

Figure 4. Mice lacking GCS activity exhibit NTDs. (A) Phenotypes of *Amt* mutant mice. NTDs were evident in the majority (88%) of *Amt*^{-/-} fetuses (examples shown are at E17.5). Various types of NTDs were observed in *Amt*^{-/-} fetuses, which principally affected the cranial region; a, no NTDs; b, small exencephaly (dotted circle); c–e, large exencephaly; f, craniorachischisis. (B) Enzymatic activity of the GCS in *Amt* knockout mice. *Amt*^{+/-} and *Amt*^{-/-} fetuses had significantly lower GCS activity in the liver than *Amt*^{+/+} fetuses, with activity in *Amt*^{-/-} samples below the level of detection (***P* < 0.01 compared with *Amt*^{+/+}).

(*n* = 66) fetuses. Defects mainly comprised exencephaly (82%), in which the cranial neural folds persistently failed to close (Fig. 4). There was also a low frequency of the more severe condition, craniorachischisis (5%), in which the neural tube remains open from the mid- and hindbrain, and throughout the spinal region (Fig. 4). Fetal liver samples were subjected to enzyme assay to determine overall activity of the GCS. In *Amt*^{-/-} mice, overall GCS activity was effectively ablated being below the detection level of the assay (0.01 nmoles of ¹⁴CO₂ formed/gram protein/h), consistent with the *Amt*⁻ allele being a functional null (22) (Fig. 4). These findings confirm that *AMT* function is essential for GCS activity, and that the latter is necessary for successful neural tube closure.

Given that GCS is a component of FOCM (Fig. 1), we evaluated the possible prevention of NTDs by folate-related metabolites. Maternal supplementation was performed with folic acid, thymidine monophosphate (TMP), methionine or methionine plus TMP (23). Neither folic acid nor TMP significantly affected the frequency of NTDs among the homozygous *Amt*^{-/-} offspring. However, we observed a significant protective effect of maternal supplementation with methionine or methionine plus TMP, compared with the non-treated group (*P* < 0.05; Fig. 5).

DISCUSSION

NTDs remain among the commonest human birth defects and understanding their genetic basis presents a considerable

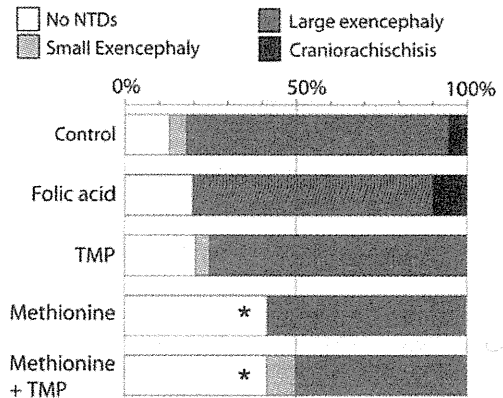


Figure 5. Maternal supplementation of *Amt* mutant embryos with folic acid, TMP or methionine. Maternal treatment with folic acid (*n* = 10 homozygous mutant fetuses) or TMP (*n* = 12) had no significant effect on NTD frequency, whereas the frequency of unaffected embryos was significantly increased following treatment with methionine (*n* = 12) or methionine plus TMP group (*n* = 12). The asterisk indicates significant difference compared with non-treated group (*P* < 0.05).

challenge owing to their multigenic inheritance and the potential influence of environmental factors, either predisposing or ameliorating. Several lines of evidence indicate a requirement for FOCM in neural tube closure and, therefore, GCS-encoding genes provide excellent candidates for possible involvement in NTD susceptibility. We identified putative mutations in *AMT* and *GLDC* which include a splice acceptor mutation and a number of non-synonymous variants that were absent from a large group of population-matched controls, as well as from public SNP databases. In the case of *GLDC*, enzymatic assay confirmed that several mutations resulted in significant loss of enzyme activity. Finally, *in vivo* functional evidence of a requirement for GCS function in neural tube closure was provided by the occurrence of NTDs in *Amt*^{-/-} mice lacking GCS activity. Together these findings indicate that mutations in *GLDC* and *AMT* predispose to NTDs in both mice and humans.

