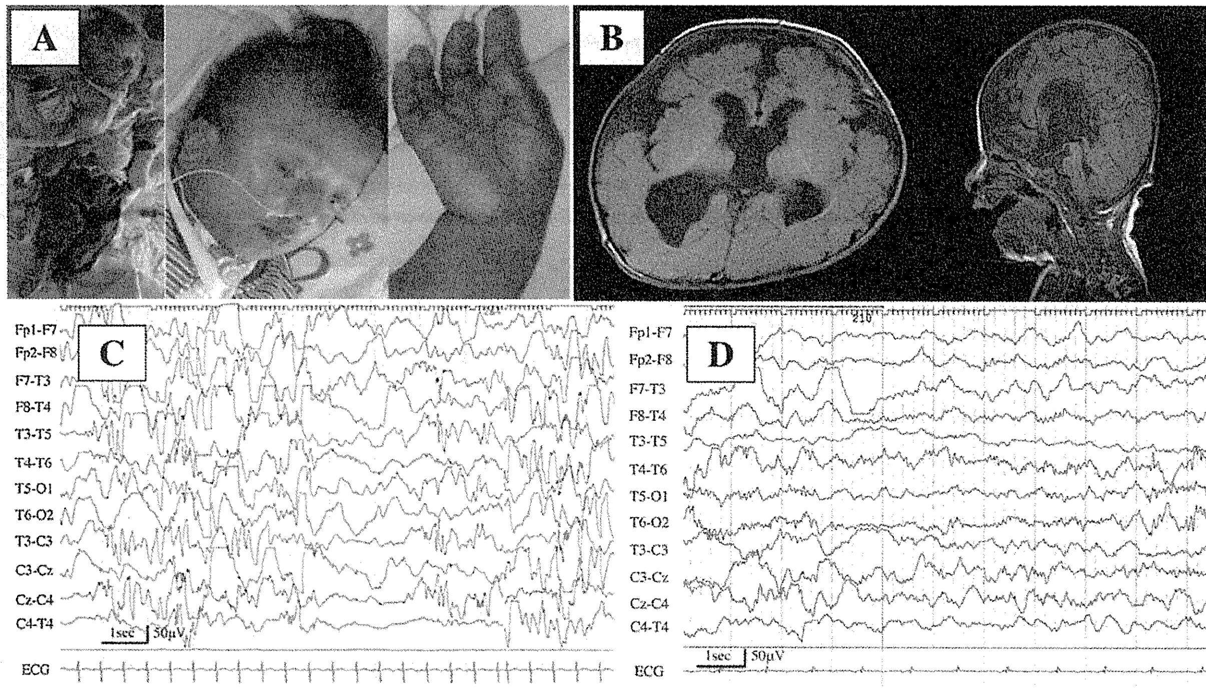

Fig. 1. Case 1. "Coarse face," curly hair, prominent forehead, dysmorphic ears, abnormal loose and pigmented skin, webbed neck, chest deformity, and micromelic dwarfism at six years (A), agenesis of the corpus callosum, ventricular dilatation, diffuse cortical atrophy and severely delayed myelination on MRI images at three years (B), hypsarrhythmia at 11 months (C), asynchronous, high-voltage slow waves with irregular spike-wave or polyspikes with/without waves dominantly in the right temporal-occipital region at six years of age (D) on EEG.

was initiated for severe dyspnea with laryngo/tracheomalacia. Percutaneous endoscopic gastrostomy (PEG) was performed for repetitive aspiration pneumonia caused by dysphagia. He has been diagnosed as mentally retarded and had not developed any expressive language. Additionally, he suffers from truncal hypotonia with increased muscle tone and joint contractures in his extremities. He has been profoundly delayed in terms of physical and mental development due to his severe motor and intellectual disabilities.

His karyotype was 46,XY and genetic screening confirmed a heterozygous nucleotide change within exon 5 of the KRAS gene (c.458A > T), causing the amino acid substitution D153V, whose phenotype was CFC/Noonan syndrome.

2.2. Case 2: four-year-old girl

An appropriate-for-date girl (35 weeks of gestational age with a birth weight of 2624 g) without asphxial episodes was born to healthy and non-consanguineous parents (mother 30 years old and father 35 years old) who had previously given birth to three healthy baby girls. Following delivery, several surface anomalies were noted, such as an odd-looking "coarse" face (prominent forehead, short nose and broad nasal bridge with anteverted nares, downslanting palpebral fissures, and low-set dysmorphic ears), curly and sparse hair, abnormal skin manifestations (loose, pigmented skin with multiple lentigo, wrinkled palms with deep palmar and plantar creases), narrow chest, and hypotonic micromelic dwarfism (Fig. 2A). Postnatal screening revealed cardiac failure due to severe hypertrophic cardiomyopathy, resulting in chronic heart failure, which necessitated the administration of diuretics and beta blockers.

On admission to our NICU, a subtle seizure occurred and was only controlled following the infusion of phenobarbital (PB) and MDL. After this episode, seizures have been severely refractory and uncontrolled despite the use of a majority of antiepileptic drugs, including PB, VPA, CZP, ZNS, carbamazepine, phenytoin, primidone, nitrazepam, clobazam, topiramate, lamotirigine, gabapentin, levetiracetam. Seizures are composed of repetitive brief tonic spasms, tonic-clonic (sometimes developing to status epileptics), myoclonic, and complex partial seizures (sometimes evolving to generalized tonic-clonic seizure [GTCS]), all occurring daily and frequently. Interictal EEG revealed modified hypsarrhythmia at one year of age (Fig. 2C), and her most recent (interictal) EEG showed continuous high-voltage spike or polyspikes with/without slow waves mainly in the left centro-temporal-parietal region at four years of age (Fig. 2D). ACTH therapy has not been introduced because of moderate cortical atrophy with delayed myelination and hypoplastic corpus callosum on cranial MRI images noted at two years of age (Fig. 2B). In addition to seizures, she has exhibited frequent involuntary movement, consisting of dystonia, athetosis, and myoclonus, all resistant to various muscle-relaxant drugs.

She had frequently developed episodes of dyspnea due to congenital laryngo/tracheomalacia, which resulted in tracheotomy and persistent mechanical ventilation during night sleep before two years of age, but recurrent aspiration pneumonia caused by dysphagia finally required PEG. She has been profoundly mentally retarded and unable to speak any words. She has been unable to sit unassisted because of general hypotonia and joint contractures in her extremities. Overall, she has exhibited severe motor and intellectual disabilities.

Her karyotype was 46,XX and advanced genetic screening confirmed a heterozygous nucleotide change within exon 12 of the BRAF gene (c.1454T > C), causing the amino acid substitution L485S, whose clinical phenotype was CFC syndrome.

3. Discussion

The different types of RAS/MAPK syndrome have many overlapping characteristics, including craniofacial manifestations, cardiac malformations, cutaneous, musculoskeletal, gastrointestinal, ocular abnormalities, and neuro-cognitive impairment,

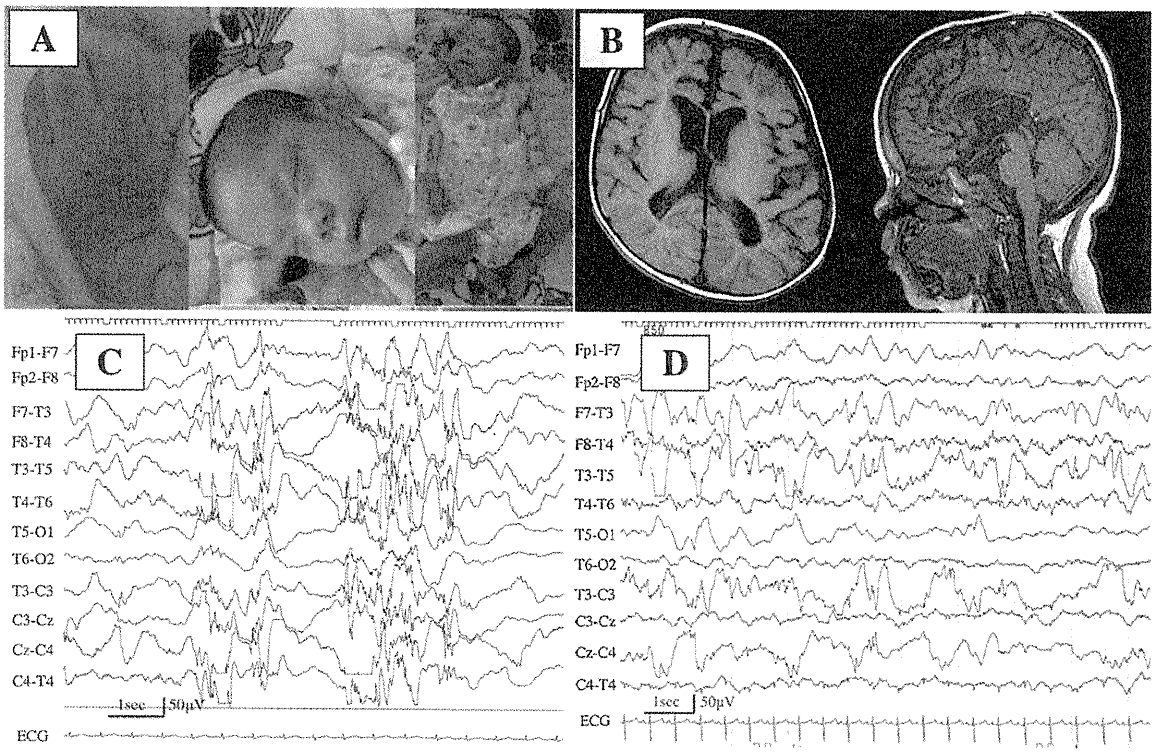

Fig. 2. Case 2. Odd looking "coarse" face (prominent forehead, short nose, and low-set dysmorphic ears, curly sparse hair), abnormal loose, pigmented skin with multiple lentigo, narrow chest, and hypotonic micromelic dwarfism at four years (A), diffuse brain atrophy with delayed myelination and hypoplastic corpus callosum on MRI images at two years (B), modified hypsarrhythmia at one year (C), and continous high-voltage spike or polyspikes with/without slow waves mainly in the left centrotemporalparietal region at four years of age (D) on EEG.

including hypotonia and seizures, caused by dysregulation of signaling in the RAS/MAPK pathway due to mutations mainly in BRAF, MEK1, or MEK2.[1–3] Correlation between confirmed mutations and non-neurological, cardiovascular, cutaneous, and musculoskeletal abnormalities in CFC patients have been discussed,[1–3] but detailed analyses of their associated neurological impairments, especially epileptic conditions, have been sorely lacking.

