Japanese patients with the XFS, and 199 normal controls were studied. Genomic DNA was extracted from the leukocytes of the peripheral blood, and 8 SNPs, rs826802, rs1404699, rs7803992, rs700308, rs4725736, rs2107856, rs2141388, and rs6970064, were amplified by polymerase chain reaction (PCR), directly sequenced, and genotyped.
Results: The allele frequencies of rs1404699 (p=8.57XE-3, odds ratio (OR)=1.59, 95% confidential intervals (CI); 1,12–2.24) and rs7803992 (p=5.43XE-4, OR=1.86, 95% CI; 1.31–2.65) were statistically significantly different between XFS and controls. In addition, there were significant differences in these genotype frequencies (p=0.0197 and 1.75XE-3). The allele and the genotype frequencies of rs2107856 and rs2141388, which were statistically significant SNPs in an earlier study, were not significantly different.
Conclusions: The variants, rs1404699 and rs7803992, of *CNTNAP2* should be associated with XFS in the Japanese population.

The exfoliation syndrome (XFS; OMIM 177650) is a generalized disorder of the extracellular matrix and is characterized clinically by the pathological accumulation of abnormal fibrillar material in the anterior segment of the eye [1-3]. This predisposes the eye to glaucomatous optic neuropathy. The XFS has also been associated with lens zonule weakness, severe chronic secondary open-angle glaucoma, cataract formation, and also a spectrum of other serious spontaneous and surgical intraocular complications.

The prevalence of XFS varies markedly between populations being highest in Scandinavian countries, while the Anglo-Celtic Caucasians have a markedly lower prevalence [4-7]. The incidence increases with age and is highest in the age group between 70 and 80 years [5]. The prevalence of XFS in Japan was reported to be 1.1% in one study [8] and 4.8% in another study [9].

Thorleifsson et al. [10] found a strong association between single-nucleotide polymorphisms (SNPs) in the lysyl oxidase–like 1 (*LOXL1*) gene and XFS in the Swedish and Icelandic populations using a genome-wide association study (GWAS). This association was replicated in the United States of America [11-13] and also in other populations [14-23].

LOXL1 is a member of the lysyl oxidase family of proteins that catalyzes the oxidative deamination of lysine residues of tropoelastin [24]. The homeostasis of elastic fibers requires the lysyl oxidase-like 1 protein [25], and *LOXL1* plays an important role in elastogenesis. Thus, it is quite possible that defects in *LOXL1* can cause features of XFS that result from an aberrant production of elastin and accumulation of fibrillar materials in the anterior segment of the eye.

A GWAS was recently performed using a DNA-pooling approach, and a single genotype at the contactin-associated protein-like 2 (*CNTNAP2*) locus had significant associations between XFS and exfoliation glaucoma and two SNPs (rs2107856 and rs2141388). These findings were confirmed in an independent German cohort but not in an Italian cohort [26]. *CNTNAP2* is a large gene spanning 2.3 mb of DNA on chromosome 7 and has 24 exons, and codes for the contactin-associated protein-like 2 (*CNTNAP2,* also called Caspr2). CNTNAP2 is member of the neurexin superfamily [27,28] and is possibly involved in stabilizing the location of the potassium channels in the juxtaparanodal region of the neuron [27]. It has been suggested to be a candidate gene for various neuropsychiatric disorders, e.g., the cortical dysplasia-focal epilepsy syndrome [29] and Pitt-Hopkins-like mental retardation [30]. However, its exact function and regulation are not known.

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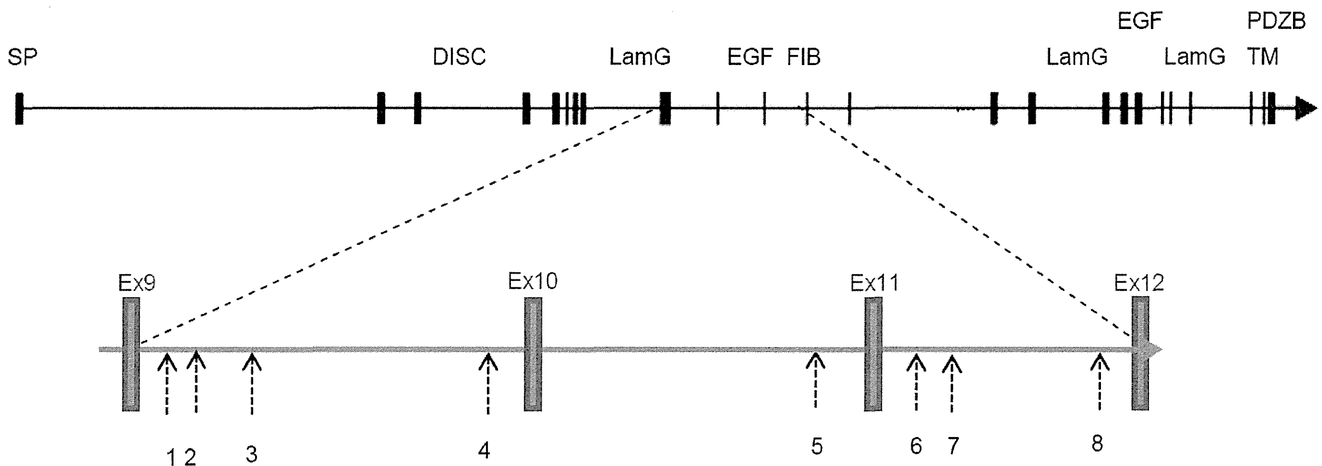