Where parental samples were available (6 of the 11 NTD cases that involved putative mutations in *GLDC*), we demonstrated parent-to-child transmission (Supplementary Material, Table S2). Six were instances of maternal transmission and one involved paternal transmission. We hypothesize that absence of an overt NTD phenotype in parents who carry a deficient *GLDC* allele may result from incomplete penetrance, or lack of additional genetic or environmental factors which are predicted to be necessary for NTDs owing to their multifactorial aetiology. We also note that partial penetrance is a feature of numerous mouse models of NTDs (5,8).

Inherited GCS deficiency, owing to mutation of *AMT* and/or *GLDC*, has been shown to cause NKH in humans (17). NKH is a rare, autosomal recessive, inborn error of metabolism, characterized by accumulation of glycine and encephalopathy-like neurological signs, including coma and convulsive seizures in neonates. GCS activity is greatly diminished in NKH patients and they would, therefore, be predicted to be at increased risk of NTDs. It is possible that NTDs may occur in combination with NKH but as anencephaly is a lethal condition, co-existing

NKH would go undetected. Lack of NTDs in NKH patients may also reflect the multigenic nature of NTDs, which require the presence of additional risk alleles in non-GCS genes. NKH is a relatively rare condition, with a prevalence of 1/63 000 births in British Columbia (24) and 1/250 000 in the USA (25). It is therefore possible that an increased risk of NTDs among carriers of GCS mutations in NKH families may not have been noted and this possibility is worthy of investigation. Based on estimated carrier frequency and the incidence of mutations among NTD patients, we predict that NTDs might be expected among 1/150 of the siblings of NKH patients (see Supplementary Material, Table S3 for estimate calculation). One case report of an NKH patient with a *GLDC* mutation describes the additional presence of spinal cord hydromyelia (19). This condition is often associated with low spinal defects (involving secondary neurulation), but it is also possible that the expanded spinal canal was also present at a higher level and might indicate a limited defect in primary neurulation.

The mutations described in the current study were all present in heterozygous form and, therefore, are hypothesized to be insufficient to cause NKH while predisposing to NTDs. For example, in the current study we found four NTD patients and one control individual to be heterozygous for the A569T mutation, which is shown to result in reduced enzyme activity. This mutation was previously identified in a Caucasian patient with typical NKH, in combination with a second mutation, P765S (26), confirming that it is deleterious *in vivo*. Hence, we predict that, depending on the co-existing genetic milieu, the A569T variant may cause NKH, predispose to NTDs or be compatible with normal development.

The high incidence of NTDs in *AMT* mutant mice is particularly notable as NTDs have not previously been found to be a common feature of mouse models deficient for folate-metabolizing enzymes. This includes null mutants that have been reported for eight other genes that encode enzymes in FOCM (Fig. 1A) (27). Four have normal morphology at birth (*Cbs*, *Mthfd1*, *Mthfr* and *Shmt1*) (28–31), *Mthfd2* null embryos die by E15.5 but neural tube closure is complete (32) and null mutants for *Mtr*, *Mtrr* and *Mthfs* die before E9.5, prior to neural tube closure (33–35). Although analysis of mouse mutants has not supported a role for single-gene mutations in FOCM as major causes of NTDs, a requirement for cellular uptake of folate for neural tube closure has been demonstrated in *Folr1* null embryos, in which NTDs occur when rescued from early lethality by folic acid supplementation (36). There is also considerable evidence for possible involvement of gene–environment and/or gene–gene interactions in NTDs. For example, in *Pax3* mutant (*spotch*) embryos, which exhibit a defect of thymidylate biosynthesis, dietary folate-deficiency increases the frequency of cranial NTDs (23,37). Similarly, a diet deficient in folate and choline causes NTDs in *Shmt1* mutant embryos, whereas *Shmt1* and *Pax3* mutations exhibit genetic interaction (38).