Only a few previous reports[4–10] of CFC syndrome mentioned associated neurological impairment, especially seizures and EEG findings, but were lacking in terms of their detailed clinical features and courses. Gross-Tsur et al. described the neurological status of 16 patients with CFC syndrome (genetically undetermined cases) in their report with a literature review,[4] including six EEG findings (generalized dysrhythmia [grade, and ungraded], low voltage, focal activity, and episodes of spike and slow 2 Hz with slow background activity). Recently, Yoon et al. mentioned the seizure types and EEG findings in 12 of 15 cases, including four cases of infantile spasms with hypsarrhythmia on EEG.[7] Moreover, Aizaki et al. reported a case of CFC syndrome with infantile spasms, suggesting that seizures with CFC syndrome were refractory despite the administration of various types of anticonvulsants and that the neuro-developmental delay caused by CFC syndrome is severe.[10]

Both cases in the present report exhibited infantile spasms with severely abnormal EEG (modified hypsarrhythmia). Case 1 has been remitted with ACTH therapy and Case 2 remains uncontrolled despite the administration of various types of anticonvulsants. Upon reviewing previous literature[4–10] describing the epileptic conditions and neuroimages in patients with cardio-facio-cutaneous syndrome (Table 1), 62 cases were discovered which mentioned their epileptic condition which included 12 cases (19.3%) with infantile spasms or brief tonic spasms, each of which were accompanied by hypsarrhythmia or modified hypsarrhythmia on EEG. Other cases also develop various types of seizures, GTCS (sometimes evolving to status epileptics), and complex partial seizure, with severe abnormal EEG, consisting of generalized or partial epileptiform activities. Most of the seizures in these patients still remain uncontrolled despite the use of various types of anticonvulsants. Based on the two present cases and the literature review, the high complication rate of infantile spasms in CFC syndrome suggests that there may be specific factors relating to refractory epilepsy, especially epileptic encephalopathy, in the RAS/MAPK signaling pathway.

As for correlations between genotype and epileptic phenotype, D153V mutation in the KRAS gene (as seen in Case 1) was previously reported[11–13] in six cases (two of CFC syndrome, three of Noonan syndrome, and one of CFC/Noonan syndrome), but in none of these cases did the patient develop seizures. Accordingly, this mutation may be unrelated to epileptic severity. On the other hand, it is noteworthy that the mutation L485S in the BRAF gene (as seen in Case 2) has been recently reported to be detected in a patient of CFC syndrome with infantile spasms following a refractory therapeutic course.[10] This point mutation may be related to the severity of epileptic conditions in RAS/MAPK syndrome. In addition, this report[10] described the efficacy of a ketogenic diet (KD) to reduce seizure frequency, but in the present Case 2 patient with the same mutation in the BRAF gene, KD has not been introduced because of severe thinness despite adequate tube nutrition.

In a recent report of CFC patients, neuroimaging played an important role in the diagnosis of this syndrome.[14] Most of the 62 patients with CFC syndrome in the present review shared severe abnormal neuroimaging, including hydrocephalus, agenesis/hypoplasia of the corpus callosum, ventricular dilatation, cortical

Table 1
Epileptic conditions and neuroimages in patients with cardio-facio-cutaneous syndrome: present cases and those from a literature review.

Ref.	Gene	Mutation	Sex	Age of seizure onset	Seizure type	Interictal EEG findings	Anticonvulsant therpay	Seizure prognosis	Neurodevelopmental delay	Neuroimaging (brain MRI)
Gross-Tsur et al.[4] ($n=1$)	N.A.	N.A.	M	1 y 9 mo	Lennox–Gastaut syndrome	Multiple episodes of spike and slow wave activity, 2 Hz. The background activity was abnormally slow.	VPA, CZP	Decrease in seizures	Hypotonia, ataxia, lack of language skill, extreme hyperactivity	Normal
Raymond and Holmes[5] ($n=2$)	N.A.	N.A.	F	–	No seizure	Decrease in anterior voltages, no epileptiform activity (postnatal screening)	–	–	No motor delay, marked language delay	External hydrocephalus with widened subarachnoid space, cortical atrophy in the frontal and temporal lobes (CT)
	N.A.	N.A.	M	–	No seizure	N.A.	–	–	No motor delay, marked language delay	Marked cortical atrophy (CT)
Sabatino et al.[6] ($n=2$)	N.A.	N.A.	M	1 y 3 mo	Tonic-clonic (SE)	Focal activity in the bilateral posterior areas	N.A.	N.A.	Moderate to severe	Cortical atrophy, ventriculomegaly
	N.A.	N.A.	F	6 y	GTCS	Irritative waves and generalized disorganization, frequent focal spikes in the right regions, sometimes in contralateral areas.	PB	Controlled	Moderate to severe	Diffuse cortical atrophy
Yoon et al.[7] ($n=15$)	MEK1	F53S	F	15 y 10 mo	GTCS, Abs, CPS	Generalized spikes/slow waves ($n=5$), hypsarrhythmia ($n=4$), focal epileptiform discharges ($n=3$)	ZNS, LEV	Not described in detail. Polytherapy required in 9 of 15 cases, suggesting that seizure control is often difficult.	Severe	Ventriculomegaly and hydrocephalus (66%), prominent Vircho- Robin spaces (20%), cortical atrophy, prominence of CFS spaces wih macrocephaly, benign extraventricular obstructive hydrocephalus. (some cases)
	BRAF	L485F	M	2 wk	CPS, sGTCS, Abs		OXC, DZP,		Severe	
	BRAF	F468S	F	11 y	GTCS		CBZ, PB		Profound	
	BRAF	Q257R	M	2 y 6 mo	Abs, focal		VPA		Mild	
	BRAF	del E11	F	1 y 6 mo	IS		TPM, CZP, VPA, PSL		Severe	
	BRAF	Q257R	M	3 y	Not specified		CBZ		Severe	
	BRAF	F595L	F	6 mo	IS, vocal motor, CPS		Felbamate, ZNS, CZP		Profound	
	BRAF	T599R	F	3 y	Not specified		OXC		Profound	
	BRAF	G534R	M	5 y	GTCS, Abs		OXC		Profound	
	BRAF	L485S	M	4 mo	GTCS, CPS, IS		TPM, CZP, VPA, DZP		N.A.	
	MEK1	Y130C	F	2y	Not specified		LEV		N.A.	
	BRAF	D638E	F	1 y 6 mo	GTCS, Abs		LEV, PHT		Profound	
	BRAF	K499N	F	7 mo	GTCS, Abs		LTG, CBZ, CZP		Severe	
	MEK1	Y130N	F	1 y	CPS		OXC		Profound	
	MEK1	G128V	F	5 mo	IS		PB, LTG, VPA, CZP		N.A.	

Table 1 (*Continued*)

Ref.	Gene	Mutation	Sex	Age of seizure onset	Seizure type	Interictal EEG findings	Anticonvulsant therpay	Seizure prognosis	Neurodevelopmental delay	Neuroimaging (brain MRI)
Armour and Allanson[8] (n=38)	BRAF (15/32 cases) MEK1(2/4 cases) MEK2(1/2 cases)	N.A.	N.A.	IS (n=5), Abs (n=4), GTCS (n=4), CPS (n=4)	N.A.	N.A.	Respondents 49%	All significant delay (available in 27cases)	Hydrocephaly (2), ventriculomegaly (9), reduced white matter (6), thin corpus callosum (3), cerebral atrophy (3), delayed myelination (3), Chiari 1 malformation (1), pachygyria (1), nodulat heterotopia (1), abnormal migration (1), cerebellar calcification (1) available on 23 cases)	
Demir et al.[9] (n=1)	BRAF	F468S	F	N.A.	Recurrent clonic seizures	Epileptiform discharges in the right front central temporal region	VPA, CBZ, TPM	Controlled	Mental/motor/ language delay	Mild frontoparietal cortical atrophy, mildly dilated ventricles, thinning of the posterior part of the corpus callosum
Aizaki et al.[10] (n=1)	BRAF	L485S	F	2 mo	Brief tonic spasms (repetitive)	Asynchronous, high-voltage slow waves with multifocal sharp waes appeared with bilateral pariet-occipital predominance	VPA, VitB6, ZNS, CLB, PB, ACTH, KD, Clorazepate dipotassium	Uncontrolled	Profound	Hypoplastic corpus callosum, moderate brain atrophy, delayed myelination, ambiguous coorticomedullary boundary in the right posterior temporal lobe
Present cases (n=2)	KRAS	D153V	M (Case 1)	3 mo/11 mo	Myo/IS	Hypsarrhythmia (at 11 mos), asynchronous, high-voltage slow waves with irregular spike-wave, or polyspikes with/without waves dominantly in the right temporal-occipital region (at 6 yrs)	MDL/VPA, CZP, ZNS, ACTH	Controlled	Profound	Diffuse cortical atriphy, ventricular dulatation, agenesis of the corpus callosum, delayed myelination
	BRAF	L485S	F (Case 2)	Day 0	Subtle, brief tonic spasms, CPS, GTCS	Modified hypsarrhythmia (at 1 yr), Continous high-voltage spike or polyspikes with/without slow waves in the left centro-temporalparietal region (at 4 yrs)	MDL, VPA, CZP, NZP, PB, CBZ, ZNS, CLB, PHT, PRM, GAP, TPM, LTG, LEV, TRH, ST	Uncontrolled	Profound	Diffuse cortical atrophy, ventricular dulatation, hypoplastic corpus callosum, delayed myelination

GTCS: generalized tonic-clonic seizure, sGTCS: secondarily generalized tonic-clonic seizure, CPS: complex partial seizure, Abs: absence seizure, Myo: myoclonic seizure, IS: infantile spasms, SE: status epileptics, Subtle: subtle seizure.