Figure 1. *CNTNAP2* gene structure. The 8 SNPs studied were; 1. rs826802, 2. rs144699, 3. rs7803992, 4. rs70308, 5. rs4725736, 6. rs2107856, 7. rs2141388, and 8. rs6970064. SP, signal peptide; DISC, discoidin-like domain; LamG, laminin-G domain; EGF, epidermal growth factor like domain; FIB, fibrinogen-like domain;TM, transmembrane region; PDZBD, PDZ-domain binding site.

The purpose of this study was to investigate 8 SNPs variations in *CNTNAP2* in Japanese patients with the XFS.

METHODS

One hundred and eight unrelated Japanese patients with XFS (mean age 73.61±6.75 years; 57 men, 51 women) and 199 controls (mean age 69.7±11.3 years; 101 men, 98 women) were studied. The controls were matched by age and gender. The XFS group included 85 exfoliation glaucoma (XFG) patients. They were examined at the ophthalmic clinic of the Tohoku University Hospital, Sendai, Japan, and the Ehime University Hospital, Ehime, Japan. The purpose and procedures were explained to all patients, and an informed consent was obtained. This study was approved by the Institutional Review Boards of the Tohoku University and Ehime University, and the procedures used conformed to the tenets of the Declaration of Helsinki.

Routine ophthalmic examinations were performed on all patients. The criteria used to classify a patient as having XFS was an open anterior chamber angle with accumulation of abnormal fibrillar material in the anterior segment of the eye. In addition, three other criteria for XFG had to be met: 1) applanation intraocular pressure (IOP) >22 mmHg in each eye; 2) glaucomatous cupping in each eye including a cup-to-disc ratio >0.7; and 3) visual field defects determined by Goldmann perimetry and/or Humphrey field analyzer consistent with the glaucomatous cupping in at least one eye. The control subjects had the following characteristics: 1) IOP less than 22 mmHg; 2) normal optic discs; and 3) no family history of glaucoma.

Genomic DNA was extracted from the leukocytes of peripheral blood and purified with the Qiagen QIAamp Blood Kit (Qiagen, Valencia, CA). Genomic DNA was extracted from the leukocytes of the peripheral blood, and the 6 SNPs, rs1404699, rs700308, rs4725736, rs2107856, rs2141388, and rs6970064, were chosen from the earlier studies. Two newly identified SNPs, rs826802 and rs7803992, were designed around intron 9 of the gene. The *CNTNAP2* gene structure with the location of the 8 SNPs is shown in Figure 1. They were amplified by polymerase chain reaction (PCR), directly sequenced, and genotyped. The amplifications were performed at 60 °C annealing temperature. The PCR fragments were purified with ExoSAP-IT (USB, Cleveland, OH), sequenced by the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA) by an automated DNA sequencer (ABI PRISM™ 3100 Genetic Analyzer, Perkin-Elmer). The allele frequencies, genotypes, and haplotypes of the *CNTNAP2* SNPs were determined.

Statistical analysis: The significance of associations between the phenotype and SNPs were determined by contingency table analysis using chi-square or Fisher's exact test. The odds ratios, approximating to relative risks, were calculated as a measure of the association between the *CNTNAP2* allele frequency and the phenotype. For each odds ratio, the 95% confidence intervals were calculated. The inferred haplotypes, quantified between all pairs of biallelic loci, were estimated using the SNPAlyze program version 7.0 (Dynacom, Yokohama, Japan). Additionally, a permutation test was performed to test the deviations of allelic frequencies of the SNPs and haplotypes. The Hardy–Weinberg equilibrium was analyzed using gene frequencies obtained by simple gene counting and the chi-square test with Yates' correction for comparing observed and expected values.

RESULTS

The allele frequencies and genotypes of the 8 *CNTNAP2* SNPs, rs826802, rs1404699, rs7803992, rs700308, rs4725736, rs2107856, rs2141388, and rs6970064, were determined in the XFS patients.

