CLINICAL REPORTS

The patient, now a 13-year-old Japanese boy, was born as the second child of healthy nonconsanguineous parents. His mother had an operation for cervical incompetency during pregnancy. At 39 weeks of gestation, he was born by spontaneous delivery. His birth weight was 2,600 g (5th centile), length 49.5 cm (50th centile), and occipital frontal circumference (OFC) 33 cm (50th centile). Hypospadias and malformed ears were noted at birth.

At age 5 months, he presented with recurrent episodes of fever. Laboratory investigations revealed thrombocytopenia (platelet count, $36 \times 10^3/\mu l$). Bone marrow examination showed normal cellular elements with increasing megakaryocytes. The serum level of platelet associated immunoglobulin G was elevated at 160.0 ng/10^7 cells (normal range, 5–25 ng/10^7 cells). His condition was diagnosed as idiopathic thrombocytopenic purpura. The administration of corticosteroid and immunoglobulin infusion improved thrombocytopenia temporarily. However, thrombocytopenia occurred again at age 10 months and persisted up to the present time with occasionally subcutaneous hemorrhage (platelet count between 70 and $100 \times 10^3/\mu l$). He had recurrent respiratory infections with frequent admission in his childhood and school age. At age 10 years, he was first noted as having moderate neutropenia (absolute neutrophil count, $0.84 \times 10^3/\mu l$).

Developmental retardation was evident at age 5 months. He sat unsupported at 12 months, walked at 25 months, and spoke his first word at 24 months. A G-banding chromosomal analysis showed a normal karyotype. Metabolic investigations, cardiac ultrasonography, cranial magnetic resonance imaging, and electroencephalography all obtained normal findings. An ophthalmologic investigation showed mild myopia. An otological examination showed severe bilateral mixed hearing impairment. The Wechsler Intelligence Scale for Children at age 7 years showed mild intellectual disability.

When seen by us at age 13 years, his height was 149.5 cm (10th–25th centile), weight 37.6 kg (10th–25th centile), and OFC 55 cm (50th–75th centile). He had a long face, broad medial eyebrows, epicanthal folds, downslanting palpebral fissures, a flat nasal bridge, a high palate, a smooth philtrum, a full lower lip, cupped ears, short fifth fingers, hypospadias, mild digital joint contractures, and mild scoliosis (Fig. 1). He also showed severe

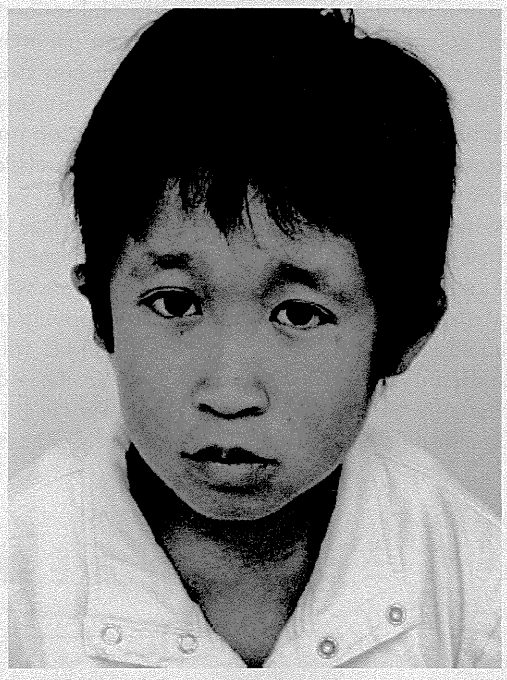

FIG. 1. A photogram of the patient at age 13-year old. A long face, broad medial eyebrows, epicanthal folds, down-slanting palpebral fissures, cupped ears, a flat nasal bridge, a smooth philtrum, and a full lower lip.

obstructive sleep apnea attributable to tonsillar hypertrophy. Hepatosplenomegaly was not observed. Though the hemoglobin level was normal at 13.3 g/dl, total white blood cell count was decreased at $1.9 \times 10^3/\mu l$, absolute neutrophil count was decreased at $0.43 \times 10^3/\mu l$, and platelet count was decreased at $68 \times 10^3/\mu l$. Serum levels of coagulation factors were all normal range. The serum immunoglobulin levels were normal. The results of the autoimmune screening were positive for anti-cardiolipin antibody (17 U/ml; normal range <10 U/ml) and negative for anti-double-stranded DNA antibody, antinuclear antibodies, anti-smith

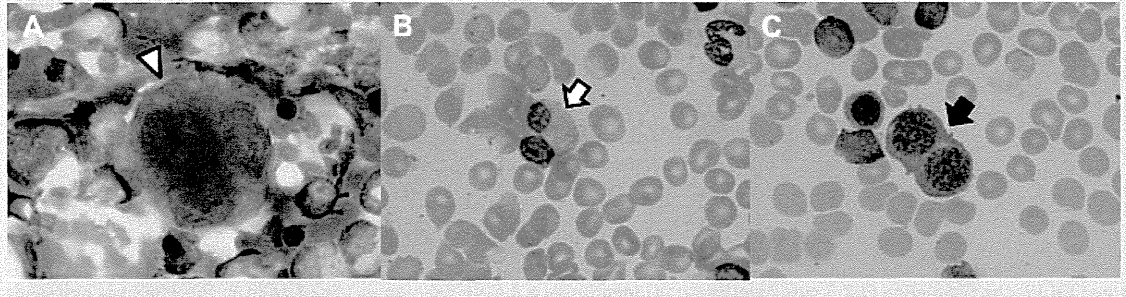

FIG. 2. Bone marrow findings of the present patient. A: Small megakaryocyte is noticeable (white arrow head). B: Pseudo-Pelger abnormality in neutrophil is shown (white arrow). C: Bilobed nuclei in erythroblast is noted (black arrow).

antibody, rheumatoid factor, antiplatelet antibody, platelet-associated immunoglobulin G, and antineutrophil antibody. Serological and polymerase chain reaction testing for parvovirus B19, cytomegalovirus, and Ebstein–Barr virus did not show an evidence of active infection. Chromosomal breakage with mitomycin C in peripheral blood lymphocytes was not increased. Monosomy 7 in bone marrow cells and telomere shortening in peripheral blood cells were not observed by cytogenetic analysis.

A bone marrow examination showed mild trilineage dysplasia with normal cellularity and increased number of megakaryocytes. Blast cell count was three percent. Megakaryocytes with single nuclei and noticeable small size, neutrophils with poor granulation and pseudo-Pelger abnormalities, and erythroblasts with binuclearity and mild hypoplasia were noted (Fig. 2A–C). According to the criteria by the fourth edition of the World Health Organization classification of hematopoietic and lymphoid neoplasms [Baumann et al., 2008], his condition was classified as childhood MDS.

MOLECULAR CYTOGENETIC INVESTIGATIONS

The study was conducted according to the Declaration of Helsinki and was approved by the Ethics Committee of Shinshu University School of Medicine, and informed consent was obtained from the parents of the patient.

DNA was extracted using the Gentra Puregene Blood Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Array CGH was performed using the CGX-3 cytogenetics arrays (Roche NimbleGen, Inc., Madison, WI). This platform included 134,829 oligonucleotide probes covering the whole genome at an average resolution of 35 kb as well as clinically significant regions at 10 kb. The procedures for DNA labeling, and hybridization

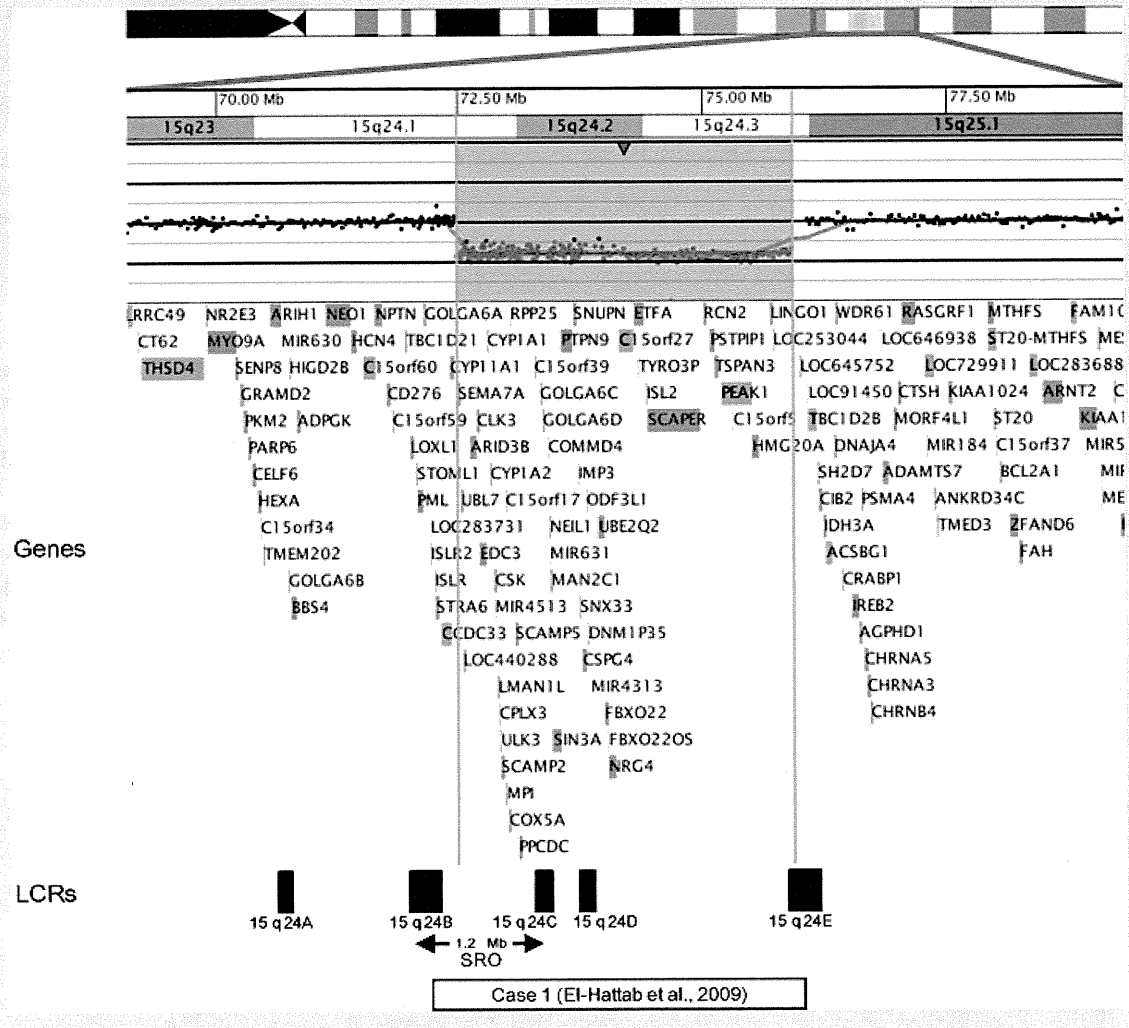

FIG. 3. Array CGH (Roche NimbleGen CGX-3 cytogenetics array) identifying a 3.4-Mb deletion at 15q24.1q24.3. The white box represent the deleted region in Case 1 (72.252–75.937 Mb) with acute lymphoblastic leukemia [El-Hattab et al., 2009]. The smallest region of overlap (SRO) by published patients and the five LCR regions represented by black boxes are shown.