PB: phenobarbital, VPA: valproic acid, CBZ: carbamazepine, ZNS: zonisamide, PHT:pheytoin, PRM: primidon, CZP: clonazepam, CLB: clobazam, NZP: nitrazepam, DZP: diazepam, MDL: midazolam, ST: sultiame, VitB6: vitamin B6, GAP: gabapentin, TPM: topiramate, LTG: lamotrigine, LEV: levetiracetam, OXC: oxcarbazepine, ACTH: adrenocorticotropic hormone, PSL: prednisone, KD: ketogenic diet, CSF: cerebrospinal fluid, MRI: magnetic resonance image, CT: computed tomography, SE: status epilepticus, N.A.: not applicable.

atrophy, and delayed myeliation, resulting in neuro-developmental delay ranging from 'moderate to severe' to 'profound', all of which distinguish CFC syndrome from the other types of RAS/MAPK syndrome (Noonan and Costello syndromes).

More cases will need to be studied in order to clarify the genotype–phenotype correlations of several genes in the RAS/MAPK signaling pathway associated with refractory epilepsy.

Conflict of interest

The authors report no conflict of interest.

Acknowledgements

We thank the two patients and their families for their willingness to be included in this report.

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

MBTPS2 Mutation Causes BRESEK/BRESHECK Syndrome

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Received 25 July 2011; Accepted 17 October 2011

BRESEK/BRESHECK syndrome is a multiple congenital malformation characterized by brain anomalies, intellectual disability, ectodermal dysplasia, skeletal deformities, ear or eye anomalies, and renal anomalies or small kidneys, with or without Hirschsprung disease and cleft palate or cryptorchidism. This syndrome has only been reported in three male patients. Here, we report on the fourth male patient presenting with brain anomaly, intellectual disability, growth retardation, ectodermal dysplasia, vertebral (skeletal) anomaly, Hirschsprung disease, low-set and large ears, cryptorchidism, and small kidneys. These manifestations fulfill the clinical diagnostic criteria of BRESHECK syndrome. Since all patients with BRESEK/BRESHECK syndrome are male, and X-linked syndrome of ichthyosis follicularis with atrichia and photophobia is sometimes associated with several features of BRESEK/BRESHECK syndrome such as intellectual disability, vertebral and renal anomalies, and Hirschsprung disease, we analyzed the causal gene of ichthyosis follicularis with atrichia and photophobia syndrome, *MBTPS2*, in the present patient and identified an p.Arg429His mutation. This mutation has been reported to cause the most severe type of ichthyosis follicularis with atrichia and photophobia syndrome, including neonatal and infantile death. These results demonstrate that the p.Arg429His mutation in *MBTPS2* causes BRESEK/BRESHECK syndrome.

Key words: BRESEK/BRESHECK syndrome; IFAP syndrome; *MBTPS2*; mutation; S2P

How to Cite this Article:
Naiki M, Mizuno S, Yamada K, Yamada Y, Kimura R, Oshiro M, Okamoto N, Makita Y, Seishima M, Wakamatsu N. 2011. *MBTPS2* mutation causes BRESEK/BRESHECK syndrome.

Am J Med Genet Part A .

INTRODUCTION

BRESEK/BRESHECK syndrome (OMIM# 300404), a multiple congenital malformation disorder characterized by brain anomalies, intellectual disability, ectodermal dysplasia, skeletal deformities, Hirschsprung disease, ear or eye anomalies, cleft palate or cryptorchidism, and kidney dysplasia/hypoplasia [Reish et al., 1997]. The acronym BRESEK refers to the common findings, whereas BRESHECK refers to all manifestations. Because the first two patients were maternally related half brothers, an X-linked disorder was proposed. Although each symptom of these patients is often observed in other congenital diseases, the combination of all symptoms is rare, and only one additional patient with BRESEK has been reported to date [Tumialán and Mapstone, 2006]. Here, we present the fourth male patient with multiple anomalies. The patient presented with a variety of clinical features that were consistent with those of the previously reported BRESHECK syndrome.

The syndrome of ichthyosis follicularis with atrichia and photophobia (IFAP, OMIM# 308205), an X-linked recessive oculocutaneous disorder, is characterized by a peculiar triad of ichthyosis follicularis, total or subtotal atrichia, and varying degrees

Grant sponsor: Takeda Science Foundation; Grant sponsor: Health Labour Sciences Research Grant.

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Published online 00 Month 2011 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/ajmg.a.34373

of photophobia [MacLeod, 1909]. Martino et al. [1992] reported a male patient with IFAP syndrome presented with short stature, intellectual disability, seizures, hypohidrosis, enamel dysplasia, congenital aganglionic megacolon, inguinal hernia, vertebral and renal anomalies, and the classic symptom triad of IFAP syndrome. This report broadened the clinical features of IFAP syndrome. It should be noted that the clinical symptoms of this patient are quite similar to those of BRESHECK syndrome, with the exception of cleft palate, cryptorchidism, and photophobia (Patient 5; Table I). The gene mutated in patients with IFAP syndrome, *MBTPS2* (GenBank reference sequence NM_015884), was identified from a variety of clinical features of IFAP syndrome, including the triad and neonatal death [Oeffner et al., 2009]. Thus, the mode of inheritance and several clinical features are common to both BRESEK/BRESHECK and IFAP syndromes. These findings prompted us to perform mutation analysis of *MBTPS2* in the present patient, resulting in the identification of a missense mutation.

MATERIALS AND METHODS

Patients

Written informed consent was obtained from the parents of the patient. Experiments were conducted after approval of the institutional review board of the Institute for Developmental Research, Aichi Human Service Center. The patient (II-1; Fig. 3) was born to a 31-year-old mother (I-2) and a 31-year-old father (I-1), both healthy Japanese individuals without consanguinity. His mother miscarried her first child at 5 weeks. The pregnancy of the patient reported here was complicated with mild oligohydramnios, and he was delivered by caesarean because of a breech position at 38 weeks of gestation. His birth weight was 1,996 g (−2.6 SD), and he measured 44 cm (−2.6 SD) in length with an occipitofrontal circumference of 32.5 cm (−0.5 SD). Apgar scores at 1 and 5 min were four and eight, respectively. The patient exhibited generalized alopecia and lacked eyelashes, scalp hair, and eyebrows (Fig. 1A). The skin on the entire body was erythematous with

TABLE I. Clinical Features of BRESEK/BRESHECK and IFAP Syndromes and *MBTPS2* Mutation

	BRESEK/BRESHECK syndrome					IFAP syndrome	
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Clinical features							
Gender	M	M	M	M	M	M	M
Gestational age (weeks)	32	40	ND	38	30	ND	ND
Birth weight (g)	990	2,230	ND	1,996	2,040	ND	ND
Intrauterine growth retardation	+	+	ND	+	−	ND	ND
Major features							
Follicular ichthyosis	−	−	ND	−	+	+	+
Atrichia	+	+	+	+	+	+	+
Photophobia	−	−	−	+	+	+	+
Brain malformation	+	+	+	+	+	−	+
Mental and growth retardation	+	+	+	+	+	+	+
Skeletal (Vertebrate) anomalies	+	+	+	+	+	+	+
Hirschsprung disease	−	+	+	+	+	+	+
Eye malformation or	+	+	+	−	+	−	−
Large ears	+	+	+	+	+	−	−
Cleft lip/palate or	−	+	−	−	−	+	−
Cryptorchidism	+	+	−	+	−	−	−
Kidney malformation	+	+	−	+	+	+	+
Other features							
Microcephaly	+	+	+	+	+	−	+
Seizures	−	+	+	+	+	−	+
Deafness	−	+	−	+	−	−	−
Hand anomalies	+	+	+	−	+	+	+
Cardiac anomalies	−	−	+	−	−	−	+
Inguinal hernia	−	−	−	−	+	+	+
Trachea anomalies	−	−	−	+	−	−	−
Regression	−	−	−	+	−	−	−
Age	6 h d	7 y	1.5 y	8 y	3 y	9 m d	14 m d
MBTPS2 mutation	NP	NP	NP	R429H	NP	R429H	R429H

+, present;−, not present; M, male; ND, not described; NP, not performed; h, hour; d, dead; m, month; y, year; R429H, Arg429His; BRESEK/BRESHECK syndrome, (Patients 1-4); IFAP syndrome, (Patients 5–7); Patients: 1, Reish et al. [1997] patient 1; 2, Reish et al. [1997] patient 2; 3, Tumialán and Mapstone [2006]; 4, present case; 5, Martino et al. [1992]; 6, Oeffner et al. [2009] 3-III:3; 7, Oeffner et al. [2009] 3-III:4.