Regarding the mechanisms by which GCS mutations affect neural tube closure, a key question is whether NTDs are caused by impairment of FOCM or by another cause such as glycine accumulation. Modelling of hepatic FOCM, based on biochemical properties of folate-metabolizing enzymes (39), predicts that loss of the mitochondrial GCS reaction

would reduce the efflux rate of formate to the cytosol by ~50%. This results in reduced synthesis of purines and thymidylate, which are essential for the rapid cell division in the closing neural folds. Interestingly, a UK patient with anencephaly who was found to carry the *GLDC* loss-of-function mutation P509A in the current study (Table 1) was previously found to have impaired thymidylate biosynthesis, assayed in cultured fibroblasts (14). These findings support the hypothetical link between diminished *GLDC* function, reduced thymidylate biosynthesis and development of NTDs. Reduced thymidylate biosynthesis and diminished cellular proliferation are proposed to underlie folate-related cranial NTDs in *spotch* (*Pax3*) mouse mutants (37,38).

As well as impairment of nucleotide biosynthesis, the predicted effect of diminished GCS activity in reducing production of methionine (39) may also be of relevance as methionine is the precursor for the methyl donor *S*-adenosylmethionine. Indeed, metabolic tracing experiments suggest that ~80% of IC units in the methylation cycle are generated within mitochondrial FOCM (40). Impairment of the methylation cycle and/or DNA methylation is known to cause NTDs in mice (41) and is proposed as a possible cause of human NTDs (7,42). It was therefore notable that we found a preventive effect of methionine supplementation in *Amt*^{-/-} mice. Together, these findings suggest that FOCM, required for both thymidylate biosynthesis and methylation reactions that are essential for neural tube closure, may be functionally deficient in individuals who have mutations in *GLDC* or *AMT*.

MATERIALS AND METHODS

Patient cohorts and sequencing

Mutation analysis by DNA sequencing was performed on all exons of *AMT*, *GCSH* and *GLDC* as described (26). Cases comprised Japanese patients with anencephaly ($n = 14$) and two separate cohorts of UK patients with a diagnosis of anencephaly (combined $n = 24$), spina bifida ($n = 122$) or craniorachischisis ($n = 22$). In addition, the exons of *AMT*, *GCSH* and *GLDC* were sequenced in 76 Swedish patients with spina bifida. Unaffected controls, completely sequenced for these genes, comprised 36 Japanese and 189 unrelated UK subjects. Exons found to contain missense mutations were also sequenced in a further cohort of 192 well-characterized UK controls (43) and in 145 Swedish controls. This study was approved by the Ethical Committees of Tohoku University School of Medicine, UCL Institute of Child Health, Newcastle University and the Karolinska Institute.

Enzymatic assay of GCS activity and *GLDC* activity

GCS activity was measured in mouse liver samples by a decarboxylation reaction using [1-¹⁴C]glycine as described (22). For analysis of *GLDC* activity, wild-type and mutant *GLDC* cDNAs were cloned into pCAG expression vector, kindly provided by Professor Jun-ichi Miyazaki (Osaka University, Japan) (44). Constructs were transfected into COS7 cells, which were harvested as described previously and cell pellets stored at -80°C prior to analysis (45). *GLDC*

enzymatic activity was determined, in triplicate, by exchange reaction between carbon dioxide and glycine using $\text{NaH}^{14}\text{CO}_3$ in the presence of excess recombinant bovine GCSH protein as described (22). An expression system of lipoylated bovine GCSH protein in *Escherichia coli* was kindly provided by Dr Kazuko Fujiwara (Tokushima University, Japan) (46). Statistical analysis was performed using SPSS software version 11.0 (SPSS, Inc., Chicago, IL, USA).

Knockout of Amt by insertion of a gene-trap vector

Mice carrying a gene-trap allele of *Amt* (here denoted *Amt*⁻) were generated at Lexicon Genetics, Inc. (Houston, TX, USA) using the OST181110 ES cell line. The genomic insertion site of the gene-trap vector was determined by inverse PCR and localized to intron 2 (Supplementary Material, Fig. S1). Total RNA was prepared from the mouse liver and brain at E18 for RT-PCR analysis (Supplementary Material, Fig. S1 and Table S1). *Amt*^{+/-} mice were backcrossed with wild-type C57BL/6 mice for nine generations to generate a congenic line of mice on the C57BL/6 background, for use in biochemical and histological analyses. This study was approved by the Animal Experiment Committee of Tohoku University.