were performed according to the manufacturer's instructions. The slides were scanned into image files using a NimbleGen MS 200 scanner. The array data analysis was performed using Genoglyphix Software (Signature Genomics Laboratories, Spokane, WA). Mapping data were analyzed on the Genoglyphix Genome Browser. All genomic locations correspond to NCBI build 36 (hg18). The array CGH analysis of the patient showed a deletion at chromosome region 15q24.1–15q24.3. The minimum size of the deletion was estimated as 3.44 Mb from the probe at 15q24.1 (chr15: 72,485,279 bp) to the probe at 15q24.3 (chr15: 75,921,984 bp) (Fig. 3).

To confirm array CGH findings, fluorescence in situ hybridization (FISH) analysis was performed on peripheral blood lymphocytes of the patient. BAC clones RP11-195A1 and RP11-91J9 were deleted and permitted to confirm the interstitial deletion on chromosome 15q24.1–q24.3 (data not shown). G-banding chromosomes of the parents were normal. The FISH analysis using two deleted BAC clones detected both two signals on the parental chromosomes. Thus, the aberration observed in present patient occurred de novo.

DISCUSSION

We report on a 13-year-old boy with characteristic facial features, hypospadias, mild developmental delay manifesting MDS. Array CGH demonstrated 15q24 deletion. This syndrome was recently delineated by Sharp et al. [2007]. Several patients had been described as having deletions encompassing the 15q24 segment, examined by standard chromosomal analysis and FISH studies [Cushman et al., 2005]. They showed many overlapping clinical features with the 15q24 deletion syndrome. However, their breakpoints had not been scrutinized. Most patients with 15q24 deletion syndrome are reported to have recurrent breakpoints, which are supposed to be mediated by nonallelic homologous recombination (NAHR) between the five low-copy repeats (LCRs) region. The deletions ranged from 1.7 to 6.1 Mb, with the smallest region of overlap (SRO) spanning approximately 1.2 Mb (chr15: 72.1–73.3 Mb) [El-Hattab et al., 2010]. Present patient had a deleted region spanning approximately 3.4 Mb, which comprises 50 genes and include the previously delineated SRO (Fig. 3).

To date, 20 patients with 15q24 deletion syndrome, including present patient, have been reported. These patients shared major clinical manifestations, including developmental delay, growth deficiency, characteristic facial dysmorphism, digital abnormalities, loose connective tissue, and genital malformations in males. The patient showed most of the major features reported in previous patients, but did not show growth deficiency, loose connective tissue, or brain anomaly (Table I). The patient manifested MDS, which has never been described in patients with the syndrome. ALL has been described in a boy reported by El-Hattab et al. [2009], whose breakpoints were similar to those of present patient (Fig. 3).

Childhood MDS is a relatively rare and complex disease. It is difficult to diagnose childhood MDS when the blast count is not elevated and clonality cannot be established. Hence, children with myelodysplasia or suspected MDS must be extensively worked up for secondary causes of dyspoiesis including nutritional deficiency, medications, toxins, metabolic diseases, infections, autoimmune diseases, growth factor therapy, and congenital disorders of hematopoiesis. Among them, congenital disorders of hematopoiesis including Fanconi anemia, dyskeratosis congenita, Diamond–Blackfan syndrome, Down syndrome, and mitochondria cytopathy is responsible for 29–44% of pediatric patients in whom MDS develops [McKenna, 2004; Yin et al., 2010]. The examinations on the present patient had no findings to suggest these secondary causes. The bone marrow examination of the patient showed mild trilineage dysplasia. His condition was classified as childhood MDS [Baumann et al., 2008].

MDS progress rapidly to leukemia or slowly over many years. Patients have a deteriorating course with 30% evolving into acute

TABLE I. Clinical Features in the Present Patient Compared to 19 Published Patients With 15q24 Deletion Syndrome

	Present patient	Nineteen previously reported cases
Deletion length	3.44 Mb 72.48–75.92	1.7–6.1 Mb 67.8–76.08
Inheritance	De novo	17 de novo, 2 unknown
Gender	Male	17 male, 2 female
Age	13y	5mo–33y
Growth retardation	–	5/19
Intellectual disability/ developmental delay	+	19/19
Facial abnormalities		
High forehead/anterior hair line	–	12/19
Long/narrow face	+	5/19
Broad medial eyebrows	+	7/19
Epicanthus folds	+	8/19
Hypertelorism	–	8/19
Downslanting palpebral fissures	+	8/19
Smooth/long philtrum	+	10/19
Full lower lip	+	7/19
Eye abnormalities		
Strabismus	–	7/19
Dysopia	+	3/19
Ear abnormalities		
Hearing impairment	+	4/19
Malformed ear	+	12/19
Brain malformation	–	9/19
Cardiac abnormalities	–	5/19
Urogenital abnormalities	+	11/19
Skeletal malformation		
Joint laxity	–	8/19
scoliosis	+	6/19
Digital abnormalities	+	14/19
Diaphragmatic hernia	–	3/19
Inguinal hernia	–	4/19
Gastrointestinal abnormalities	–	3/19
Recurrent infection	+	7/19
Hematopoietic disorder	+	1/19
Autism spectrum	–	3/19

mo, month; y, year.

leukemia usually of myeloid phenotype. Evolution into ALL from MDS is rare and seen in <1% adult cases and extremely rare in pediatric population. However, 26 patients with MDS progress to ALL were reported [Gupta and Bhatia, 2010]. El-Hattab et al. [2009] reported a boy with 15q24 deletion and ALL. They hypothesized some tumor-associated genes which located in 15q24 region, *C-Src* tyrosine kinase (*CSK*) and *SIN3A* may lead to increased risk of developing neoplasm. *UBL7/BMSC-UbP* located at 15q24.1 isolated from the bone marrow stromal cell cDNA library encodes a bone marrow stromal cell-derived ubiquitin-like protein. *UBL7* was suggested to a play role in the regulation of bone marrow stromal cell function or cell differentiation through an evocator and cell specific pattern [Liu et al., 2003]. The development of MDS and hematological malignancy in the syndrome might be caused by the haploinsufficiency of deleted 15q24 segment either alone or in combination with other genetic abnormalities in hematopoietic cells.

In conclusion, we report a patient with MDS and 15q24 deletion syndrome. This syndrome might be prone to have hematological malignancy. A careful hematological follow-up of the present patient is required. Further hematological investigation is recommended to be beneficial if physical and hematological examination results are suggestive of hematopoietic disturbance in patients with 15q24 deletion syndrome.

ACKNOWLEDGMENTS

The authors appreciate a patient and his families for their cooperation. The authors are grateful to A. Manabe, St Luke's International Hospital, M. Ito, Japanese Red Cross Nagoya Daiichi Hospital, for providing bone-marrow pathology. This work was supported by Research on Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (#22790972) (Y.N.), Research on Intractable Diseases from Japanese Ministry of Health, Welfare, and Labor (Y.F.).

REFERENCES

Andrieux J, Dubourg C, Rio M, Attie-Bitach T, Delaby E, Mathieu M, Journel H, Copin H, Blondeel E, Doco-Fenzy M, Landais E, Delobel B, Odent S, Manouvrier-Hanu S, Holder-Espinasse M. 2009. Genotype–phenotype correlation in four 15q24 deleted patients identified by array-CGH. Am J Med Genet Part A 149A:2813–2819.

Baumann I, Niemeyer CM, Benett JM, Shannon K. 2008. Childhood myelodysplastic syndrome. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Bardiman JW, editors. WHO classification of tumors of haematopoietic and lymphoid tissues, 4th edition. Lyon: IARC. pp 104–107.

Cushman LJ, Torres-Martinez W, Cherry AM, Manning MA, Abdul-Rahman O, Anderson CE, Punnett HH, Thurston VC, Sweeney D, Vance GH. 2005. A report of three patients with an interstitial deletion of chromosome 15q24. Am J Med Genet Part A 137A:65–71.

El-Hattab AW, Smolarek TA, Walker ME, Schorry EK, Immken LL, Patel G, Abbott MA, Lanpher BC, Ou Z, Kang SH, Patel A, Scaglia F, Lupski JR, Cheung SW, Stankiewicz P. 2009. Redefined genomic architecture in 15q24 directed by patient deletion/duplication breakpoint mapping. Hum Genet 126:589–602.

El-Hattab AW, Zhang F, Maxim R, Christensen KM, Ward JC, Hines-Dowell S, Scaglia F, Lupski JR, Cheung SW. 2010. Deletion and duplication of 15q24: Molecular mechanisms and potential modification by additional copy number variants. Genet Med 12: 573–586.

Gupta V, Bhatia B. 2010. Transformation of myelodysplastic syndrome to acute lymphoblastic leukemia in a child. Indian J Hematol Blood Transfus 26:111–113.

Klopocki E, Graul-Neumann LM, Grieben U, Tönnies H, Ropers HH, Horn D, Mundlos S, Ullmann R. 2008. A further case of the recurrent 15q24 microdeletion syndrome, detected by array CGH. Eur J Pediatr 167:903–908.

Liu S, Yu Y, An H, Li N, Lin N, Wang W, Zhang W, Wan T, Cao X. 2003. Cloning and identification of a novel ubiquitin-like protein, BMSC-UbP, from human bone marrow stromal cells. Immunol Lett 68:169–175.