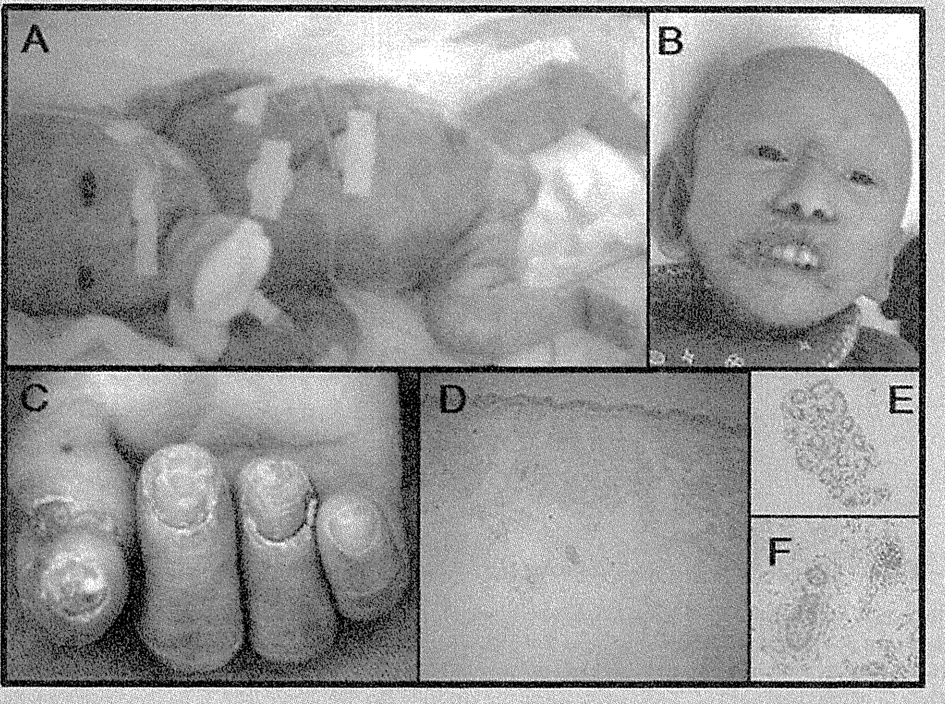

FIG. 1. Clinical appearance and dermatological findings of the patient. A: Lateral view of the patient at birth. Note the generalized alopecia with an absence of scalp hair, eyebrows, and eyelashes. The skin was dry and scaly, and an itchy erythema was observed over the entire body. B: Frontal view of the patient at 4 years of age. Note the characteristic facial appearance with long, malformed ears, a relatively high nasal bridge, and a wide nasal base. C: The patient had normal-sized but deformed and thickened nails. D–F: Histologic examination of the abdominal skin at the age of 15 months showed a reduced number of hair follicles (D), normal eccrine glands (E), and hypoplastic hair follicles (F).

continuous desquamation (Fig. 1A). He had malformed large ears, an inferiorly curved penis, and a bifid scrotum. The testicles were not palpable. He experienced persistent constipation, and total colonic Hirschsprung disease was confirmed through barium enema (Fig. 2E) and rectal biopsy at 2 months. A bone survey performed using three-dimensional (3D) computed tomography (CT) showed abnormal imbalanced hemivertebrae in the two lowest thoracic vertebral bodies (Fig. 2C). The patient's right kidney was smaller than normal. Brain magnetic resonance imaging (MRI) at 3 years of age demonstrated decreased volumes of the frontal and parietal lobes and thinning of the corpus callosum with dilatation of the ventricles (Fig. 2A,B). There were no abnormalities of the eyes or optic nerves. We concluded that the patient had BRESHECK syndrome. The patient had seizures at 5 months of age with an apneic episode and cyanosis. Electroencephalographic (EEG) analysis showed abnormal patterns of sharp waves in the posterior lobe. The seizures were almost completely controlled with phenobarbital. The patient was allergic to milk. At 7 months, tracheal endoscopy revealed subglottic tracheal stenosis and abnormal segmentation of the left lung. A chest CT performed at 3 years of age showed a congenital cystic adenomatoid malformation (CCAM) in the right upper lobe (Fig. 2D). Auditory brain stem responses showed bilateral 80 dB hearing loss at 8 months of age.

The patient exhibited delayed psychomotor development during his infancy. He could drink from a bottle at the age of 3 months and could sit up unsupported at 15 months. Abdominal skin biopsy at 15 months revealed reduced number of hair follicles (Fig. 1D). The eccrine glands were normal (Fig. 1E), and most of his hair follicles appeared to be hypoplastic (Fig. 1F). These findings were similar to ichthyosiform erythroderma. Photophobia was noted when the patient left the hospital and first went outside at 18 months of age. At 2 years and 6 months of age, he had a series of epileptic episodes. He experienced a maximum of 100 seizures per day, and EEG analysis showed continual abnormal spikes in the posterior lobe. The seizures were controlled with clonazepam therapy. At 2 years and 9 months of age, he could stand with support and displayed social smiles when interacting with other people. However, the patient developed psychomotor regression at the age of 3 years. He exhibited a progressive loss of emotional response to others, developed hypotonia, and could not stand or sit alone. At 4 years of age, he became bedridden and showed almost no response to people. He had highly desquamated skin, similar to that seen in ichthyosis (Fig. 1B), and easily developed erythema on the skin of the entire body. The patient had deformed and thickened nails (Fig. 1C). He had persistent corneal erosions, but ophthalmoscopy could not be performed at the age of 4 years because of corneal opacification.

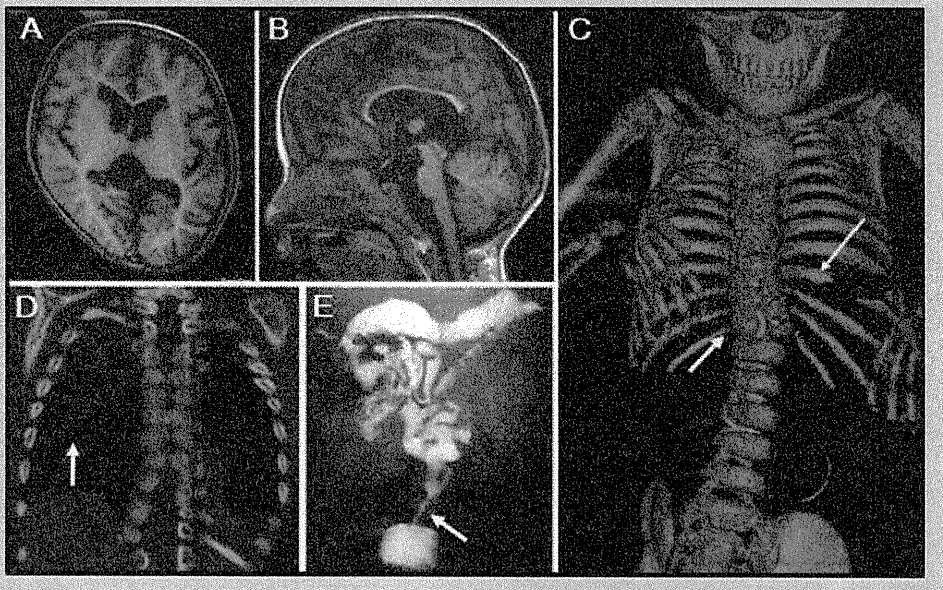

FIG. 2. CT and MRI findings of the patient. A,B: Brain MRI (T1-weighted image) at 3 years of age showed decreased volume of the cortex in the frontal and parietal lobes, the presence of a subdural cyst in the corpora quadrigemina, and dilatation of the lateral and fourth ventricle. C: A bone survey performed using 3D CT showed abnormal segmentation of the ninth rib and an imbalanced hemivertebrae in the two lowest thoracic vertebral bodies (shown with arrows). D: CT of the chest showed CCAM (indicated by the arrow) in the right upper lobe. E: Barium enema showed a reduced caliber rectum (indicated by the arrow), suggesting that the patient had Hirschsprung disease.

Chromosomal and Molecular Genetic Studies

Genomic DNA isolated from the patient's peripheral white cells by phenol/chloroform extraction was used for *MBTPS2* mutation analysis. PCR-amplified DNA fragments were isolated using the QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA) and purified using polyethylene glycol 6000 precipitation. PCR products were sequenced with the Big Dye Terminator Cycle Sequencing Kit V1.1 and analyzed with the ABI PRISM 310 Genetic Analyzer (Life Technologies, Carlsbad, CA). We also performed G-banded chromosome analysis at a resolution of 400–550 bands, genome-wide subtelomere fluorescence in situ hybridization (FISH) analysis, and array comparative genomic hybridization (array CGH) using Whole Human Genome Oligo Microarray Kits 244K (Agilent Technologies Inc., Palo Alto, CA) to identify genomic abnormalities.

RESULTS

G-banded chromosome analysis and genome-wide subtelomere FISH analyses did not show chromosomal rearrangements in the patient. Array CGH analysis did not show copy number changes in the patient's genome with the exception of known copy-number variations (CNVs). Since some patients with IFAP syndrome have been reported to present with several clinical features of BRESEK/BRESHECK syndrome, including severe intellectual disability, vertebral and renal anomalies, and Hirschsprung disease, we conducted a comprehensive sequencing analysis of all exons and intron–exon boundaries of *MBTPS2*. This analysis identified a missense mutation (c.1286G>A, [p.Arg429His]) in exon 10, which was previously reported for IFAP syndrome (Fig. 3). The mutation was also found in one allele of the mother (I-2), indicating that the mutation was of maternal origin and that the mother was a heterozygous carrier (Fig. 3).