Table 1. *CNTNAP2* allele frequencies in patients with exfoliation syndrome and in controls in Japanese.

dbSNP	Allele	MAF in this study			MAF in previous study*		
		XFS (n=108) XFG (n=85)	Control (n=199)	p-value	XFS (n=770)	Control (n=444)	p-value
rs826802	T	0.435 0.429	0.372	0.0884 0.0198	N/A	N/A	N/A
rs1404699	T	0.412 0.388	0.307	8.57XE-3 0.0581	0.445	0.397	0.0225
rs7803992	G	0.394 0.359	0.259	5.43XE-4 0.016	N/A	N/A	N/A
rs700308	A	0.407 0.412	0.432	0.553 0.653	0.138	0.103	0.0117
rs4725736	A[1]	0.472 0.441	0.402	0.093 0.385	0.585	0.637	0.0121
rs2107856	G[2]	0.450 0.441	0.432	0.687 0.843	0.709	0.776	0.0003
rs2141388	C[3]	0.444 0.441	0.437	0.863 0.930	0.709	0.777	0.0002
rs6970064	A[4]	0.181 0.182	0.123	0.0524 0.0631	0.418	0.463	0.0306

*reported by Krumbiegel et al. [26]. MAF; minor allele frequency, XFS; Exfoliation Syndrome, XFG; Exfoliation Glaucoma. The significance of the association was determined by a contingency table analysis using the χ^2 test. Upper columns show XFS data, and lower columns show XFG data. 1. There was a difference between the Caucasian and Japanese. Minor allele in previous study was C. 2. Minor Allele in previous study was T. 3. Minor Allele in previous study was T. 4. Minor Allele in previous study was G.

Distribution of CNTNAP2 variants in XFS patients and control subjects: The allele frequencies of rs1404699 (p=8.57XE-3, odd ratio (OR)=1.59, 95% confidential intervals (CI); 1,12–2.24) and rs7803992 (p=5.43XE-4, OR=1.86, 95% CI; 1.31–2.65) were statistically significant between the XFS group and the control group (Table 1). There were also significant differences in these genotype frequencies (p=0.0197 and 1.75XE-3; Table 2). Only the rs7803992 was significantly different between the XFG group and the control group (p=0.016; Table 1). Compared with the allele frequencies of rs2107856 and rs2141388 statistically significant SNPs in a previous study [26], our results showed no significantly difference between the XFS group and the control group (Table 1). Also, the genotype frequencies of those in *CNTNAP2* were not significantly higher in the two groups than in the control group (Table 2).

The genotype frequencies of rs700308 and rs6970064 were statistically significant (p=0.0402 and 0.0315), but the allele frequencies were not significantly different (p=0.553 and 0.0524) between the XFS group and the control group. All SNPs adhered to the Hardy–Weinberg expectations (p>0.05).

Haplotype analyses at CNTNAP2 LD block in the Japanese population: The inferred haplotypes between all pairs of biallelic loci on rs1404699 and rs7803992 were estimated (Table 3). The haplotype-based associations were tested with a 1,000 iterated permutation test. Four major haplotypes; C-A, T-G, T-A, C-G (each frequency >5%) were found in the XFS subjects and normal controls. T-G was over-represented in the XFS subjects with a highly significant difference in frequency compared to the control group (0.327 versus 0.202; p=0.003). In addition, the C-A haplotype was significantly less represented in the XFS subjects (0.522 versus 0.637; p=0.003).

Two locus analyses: A strong correlation between variants in *LOXL1* and XFS has been reported [10], *LOXL1* common risk haplotype is T-G (the major alleles T of the coding SNPs rs1048661 and major alleles G of the coding SNPs rs3825942) in Japan, instead of G-G in Europeans. We investigated how the variants in *LOXL1* gene were related to *CNTNAP2*. We sorted our subjects for carriers and non-carriers of the risk haplotype T-G (Table 4). The numbers in the subgroup of non-T-G carriers was quite small, and there was no association of *CNTNAP2* SNPs with the *LOXL1* non-risk haplotype (Table 4; p=0.53 and 0.69, respectively). Besides the subgroups risk of T-G carriers, there was no significant association (Table 4; p=0.072 and 0.084, respectively).

DISCUSSION

Association between CNTNAP2 and XFS: We compared the findings of Krumbiegel and colleagues [26] to that obtained

Table 2. Frequency of genotypes *CNTNAP2* gene in patients with exfoliation syndrome and in controls in Japanese.

dbSNP	Allele	XFG (n=108)	p value*	XFG (n=85)	p value*	Control (n=199)
rs826802	G/G	36 (33.3)	0.224	27 (31.8)	0.425	77 (38.7)
	G/T	50 (46.3)		43 (50.6)		96 (48.2)
	T/T	22 (20.4)		15 (17.6)		26 (13.1)
rs1404699	C/C	38 (35.2)	0.0197	32 (37.6)	0.121	93 (46.7)
	C/T	51 (47.2)		40 (47.1)		90 (45.2)
	T/T	19 (17.6)		13 (15.3)		16 (8.1)
rs7803992	A/A	38 (35.2)	1.75XE-3	31 (36.5)	6.22XE-3	112 (56.3)
	A/G	55 (50.9)		47 (55.3)		71 (35.7)
	G/G	15 (13.9)		7 (8.2)		16 (8.0)
rs700308	G/G	45 (41.7)	0.0402	33 (38.8)	0.282	63 (31.7)
	G/A	38 (35.2)		34 (40.0)		100 (50.3)
	A/A	25 (23.1)		18 (21.2)		36 (18.1)
rs4725736	C/C	34 (31.5)	0.0659	27 (31.8)	0.385	69 (34.7)
	C/A	46 (42.6)		41 (48.2)		100 (50.3)
	A/A	28 (25.9)		17 (20.0)		30 (15.1)
rs2107856	T/T	39 (36.1)	0.091	29 (34.1)	0.541	63 (31.7)
	T/G	41 (38.0)		37 (43.5)		100 (50.3)
	G/G	28 (25.9)		19 (22.4)		36 (18.1)
rs2141388	T/T	39 (36.1)	0.100	29 (34.1)	0.470	61 (30.7)
	T/C	42 (38.9)		37 (43.5)		106 (53.3)
	C/C	27 (25.0)		19 (22.4)		32 (16.1)
rs6970064	G/G	74 (68.5)	0.0315	58 (68.2)	0.0345	151 (75.9)
	G/A	29 (26.9)		23 (27.1)		47 (23.6)
	A/A	5 (4.6)		4 (4.7)		1 (0.5)