Maternal supplementation with folic acid and related metabolites

Dams were treated with folic acid (25 mg/kg), thymidine-1-phosphate (TMP; 30 mg/kg) or L-methionine (70 mg/kg) by intra-peritoneal injection, 2 h prior to mating and daily from E7.5–10.5. Doses were based on previous studies (23,47,48).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

Conflict of Interest statement. None declared.

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

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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 medicine². 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 countries³. The third HVP meeting at UNESCO Headquarters in 2010 allowed the election of an International Scientific Advisory Committee and affirmation of a Roadmap⁴. Most recently, China has committed \$300 million to the project⁵,

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 countries¹⁰ 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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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

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

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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. © 2011 Wiley-Liss, Inc.

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

INTRODUCTION

LEOPARD syndrome (LS) (OMIM#151100) is a rare autosomal-dominant multiple congenital anomaly condition, characterized by multiple lentiginos, 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

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[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.

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CLINICAL REPORT

The probanda 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 lentiginos (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 provokable 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), torsemide (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

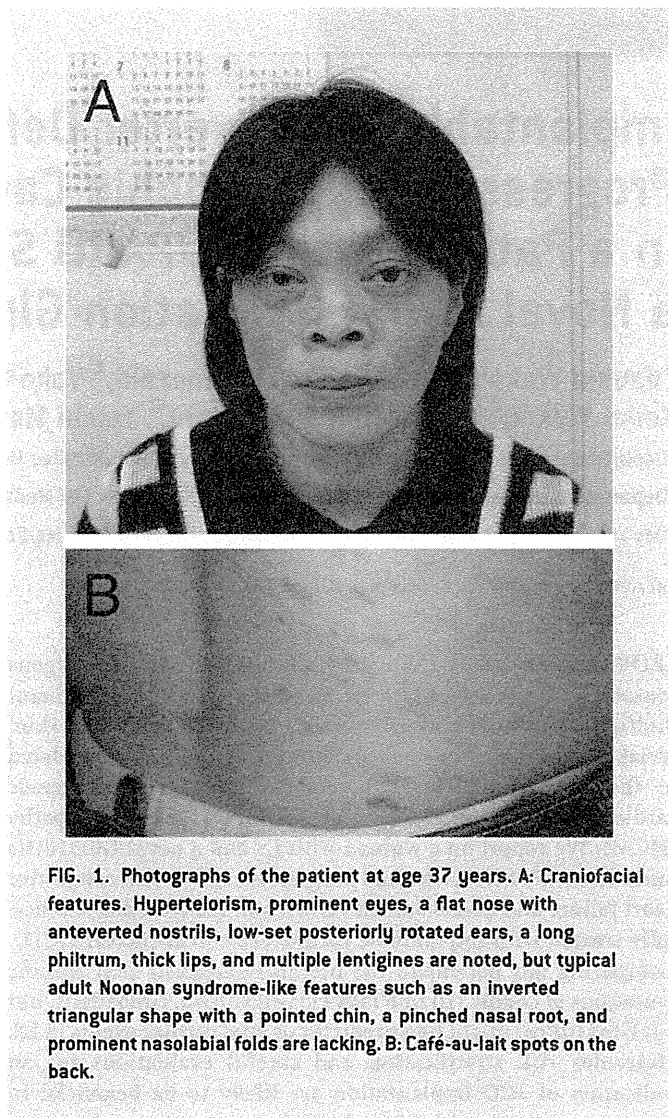


FIG. 1. Photographs of the patient at age 37 years. **A:** Craniofacial features. Hypertelorism, prominent eyes, a flat nose with anteverted nostrils, low-set posteriorly rotated ears, a long philtrum, thick lips, and multiple lentiginos are noted, but typical adult Noonan syndrome-like features such 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.

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.

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 Patient	1			2			3	4	5	6	7	8
	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
Lentiginos	+	+	+	+	+	- ^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 abnormalities	-	+	+	-	-	-	-	-	+	-	-	-
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.

^aabsent 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 lentiginos and be diagnosed with LS, like the family described by Kalidas et al. [2005] (the 4-year-old boy showed no lentiginos, while his mother and grandmother with the same mutation showed multiple lentiginos).

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 lentiginos 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.