DISCUSSION

In this report, we describe the fourth male patient with BRESHECK syndrome in whom we identified a missense mutation (c.1286G>A, [p.Arg429His]) in *MBTPS2*, which is the causal gene for IFAP syndrome. *MBTPS2* encodes a membrane-embedded zinc metalloprotease, termed site-2 protease (S2P). S2P cleaves and activates cytosolic fragments of sterol regulatory element binding proteins (SREBP1 and SREBP2) and a family of bZIP membrane-bound transcription factors of endoplasmic reticulum (ER) stress sensors (ATF6, OASIS), after a first luminal proteolytic cut by site-1 protease (S1P) within Golgi membranes [Sakai et al., 1996; Ye et al., 2000; Kondo et al., 2005; Asada et al., 2011]. The SREBPs control the expression of many genes involved in the biosynthesis and uptake of cholesterol, whereas ATF6 and OASIS induce many genes that clean up accumulated unfolded proteins in the ER. Dysregulated SREBP activation, impaired lipid metabolism, and accumulation of unfolded proteins in the ER caused by *MBTPS2* mutations could lead to disturbed differentiation of epidermal structures, resulting in the symptom triad of IFAP syndrome [Cursiefen et al., 1999; Traboulsi et al., 2004; Elias et al., 2008]. Oeffner et al. [2009] first identified five missense mutations in *MBTPS2* in patients with IFAP

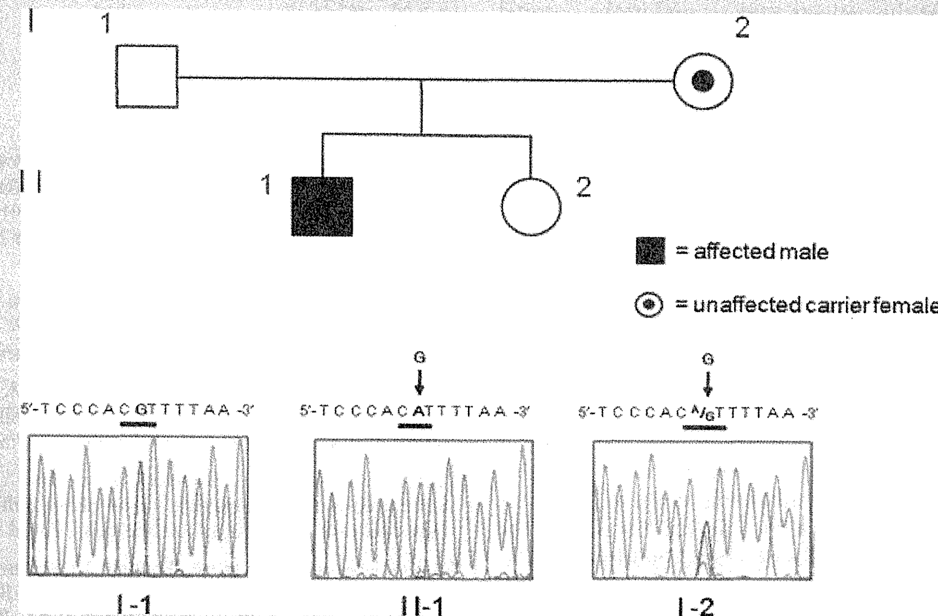

FIG. 3. Identification of a disease mutation. The sequence analyses of the patient (II-1) showed a c.1286G>A variant in exon 10 of *MBTPS2*, which predicts p.Arg429His, as indicated by the arrow (middle panel). The mother (I-2) was heterozygous for the mutation (C^{A}/$_{G}$T) (right panel).

syndrome. Transfection studies using wild type and mutant *MBTPS2* expression constructs demonstrated that the five *MBTPS2* mutations did not affect S2P protein amount and localization in the ER. However, enzyme activities, as measured by sterol responsiveness, were decreased in S2P-deficient M19 cells when the mutant *MBTPS2* was transiently expressed. Interfamilial phenotypic differences between male IFAP patients and the properties of mutants in functional assays predict a genotype–phenotype correlation, ranging from mild forms of the triad with relatively high enzyme activity (~80%) to severe manifestations of intellectual disability, various developmental defects, and early death with low enzyme activity (~15%). The identified p.Arg429His mutation in the patient reported here is one of the five missense mutations with the lowest enzyme activity. It was previously reported that all four patients harboring the p.Arg429His mutation died within 14 months of birth. The five mutations were not located in the HEIGH motif (amino acids [aa] 171–175) or in the $LD_{467}G$ sequence, both of which are regions important for coordinating the zinc atom at the enzymatic active site for protease activity in the Golgi membrane [Zelenski et al., 1999]. However, among the five mutations, the p.Arg429His mutation is located closest to the intramembranous domain, and it strongly reduced the enzymatic activity and caused a severe phenotype. This finding suggests that mutations in the HEIGH motif or in the $LD_{467}G$ sequence are fatal because they lead to a null function of the S2P. Although the detailed skin findings of the four patients with the p.Arg429His mutation have not been reported, it should be noted that one of the four patients (3-III:4) with the p.Arg429His mutation had brain anomaly, seizures, psychomotor retardation, vertebrae anomaly, Hirschsprung disease, absence of a kidney, atrial septum defect, and inguinal hernia, in addition to the symptom triad of IFAP syndrome [Oeffner et al., 2009]. These symptoms overlap with the majority of symptoms observed in BRESHECK syndrome (BRESHK; six of eight symptoms observed in BRESHECK) (Table I), and the present patient has BRESHECK syndrome. Collectively, these observations suggest that the most severe form of the syndrome caused by the p.Arg429His mutation in *MBTPS2* shows features quite similar or identical to those of BRESEK/BRESHECK syndrome.

There are two major differences in the definitions of IFAP syndrome and BRESEK/BRESHECK syndrome. Ichthyosis follicularis, one of the triad symptoms of IFAP syndrome, is a clinical condition of the skin. However, several studies on IFAP syndrome have reported various skin eruptions such as psoriasis-like and ichthyosis-like eruptions [Martino et al., 1992; Sato-Matsumura et al., 2000]. In contrast, patients with BRESEK/BRESHECK syndrome showed severe lamellar desquamation with diffuse scaling [Reish et al., 1997], similar to that observed in the present patient. This could be because of the difference in features of the skin, namely, ichthyosiform erythroderma-like appearance versus ichthyosis follicularis, in patients with the most severe forms of *MBTPS2* mutation and patients with IFAP syndrome who were described earlier, respectively.

The second difference is that photophobia was not described in the reported three male patients with BRESEK/BRESHECK syndrome [Reish et al., 1997; Tumialán and Mapstone, 2006]. In the present patient, photophobia became evident after he was diagnosed with BRESHECK syndrome. Photophobia is a symptom of epithelial disturbances of the cornea, such as ulceration and vascularization, which result in corneal scarring [Traboulsi et al., 2004]. In the most severe cases of *MBTPS2* mutation, such as patients with severe intellectual disability who are bedridden and die early, it is likely that the patients were treated in the hospital without being exposed to sunlight. Therefore, it would be difficult to observe photophobia as a main symptom in those cases. Moreover, two previously described patients with BRESEK/BRESHECK syndrome had initial maldevelopment of one eye or small optic nerves. In these patients, photophobia may not have been obvious because of malformations of the eyes and optic nerves [Reish et al., 1997]. In our study, the patient showed clinical features of BRESHECK syndrome and photophobia with *MBTPS2* mutation, indicating that the clinical features of the present patient are extremely broad compared to the features of IFAP syndrome caused by *MBTPS2* mutation that have been previously reported [MacLeod, 1909].

Recently, a missense mutation (c.1523A>G, [p.Asn508Ser]) in *MBTPS2* was identified from 26 cases of three independent families with keratosis follicularis spinulosa decalvans (KFSD; OMIM# 308800), which is characterized by the development of hyperkeratotic follicular papules on the scalp followed by progressive alopecia of the scalp, eyelashes, and eyebrows in addition to childhood photophobia and corneal dystrophy [Aten et al., 2010]. A significant association was found between KFSD and the p.Asn508Ser mutation. The specific localization of alopecia to the scalp, eyelashes, and eyebrows and the limited childhood photophobia of KFSD indicate that KFSD has a relatively mild phenotype. The authors postulate that IFAP syndrome and KFSD are within the spectrum of one genetic disorder with a partially overlapping phenotype and propose that a new name should be chosen for KFSD/IFAP syndrome with an *MBTPS2* mutation. In contrast, the BRESHECK syndrome observed in the present patient has a severe phenotype caused by the p.Arg429His mutation. The present patient and the two patients (3-III:3 and 3-III:4) with the p.Arg429His mutation displayed broader clinical features, including eight features (BRESHECK) and six features (RESHCK and BRESHK) of BRESEK/BRESHECK syndrome, respectively (patients 4, 6, and 7; Table I) [Oeffner et al., 2009]. There is a debate regarding whether the two patients harboring six features were correctly diagnosed with BRESEK/BRESHECK syndrome since the patients did not have "BRESEK" but rather a combination of six other clinical features. To better understand and clearly distinguish the clinical features of the present patient from those of the reported patients with *MBTPS2* mutations, we propose the nomenclature of "BRESHECK/IFAP syndrome" for the present patient because he has clinical features of BRESHECK syndrome. We also suggest that the BRESHECK/IFAP syndrome be used for a broader definition that would include patients harboring most features of BRESHECK syndrome, including the previously reported two patients (3-III:3 and 3-III:4) with p.Arg429His mutation in *MBTPS2* [Oeffner et al., 2009]. Data from further genetic and clinical studies on more patients are required to determine which genes or *MBTPS2* mutations are associated with BRESEK/BRESHECK or BRESHECK/IFAP syndrome, respectively.

ACKNOWLEDGMENTS

We thank the patient and his family for participating in the study. This study was supported by the Takeda Science Foundation (to N.W.) and by the Health Labour Sciences Research Grant (to S.M. and N.W.).

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LINK TO ORIGINAL ARTICLE
LINK TO RESPONSE

Mutation (variation) databases and registries: a rationale for coordination of efforts

Arleen D. Auerbach, John Burn, Jean-Jacques Cassiman, Mireille Claustres, Richard G. H. Cotton, Garry Cutting, Johan T. den Dunnen, Mona El-Ruby, Aida Falcon Vargas, Marc S. Greenblatt, Finlay Macrae, Yoichi Matsubara, David L. Rimoin, Mauno Vihinen and Christine Van Broeckhoven

The importance of gene- or locus-specific databases (LSDBs) has recently been extolled in this journal (The case for locus-specific databases. *Nature Reviews Genetics* **12**, 378–379 (2011))[1]. Here we argue that coordination of international efforts for developing comprehensive mutation databases and patient phenotype registries is essential for optimal genetic health care.

Well-funded international efforts for setting up mutation databases or registries are crucial for several reasons. Many variants that are found during clinical testing worldwide are not submitted to databases, where they could form an important resource for patient care. Many laboratories and clinicians do not have the capacity or incentive to submit data to databases. This is especially the case in developing countries owing mainly to technical insufficiency, lack of public awareness, lack of international communications, the absence of the concept of DNA biobanking, national authority restrictions and lack of translation from original languages to English.