XFS; Exfoliation Syndrome, XFG; Exfoliation Glaucoma. Data presented are number of patients, unless otherwise indicated. The significance of the association was determined by a contingency table analysis using the χ^2 test.

Table 3. Haplotype analysis with rs1404699 and rs7803992 in patients with exfoliation syndrome and in controls in Japanese.

Haplotype	Overall	XFS	Control	p-value
C-A	0.5966	0.5217	0.637	0.003
T-G	0.2464	0.3273	0.2024	0.003
T-A	0.0972	0.0847	0.1041	0.489
C-G	0.0597	0.0662	0.0564	0.708

XFS; Exfoliation Syndrome. The significance of the association was determined by a contingency table analysis using the χ^2 test.

from our Japanese cohorts. We found that two SNPs in *CNTNAP2* were strongly associated with XFS. In an earlier study [26], the frequencies of rs2107856 and rs2141388 SNPs in *CNTNAP2* were confirmed in an independent German cohort but not in the Italian cohort. Although neither the rs2107856 or rs2141388 SNPs was significant in our study, rs1404699 and nearby rs7803992 were statistically significant between the XFS group and the control group. Thus, it is possible that *CNTNAP2* could be associated with XFS. Like other susceptible variants of a complex disease, the OR in the earlier study was modest at about 1.4. In our study, the highest OR was 1.86 for rs7803992. This difference can be explained by racial differences and heterogeneities. Because the number of XFG patients was small, it seemed that the statistical power was weak.

No association between CNTNAP2 and LOXL1 in Japanese: Because a strong association of variants in *LOXL1* in XFS has been reported [10], we compared the allele frequencies at *CNTNAP2* locus based on the presence of the identified Japanese *LOXL1* common risk haplotype T-G. We found no significant association to allele T of the rs1404699 and rs7803992 SNPs of *CNTNAP2* in carriers of *LOXL1* the risk T-G haplotype (Table 4), and also in non-risk haplotypes. These findings suggest that there is no association between

TABLE 4. ASSOCIATION OF *LOXL1* COMMON-RISK HAPLOTYPE T-G, COMPOSED OF RS1048661 AND RS3825942, WITH *CNTNAP2* SNPS RS1404699 AND RS7803992.

LOXL1 haplotype	Cases	Control	*CNTNAP2* SNP	Cases MAF	Control MAF	p-value
T-G carriers	103	52	rs1404699	0.413	0.308	0.072
			rs7803992	0.398	0.298	0.084
Non T-G carriers	5	147	rs1404699	0.400	0.306	0.53
			rs7803992	0.300	0.245	0.69

LOXL1; lysyl oxidase–like 1, MAF; minor allele frequency.

CNTNAP2 and *LOXL1* in the Japanese. This would then mean that a *LOXL1*-independent mechanism is involved in *CNTNAP2* function.

In a molecular genetic study, the most promising loci at 18q12.1–21.33 and 2q, 17p, and 19q have been proposed to be the susceptible loci in a Finnish population in an autosomal dominant mode of inheritance [31]. In a microarray study, 23 genes with different expression patterns in the anterior segment tissues of eyes with XFS have recently been reported [32]. This strongly suggests that an unidentified gene or environmental factors independent of the *LOXL1* gene strongly influence the phenotypic expression of the XFS.

CNTNAP2 function and molecular genetics: CNTNAP2 is a single-pass transmembrane protein with multiple protein-interaction motifs typical of the neurexins, e.g., epidermal growth factor repeats, laminin globular domains, and F5/8-type C domain, and a putative PDZ-binding site. Poliak et al. [33] reported that CNTNAP2 is necessary to maintain the potassium channels at the juxtaparanodal region in myelinated axons. The SNPs we selected were located in introns 9, 10, and 11 (Figure 1), while several SNPs related to autism were located in intron 2 [34] and intron 13 [35]. The cortical dysplasia-focal epilepsy syndrome is caused by a single nucleotide deletion in Exon 22. Therefore, it seems that our SNPs have nearly no correlation with neuropsychiatric disorders. The rs1404699 and rs7803992 SNPs are located in intron 9 of the *CNTNAP2* gene. Exon 9, nearby to intron 9, codes for the laminin globular domain, which contains proteins that play a wide variety of roles in cell adhesion, signaling, migration, assembly, and differentiation of cells. We suggest that alterations in membrane stabilization may contribute to the abnormal exfoliation matrix processes, which are associated with cell-surface irregularities, basement membrane destruction and degenerative alterations.