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Case report

Epilepsy in RAS/MAPK syndrome: Two cases of cardio-facio-cutaneous syndrome with epileptic encephalopathy and a literature review

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ABSTRACT

We report two individual cases of cardio-facio-cutaneous (CFC) syndrome with severe neurological impairment consisting of infantile spasms with hypsarrhythmia and refractory epilepsy with multifocal epileptic paroxysms such as modified hypsarrhythmia. Both cases shared diffuse brain atrophy and severely delayed myelination on neuroimaging. Genetic analysis revealed individual heterozygous mutations in the KRAS (phenotype of CFC/Noonan syndrome) and BRAF genes (phenotype of CFC syndrome). Neurological impairment in cases with mutations in the RAS/MAPK (mitogen activated protein kinase) signal pathway may be more severe, and could be linked to some forms of refractory epilepsy, especially epileptic encephalopathy that includes infantile spasms.

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1. Introduction

Cardio-facio-cutaneous (CFC) syndrome is a very rare and sporadic disease that includes the characteristics of dysmorphic facial appearance, ectodermal abnormalities, cardiac abnormalities, growth retardation and neuro-developmental delay. This syndrome is categorized as one of the RAS/MAPK syndromes, which cause altered signal transduction of the RAS/MAPK (mitogen activated protein kinase) pathway, including BRAF, MEK1/2, and KRAS.^{1–3} Compared with other RAS/MAPK syndromes, such as Costello syndrome and Noonan syndrome, CFC syndrome exhibits a more severe phenotype including severe neurological impairment, seizures, and developmental delay. We describe the clinical details of neurological findings in two cases of genetically determined CFC syndrome which displayed refractory epilepsies diagnosed as infantile spasms and other epileptic encephalopathy, and we then compare our results with those of similar literature findings.

2. Case reports

2.1. Case 1: six-year-old boy

A large-for-date boy was delivered as the first child to healthy and non-consanguineous Japanese parents (mother 42 years old and father 31 years old) at 32 weeks of gestational age with a birth weight of 3758 g and with moderate neonatal asphyxia (an Apgar

score of 6 at 5 min after birth) following a normal pregnancy. The patient was intensively treated in the neonatal intensive care unit (NICU) in our hospital. Postnatal screening showed fetal hydrops with heart failure due to severe pulmonary valve stenosis which was treated with diuretics and beta blockers. Peculiar craniofacial features included “coarse face,” curly hair, prominent forehead, downslanting palpebral fissures, short nose and broad nasal tip with anteverted nares, low-set dysmorphic and posteriorly angulated ears, abnormal skin (loose and pigmented skin with deep furrows and multiple lentigo, wrinkled palms with deep palmar and plantar creases), webbed neck, chest deformity, and micromelic dwarfism (Fig. 1A).

At three days postnatal, myoclonic seizures of the extremities occurred which were controlled by administration of bolus midazolam (MDL). At the age of 11 months, he developed repetitive series-formed tonic spasms, and the interictal electroencephalogram (EEG) showed hypsarrhythmia (Fig. 1C). Valproic acid (VPA), clonazepam (CZP), and zonisamide (ZNS) were ineffective in reducing seizure frequency and improving EEG findings, and complete remission was only obtained by one course of low dose (0.025 mg/kg) adrenocorticotropic hormone (ACTH). Since undergoing ACTH therapy, he has had no episodes of epileptic seizures while undergoing VPA monotherapy until the present age of six years, but his most recent (interictal) EEG showed asynchronous, high-voltage slow waves with irregular spike-waves, or polyspikes with/without waves dominantly in the right temporal-occipital region (Fig. 1D). Magnetic resonance imaging (MRI) at three years of age revealed agenesis of the corpus callosum, ventricular dilatation, diffuse cortical atrophy and severely delayed myelination (Fig. 1B). Tracheotomy was performed and persistent mechanical ventilation