The Human Variome Project (HVP) was initiated to facilitate the collection of all variants in all genes from all countries and to include annotation of these variants for pathogenicity and relevance to clinical medicine[2]. It was established at a meeting in 2006 that was attended by representatives of the World Health Organization (WHO), the United Nations Educational, Scientific and Cultural Organization (UNESCO), the Organisation for Economic Co-operation and Development (OECD), the European Commission, March of Dimes, the US National Center for Biotechnology Information, the European Bioinformatics Institute (EBI) and 30 countries[3]. The third HVP meeting at UNESCO Headquarters in 2010 allowed the election of an International Scientific Advisory Committee and affirmation of a Roadmap[4]. Most recently, China has committed $300 million to the project[5], and UNESCO has awarded the HVP the status of 'NGO in operational relations with UNESCO'. Many working groups are establishing standards for collecting, presenting and sharing variation information.

Registries for inherited diseases have been developed in some countries, especially where therapies are available (for example, see REF. 6). Recently, there has been a call for global registries of rare diseases (more than 80% of which are genetic)[7,8]. Most recently, the US National Institutes of Health and the European Commission have developed the International Rare Diseases Research Consortium (IRDiRC)[9].

These two initiatives, the HVP and IRDiRC, have been developing essentially independently and in parallel. The HVP was driven by clinicians and laboratories wishing to have access to complete disease-associated variation information to support diagnostic advice and to facilitate the publication of novel mutations of interest. Recently, the focus has moved to collecting all mutations in all genes from all countries[10] as a means of assisting the interpretation of functional effects of genetic variations. The IRDiRC has been driven by patient groups who are anxious to achieve therapy for their families' diseases and to recruit cohorts for clinical trials in registries.

Practically, the promised funds from China in support of the HVP will allow 5,000 databases to be properly set up. If the decision is to set up these databases as both mutation and patient registries, this will assist both initiatives and avoid duplication.

Each group has their own networks, methodology, experts, data content and specifications. It would seem wasteful if two parallel systems were developed when many data are in common and when global reach is needed by both. In the case of the HVP, key components that are in place are a federated model, forums for sharing experiences, development of best informatics practices that are relevant to the task, and leadership. Clearly in the case of the IRDiRC, the key components are model registries, Orphanet experience and Genetic Alliance experience.

Future generations will pay the price for a failure to establish a joint international approach to the recording of and provision of access to human molecular variation, as such access is the most important step in approaching the diagnosis, and thus prevention, of inherited disorders.

The authors are all members of the International Scientific Advisory Committee of the Human Variome Project.

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doi:10.1038/nrg3011-c1
Published online 25 October 2011

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Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
The Human Genome Variation Society: www.hgvs.org
The Human Variome Project: www.humanvariomeproject.org
ALL LINKS ARE ACTIVE IN THE ONLINE PDF

CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Implantable Cardioverter Defibrillator for Progressive Hypertrophic Cardiomyopathy in a Patient With LEOPARD Syndrome and a Novel *PTPN11* Mutation Gln510His

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Received 16 January 2011; Accepted 8 June 2011

LEOPARD syndrome (LS), generally caused by heterozygous mutations in the *PTPN11* gene, is a rare autosomal-dominant multiple congenital anomaly condition, characterized by skin, facial, and cardiac abnormalities. Prognosis appears to be related to the type of structural, myocardial, and arrhythmogenic cardiac disease, especially hypertrophic cardiomyopathy (HCM). We report on a woman with LS and a novel Gln510His mutation in *PTPN11*, who had progressive HCM with congestive heart failure and nonsustained ventricular tachycardia, successfully treated with implantable cardioverter defibrillator (ICD). Comparing our patient to the literature suggests that specific mutations at codon 510 in *PTPN11* (Gln510Glu, Gln510His, but not Gln510Pro) might be a predictor of fatal cardiac events in LS. Molecular risk stratification and careful evaluations for an indication of ICD implantation are likely to be beneficial in managing patients with LS and HCM.

Key words: LEOPARD syndrome; *PTPN11*; codon 510; hypertrophic cardiomyopathy; nonsustained ventricular tachycardia; implantable cardioverter defibrillator

How to Cite this Article:
Wakabayashi Y, Yamazaki K, Narumi Y, Fuseya S, Horigome M, Wakui K, Fukushima Y, Matsubara Y, Aoki Y, Kosho T. 2011. Implantable cardioverter defibrillator for progressive hypertrophic cardiomyopathy in a patient with LEOPARD syndrome and a novel *PTPN11* mutation Gln510His.

Am J Med Genet Part A 155:2529–2533.

INTRODUCTION

LEOPARD syndrome (LS) (OMIM#151100) is a rare autosomal-dominant multiple congenital anomaly condition, characterized by multiple lentigines, electrocardiographic (ECG) abnormalities, ocular hypertelorism, pulmonary stenosis, genital abnormalities, growth retardation, and sensorineural deafness [Sarkozy et al., 2008]. LS is caused by heterozygous missense mutations in the protein tyrosine phosphates, non-receptor type 11 gene (*PTPN11*) in roughly 85% of the cases [Digilio et al., 2002; Sarkozy et al., 2008]. The protein encoded by *PTPN11* functions as a cytoplasmic signaling transducer downstream of multiple receptors for growth factors, cytokines, and hormones, with a particular role through the RAS/mitogen activated protein kinase (MAPK) pathway [Sarkozy et al., 2008]. Disorders caused by mutations in various RAS/MAPK pathway components have recently been coined as "RASopathies", including Noonan syndrome, neurofibromatosis 1, cardio-facio-cutaneous syndrome, Costello syndrome, and LS [Rauen et al., 2010; Marin et al., 2011].

The prognosis of LS depends on the type of cardiovascular abnormality, especially hypertrophic cardiomyopathy (HCM) [Limongelli et al., 2008; Lehmann et al., 2009], however there have been few guidelines to manage complications. We report on a woman with LS and a novel Gln510His mutation in *PTPN11*, who had progressive HCM with congestive heart failure and nonsustained ventricular tachycardia, successfully treated with implantable cardioverter defibrillator (ICD) as for primary prevention of sudden death.

Grant sponsor: Ministry of Health, Labor and Welfare, Japan; Grant sponsor: Japan Society for the Promotion of Science.
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Published online 9 September 2011 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/ajmg.a.34194

CLINICAL REPORT

The proposita is a 38-year-old Japanese woman who underwent intracardiac repair of an atrial septal defect and pulmonary stenosis at age 2 years, when cardiac hypertrophy was detected. In childhood, she was easily exhausted after exercise and had growth retardation. At age 8 years, she was diagnosed with HCM with heart failure, though detailed laboratory data was not available. Oral administration of disopyramide and atenolol was initiated. In her 30s, she had generalized edema. Her plasma brain natriuretic peptide level was elevated at around 2,000 pg/ml (normal values, <18 pg/ml).

At age 37 years, she showed dyspnea, and was referred to our hospital. Her height was 143 cm (−3.0 SD) and weight was 40.8 kg (−1.6 SD). Her craniofacial features included hypertelorism, prominent eyes, a flat nose with anteverted nostrils, low-set posteriorly rotated ears, a long philtrum, thick lips, a high palate, and multiple caries (Fig. 1A). Her skeletal features included a short neck and short fingers with mild flexion contractures at the distal interphalangeal joints. She had numerous lentigines (congenital freckles) on the face (Fig. 1A) and café-au-lait spots on the back (Fig. 1B). She had no apparent hearing impairment. Her blood pressure was 130/66 mmHg, heart rate was 80 beats per minute, and SpO2 was 97% under administration of 1 L/min oxygen. Grade 2 systolic murmurs were heard at the 4th left intercostal space. The plasma brain natriuretic peptide level was 3,450 pg/ml. A chest radiograph showed cardiomegaly with a cardiothoracic ratio as 62% and pulmonary congestion. An ECG showed complete right bundle branch block and left axis deviation. Echocardiograph showed thickening of the interventricular septum as 23 mm (normal values, 7–12) and of the posterior wall of the left ventricle as 30 mm (normal values, 7–12), and tricuspid valve regurgitation with a pressure gradient as 35 mmHg. Pressure gradient of the left ventricular outlet was 21 mmHg at rest (stress echo was not performed to look for a provocable pressure gradient). Left ventricular end-diastolic volume was 20 ml (normal values, 56–136) and ejection fraction was 75% (normal values, >55). These findings were consistent with non-obstructive HCM with left ventricular hypertrophy and without low ejection fraction. The patient was treated with candesartan (angiotensin II receptor blocker), torasemide (diuretics), carvedilol (beta blocker), and amiodarone (antiarrhythmic agent), and her symptoms were improved with a decreased brain natriuretic peptide level to 1,720 pg/ml.

A delayed enhanced cardiac magnetic resonance imaging revealed severe concentric left ventricular hypertrophy with narrowing of the internal cavity and scattered hyper-enhancement regions that were suggested to be fibrosed myocardium [Moon et al., 2003]. A 24-hour Holter ECG showed 1,406 multifocal premature ventricular contractions and eight series of multifocal nonsustained ventricular tachycardia. An electrophysiological study, through a cardiac catheterization, demonstrated that polymorphic ventricular tachycardia was induced by programmed extrastimuli from the right ventricular apex with 400–250–240–230 ms, resulting in consciousness loss. According to the American College of Cardiology/the American Heart Association/the Heart Rhythm Society guidelines for device-based therapy [Epstein et al., 2008], she has two major risk factors (left ventricular wall thickness greater than or equal to 30 mm, nonsustained ventricular tachycardia on Holter ECG) for sudden death in HCM, and was considered to have a class IIa indication for ICD implantation. We placed ICD (Atlas™ + DR, St Jude Medical), which subsequently terminated several ventricular tachycardia episodes with anti-tachycardia pacing.