Marshall CR, Noor A, Vincent JB, Lionel AC, Feuk L, Skaug J, Shago M, Moessner R, Pinto D, Ren Y, Thiruvahindrapduram B, Fiebig A, Schreiber S, Friedman J, Ketelaars CE, Vos YJ, Ficicioglu C, Kirkpatrick S, Nicolson R, Sloman L, Summers A, Gibbons CA, Teebi A, Chitayat D, Weksberg R, Thompson A, Vardy C, Crosbie V, Luscombe S, Baatjes R, Zwaigenbaum L, Roberts W, Fernandez B, Szatmari P, Scherer SW. 2008. Structural variation of chromosomes in autism spectrum disorder. Am J Hum Genet 82:477–488.

Masurel-Paulet A, Callier P, Thauvin-Robinet C, Chouchane M, Mejean N, Marle N, Mosca AL, Ben Salem D, Giroud M, Guibaud L, Huet F, Mugneret F, Faivre L. 2009. Multiple cysts of the corpus callosum and psychomotor delay in a patient with a 3.1 Mb 15q24.1q24.2 interstitial deletion identified by array-CGH. Am J Med Genet Part A 149A: 1504–1510.

McInnes LA, Nakamine A, Pilorge M, Brandt T, Jiménez González P, Fallas M, Manghi ER, Edelmann L, Glessner J, Hakonarson H, Betancur C, Buxbaum JD. 2010. A large-scale survey of the novel 15q24 microdeletion syndrome in autism spectrum disorders identifies an atypical deletion that narrows the critical region. Mol Autism 1:5–16.

McKenna RW. 2004. Myelodysplasia and myeloproliferative disorders in children. Am J Clin Pathol 122:S58–S69.

Niemeyer CM, Kratz CP, Hasle H. 2005. Pediatric myelodysplastic syndromes. Curr Treat Options Oncol 6:209–214.

Sharp AJ, Selzer RR, Veltman JA, Gimelli S, Gimelli G, Striano P, Coppola A, Regan R, Price SM, Knoers NV, Eis PS, Brunner HG, Hennekam RC, Knight SJ, de Vries BB, Zuffardi O, Eichler EE. 2007. Characterization of a recurrent 15q24 microdeletion syndrome. Hum Mol Genet 16: 567–572.

Smith M, Filipek PA, Wu C, Bocian M, Hakim S, Modahl C, Spence MA. 2000. Analysis of a 1-megabase deletion in 15q22–q23 in an autistic patient: Identification of candidate genes for autism and of homologous DNA segments in 15q22–q23 and 15q11–q13. Am J Med Genet 96: 765–770.

Van Esch H, Backx L, Pijkels E, Fryns JP. 2009. Congenital diaphragmatic hernia is part of the new 15q24 microdeletion syndrome. Eur J Med Genet 52:153–156.

Yin CC, Medeiros LJ, Bueso-Ramos CE. 2010. Recent advances in the diagnosis and classification of myeloid neoplasms—Comments on the 2008 WHO classification. Int J Lab Hematol 32:461–476.

Molecular Genetics and Metabolism 107 (2012) 87–91

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Bezafibrate can be a new treatment option for mitochondrial fatty acid oxidation disorders: Evaluation by in vitro probe acylcarnitine assay

Seiji Yamaguchi [a,*], Hong Li [a,b], Jamiyan Purevsuren [a], Kenji Yamada [a], Midori Furui [a], Tomoo Takahashi [a], Yuichi Mushimoto [a], Hironori Kobayashi [a], Yuki Hasegawa [a], Takeshi Taketani [a], Toshiyuki Fukao [c], Seiji Fukuda [a]

[a] Department of Pediatrics, Shimane University School of Medicine, Izumo, Shimane 693-8501, Japan
[b] Department of Pediatrics, the Affiliated Hospital of Ningxia Medical University, Yinchuan 750004, China
[c] Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, 501-1194, Japan

ARTICLE INFO

Article history:
Received 29 May 2012
Received in revised form 5 July 2012
Accepted 5 July 2012
Available online 14 July 2012

Keywords:
Mitochondrial fatty acid oxidation disorder
Bezafibrate
New treatment
Hypolipidemic drug
In vitro probe acylcarnitine assay
Peroxisome proliferation activator receptor

ABSTRACT

Background: The number of patients with mitochondrial fatty acid oxidation (FAO) disorders is recently becoming larger with the spread of newborn mass screening. Despite the advances in metabolic and molecular characterization of FAO disorders, the therapeutic studies are still limited. It was reported recently that bezafibrate (BEZ), an agonist of peroxisome proliferating activator receptor (PPAR), can restore FAO activity in cells from carnitine palmitoyltransferase-2 (CPT2) and very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiencies as well as clinical symptoms in the adult patients.
Methods: In this study, the therapeutic effect of BEZ was determined by in vitro probe acylcarnitine (IVP) assay using cultured fibroblasts and tandem mass spectrometry on various FAO disorders. The clinical trial of BEZ treatment for a boy with the intermediate form of glutaric acidemia type 2 (GA2) was also performed.
Results: The effect of BEZ was proven in cells from various FAO disorders including GA2, deficiencies of VLCAD, medium-chain acyl-CoA dehydrogenase, CPT2, carnitine acylcarnitine translocase and trifunctional protein, by the IVP assay. The aberrantly elevated long- or medium-chain acylcarnitines that are characteristic for each FAO disorder were clearly corrected by the presence of BEZ (0.4 mmol/L) in culture medium. Moreover, daily administration of BEZ in a 2-year-old boy with GA2 dramatically improved his motor and cognitive skills, accompanied by sustained reduction of C4, C8, C10 and C12 acylcarnitines in blood, and normalized the urinary organic acid profile. No major adverse effects have been observed.
Conclusion: These results indicate that BEZ could be a new treatment option for FAO disorders.

1. Introduction

Mitochondrial β-oxidation (FAO) is an essential energy producing pathway, particularly during the reduced energy supply from carbohydrate due to prolonged starvation or low caloric intake during infection, diarrhea or febrile illness. A number of FAO disorders have been recognized with the spread of tandem mass spectrometry (MS/MS) in the field of study of inborn metabolic disease as well as neonatal mass screening [1,2]. Many of them show episodic attacks like lethargy, acute encephalopathy or even sudden death due to energy production insufficiency.

It is considered that the FAO system consists of the following four groups : 1) carnitine cycle, which activates long-chain fatty acids for undergoing β-oxidation, including carnitine transporter (OCTN2), carnitine palmitoyltransferase-1 or -2 (CPT1 or CPT2, respectively, EC 2.3.1.21), or carnitine acylcarnitine translocase (CACT, EC 2.3.1.21); 2) long-chain FAO, whose enzymes are connected to the mitochondrial inner membrane, including very-long-chain acyl-CoA dehydrogenase (VLCAD, EC 1.3.99.13) deficiency, and trifunctional protein (TFP, EC 1.1.1.211 and EC 2.3.1.16); 3) medium-chain FAO, whose enzymes are located in the mitochondrial matrix, including medium- and short-chain acyl-CoA dehydrogenases (MCAD, EC 1.3.99.3 and SCAD, EC 1.3.8.1) respectively), enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, or medium- and short-chain 3-ketothiolase (MCKAT and SCKAT, respectively); and 4) electron transfer system, from the dehydrogenases to respiratory chain, including electron transferring flavoprotein (ETF, EC 1.5.8.2) and ETF dehydrogenase (ETFDH, EC 1.5.5.1) [3–5].

Clinical features of FAO disorders can be roughly divided into the following three types: 1) severe form (neonatal form): patients present life-threatening illness with profound hypoglycemia, liver failure or hyperammonemia, and are often fatal in early infancy; 2) intermediate

* Corresponding author at: Department of Pediatrics, Shimane University School of Medicine, 89-1 En-ya-cho, Izumo, Shimane 693-8501, Japan. Fax: +81 853 20 2215.
E-mail address: seijiyam@med.shimane-u.ac.jp (S. Yamaguchi).

1096-7192/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymgme.2012.07.004

form (juvenile form): patients have intermittent episodic attacks like lethargy, encephalopathy, or even sudden death often onset in infancy or young childhood; 3) mild form (myopathic form): the patients may often show late onset after school ages or adulthood with episodes of hypotonia, myalgia, lethargy, myopathy-like symptoms, or liver dysfunction [6].

In vitro probe acylcarnitine profiling (IVP) assay was developed to evaluate FAO disorders recently [7,8]. Acylcarnitine (AC) profiles in the special culture medium as below after incubating with fatty acids as substrates are determined by MS/MS. Bezafibrate (BEZ) is a hypolipidemic drug, which is an agonist of peroxisome proliferating activator receptor (PPAR), and is claimed to act for induction of several FAO enzymes [9–11].

In this study, the effect of BEZ on various FAO disorders was evaluated using the IVP assay. Furthermore, we report an in vivo trial of BEZ on a boy with the intermediate form of GA2, presenting dramatic improvement with BEZ.

2. Materials and methods

2.1. Subjects and skin fibroblasts

Fibroblasts from 10 Japanese children with FAO disorders, one each of severe and intermediate forms of GA2, 2 each of severe and myopathic (mild) forms of VLCAD deficiency, one each of deficiencies of MCAD, CPT2, CACT, and TFP as well as 6 controls (healthy volunteers, passages 3 to 16) were used. The clinical types and genotypes are shown in Table 1. The child with MCAD deficiency was detected in a newborn mass screening and non-symptomatic, while one with the intermediate form of CPT2 deficiency had liver dysfunction in infancy. The child with the intermediate form of CACT deficiency had two life-threatening episodes in infancy, and after that no episodes were noted with normal development [12]. The child with TFP deficiency had an episode of liver failure in infancy, and then intermittent episodes of myalgia or hypotonia particularly following infection.

The clinical types and genotypes are shown in Table 1. In all cases, at least one allele has missense mutation, although the other alleles had missense or truncated mutations. In CACT deficiency (case 9), a missense mutation in an initiation codon (c.3G>A) in SLC25A29 was detected, but this could harbor a residual activity (Fukao et al., unpublished data).

Table 1
Clinical types and genotypes of patients with mitochondrial fatty acid oxidation disorders investigated.