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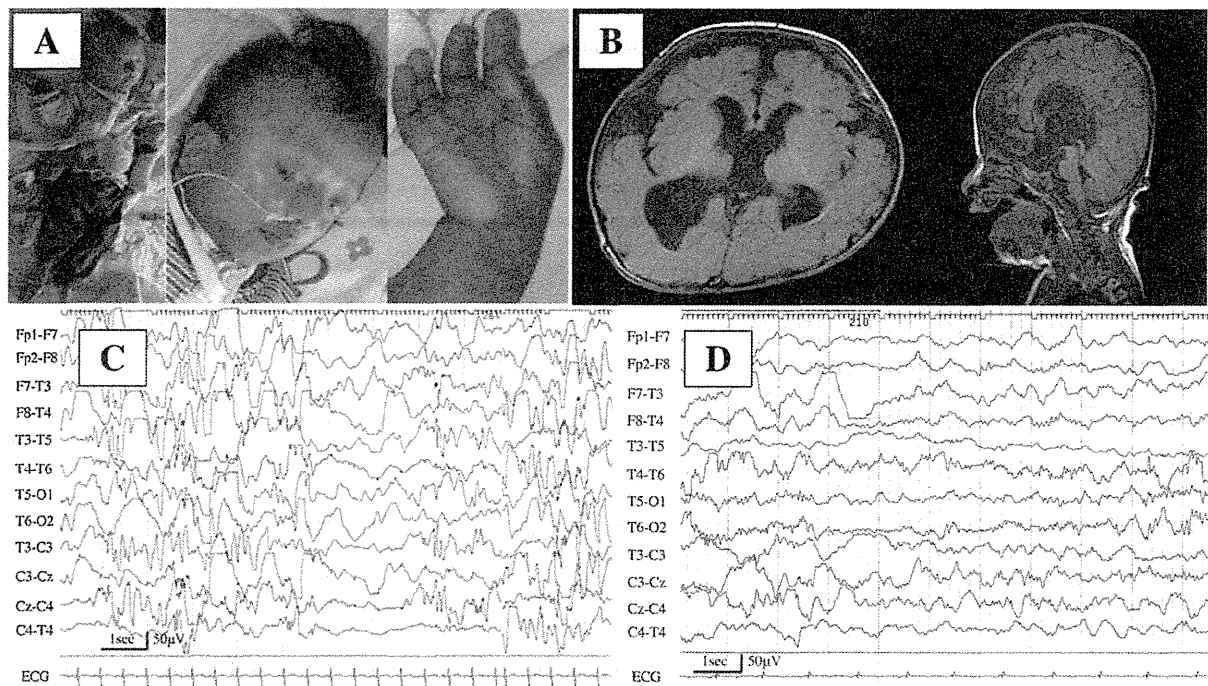


Fig. 1. Case 1. "Coarse face," curly hair, prominent forehead, dysmorphic ears, abnormal loose and pigmented skin, webbed neck, chest deformity, and micromelic dwarfism at six years (A), agenesis of the corpus callosum, ventricular dilatation, diffuse cortical atrophy and severely delayed myelination 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 asphyxial 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, lamotrigine, gabapentin, levetiracetam. Seizures are composed of repetitive brief tonic spasms, tonic-clonic (sometimes developing to status epilepticus), 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,