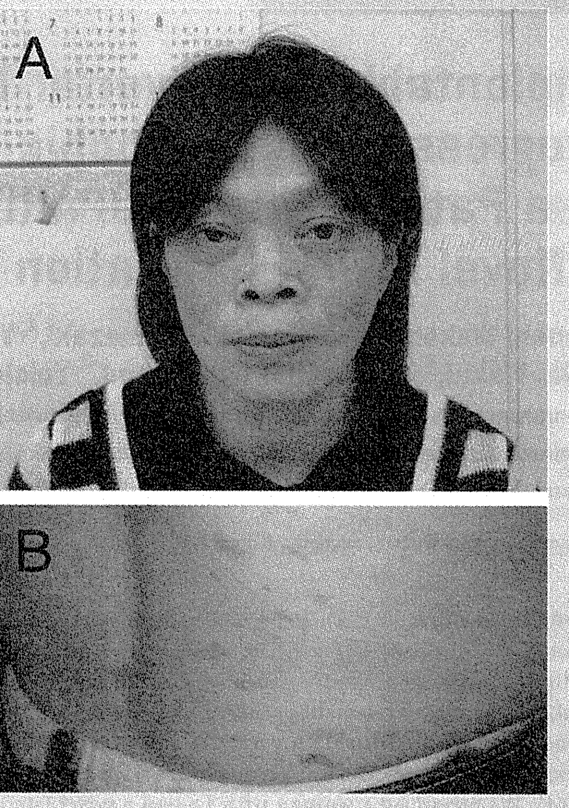

FIG. 1. Photographs of the patient at age 37 years. A: Craniofacial features. Hypertelorism, prominent eyes, a flat nose with anteverted nostrils, low-set posteriorly rotated ears, a long philtrum, thick lips, and multiple lentigines are noted, but typical adult Noonan syndrome-like features such as an inverted triangular shape with a pointed chin, a pinched nasal root, and prominent nasolabial folds are lacking. B: Café-au-lait spots on the back.

MUTATION ANALYSIS

Genomic DNA was isolated from the peripheral blood leukocytes of the patient. Each exon with flanking intronic sequences in *PTPN11* was amplified by polymerase chain reaction (PCR) with primers based on GenBank sequences. The primer sequences are available on request. PCR amplification was performed under standard condition using Taq DNA polymerase. After amplification, the PCR products were gel-purified and sequenced on an ABI PRISM 310 automated DNA sequencer (Applied Biosystems, California). A heterozygous missense mutation (c. 1,530 G > C; p. Gln510His) was identified in exon 13 (data not shown).

TABLE I. Patients With Mutations at Codon 510 of *PTPN11*

Family	**1**			**2**			**3**	**4**	**5**	**6**	**7**	**8**
Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Mutation		Gln510Pro			Gln510Pro		Gln510Glu	Gln510Glu	Gln510Glu	Gln510Glu	Gln510Glu	Gln510His
Sex	F	M	F	F	F	M	M	F	M	M	F	F
Age at publication (y, years; m, months)	?	12y	25y	?	?	4y	1y 3m	2y	2.3y	2m	37y	38y
Lentigines	+	+	+	+	+	−[a]	−	+	−	−	+	+
Café-au-lait spots	−	+	+					+	+	−		+
Congenital heart defects	−	+ (non-PS)	+ (PS, MVP)	−		+ (PS, ASD)	−	+ (MVA)	+ (PS)	+ (MR, VSD)	−	+ (PS, ASD)
Cardiomyopathy	−	−	−				HCM	HCM	HCM	HCM	HCM	HCM
ECG conduction abnormalities	?	+	−	+	−	+					+	+
Hypertelorism	−	−	−	+?	+	+	+	+	−	+	+	+
Prominent eyes									+	+		+
Ptosis	−	−	−					+	+			−
Low-set ears						+	+	+		+		+
Dysmorphic ears				+?	+	+	+	+	+	+		+
Hearing impairment	−	+	+	−	+	−	+	−	−	−	+	−
Genital abnormalities	−	C	−	−	−	−				C		
Scoliosis	−	−	−	−	+	−						
Coagulation abnormalies	−	+	+									
Growth retardation	−	−	−	+	−	+	−	+	+	−	+	+
Mental retardation	−	−	−			MDD	MDD	MDD	MDD			+
References	Keren et al. [2004]			Kalidas et al. [2005]			Takahashi et al. [2005]	Digilio et al. [2006]		Faienza et al. [2009]	Lehmann et al. [2009]	Present patient

Patient 1 was the mother of Patient 2 and Patient 3. Patient 4 and Patient 5 were the maternal grandmother and mother of Patient 6, respectively.
F, female; M, male; +, present; −, absent.
PS, pulmonary stenosis; MVP, mitral valve prolapse; ASD, atrial septal defect; MVA, mitral valve anomaly; MR, mitral valve regurgitation; VSD, ventricular septal defect; HCM, hypertrophic cardiomyopathy; C, cryptorchidism; MDD, motor developmental delay.
[a]absent at age 1 year.

DISCUSSION

This patient fulfills the clinical diagnostic criteria of LS proposed by Voron et al. [1976] with a novel heterozygous mutation Gln510His in *PTPN11*, the major causative gene for LS. *PTPN11* mutations in patients with LS are clustered in exons coding the protein tyrosine phosphatase domain, with two recurrent mutations in exons 7 (Tyr279Cys) and 12 (Thr468Met) in about 65% of *PTPN11*-positive cases, and other rare mutations [Digilio et al., 2002; Sarkozy et al., 2008]. Heterozygous missense mutations at codon 510 in exon 13 have been reported in 12 patients from eight families including this patient (Table I) [Keren et al., 2004; Kalidas et al., 2005; Takahashi et al., 2005; Digilio et al., 2006; Faienza et al., 2009; Lehmann et al., 2009]. A Gln510Glu mutation was found in five sporadic patients, who all manifested HCM with or without congenital heart defects. HCM was detected prenatally in two patients [Digilio et al., 2006], on the first day of life in one [Faienza et al., 2009], at age 1 month in one [Takahashi et al., 2005], and at age 23 years in one [Lehmann et al., 2009]. Pharmacotherapy including diuretics and propranolol was effective in two patients with progressive HCM with left ventricular outflow tract obstruction and congestive heart failure [Takahashi et al., 2005; Digilio et al., 2006; Limongelli et al., 2008]. Septal myectomy was required in one [Digilio et al., 2006; Limongelli et al., 2008] and sudden death occurred in one [Faienza et al., 2009]. On the other hand, a Gln510Pro mutation was found in six patients from two families, none of whom was described to manifest HCM, though three had congenital heart defects and two were elders at publication [Keren et al., 2004; Kalidas et al., 2005]. Limongelli et al. [2008] reviewed 24 LS patients with (n = 16) and without (n = 8) *PTPN11* mutations. They proposed mutations in exon 13 and codon 510 as molecular predictors of adverse cardiac events (life-threatening arrhythmic events, cardiac arrest, and heart failure), as well as LVH at ECG, New York Heart Association class >2, maximal wall thickness z-score > + 10, LVOT gradient >50 mmHg, and NSVT as clinical predictors of these events. However, six patients from two families with a Gln510Pro mutation did not show HCM (Table I) [Keren et al., 2004; Kalidas et al., 2005]. Thus, presence of specific missense mutations at codon 510 (Gln510Glu and Gln510His, not Gln510Pro) would be a molecular risk factor of adverse cardiac events. The boys described by Takahashi et al. [2005] and Faienza et al. [2009] were diagnosed with Noonan syndrome because of no pigmented spots at the time of publication. They might develop lentigines and be diagnosed with LS, like the family described by Kalidas et al. [2005] (the 4-year-old boy showed no lentigines, while his mother and grandmother with the same mutation showed multiple lentigines).

Management of each "RASopathy" might depend on the cardiac phenotype. Whereas pulmonary valve stenosis with dysplastic leaflets and atrial/ventricular septal defects are the most prevalent cardiac defects in patients with Noonan syndrome caused by gain-of-function mutations in *PTPN11*, HCM is the most frequent cardiac complication and represents the only life-threatening problem in patients with LS caused by dominant-negative mutations in *PTPN11* [Sarkozy et al., 2008; Marin et al., 2011]. Indeed, the present patient could return to work under an appropriate cardiac management including intensive pharmacotherapy for controlling heart failure and ICD for preventing fatal arrhythmias. HCM in LS patients, which in general is asymptomatic and involves the left ventricle, is complicated by left ventricular outflow tract obstruction in up to 40% of the cases and frequently manifests during the second infancy before multiple lentigines occur [Sarkozy et al., 2008]. Therefore, those with LS, as well as those clinically diagnosed with Noonan syndrome and having HCM, are recommended to have molecular testing of *PTPN11* for genotype-based risk stratification of fatal cardiac events. LS patients with symptomatic HCM should receive intensive pharmacotherapy including beta blockers, calcium channel blockers, digoxin, diuretics, antiarrhythmic drugs, and angiotensin-converting enzyme inhibitors, depending on their symptoms and cardiac features; and for drug-refractory patients with obstructive HCM, surgical relief of left ventricular outflow obstruction is considered [Maron et al., 2003; Biagas and Hsu, 2006]. LS patients with symptomatic or asymptomatic HCM are recommended to have regular cardiac ultrasonography to measure left ventricular wall thickness and Holter ECG to detect nonsustained ventricular tachycardia for an indication of ICD implantation. Furthermore, etiology-based therapy might be realized, as recently published study by Marin et al. [2011], proposing effectiveness of TOR inhibitors such as rapamycin for the treatment of HCM in LS patients based on an evidence that dominant-negative *PTPN11* mutations in LS would enhance mTOR activity as critical for causing LS-associated HCM in a mouse model.

In conclusion, we have reported successful intervention through ICD implantation on a woman with LS and progressive HCM accompanied by congestive heart failure and nonsustained ventricular tachycardia, who was found to have a novel Gln510His mutation in *PTPN11*. Review of patients with mutations at codon 510 in *PTPN11* suggested that specific mutations (Gln510Glu, Gln510His, not Gln510Pro) would be a predictor of fatal cardiac events in LS. Molecular risk stratification and careful evaluations for an indication of ICD implantation are likely to be beneficial in managing patients with LS and HCM. Continued molecular characterization with cardiac phenotypes of these patients is crucial in further delineation of the risks as well as future etiology-based therapy.