Disease & case No.	Phenotype	Gene	Genotype, nucleotides (amino acids)	
			Allele 1	Allele 2
GA2				
1 (B)	Severe	*ETFA*	c.799G>A (G267R)	c.7C>T (R3X)
2 (C)	Intermediate	*ETFDH*	c.1217G>A (S406N)	c.1675C>T (R559X)
VLCAD deficiency				
3 (D)	Severe	*ACADV*	c.553G>A (G185S)	IVS9+1g>c
4 (E)	Severe	*ACADV*	c.454G>A (G152S)	c.997insT (A333fsX358)
5 (F)	Myopathic	*ACADV*	c.790A>G (K264E)	c.997insT (A333fsX358)
6 (G)	Myopathic	*ACADV*	c.1144A>C (K382Q)	c.1339G>A (G447R)
MCAD deficiency				
7 (H)	Non-symptomatic	*ACADM*	c.134A>G (Q45R)	c.449delCTGA (T150fsX153)
CPT2 deficiency				
8 (I)	Intermediate	*CPT2*	c.151A>G (R51G)	c.520G>A (E174K)
CACT deficiency				
9 (J)	Intermediate	*SLC25A29*	c.3G>A (M1I)	IVS4+1g>t
TFP deficiency				
10 (K)	Intermediate	*HADHB*	c.739C>T (R247C)	c.817delG (D273fsX292)

Abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase; GA2, glutaric acidemia type 2; VLCAD, very-long-chain acyl-CoA dehydrogenase; CPT2, carnitine palmitoyltransferase-2; TFP, mitochondrial trifunctional protein; CACT, carnitine acylcarnitine translocase. Case 2 (C) is a boy with GA2 who underwent the clinical trial of BEZ. Non-symptomatic case 7 (H) was detected in the newborn mass screening. Severe, intermediate, and myopathic forms are mentioned in the text. (B) to (K) correspond to those of Fig. 1.

2.2. In vitro probe assay with BEZ

Fibroblasts were cultured in 75 cm^2 flasks (Iwaki, Tokyo, Japan) containing modified Eagle's minimal essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 2 mmol/L of L-glutamine (Nacalai Tesque, Kyoto, Japan), 10% FBS (Sigma, St Louis, MO, USA) and 1% penicillin/streptomycin (Sigma) at 37 °C in a humidified 5% CO_2/95% air incubator [13].

Fibroblasts harvested by trypsinization were seeded onto 6-well microplates (35 mm i.d., Iwaki, Japan) with the fresh above medium (2 mL/per well) until they reached confluence. Thereafter, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA) and cultured for 96 h in 1 mL of experimental substrate (experimental medium). The experimental medium is MEM containing bovine serum albumin (0.4% essential fatty acid-free BSA; Sigma), L-carnitine (0.4 mmol/L; Sigma), unlabeled palmitic acid (0.2 mmol/L; Nacalai Tesque) and 1% penicillin/streptomycin without L-glutamine, in the presence or absence of BEZ (0.4 mmol/L; Sigma). AC profiles in the culture medium were analyzed after 96 h. The experiments for each case were performed in triplicate.

2.3. Quantitative acylcarnitine analysis

ACs in culture medium supernatants were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA) as described previously [13]. Briefly, methanol (200 μL) including an isotopically-labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to 10 μL of the supernatant from culture medium. The portions were placed on ice for 30 min, and centrifuged at 1000×g for 10 min. Then, 150 μL of the supernatant was dried under a nitrogen stream, and butyl-derivatized with 50 μL of 3N n-butanol-HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100 μL of 80% acetonitrile:water (4:1 v/v). The ACs in 10 μL of the resultant aliquots were analyzed using MS/MS and quantified using ChemoView™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

Protein concentrations were measured by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instruction. The AC concentrations are expressed as nmol/mg protein.

2.4. Organic acid analysis using GC/MS

Urinary organic acids were analyzed according to the previous method [14]. Briefly, 40 μg of tropate (IS-2) and 20 μg each of heptadecanoate (IS-1) and tetracosane (C24) as internal standards were added to a urine specimen containing 0.2 mg creatinine. The samples were oxime-derivatized, and solvent extracted with ethylacetate, and trimethylsilylated (TMS-derivatization). The resultant aliquots were subjected to GC/MS (Shimadzu GC/MS QP2010 Plus, Kyoto, Japan), with a DB-5 column of 0.25 mm I.D×30 m, 1 μm film thickness (J&W, Folsom, CA). The temperature program was from 100 °C to 290 °C at a rate of 4 °C/min).

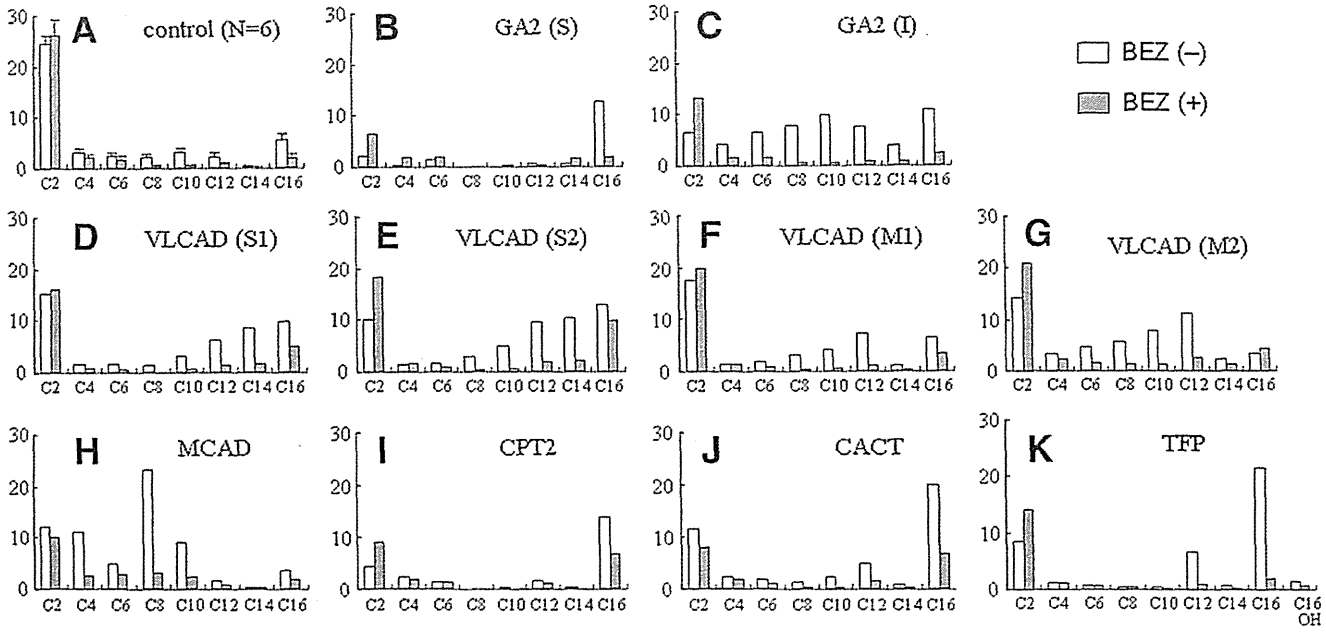

Fig. 1. Acylcarnitine profiles of in vitro probe assay in the presence and absence of bezafibrate. A, normal control; B, severe form of GA2; C, intermediate form of GA2 (the boy who underwent the clinical trial) (S and I, the clinically severe and intermittent form, respectively); D and E, severe form of VLCAD deficiency (S1 and S2, two cases, respectively); F and G: myopathic (mild) form of VLCAD deficiency (M1 and M2, two cases, respectively); H, I, J, and K: deficiencies of MCAD, CPT, CACT, and TFP, respectively. Unit of vertical lines, nmol /mg protein of acylcarnitines; the horizontal lines represent acylcarnitines from C2, C4, C6, C8, C10, C12, C14, C16, and C16-OH. The experiments for each were performed in triplicate, and the mean values of ACs are illustrated with bars. In control (A), the mean plus SD values of 6 controls are shown.

2.5. BEZ trial on a child with the intermittent form of GA2

A Japanese boy with GA2 was detected in the newborn mass screening using MS/MS, and had no special symptoms in infancy with therapies of special formula and carnitine (approximately100 mg/kg/day, div. 3). After 1 year of age, however, he sometimes experienced episodes of hypotonia or lethargy following infection, and muscle weakness, often falling. At the age 2 years and 1 month, he was hospitalized for 2 and a half months, because of infection and lethargy, receiving treatments including artificial respiration to repeated aspiration pneumonia and unconsciousness in intensive care unit (ICU). At discharge, he could not walk alone, and could speak only a few words. So, his family consulted us, and strongly expressed a desire for any new therapies that might help their son.

Thereafter, under the approval by the ethical committee of Shimane University, we started a clinical trial of BEZ, continuing the dietary and carnitine therapies as before, since 2 years and 9 months of his age. His body weight ranged from 12 to 14 kg during the treatment, and 200 to 300 mg/day (approximately 17 to 25 mg/kg/day, div. 3) of BEZ was used in the trial. BEZ was purchased from Kissei Co Ltd, Tokyo, Japan. The study had no potential conflicts of interest (COI) to the authors.

3. Results

3.1. Effects of BEZ on FAO disorders by IVP assay

The AC profiles in the culture medium of fibroblasts from various FAO disorders in the presence and absence of BEZ are illustrated in Fig. 1. In control cells, C2 (acetylcarnitine) is the only prominent peak, and many of ACs further decreased in the presence of BEZ (Fig. 1A).

In the severe form of GA2 (Fig. 1B/S), C16 was apparently decreased, and C2 increased in the presence of BEZ, while C16 was extremely high before BEZ addition. The increase of C2 may indicate the acceleration of FAO, namely an increase of acetyl-CoA production. In the intermediate form of GA2 (Fig. 1C/I), all elevated ACs clearly decreased and normalized in the presence of BEZ, although broad ranges of ACs from C4 to C16 were extremely high before adding BEZ. This patient is the case 3 in Table 1, who underwent the clinical trial of BEZ treatment as illustrated in Fig. 2.