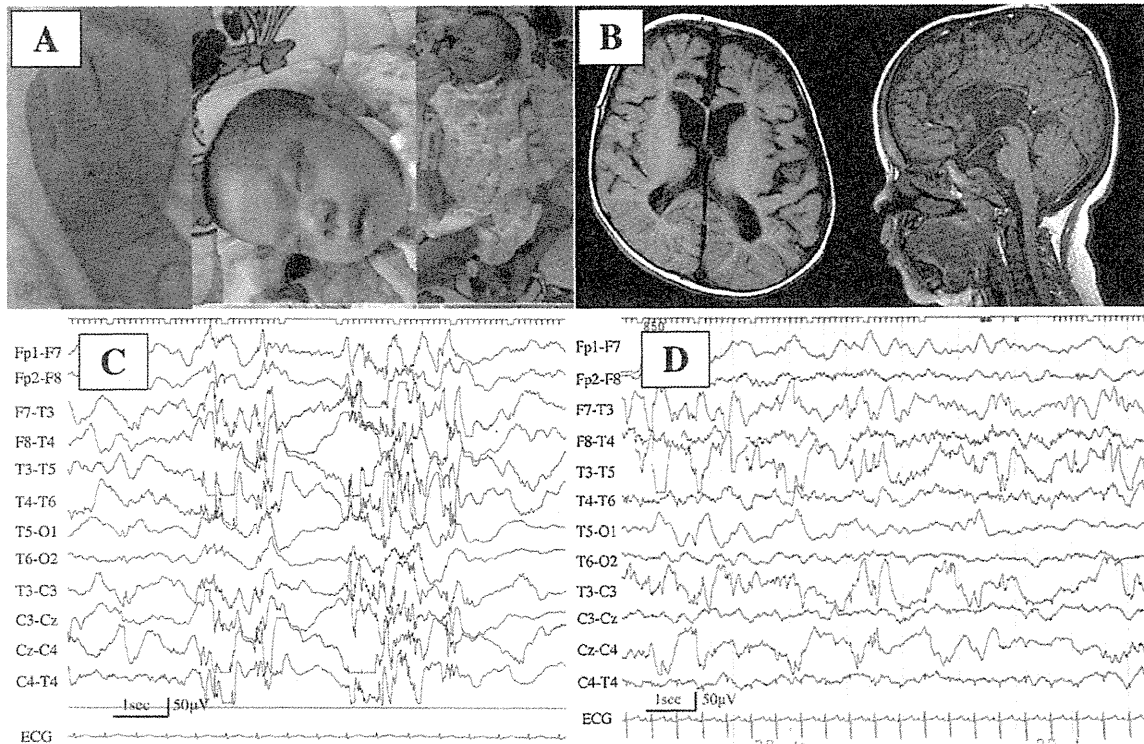


Fig. 2. Case 2. Odd looking “coarse” face (prominent forehead, short nose, and low-set dysmorphic ears, curly sparse hair), abnormal loose, pigmented skin with multiple lentigo, narrow chest, and hypotonic micromelic dwarfism at four years (A), diffuse brain atrophy with delayed myelination and hypoplastic corpus callosum on MRI images at two years (B), modified hypsarrhythmia at one year (C), and continuous high-voltage spike or polyspikes with/slow waves mainly in the left centrotemporoparietal 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 epilepticus), 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 report¹⁰ 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 therapy	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 with 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	2 y	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 therapy	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 corticomedullary 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 atrophy, ventricular dulation, 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), Continuous 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 dulation, 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: adrenocorticotrophic 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 myelination, 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.

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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.

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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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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 μ l reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 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 μ g/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 μ g/ml streptomycin. To characterize the phenotypes of cells overexpressing wild-type or mutant *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 μ g 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 pRLnull-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 pRLnull-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

dodecyl sulfate). Lysates were boiled for 5 min and centrifuged at 13 000 g for 10 min at 4 °C. Protein concentrations were estimated using the Lowry or Bradford method (BioRad, Hercules, CA, USA), and each lysate was adjusted to equalize the protein concentrations. Equal volumes of lysates were mixed with 2×sodium dodecyl sulfate sample buffer and boiled for 5 min. Electrophoresis was performed on 5–15% sodium dodecyl sulfate–polyacrylamide gels. After separation, proteins were transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature and incubated overnight at 4 °C with one of the following primary antibodies: HRAS (sc-520, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-p44/42MAPK, p44/42MAPK (#9102 and #9101, respectively, Cell Signaling Technology, Danvers, MA, USA), p16 (sc-468, Santa Cruz Biotechnology), phospho-p53 (Ser15) (#9284, Cell Signaling Technology) or β-actin (A5316, Sigma, St. Louis, MO, USA). Detection was performed using the enhanced chemiluminescence method (Amersham, GE Healthcare UK, Amersham, UK), with the appropriate peroxidase-conjugated secondary antibody.

Retroviral gene transfer

We generated cells that stably overexpressed wild-type or mutant HRAS by retroviral gene transfer. Phoenix cells (5×10^6) were plated in a 10 cm dish, incubated for 24 h and then transfected with 18 μg of retroviral plasmid using Fugene6 (Roche Applied Science, Mannheim, Germany). After 48 h, the virus-containing medium was filtered through a 0.45-μm filter and supplemented with 4 μg/ml polybrene (Sigma) to collect the virus (first supernatant). Viruses were collected after an additional 24 h as before (second supernatant). BJ fibroblasts were plated at 6×10^5 cells per 10 cm dish and incubated overnight. For infections, the culture medium was replaced with the first viral supernatant and incubated at 37 °C for 8 h, after which the second viral supernatant was added. Infected cell populations were selected 40 h later, using 2 μg/ml puromycin or 200 μg/ml zeocin. The ecotropic retrovirus receptor was introduced into the BJ human fibroblasts by infecting cell populations with an amphotropic vector (pBabe-zeo-ecotropic receptor produced in Phoenix Ampho cells), allowing subsequent infection with ecotropic viruses.