Conclusions: Identification of XFS-associated SNPs that will allow early detection of an increase in the IOP, or even before an elevation of IOP, would be desirable. Our findings showed that variants of *CNTNAP2* rs1404699 and rs7803992 are significantly associated with XFS in the Japanese population. More studies of the functions and genotype-phenotype correlation of *CNTNAP2* are required to determine the pathophysiology of XFS. In addition, further studies searching for secondary genetic and environmental factors that contribute to XFS is required to gain better understanding of the complex etiology of XFS.

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Association of Toll-like Receptor 4 Gene Polymorphisms in Japanese Subjects With Primary Open-Angle, Normal-Tension, and Exfoliation Glaucoma

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• PURPOSE: To determine whether polymorphisms in the Toll-like receptor 4 (*TLR4*) gene are associated with primary open-angle glaucoma (POAG), normal-tension glaucoma (NTG), and exfoliation glaucoma (XFG) in Japanese individuals.
• DESIGN: Genetic association study.
• METHODS: SETTING: Multicenter study. STUDY POPULATION: One hundred eighty-four unrelated Japanese patients with POAG, 365 unrelated patients with NTG, and 109 unrelated patients with XFG from 5 hospitals. PROCEDURES: Genomic DNA was extracted from leukocytes of the peripheral blood, and 8 polymorphisms in the *TLR4* genes were amplified by polymerase chain reaction (PCR) and directly sequenced. Allele and genotype frequencies and the inferred haplotypes were estimated. MAIN OUTCOME MEASURES: Differences in allele and genotype frequencies and haplotypes between subjects with POAG, NTG, and XFG.
• RESULTS: The allele frequency of rs2149356 of the *TLR4* gene in the POAG, NTG, and XFG groups was the most significantly different from that of the control group (minor allele frequency 0.446, 0.395, 0.404, vs 0.308; *P* = .000058, *P* = .0030, and *P* = .015). The allele frequencies of the 5 *TLR4* SNPs were higher in all of the glaucoma groups than that in the control group. The statistics of genotypes of *TLR4* were approximately the same for all allele frequencies. The haplotypic frequencies with Tag SNPs studied earlier showed that only POAG was statistically significant. Other haplotypes, such as rs10759930, rs1927914, rs1927911, and rs2149356, had higher statistical significance (overall *P* = .00078 in POAG, overall *P* = .018 in NTG, and overall *P* = .014 in XFG).
• CONCLUSIONS: This study demonstrated that TLR4 polymorphisms are associated with NTG in the Japanese, and they also play a role in the pathogenesis of POAG and XFG. (Am J Ophthalmol 2012;154:825–832. © 2012 by Elsevier Inc. All rights reserved.)

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GLAUCOMA IS A COMPLEX, HETEROGENEOUS DISease characterized by a progressive degeneration of the axons of the retinal ganglion cells (RGCs). It is the second-highest cause of blindness worldwide, affecting approximately 70 million people.[1] Primary open-angle glaucoma (POAG), the most common type of glaucoma, is associated with an elevated intraocular pressure (IOP). However, there are some POAG patients who have normal IOPs of <22 mm Hg, and they are classified as having normal-tension glaucoma (NTG).[2] The prevalence of NTG is higher among the Japanese than among whites.[3,4] POAG and NTG appear to be a continuum of glaucoma with overlapping causative factors in addition to the IOPs. It is believed that the mechanism shifts from predominantly elevated IOP in POAG to that of independent factors in eyes with NTG. Although the precise molecular basis of POAG and NTG has not been determined, the glaucoma in patients with POAG and NTG is probably heterogeneous and is caused by the interaction of multiple genes and environmental factors.

Several genetic loci that contribute to the susceptibility of eyes to POAG/NTG have been identified, and at least 15 loci, from GLC1A to GLC1O, have been linked to POAG.[5] Three genes have been identified worldwide: the myocilin (*MYOC*) gene,[6] the optineurin (*OPTN*) gene,[7] and the WD repeat domain 36 (*WDR36*) gene,[8] with a

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TABLE 1. Toll-like Receptor 4 Single-Nucleotide Polymorphisms Allele Frequencies in Patients With Primary Open-Angle, Normal-Tension, and Exfoliation Glaucoma and in Controls in Japanese