In 2 cases of the severe form of VLCAD deficiency (Figs. 1D/S1, and 1E/S2), elevation of C14 and C16 was larger, compared with that in 2 cases of the mild form (Figs. 1F/M1, and 1G/M2). The elevated ACs

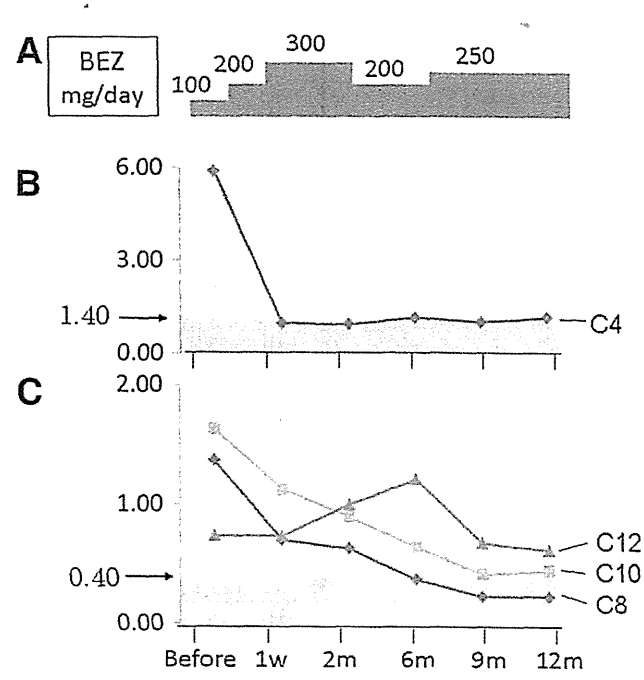

Fig. 2. Bezafibrate administration and changes in blood acylcarnitines. A, dose of bezafibrate, mg/day (approximately 17 to 25 mg/kg/day, div. 3); B, change of C4 acylcarnitine; C, changes in C8, C10, and C12. Arrows with the 1.40 and 0.40 indicate the cutoff values of blood acylcarnitines. Unit of acylcarnitine is nmol/mg protein.

Table 2
Time course of biochemical findings after initiation of bezafibrate administration.

	(Unit)	Before	After the start of BEZ treatment					Reference value*
			1w	2 m	6 m	9 m	12 m	
AST	(IU/L)	47	35	44	43	26	42	10–38
ALT	(IU/L)	27	17	22	24	20	21	5–40
LDH	(IU/L)	448	426	392	384	341	371	100–215
CK	(IU/L)	496	185	187	324	174	207	36–216
TChol	(mg/dL)	161	127	117	141	127	140	150–219

* : used in Shimane University Hospital. Abbreviations: AST, aspartate amino transferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; and TChol, total cholesterol.

such as C10, C12, C14, or C16 in both the severe and mild forms apparently decreased in the presence of BEZ.

In MCAD deficiency (Fig. 1H), the AC peaks of C4 to C10 were significant, but in the presence of BEZ, these AC peaks were almost normalized. In cases of CPT2 deficiency (Fig. 1I), CACT deficiency (Fig. 1J) and TFP deficiency (Fig. 1K), the extremely high AC peaks of C16 and/or C12 apparently decreased to an almost normal level, in the presence of BEZ.

3.2. Clinical trial of BEZ to a GA2 patient

Since the start of BEZ treatment, his motor and social development, and languages remarkably improved, and no metabolic episodes were noted. He became able to walk alone, showed improved muscle strength, and could speak markedly more words in a few weeks. Furthermore, several months later, he could ride a kid's tricycle by himself, although his intellectual ability was on the borderline for entrance into a kindergarten. For at least 1 year of the administration, no adverse effects of BEZ such as hypolipidemia or rhabdomyolysis have been observed.

The routine laboratory data such as blood AST, ALT, LDH or CK were in normal or subnormal ranges as shown in Table 2, showing stable levels of each test, although these laboratory data had sometimes fluctuated, in particular, when his condition was unstable before the initiation of BEZ. For example, during the stay in the ICU at the age of 2 years, the maximum levels of AST, ALT, LDH or CK were 1450 IU/L, 825 IU/L, 5200 IU/L, or 10,750 IU/L, respectively. The maximum level of blood ammonia at the ICU was 126 μg/dL, while no significant elevation was observed after that. Hypoglycemic attacks have not been noted.

BEZ is a hypolipidemic drug, and we have paid attention to the blood level of Cholesterol (TChol), because of the potential adverse effects. The dose of BEZ was 100 mg/day for the first 3 days, 200 mg/day for 4 days, and 300 mg/day for 2 months, respectively, as shown in Fig. 2A. At 2 months after starting BEZ of 300 mg/day, TChol level was a bit low, 117 mg/dL. Since then the dose has been lowered to 200 or 250 mg/day, and the TChol level has ranged between around 130 to 150 mg/mL, as shown in Table 2.

The changes in the AC levels of C4, C8, C10, and C12 are illustrated in Figs. 2B and C, respectively. All the increased ACs returned to approximately normal levels with the administration of BEZ after several months. In particular, C4 decreased to the normal range within a few weeks. Urinary organic acid analysis showed remarkable increases of ethylmalonate, methylsuccinate, adipate, 2-hydroxyglutarate, hexanoylglycine, suberate, and suberylglycine, before the BEZ treatment as shown in Fig. 3. The abnormalities in urinary organic acids were markedly corrected as early as 2 weeks after the initiation of BEZ therapy. The profile was almost normal but for a slight increase of ethylmalonate, and/or hexanoylglycine as illustrated in Fig. 3B.

4. Discussion

The treatments for FAO disorders have generally been described as follows: 1) avoiding a "long fasting": it prevents the increased requirement of fuel from FAO; 2) early infusion of glucose: it should be performed during the metabolic stress resulting from infection, diarrhea or overexercise, to prevent hypercatabolism; 3) carnitine therapy: it may be effective in many cases, although controversy

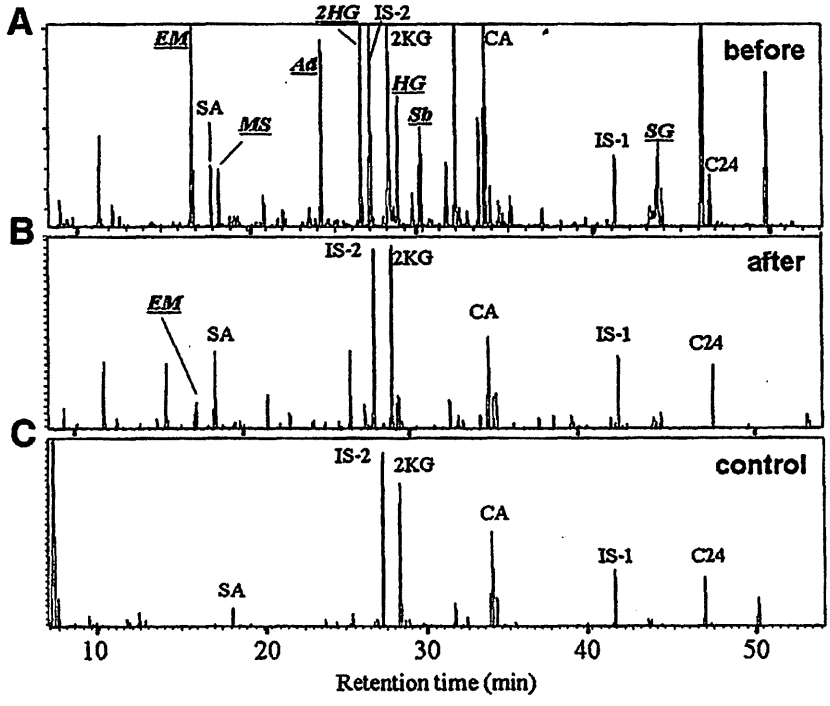

Fig. 3. Urinary organic acid profiles before and after bezafibrate administration. A, The total ion chromatogram (GC/MS) of urinary organic acids just before the start of BEZ; B, One year after the treatment; C, Normal control. Abbreviations: IS-2, IS-1 and C24 are tropate, heptadecanoate, and tetracosane, respectively, as internal standards; EM, ethylmalonate; SA, succinate; MS, methylsuccinate; Ad, adipate; 2HG, 2-hydroxyglutarate; 2KG, 2-ketoglutarate; HG, hexanoylglycine; Sb, suberate; CA, citrate; SG, suberylglycine. Metabolites judged as abnormal are shown in bold letters underlined.

remains in some cases; and 4) dietary therapy, including high carbohydrate/low lipid diet: Dietary restriction in FAO disorders may be less strict [15–18].

In this study, we demonstrated the effect of BEZ on various FAO disorders at both in vitro and in vivo levels. It was indicated by the IVP assay that FAO capacity was corrected by BEZ in various FAO disorders, and a clinical trial of BEZ in a boy with the intermediate form of GA2 showed a favorable consequence. Bastin, Djourdi and their colleagues reported the potential effect of BEZ for FAO disorders showing the increase of enzyme activity and mRNA production in several FAO enzymes from normal individuals, or reduced ACs in cells from VLCAD deficiency by the IVP assay using stable isotope-labeled palmitate [19]. Furthermore, they are performing a clinical trial on adult cases of mild form of CPT2 deficiency [20,21]. We should continue to pay attention to potential adverse effects of BEZ, including hypolipidemia or rhabdomyolysis, although such signs have never seen up to now.

We used the IVP assay to investigate the effect of BEZ in the other FAO disorders including GA2, deficiencies of MCAD, CACT, and TFP as well as CPT2 or VLCAD deficiencies. The beneficial effect of BEZ was clearly demonstrated in all these cases tested in this study, which included the clinically intermediate or severe forms as well as the mild form, having missense mutation of at least one allele. However, it is not yet clear whether the effect of BEZ is due to induction of mutant enzyme itself, or due to stimulation of the other FAO enzymes. If the effect is due to the latter mechanism, BEZ could potentially induce a "high pressure" on the FAO pathway, even resulting in devastating outcomes. We should further investigate the effect on the other severe forms of FAO disorders, the relation with the genotypes, or the dose dependency.

BEZ is an agonist of PPAR, which facilitates transcription of genes encoding FAO enzymes, and subsequently induces FAO enzyme production. Eventually, it can be considered to correct the FAO capacity in FAO disorders. Recently, it was reported that resveratrol which is a natural polyphenol and an activator of Sirtuin 1, is also expected to be a novel treatment option for FAO disorders [22]. The effect of resveratrol on FAO capacity can also be evaluated by the IVP assay like this study.