ACKNOWLEDGMENTS

The authors are grateful to the patient for her cooperation. This work was supported by Research on Intractable Diseases, Ministry of Health, Labor and Welfare, Japan (to Y.F., Y.M., Y.A., T.K.), and Grants-in-Aids from the Japan Society for the Promotion of Science (to Y.M., Y.A.).

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Journal of Human Genetics (2011) 56, 707–715

www.nature.com/jhg

ORIGINAL ARTICLE

HRAS mutants identified in Costello syndrome patients can induce cellular senescence: possible implications for the pathogenesis of Costello syndrome

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Costello syndrome (CS) is a congenital disease that is characterized by a distinctive facial appearance, failure to thrive, mental retardation and cardiomyopathy. In 2005, we discovered that heterozygous germline mutations in *HRAS* caused CS. Several studies have shown that CS-associated *HRAS* mutations are clustered in codons 12 and 13, and mutations in other codons have also been identified. However, a comprehensive comparison of the substitutions identified in patients with CS has not been conducted. In the current study, we identified four mutations (p.G12S, p.G12A, p.G12C and p.G12D) in 21 patients and analyzed the associated clinical manifestations of CS in these individuals. To examine functional differences among the identified mutations, we characterized a total of nine HRAS mutants, including seven distinct substitutions in codons 12 and 13, p.K117R and p.A146T. The p.A146T mutant demonstrated the weakest Raf-binding activity, and the p.K117R and p.A146T mutants had weaker effects on downstream c-Jun N-terminal kinase signaling than did codon 12 or 13 mutants. We demonstrated that these mutant HRAS proteins induced senescence when overexpressed in human fibroblasts. Oncogene-induced senescence is a cellular reaction that controls cell proliferation in response to oncogenic mutation and it has been considered one of the tumor suppression mechanisms *in vivo*. Our findings suggest that the *HRAS* mutations identified in CS are sufficient to cause oncogene-induced senescence and that cellular senescence might therefore contribute to the pathogenesis of CS.
Journal of Human Genetics (2011) **56,** 707–715; doi:10.1038/jhg.2011.85; published online 18 August 2011

Keywords: Costello syndrome; HRAS; phenotype-genotype; RAS/MAPK; senescence

INTRODUCTION

Costello syndrome (CS, OMIM 218040) is a genetic disorder that is characterized by a distinctive facial appearance, loose skin, failure to thrive, mental retardation, cardiomyopathy and a predisposition to tumor formation.[1] Patients with CS have an estimated 13% chance of developing tumors, usually rhabdomyosarcoma, neuroblastoma or bladder cancer.[2] Previously, we identified heterozygous germline *HRAS* mutations in patients with CS.[3] It has been suggested that the CS diagnosis should be applied only to patients with a mutation in *HRAS* because of the high risk of malignancies associated with *HRAS* mutations and the relative homogeneity of the CS phenotype.[4]

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Received 4 April 2011; revised and accepted 15 June 2011; published online 18 August 2011

A total of 14 *HRAS* missense mutations and one duplication mutation have been reported in 185 patients with CS[3,5–23] or congenital myopathy with excess of muscle spindles.[24] Most of these mutations have previously been reported as somatic and oncogenic mutations in various tumors. More than 90% of the mutations found in CS patients are clustered in codons 12 and 13 (p.G12A/S/V/C/D/E and p.G13C/D). Other mutations, including p.Q22K, p.E37dup, p.T58I, p.E63K, p.K117R, p.A146V and p.A146T, have also been identified, albeit rarely. Although the clinical manifestations of CS appear to be homogeneous, several genotype-phenotype correlations have been reported. Previous studies have also suggested that CS patients with the p.G12A mutation may have an increased risk of malignancy, compared with patients with p.G12S.[7] Patients with the p.G12C mutation had a more severe CS phenotype; these individuals developed severe hypertrophic cardiomyopathy and died in the neonatal period. Patients with p.K117R or p.A146V had a milder and more unusual CS phenotype, compared with patients with mutations in codon 12 or 13. Though detailed analyses of some mutants have been performed,[13,25–28] a comprehensive comparison of the substitutions identified in patients with CS has not been conducted.

The activated RAS/mitogen-activated protein kinase (MAPK) pathway generally stimulates cell proliferation, but it can also result in antiproliferation under certain conditions. Overexpressing HRAS p.G12V in human and murine fibroblasts caused oncogene-induced senescence (OIS),[29–31] which protects cells from proliferating in the presence of oncogene-induced damage.[32,33] OIS is a cellular reaction that controls cell proliferation in response to oncogenic mutation and is considered a tumor suppression mechanism *in vivo*.[34,35] Studies of a zebrafish model of CS, which expresses *HRAS* p.G12V, have shown that progenitor cells in the adult heart and brain undergo cellular senescence, suggesting that OIS in adult progenitor cells contributes to the development of CS. We hypothesized that OIS would be a key mechanism of the clinical manifestations in patients with CS, including short stature, osteoporosis and tumor suppressive effects. However, it has not been verified that HRAS mutants other than p.G12V cause cellular senescence.

The three aims of this study were the following: (1) to examine the detailed clinical manifestations of CS in patients with *HRAS* mutations, (2) to characterize a large panel of HRAS mutants to look for differences among various mutations located in codon 12/13 and to compare the effects of mutants in codon 12/13 with those of p.K117R/p.A146T, and (3) to clarify whether *HRAS* mutants other than p.G12V can cause OIS. To address these issues, we analyzed the *HRAS* mutations in CS patients and studied the Raf-binding activity, downstream signaling and ability to cause senescence of a large panel of HRAS mutants.

MATERIALS AND METHODS

Patients

A total of 31 patients suspected of having CS were recruited to the study. The diagnosis of CS was evaluated by clinical geneticists. All patients had sporadic cases. The study was approved by the Ethics Committee of the Tohoku University School of Medicine.

Mutation analysis

We sequenced the *HRAS* genes of all patients in the study to confirm the diagnosis of CS. After obtaining written informed consent, genomic DNA was isolated from the peripheral leukocytes of patients. Four coding exons of *HRAS* from 31 CS patients were sequenced. Each *HRAS* exon with flanking intronic sequences was amplified using primers based on sequences obtained from GenBank (GenBank accession no. NT035113). The M13 reverse or forward sequence was added to the 5′ end of the polymerase chain reaction primers for use, as a sequencing. polymerase chain reaction was performed in a 30 μl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphate, 10% (v/v) dimethyl sulfoxide, 0.4 pmol each primer, 100 ng genomic DNA and 2.5 units of Taq DNA polymerase. The reaction consisted of 35 cycles of denaturation at 94 °C for 15 s, annealing at 57 °C for 15 s and extension at 72 °C for 30 s. The products were gel-purified and sequenced on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Plasmids

To introduce exogenous wild-type or mutated HRAS into cultured cells, we constructed plasmids encoding wild-type or mutant *HRAS* cDNAs. Human *HRAS* cDNA in pUSEamp was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The plasmid was digested with *Eco*RI and subcloned into pBluescript KSII+ (Stratagene, La Jolla, CA, USA). Substitutions generating p.G12V (c.35G>T), p.G12A (c.35G>C), p.G12S (c.34G>A), p.G12C (c.34G>C), p.G12D (c.35G>A), p.G13C (c.37G>C), p.G13D (c.38G>A), p.K117R (c.350A>G) or p.A146T (c.436G>A) were introduced using the QuikChange Site-Directed mutagenesis kit (Stratagene). All mutant and wild-type constructs were verified by sequencing. The full-length wild-type and mutant *HRAS* cDNAs were digested with *Eco*RI and subcloned into the pBabe-puro retroviral vector (GenHunter, Nashville, TN, USA) and the pCAGGS expression vector (gifted by Dr Jun-ichi Miyazaki of Osaka University). The pBabe-zeo-Ecotropic Receptor plasmid (Addgene plasmid 10687, Addgene Inc., Cambridge, MA, USA) was obtained from Addgene.

Cell culture and senescence-associated β-galactosidase staining

NIH 3T3 cells, human fibroblast BJ cells and the Phoenix Ampho and Eco packaging cell lines were purchased from the American Tissue Culture Collection (Manassas, VA, USA). NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium containing 10% calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. BJ and Phoenix cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. To characterize the phenotypes of cells overexpressing wild-type or mutated HRAS, senescence associated β-galactosidase staining was performed with the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Beverly, MA, USA) according to the manufacturer's protocol.

Ras activation assay

We performed RAS activation assays to clarify the functional differences among the HRAS mutants identified in patients with CS. The Ras activation assay kit was purchased from Millipore (Billerica, MA, USA). NIH 3T3 cells were plated in 6-well plates at 1.5×10^5 cells per well. Cells were transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA) with 1 μg wild-type or mutant *HRAS* construct. The assay was performed according to the manufacturer's protocol.

Luciferase assay

We used luciferase assays to examine the effect of the identified mutations on the RAS pathway. NIH 3T3 cells were plated in 12-well plates at 1×10^5 cells per well. After 24 h, cells were transiently transfected with 700 ng pFR-luc, 10 ng pFA2-Elk1 or 10 ng pFA2-cJun, 7 ng phRLnull-luc and 35 ng wild-type or mutant *HRAS* construct, using Lipofectamine Plus (Invitrogen). At 18 h after transfection, the cells were serum starved in Dulbecco's modified Eagle medium for 24 h. Cells were then harvested in passive lysis buffer, and luciferase activity was assayed using the Promega Dual-Luciferase assay kit (Promega, Madison, WI, USA). Renilla luciferase expressed by phRLnull-luc was used to normalize the transfection efficiency. The experiments were performed in triplicate. Statistical analysis was performed with Tukey's multiple comparison test.

Western blotting

We performed western blotting against molecular markers of premature senescence to confirm their expression in cells overexpressing HRAS. Cells were harvested at the indicated times, washed in ice-cold phosphate-buffered saline and lysed on ice in lysis buffer (10 mM Tris-HCl, pH 7.5 and 1% sodium