	This Study							Previous Study		
SNP	POAG (n = 184)	P Value	NTG (n = 365)	P Value	XFG (n = 109)	P Value	Control (n = 216)	NTG (n = 250)	Control (n = 318)	P Value
rs10759930	0.454	.00022	0.396	.018	0.404	.052	0.326	0.422	0.347	.010
rs1927914	0.457	.00012	0.400	.0096	0.417	.019	0.324	0.420	0.347	.012
rs1927911	0.448	.000076	0.392	.0066	0.404	.021	0.313	0.408	0.344	.028
rs12377632	0.467	.000014	0.364	.10	0.399	.038	0.317	0.412	0.343	.017
rs2149356	0.446	.000058	0.395	.0030	0.404	.015	0.308	0.408	0.343	.024
rs11536889	0.258	.471	0.264	.28	0.248	.74	0.236	0.232	0.256	.35
rs7037117	0.223	.045	0.221	.027	0.225	.072	0.167	0.252	0.182	.0044
rs7045953	0.090	.112	0.074	.37	0.078	.39	0.060	0.098	0.078	.21

NTG = normal-tension glaucoma; POAG = primary open-angle glaucoma; SNP = single nucleotide polymorphisms; XFG = exfoliation glaucoma.
The significance of the association was determined the χ^2 test.

diverse mutation spectrum. Other studies have reported that the *OPTN* and *WDR36* variants do not predispose individuals to POAG and NTG.[9,10] The pseudoexfoliation syndrome (XFS; OMIM:177650) is a generalized disorder of the extracellular matrix and is characterized by the pathologic accumulation of abnormal fibrillar material in the anterior segment of the eye.[11] A recent genome-wide association study (GWAS) showed a strong association between single nucleotide polymorphisms (SNPs) in the *lysyl oxidase–like 1* (*LOXL1*) gene and XFS in the Swedish and Icelandic populations.[12] The association between the *LOXL1* gene and XFS and exfoliation glaucoma (XFG) has also been found in the Japanese population.[13,14] XFG is a common identifiable cause of open-angle glaucoma worldwide, affecting an estimated 60 to 70 million people.[15] Inflammation and oxidative stress may be a modifiable risk factor in the management of patients with XFS and XFG.

An IOP elevation is considered a major risk factor for glaucoma, but an elevated IOP is not associated with glaucomatous characteristics in all glaucoma patients. Other possible pathogenetic factors, such as autoimmune mechanisms including apoptosis, may be involved in some patients with glaucoma.[16] Wax and associates were the first to report an elevation of antibody titers in patients with NTG (eg, an increase in the level of heat shock protein 60 [HSP60] antibodies)[17] and also higher levels of antibodies against small HSPs (eg, [alpha] A-crystalline, [alpha] B-crystalline, and HSP27) in NTG patients.[18] A number of other autoantibodies against retinal or optic nerve proteins have been identified in many NTG patients. Because some glaucoma patients have increased titers of serum antibodies against these proteins, the degeneration of the RGCs in glaucoma may be attributable to a failure of immune regulation of both pro-apoptotic and protective pathways.

The Toll-like receptor (*TLR*) family, an anchor of innate immunity system, recognizes external ligands and differentiates self from nonself proteins. The ability of a tissue to recognize pathogens is mediated by a set of receptors that are referred to as pattern-recognition receptors (PRRs). To date, 13 members of the *TLR* family have been identified in mammals. *TLR4* is a transmembrane receptor that mediates immune responses to exogenous and endogenous ligands, and not only recognizes bacterial lipopolysaccharides (LPSs) but is also activated by endogenous ligands such as heat shock proteins (HSPs).[19] Toll-like receptors (TLRs) can also recognize endogenous ligands that are induced during inflammatory responses.[20] Recently, the *TLR4* (OMIM 603030) gene was implicated in NTG in the Japanese population,[21] but not in the South Korean population.[22]

Glaucoma is a neurodegenerative disease, but the mechanisms causing the RGC loss are still undetermined. Several studies have pointed to a possible involvement of autoimmune mechanisms in the pathogenesis of glaucoma, especially NTG. On the other hand, it is believed that the mechanisms shift from predominantly elevated IOP in the POAG and XFG to other factors such as autoimmune reactions in NTG.

Thus, the purpose of this study was to determine whether mutations in the *TLR4* gene contributed to POAG, NTG, and XFG in unrelated Japanese patients.

PATIENTS AND METHODS

• **PATIENTS:** One hundred eighty-four unrelated Japanese patients with POAG (119 men and 65 women; mean age 64.6 ± 14.3 years), 365 unrelated Japanese patients with NTG (171 men and 194 women; mean age 58.6 ± 13.1 years), and 109 unrelated Japanese patients with XFG (57 men and 52 women; mean age 77.6 ± 6.2 years) were studied. They were diagnosed with glaucoma in the ophthalmological clinic of the Tohoku University Hospital, Sendai; Niigata University Hospital, Niigata; Tokyo Met-

TABLE 2. Frequency of Genotypes of Toll-like Receptor 4 Gene in Patients with Primary Open-Angle, Normal-Tension, and Exfoliation Glaucoma and in Controls in Japanese[a]