In conclusion, BEZ could be a new promising treatment option for FAO disorders. Many of patients with FAO disorders, particularly children with the milder form or adult cases, are intellectually normal, and their life prognosis is favorable if they can be prevented from severe episodes like encephalopathy. Symptoms or severity of FAO disorders are very heterogeneous depending on the disease, genetic background or lifestyle. Additional clinical studies of BEZ treatment will be essential for confirmation of its safety and practical utility.

Acknowledgments

The authors thank Dr. M Ito, Kagawa Children's Hospital, Japan, for kindly providing clinical data before the BEZ treatment of this patient, Dr. T. Hashimoto, professor emeritus of Shinshu University, for helpful comments on our study, and also thank MS. M. Hattori, Y. Ito, E. Mizuno, N. Tomita and T. Esumi, for their technical assistance. Finally, the authors thank Dr. Paul Langman, Iwate University, Japan for his kind assistance with English usage. This study was supported by grants from the Ministry of Science, Culture, and Sports (S.Y. and J.P.), and from the Ministry of Health, Labour and Welfare (S.Y.), of Japan. The authors had no potential conflicts of interest (COI) associated with this work. This study was approved by the ethics committee of Shimane University.

References

[1] L.L. McCabe, E.R.B. McCabe, Expanded newborn screening: implications for genomic medicine, Annu. Rev. Med. 59 (2008) 163–175.
[2] B. Wilcken, M. Haas, P. Joy, V. Wiley, F. Bowling, K. Carpenter, J. Christodoulou, D. Cowley, C. Ellaway, J. Fletcher, E.P. Kirk, B. Lewis, J. McGill, H. Peters, J. Pitt, E. Ranieri, J. Yaplito-Lee, A. Boneh, Expanded newborn screening: outcome in screened and unscreened patients at age 6 years, Pediatrics 124 (2009) e241–e248.
[3] P. Rinaldo, D. Matern, M.J. Bennett, Fatty acid oxidation disorders, Annu. Rev. Physiol. 64 (2002) 477–502.
[4] M. Kompare, W.B. Rizzo, Mitochondrial fatty-acid oxidation disorders, Semin. Pediatr. Neurol. 15 (2008) 140–149.
[5] M.J. Bennett, Pathophysiology of fatty acid oxidation disorders, J. Inherit. Metab. Dis. 33 (2010) 533–537.
[6] B.S. Andresen, S. Olpin, B.J. Poorthuis, H.R. Scholte, C. Vianey-Saban, R. Wanders, L. Ijlst, A. Morris, M. Pourfarzam, K. Bartlett, E.R. Baumgartner, J.B. deKlerk, L.D. Schroeder, T.J. Corydon, H. Lund, V. Winter, P. Bross, L. Bolund, N. Gregersen, Clear correlation of genotype with disease phenotype in very-long-chain acyl-CoA dehydrogenase deficiency, Am. J. Hum. Genet. 64 (1999) 479–494.
[7] J.G. Okun, S. Kolker, A. Schulze, D. Kohlmuller, K. Olgemoller, M. Linder, G.F. Hoffmann, R.J.A. Wanders, E. Mayatepek, A method for quantitative acylcarnitine profiling in human skin fibroblasts using unlabelled palmitic acid: diagnosis of fatty acid oxidation disorders and differentiation between biochemical phenotypes of MCAD deficiency, Biochim. Biophys. Acta 1584 (2002) 91–98.
[8] K.G. Sim, K. Carpenter, J. Hammond, J. Christodoulou, B. Wilcken, Quantitative fibroblast acylcarnitine profiles in mitochondrial fatty acid beta-oxidation defects: phenotype/metabolite correlations, Mol. Genet. Metab. 76 (2002) 327–334.
[9] T. Aoyama, J.M. Peters, N. Iritani, T. Nakajima, K. Furihata, T. Hashimoto, F.J. Gonzalez, Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor alpha (PPARalpha), J. Biol. Chem. 273 (1998) 5678–5684.
[10] F. Djouadi, J.P. Bonnefont, L. Thuillier, V. Droin, N. Khadom, A. Munnich, J. Bastin, Correction of fatty acid oxidation I carnitine palmitoyl transferase 2-deficient cultured skin fibroblasts by bezafibrate, Pediatr. Res. 54 (2003) 446–451.
[11] S. Gobin-Limballe, F. Djouadi, F. Aubey, S. Olpin, B.S. Andresen, S. Yamaguchi, H. Mandel, T. Fukao, J.P. Ruiter, R.J. Wanders, R. McAndrew, J.J. Kim, J. Bastin, Genetic basis for correction of very-long-chain acyl-coenzyme A dehydrogenase deficiency by bezafibrate in patient fibroblasts: toward a genotype-based therapy, Am. J. Hum. Genet. 81 (2007) 1133–1143.
[12] E. Lopriore, R.J. Gemke, N.M. Verhoeven, C. Jakobs, R.J. Wanders, A.B. Roeleveld-Versteeg, B.T. Poll-The, Carnitine-acylcarnitine translocase deficiency: phenotype, residual enzyme activity and outcome, Eur. J. Pediatr. 160 (2001) 101–104.
[13] H. Li, S. Fukuda, Y. Hasegawa, H. Kobayashi, J. Purevsuren, Y. Mushimoto, S. Yamaguchi, Effect of heat stress and bezafibrate on mitochondrial β-oxidation: comparison between cultured cells from normal and mitochondrial fatty acid oxidation disorder children using in vitro probe acylcarnitine profiling assay, Brain Dev. 32 (2010) 362–370.
[14] S. Yamaguchi, M. Iga, M. Kimura, Y. Suzuki, N. Shimozawa, T. Fukao, N. Kondo, Y. Tazawa, T. Orii, Urinary organic acids in peroxisomal disorders: a simple screening method, J. Chromatogr. B 758 (2001) 81–86.
[15] U. Spiekerkoetter, M. Lindner, R. Santer, M. Grotzke, M.R. Baumgartner, H. Boehles, A. Das, C. Haase, J.B. Hennermann, D. Karall, H. de Klerk, I. Knerr, H.G. Koch, B. Plecko, W. Röschinger, K.O. Schwab, D. Scheible, F.A. Wijburg, J. Zschocke, E. Mayatepek, U. Wendel, Management and outcome in 75 individuals with long-chain fatty acid oxidation defects: results from a workshop, J. Inherit. Metab. Dis. 32 (2009) 488–497.
[16] U. Spiekerkoetter, J. Bastin, M. Gillingham, A. Morris, F. Wijburg, B. Wilcken, Current issues regarding treatment of mitochondrial fatty acid oxidation disorders, J. Inherit. Metab. Dis. 33 (2010) 555–561.
[17] P. Laforêt, C. Vianey-Saban, Disorders of muscle lipid metabolism: diagnostic and therapeutic challenges, Neuromuscul. Disord. 20 (2010) 693–700.
[18] J. Vockley, D.A. Whiteman, Defects of mitochondrial beta-oxidation: a growing group of disorders, Neuromuscul. Disord. 12 (2002) 235–246.
[19] F. Djouadi, F. Aubey, D. Schlemmer, J.P. Ruiter, R.J. Wanders, A.W. Strauss, J. Bastin, Bezafibrate increases very-long-chain acyl-CoA dehydrogenase protein and mRNA expression in deficient fibroblasts and is a potential therapy for fatty acid oxidation disorders, Hum. Mol. Genet. 14 (2005) 2695–2703.
[20] J.P. Bonnefont, J. Bastin, P. Laforet, F. Aubey, A. Mogenet, S. Romano, D. Ricquier, S. Gobin-Limballe, A. Vassault, A. Behin, B. Eymard, J.L. Bresson, F. Djouadi, Long-term follow-up of bezafibrate treatment in patients with the myopathic form of carnitine palmitoyltransferase 2 deficiency, Clin. Pharmacol. Ther. 88 (2010) 101–108.
[21] J.P. Bonnefont, J. Bastin, A. Behin, F. Djouadi, Bezafibrate for treatment of an inborn mitochondrial b-oxidation defect, N. Engl. J. Med. 360 (2009) 838–840.
[22] J. Bastin, A. Lopes-Costa, F. Djouadi, Exposure to resveratrol triggers pharmacological correction of fatty acid utilization in human fatty acid oxidation-deficient fibroblasts, Hum. Mol. Genet. 20 (2011) 2048–2057.

Journal of Human Genetics (2012) 57, 207–211

www.nature.com/jhg

ORIGINAL ARTICLE

Missense mutations in the DNA-binding/dimerization domain of *NFIX* cause Sotos-like features

Yuriko Yoneda[1], Hirotomo Saitsu[1], Mayumi Touyama[2], Yoshio Makita[3], Akie Miyamoto[4], Keisuke Hamada[5], Naohiro Kurotaki[6], Hiroaki Tomita[7], Kiyomi Nishiyama[1], Yoshinori Tsurusaki[1], Hiroshi Doi[1], Noriko Miyake[1], Kazuhiro Ogata[5], Kenji Naritomi[8] and Naomichi Matsumoto[1]

Sotos syndrome is characterized by prenatal and postnatal overgrowth, characteristic craniofacial features and mental retardation. Haploinsufficiency of *NSD1* causes Sotos syndrome. Recently, two microdeletions encompassing *Nuclear Factor I-X (NFIX)* and a nonsense mutation in *NFIX* have been found in three individuals with Sotos-like overgrowth features, suggesting possible involvements of *NFIX* abnormalities in Sotos-like features. Interestingly, seven frameshift and two splice site mutations in *NFIX* have also been found in nine individuals with Marshall–Smith syndrome. In this study, 48 individuals who were suspected as Sotos syndrome but showing no *NSD1* abnormalities were examined for *NFIX* mutations by high-resolution melt analysis. We identified two heterozygous missense mutations in the DNA-binding/dimerization domain of the NFIX protein. Both mutations occurred at evolutionally conserved amino acids. The c.179T>C (p.Leu60Pro) mutation occurred *de novo* and the c.362G>C (p.Arg121Pro) mutation was inherited from possibly affected mother. Both mutations were absent in 250 healthy Japanese controls. Our study revealed that missense mutations in *NFIX* were able to cause Sotos-like features. Mutations in DNA-binding/dimerization domain of NFIX protein also suggest that the transcriptional regulation is abnormally fluctuated because of *NFIX* abnormalities. In individuals with Sotos-like features unrelated to *NSD1* changes, genetic testing of *NFIX* should be considered.

Journal of Human Genetics (2012) **57,** 207–211; doi:10.1038/jhg.2012.7; published online 2 February 2012

Keywords: DNA-binding/dimerization domain; missense mutation; *NFIX*; Sotos syndrome

INTRODUCTION

Sotos syndrome (MIM #117550) is an overgrowth syndrome characterized by tall stature and/or macrocephaly, distinctive facial appearance and mental retardation.[1] A *de novo* t(5;8)(q35;q24.1) translocation in a patient with Sotos syndrome revealed disruption of *NSD1* at 5q35. Subsequent identification of nonsense, frameshift and submicroscopic deletion mutations of *NSD1* in patients with Sotos syndrome clearly showed that haploinsufficiency of *NSD1* causes Sotos syndrome.[2] *NSD1* encodes nuclear receptor-binding SET domain protein 1, which functions as a histone methyltransferase that activates and represses transcription through chromatin modification.[3] The diagnosis of Sotos syndrome is established by confirming *NSD1* abnormalities,[4] and abnormalities of *NSD1* causes up to 90% of Sotos syndrome cases. However, a part of patients with suspected Sotos syndrome are known to show no abnormalities in *NSD1*,[5] suggesting involvement of another gene.

Recently it was reported that two patients with Sotos-like overgrowth features possessed microdeletions encompassing *Nuclear Factor I-X (NFIX)* at 19p13.2. In addition, a nonsense mutation in *NFIX* was identified in one patient with Sotos-like features.[6] Interestingly, frameshift and donor-splice site mutations were also identified in Marshall–Smith syndrome (MIM 602535) that is osteochondysplasia syndrome characterized by accelerated skeletal maturation, relative failure to thrive, respiratory difficulties, mental retardation and unusual facial features.[7] Therefore, *NFIX* mutations could cause either Sotos-like features or Marshall–Smith syndrome. Whereas the transcripts possessing the nonsense mutation in a patient with Sotos-like features suffered from the nonsense-mediated mRNA decay, transcripts of mutated alleles (by a donor-spice site and two frameshift mutations) in patients with Marshall–Smith syndrome escaped from the nonsense-mediated mRNA decay surveillance and could be translated, suggesting that haploinsufficiency of *NFIX* leads to

[1]Department of Human Genetics, Yokohama City University Graduate School of Medicine, Yokohama, Japan; [2]Department of Pediatrics, Okinawa Child Development Center, Okinawa, Japan; [3]Education Center, Asahikawa Medical University, Asahikawa, Japan; [4]Department of Pediatrics, Hokkaido Asahikawa Habilitation Center for Disabled Children, Asahikawa, Japan; [5]Department of Biochemistry, Yokohama City University Graduate School of Medicine, Yokohama, Japan; [6]Department of Neuropsychiatry, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [7]Department of Biological Psychiatry, Tohoku University Graduate School of Medicine, Sendai, Japan and [8]Department of Medical Genetics, University of the Ryukyus Faculty of Medicine, Nishihara, Japan
Correspondence: Dr N Matsumoto, Department of Human Genetics, Yokohama City University Graduate School of Medicine, Fukuura 3-9, Kanazawa-ku, Yokohama 236-0004, Japan.
E-mail: naomat@yokohama-cu.ac.jp
Received 9 September 2011; revised 21 November 2011; accepted 5 January 2012; published online 2 February 2012

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,

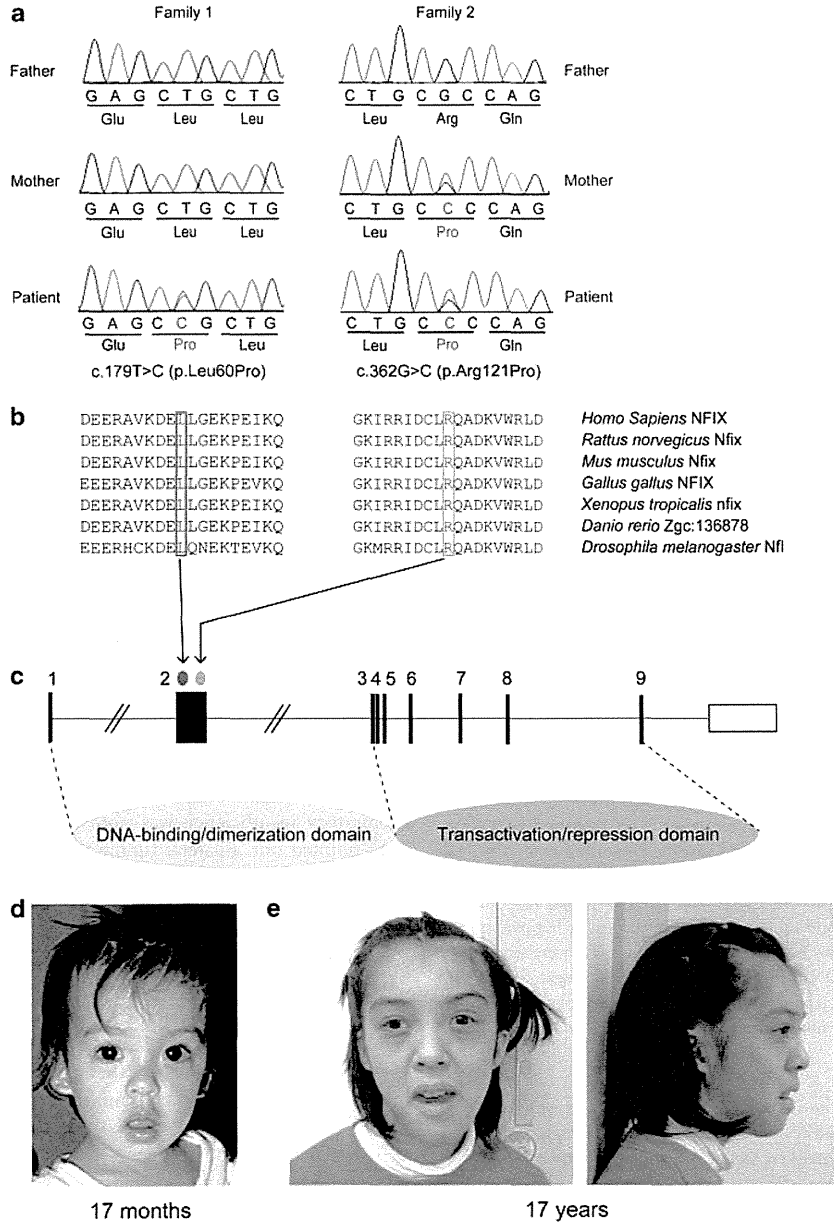

Figure 1 Missense mutations in *NFIX* in individuals with Sotos-like features. (**a**) Electropherogram of family 1 (left) and family 2 (right). The c.179T>C (p.Leu60Pro) mutation occured *de novo*. The c.362G>C (p.Arg121Pro) mutation was inherited from his mother. (**b**) An amino-acid sequence alignments 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, underdeveloped 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.

ACKNOWLEDGEMENTS

We thank the patients and their family members for their participation in this study. This work was supported by Research Grants from the Ministry of Health, Labour and Welfare (HS, N Miyake and N Matsumoto) and the Japan Science and Technology Agency (N Matsumoto), a Grant-in-Aid for Young Scientist from the Japan Society for the Promotion of Science (HS, HD and N Miyake) and a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (N Matsumoto).

1 Leventopoulos, G., Kitsiou-Tzeli, S., Kritikos, K., Psoni, S., Mavrou, A., Kanavakis, E. *et al.* A clinical study of Sotos syndrome patients with review of the literature. *Pediatr. Neurol.* **40,** 357–364 (2009).
2 Kurotaki, N., Imaizumi, K., Harada, N., Masuno, M., Kondoh, T., Nagai, T. *et al.* Haploinsufficiency of *NSD1* causes Sotos syndrome. *Nat. Genet.* **30,** 365–366 (2002).
3 Rayasam, G. V., Wendling, O., Angrand, P. O., Mark, M., Niederreither, K., Song, L. *et al. NSD1* is essential for early post-implantation development and has a catalytically active SET domain. *EMBO J.* **22,** 3153–3163 (2003).
4 Visser, R. & Matsumoto, N. in *Inborn Errors of Development* (eds Epstein, C. J., Erickson, R. P., Wynshaw-Boris, A.) 1032–1037 (Oxford University Press, New York, 2008).
5 Tatton-Brown, K. & Rahman, N. Sotos syndrome. *Eur. J. Hum. Genet.* **15,** 264–271 (2007).
6 Malan, V., Rajan, D., Thomas, S., Shaw, A. C., Louis Dit Picard, H., Layet, V. *et al.* Distinct effects of allelic *NFIX* mutations on nonsense-mediated mRNA decay engender either a Sotos-like or a Marshall-Smith syndrome. *Am. J. Hum. Genet.* **87,** 189–198 (2010).
7 Adam, M. P., Hennekam, R. C., Keppen, L. D., Bull, M. J., Clericuzio, C. L., Burke, L. W. *et al.* Marshall-Smith syndrome: natural history and evidence of an osteochondrodysplasia with connective tissue abnormalities. *Am. J. Med. Genet. A.* **137,** 117–124 (2005).
8 Saitsu, H., Kato, M., Mizuguchi, T., Hamada, K., Osaka, H., Tohyama, J. *et al. De novo* mutations in the gene encoding STXBP1 (MUNC18-1) cause early infantile epileptic encephalopathy. *Nat. Genet.* **40,** 782–788 (2008).
9 Gronostajski, R. M. Roles of the NFI/CTF gene family in transcription and development. *Gene* **249,** 31–45 (2000).
10 Kruse, U. & Sippel, A. E. Transcription factor nuclear factor I proteins form stable homo- and heterodimers. *FEBS Lett.* **348,** 46–50 (1994).
11 Armentero, M. T., Horwitz, M. & Mermod, N. Targeting of DNA polymerase to the adenovirus origin of DNA replication by interaction with nuclear factor I. *Proc. Natl. Acad. Sci. USA* **91,** 11537–11541 (1994).
12 Stefancsik, R. & Sarkar, S. Relationship between the DNA binding domains of SMAD and NFI/CTF transcription factors defines a new superfamily of genes. *DNA Seq.* **14,** 233–239 (2003).
13 MacArthur, M. W. & Thornton, J. M. Influence of proline residues on protein conformation. *J. Mol. Biol.* **218,** 397–412 (1991).
14 Shaw, A. C., van Balkom, I. D., Bauer, M., Cole, T. R., Delrue, M. A., Van Haeringen, A. *et al.* Phenotype and natural history in Marshall-Smith syndrome. *Am. J. Med. Genet. A* **152A,** 2714–2726 (2010).
15 Cole, T. R. & Hughes, H. E. Sotos syndrome: a study of the diagnostic criteria and natural history. *J. Med. Genet.* **31,** 20–32 (1994).