	This Study				Previous Study	
	POAG (n = 184)	NTG (n = 365)	XFG (n = 109)	Control (n = 216)	NTG (n = 250)	Control (n = 318)
rs10759930 T/C						
T/T	49 (26.6%)	141 (38.6%)	40 (36.7%)	103 (47.7%)	81 (32.4%)	137 (43.1)
T/C	103 (56.0%)	159 (43.6%)	50 (45.9%)	85 (39.4%)	127 (50.8%)	141 (44.3%)
C/C	32 (17.4%)	65 (17.8%)	19 (17.4%)	28 (12.9%)	42 (16.8%)	40 (12.6%)
P value[b]	.000085	.074	.16		.028	
P value[c] (dominant)	.000015	.032	.060			
rs1927914 A/G						
A/A	47 (25.5%)	137 (37.5%)	38 (34.9%)	105 (48.6%)	82 (32.8%)	137 (43.1%)
A/G	106 (57.6%)	164 (44.9%)	51 (46.8%)	82 (38.0%)	126 (50.4%)	141 (44.3%)
G/G	31 (16.9%)	64 (17.5%)	20 (18.3%)	29 (13.4%)	42 (16.8%)	40 (12.6%)
P value[b]	.000011	.030	.059		.036	
P value[c] (dominant)	.0000022	.0089	.018			
rs1927911 G/A						
G/G	51 (27.7%)	139 (38.1%)	40 (36.7%)	106 (49.1%)	87 (34.8%)	141 (44.3%)
G/A	101 (54.9%)	166 (45.5%)	50 (45.9%)	85 (39.4%)	122 (48.8%)	135 (42.5%)
A/A	32 (17.4%)	60 (16.4%)	19 (17.4%)	25 (11.5%)	41 (16.4%)	42 (13.2%)
P value[b]	.000072	.027	.080		.067	
P value[c] (dominant)	.000013	.0095	.034			
rs12377632 C/T						
C/C	53 (28.8%)	137 (37.5%)	41 (37.6%)	104 (48.1%)	86 (34.4%)	140 (44.0%)
C/T	90 (48.9%)	190 (52.1%)	49 (45.0%)	87 (40.3%)	122 (48.8%)	138 (43.4%)
T/T	41 (22.3%)	38 (10.4%)	19 (17.4%)	25 (11.6%)	42 (16.8%)	40 (12.6%)
P value[b]	.00012	.020	.13		.053	
P value[c] (dominant)	.000079	.012	.071			
rs2149356 G/T						
G/G	53 (28.8%)	139 (38.1%)	40 (36.7%)	107 (49.5%)	87 (34.8%)	140 (44.0%)
G/T	98 (53.3%)	164 (44.9%)	50 (45.9%)	85 (39.4%)	122 (48.8%)	138 (43.4%)
T/T	33 (17.9%)	62 (17.0%)	19 (17.4%)	24 (11.1%)	41 (16.4%)	40 (12.6%)
P value[b]	.00012	.015	.062		.070	
P value[c] (dominant)	.000025	.0069	.028			
rs11536889 G/C						
G/G	95 (51.6%)	196 (53.7%)	62 (56.9%)	127 (58.8%)	146 (58.4%)	177 (55.6%)
G/C	83 (45.1%)	145 (39.7%)	40 (36.7%)	76 (35.2%)	93 (37.2%)	119 (37.4%)
C/C	6 (3.3%)	24 (6.6%)	7 (6.4%)	13 (6.0%)	11 (4.4%)	22 (6.9%)
P value[b]	.083	.49	.95		.42	
P value[c] (dominant)	.15	.23	.74			
rs7037117 A/G						
A/A	111 (60.3%)	222 (60.8%)	65 (59.6%)	153 (70.8%)	138 (55.2%)	213 (67.0%)
A/G	64 (34.8%)	125 (34.2%)	39 (35.8%)	54 (25.0%)	98 (39.2%)	94 (29.6%)
G/G	9 (4.9%)	18 (4.9%)	5 (4.6%)	9 (4.2%)	14 (5.6%)	11 (3.5%)
P value[b]	.082	.049	.12		.015	
P value[c] (dominant)	.027	.015	.043			
rs7045953 A/G						
A/A	152 (82.6%)	313 (85.8%)	93 (85.3%)	191 (88.4%)	203 (81.2%)	269 (84.6%)
A/G	31 (16.8%)	50 (13.7%)	15 (13.8%)	24 (11.1%)	45 (18.0%)	49 (15.4%)
G/G	1 (0.6%)	2 (0.5%)	1 (0.9%)	1 (0.5%)	2 (0.8%)	0 (0.0%)
P value[b]	.25	.66	.69		.19	
P value[c] (dominant)	.097	.36	.43			

NTG = normal-tension glaucoma; POAG = primary open-angle glaucoma; XFG = exfoliation glaucoma.
[a]Data presented are number of patients, unless otherwise indicated.
[b]Significance of the association determined by a contingency table analysis using the χ^2 test.
[c]Significance by a dominant model.

ropolitan Police Hospital, Tokyo; Ideta Eye Hospital, Kumamoto; and Ehime University Hospital, Ehime, Japan. All of the subjects were enrolled from 2004 through 2010.