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

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

YOSHIMASA TAKANO, DONG SHI, AI SHIMIZU, TOMOYO FUNAYAMA, YUKIHIKO MASHIMA, NORIKO YASUDA, TAKEO FUKUCHI, HARUKI ABE, HIDENAO IDETA, XIAODONG ZHENG, ATSUSHI SHIRAISHI, YUICHI OHASHI, KOHJI NISHIDA, TORU NAKAZAWA, AND NOBUO FUSE

• 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.)

AJO.com Supplemental Material available at AJO.com.

Accepted for publication Mar 29, 2012.

From the Department of Ophthalmology, Tohoku University Graduate School of Medicine, Miyagi, Japan (Y.T., D.S., Ai.S., T.N., N.F.); Department of Ophthalmology, the Fourth Affiliated Hospital, China Medical University, Liaoning, China (D.S.); Department of Chemistry, Faculty of Education, Bunkyo University, Saitama, Japan (To.F.); Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan (To.F., Y.M.); Department of Ophthalmology, Tokyo Metropolitan Police Hospital, Tokyo, Japan (N.Y.); Division of Ophthalmology and Visual Science, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan (Ta.F., H.A.); Ideta Eye Hospital, Kumamoto, Japan (H.I.); Department of Ophthalmology, Ehime University School of Medicine, Ehime, Japan (X.Z., At.S., Y.O.); Department of Ophthalmology, Osaka University Graduate School of Medicine, Osaka, Japan (K.N.); and Department of Integrative Genomics, Tohoku Medical Megabank Organization, Miyagi, Japan (N.F.).

Inquiries to N. Fuse, Department of Integrative Genomics, Tohoku Medical Megabank Organization & Department of Ophthalmology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan; e-mail: fusen@oph.med.tohoku.ac.jp

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

0002-9394/$36.00
http://dx.doi.org/10.1016/j.ajo.2012.03.050

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

TABLE 3. Haplotype Frequencies of Tag Single Nucleotide Polymorphisms of the Toll-like Receptor 4 Gene Compared with Previous Study

	This Study										Previous Study			
Tag SNPs rs10759930, rs11536889, rs7037117, and rs7045953	POAG (n = 184)	*P* Value	Overall *P* Value (POAG)	NTG (n = 365)	*P* Value	Overall *P* Value (NTG)	XFG (n = 109)	*P* Value	Overall *P* Value (XFG)	Control (n = 216)	NTG (n = 250)	Control (n=318)	*P* Value	Overall *P* Value (NTG)
TGAA	0.311	.000072	.00097	0.360	.003	.057	0.362	.036	0.134	0.448	0.350	0.402	.070	.044
TCAA	0.228	.882		0.242	.465		0.229	.863		0.223	0.226	0.247	.41	
CGAA	0.208	.030		0.164	.519		0.173	.439		0.150	0.166	0.159	.75	
CGGA	0.125	.406		0.137	.126		0.146	.141		0.107	0.154	0.102	.0090	
CGGG	0.090	.080		0.074	.280		0.067	.664		0.058	0.096	0.077	.26	
Tag SNPs rs10759930 and rs7037117														
TA	0.539	.00014	.0017	0.603	.020	.085	0.591	.044	0.201	0.674	0.575	0.649	.0044	.010
CG	0.216	.063		0.219	.023		0.219	.086		0.164	0.249	0.179	.21	
CA	0.238	.0073		0.178	.524		0.185	.479		0.162	0.173	0.169		

NTG = normal-tension glaucoma; POAG = primary open-angle glaucoma; SNP = single nucleotide polymorphism; XFG = exfoliation glaucoma.

TABLE 4. Haplotype Frequencies of Tag Single Nucleotide Polymorphisms of the Toll-like Receptor 4 Gene Between Primary Open-Angle, Normal-Tension, and Exfoliation Glaucoma and Control Subjects

Tag SNPs rs10759930, rs1927914, rs1927911, and rs2149356	POAG (n = 184)	*P* Value	Overall *P* (POAG)	NTG (n = 365)	*P* Value	Overall *P* (NTG)	XFG (n = 109)	*P* Value	Overall *P* (XFG)	Control (n = 216)
TTCC	0.516	.000014	.00078	0.589	.009	0.018	0.573	.020	.014	.667
CCTA	0.418	.00033		0.384	.003		0.395	.012		.296
CCCC	0.005	.138		0.008	.211		NA	NA		.016

NTG = normal-tension glaucoma; POAG = primary open-angle glaucoma; XFG = exfoliation glaucoma.

rs7045953) were studied earlier.[21] The results showed that NTG and XFG were not statistically significant (overall $P = .057$, $P = .134$), but POAG was statistically significant (overall $P = .00097$; Table 3). The tag SNPs, rs10759930 and rs7037117, used in that study were similar with these haplotypes (Table 3).

Other haplotypes, 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; Table 4).

DISCUSSION

• **TOLL-LIKE RECEPTOR 4 POLYMORPHISMS IN PRIMARY OPEN-ANGLE GLAUCOMA, NORMAL-TENSION GLAUCOMA, AND EXFOLIATION GLAUCOMA SUBJECTS:** Shibuya and associates showed that rs7037117, located in the 3′-untranslated region of *TLR4*, was most strongly associated with NTG.[21] Compared to earlier reports, the intragenic SNP rs2149356 could be more associated with NTG and also with POAG and XFG in this study. The statistics of all 8 genotypes showed that *TLR4* had approximately the same tendency for all corresponded allele frequencies (Table 2). The haplotypes rs10759930, rs1927914, rs1927911, and rs2149356 had the higher statistically significant values in both groups (overall $P = .00078$, $P = .018$, and $P = .014$, respectively; Table 4). On the other hand, this haplotype was shown to be not significant, and even in the original study,[21] it was statistically marginal ($P = .044$ for 4 SNPs and $P = .010$ for 2 SNPs). Thus, these haplotypes and/or SNPs are valuable for screening for glaucoma in the Japanese.

Subjects enrolled in this study and those reported by Shibuya and associates[21] were from across Japan; however, the subjects from our study were predominantly from northern Japan. The difference in the heterogeneity may explain the slight differences between the 2 studies. An association between the SNPs and POAG and XFG was not expected before this study because the IOP has a predominant effect on these diseases. So it is interesting that *TLR4* would be associated with those phenotypes of POAG and XFG, and the risk associations were stronger in POAG than in NTG. Recently, Suh, and associates showed that *TLR4* gene polymorphisms do not associate significantly with NTG in a Korean population,[22] but they did not examine it in POAG and XFG subjects. It should be evaluated in various types of glaucoma in different populations.

• **FUNCTION OF *TLR4* GENE:** Innate immunity produces antimicrobial peptides against many kinds of pathogens in the host defense system, and these induce adaptive immunity secondarily. Together, they play important roles in the total immune system.[24] Targeting TLR signaling has implications in the control of infection, vaccine design, desensitization to allergens, and downregulation of inflammation. *TLR4*-deficient mice were reported to have an upregulation of NADPH oxidase (Nox3), which increased the oxidative stress.[25] Although the function of the 8 SNPs on the *TLR4* gene was not examined, rs10759930 and rs1927914 exist within the 5′ untranslated region, rs1927911, rs12377632, and rs2149356 exist within introns, and rs11536889, rs7037117, and rs7045953 exist within the 3′ untranslated region. There is a possibility that these SNPs influence the stability of the mRNA and expression of the *TLR4* gene because rs11536889 exists near exon3.

TLR4 is expressed in the conjunctiva, cornea, iris, ciliary body, choroid, retina, and retinal pigment epithelium. In the retina, changes in the glial cells may be associated with glaucoma, especially NTG, which is not so dependent on the IOP. Widespread chronic stress is evident in the retina and optic nerve head by the strong upregulation of the HSPs in glaucomatous eyes.[26] Recently, an upregulation of toll-like receptors TLR2, TLR3, and TLR4 was found in human glaucoma donor eyes, which is consistent with the strongly increased level of expression of HSPs.[27] Immunohistochemical analyses supported an upregulated expression of TLRs in both microglia and astrocytes in glaucomatous retinas. It has been postulated that changes in the microenvironment of injured axons will alter the glycosaminoglycan composition in the lamina cribrosa, and this may account for the increased vulnerability of the remaining axons to sustain further damage independent of the IOP.

The significance of these findings in POAG more than NTG raises further speculation. Chronic stress could influence the aqueous humor and may adversely affect the outflow structure to increase resistance to outflow, with alterations of the trabecular meshwork and intrascleral channels and collapse of the Schlemm canal. There is a possibility that *TLR4* might have an effect on the alterations of the aqueous humor dynamics and injury to the glaucomatous retina in eyes with POAG and XFG.

• **AUTOIMMUNE DISEASES, CHRONIC INFLAMMATION, AND GLAUCOMA:** To date, the genes of the TLR family have not been candidates as genetic modifiers of glaucoma susceptibility, but they have been implicated in other autoimmune diseases and allergic diseases, including rheumatoid arthritis[28,29] and bronchical asthma.[30,31] It is interesting that the net *TLR4* sequence variants and the *TLR4* signaling network would affect not only the development of NTG but also POAG and XFG. Chronic infection by certain bacteria and viruses may play a role in inflammation.[32] More specifically, chronic infection by *Helicobater pyori* may induce a persistent systemic and vascular inflammation and endothelial dysfunction.[33] The results of one study showed that the specific IgG antibody levels of *H. pylori* were significantly increased in the aqueous humor and serum of patients with POAG and