Routine ophthalmic examinations were performed on all patients. The criteria for classifying a patient as having POAG were: applanation IOP >22 mm Hg in each eye; glaucomatous cupping including cup-to-disc ratio >0.7 in each eye; visual field defects determined by Goldmann perimetry and/or Humphrey visual field analysis consistent with the glaucomatous cupping in at least 1 eye; and an open anterior chamber angle. Patients with glaucoma of secondary causes (eg, trauma-, uveitis-, or steroid-induced) were excluded. The criteria for NTG were applanation IOP <22 mm Hg in both eyes at each examination and the same characteristics as that of the POAG group. The IOP used for the statistical analyses was the clinic-based value. We checked the IOP in at least 3 visits and the measurements were made during the daylight hours. Patients were excluded if the IOP was 22 mm Hg or more for any of the measurements. The criteria for XFG were an open anterior chamber angle with accumulation of abnormal fibrillar material in the anterior segment of the eye and the same characteristics as the POAG group.

The control subjects (116 men and 100 women; age, 69.7 ± 11.3 years) had the following characteristics: IOP <22 mm Hg, normal optic discs, and no family history of glaucoma. To decrease the chance of studying individuals with presymptomatic glaucoma, we studied individuals who were older than 60 years in this group.

• **SAMPLE PREPARATION AND MUTATION SCREENING:** Genomic DNA was extracted from leukocytes of peripheral blood and purified with the Qiagen QIAamp DNA Blood Kit (Qiagen, Valencia, California, USA). Eight SNPs were amplified by polymerase chain reaction (PCR) using 0.5 μM intronic primers, 0.2 mM dNTPs, and 0.5 U Ex Taq polymerase (Takara, Shiga, Japan) with 30 ng template DNA in the amplification mixture (25 μL). The annealing temperature and sequence of primer set are given in the Supplemental Table (available at AJO.com).

Oligonucleotides for the amplification and sequencing were selected using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/, provided in the public domain by the Massachusetts Institute of Technology, Cambridge, Massachusetts, USA). The PCR fragments were purified with ExoSAP-IT (USB, Cleveland, Ohio, USA), sequenced by the BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Foster City, California, USA) on an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer, Perkin-Elmer).

• **STATISTICAL ANALYSES:** Differences in the genotype frequencies among the cases and controls were tested by Fisher exact test or χ^2 depending on the cell counts. The inferred haplotypes and LD (linkage dysequilibrium), expressed as D',[23] quantified between all pairs of biallelic loci, were estimated using the SNPAlyze program version 5.0.3 (Dynacom, Yokohama, Japan). The significance of an association was determined by contingency table analysis using χ^2 or Fisher exact tests. The Hardy-Weinberg equilibrium was analyzed using gene frequencies obtained by simple gene counting and the χ^2 test with Yates' correction for comparing observed and expected values.

RESULTS

• **HAPLOTYPE BLOCK:** AII of the 8 SNPs in the *TLR4* gene were genotyped, and all were in Hardy-Weinberg equilibrium in the glaucoma cases and control subjects. All SNPs were located in 1 haplotype block, and the magnitude of the LD between each SNP was very high, with a pairwise D' of more than 0.90. However, rs11536889 had a pairwise D' less than 0.80.

• **ALLELE AND GENOTYPE FREQUENCIES IN *TLR4* VARIANTS DETECTED IN SUBJECTS:** The allele frequencies of the 8 SNPs in the glaucoma cases and control subjects are shown in Table 1. The frequencies of the minor alleles of all SNPs were higher in the glaucoma cases than in control subjects. In the POAG subjects, the allele frequencies of 6 SNPs (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, and rs7037117) were significantly different from the control group ($P < .05$). In addition, 5 SNPs (rs10759930, rs1927914, rs1927911, rs2149356, and rs7037117) in NTG subjects and 4 SNPs (rs1927914, rs1927911, rs12377632, and rs2149356) in XFG subjects were significantly different from that in the control group ($P < .05$; Table 1). Three SNPs, rs1927914, rs1927911, and rs2149356, were identical for the POAG, NTG, and XFG groups. Among these 3 SNPs, the minor allele of rs2149356, located in intron 2 of *TLR4*, conferred the highest increased risk of POAG ($P = .000058$, OR = 1.77, 95% CI = 1.31–2.39), NTG ($P = .0030$, OR = 1.51, 95% CI = 1.17–1.95), and XFG ($P = .015$, OR = 1.56, 95% CI = 1.11–2.20).

The genotype frequencies of 8 SNPs are shown in Table 2. The genotype frequency of 5 SNPs was significantly higher in the POAG and NTG subjects than in the controls, and none of the SNPs was significantly higher in the XFG subjects than in the control group ($P = .16$, $P = .059$, $P = .080$, $P = .13$, $P = .062$, $P = .95$, $P = .12$, $P = .69$, respectively; χ^2 test). Considering the dominant model, 4 SNPs in the XFG group were significant compared with the genotype frequencies of the control group. In POAG, NTG and XFG individuals bearing the minor allele of rs2149356 had the most significantly increased risk for glaucoma over that of control subjects ($P = .00014$, $P = .015$, $P = .062$, respectively).

• **HAPLOTYPE ANALYSIS:** The haplotype frequencies of the Tag SNPs (rs10759930, rs11536889, rs7037117, and