RESULTS

Mutation analysis in patients with CS

Genomic sequencing analysis of 32 individuals with confirmed or suspected CS revealed four different missense mutations in 21 patients: a heterozygous 34G>A mutation (p.G12S) in 16 patients, a heterozygous 35G>C mutation (p.G12A) in three patients, a heterozygous 34G>T change (p.G12C) in one patient, and a 35G>A change (p.G12D) in one patient.

The clinical data for 21 CS mutation-positive patients are shown in Table 1. Curly and/or sparse hair (21/21), failure to thrive (21/21), coarse facial appearance (20/20), deep palmar/plantar creases (20/21), soft, loose skin (18/21) and relative macrocephaly (17/21) were observed at high frequency in patients with CS, as previously reported.^{1,3} Laryngomalacia (soft larynx), which has been reported in several patients with CS,^{36–38} was observed in three patients. One patient had hypertension, which was also observed in a mouse model of CS.³⁹ One patient had glycogen storage disease type III, as previously reported by Kaji *et al.*,⁴⁰ accompanied by a p.G12S mutation. Bladder cancer was observed in one patient.

One patient (NS 223) with HRAS p.G12C had severe clinical manifestations of CS and was treated with pravastatin.⁴¹ She was born at 23 weeks of gestation with extremely low birth weight (766 g, >90th percentile), even though her mother had received tocolytic therapy. Her Apgar scores were 3 and 7 at 1 and 5 min, respectively. She required mechanical ventilation. Extubation was attempted periodically beginning at day 70, but it was unsuccessful until she turned 2 years old, because of her laryngomalacia and increased mucus secretion. Hypertrophic cardiomyopathy was first observed on day 38. The patient was given propranolol and cibenzoline to control the

gradual progression of hypertrophic cardiomyopathy. Cardiac arrest after extubation occurred on day 192 and the patient was successfully resuscitated. Papillomas developed at approximately 11 months of age. Erosion and itching of skin were not well controlled by topical steroids or antihistamines. Pravastatin (0.2 ~ 0.4 mg/kg/day) was administered in anticipation of its suppressive effect on RAS, beginning when she was 16 months old. Thereafter, the papillomas disappeared once and appeared again, but were less numerous than when they first appeared. The effects of pravastatin on hypertrophic cardiomyopathy were not obvious. The patient was discharged from the hospital at 2 years of age.

Analysis of mutant HRAS activation states and effects on the downstream pathway

We performed RAS activation assays to elucidate functional differences among the mutants identified in patients with CS. We transfected NIH 3T3 cells with wild-type HRAS or one of the nine HRAS mutants identified in patients with CS. We found an increase in guanosine triphosphate (GTP)-bound HRAS in all cells transfected with HRAS p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C, p.G13D, p.K117R and p.A146T. We did not detect any differences among the increases of GTP-bound HRAS in the cells transfected with HRAS p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C, p.G13D and p.K117R. The increase in the level of GTP-bound HRAS-p.A146T was milder than that of other mutants.

Next, we examined the effect of the identified mutations on the RAS pathway by studying the activation of ELK1 and c-Jun in transfected NIH 3T3 cells. ELK1 and c-Jun are the main nuclear targets of extracellular signal-regulated kinase and c-Jun N-terminal kinase, respectively. We transfected the pFR-luc trans-reporter vector, the pFA2-ELK1 or pFA2-cjun vector and the pRLnull-luc vector into NIH 3T3 cells and determined the relative luciferase activity (RLA) in each cell line. The basal RLA in cells transfected with active MEK1 or MEK1 constructs showed a three-fold increase, compared with cells transfected with wild-type HRAS cDNA (Figure 1a). A significant increase in RLA was observed upon transfection with ELK1 and HRAS p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C, p.G13D, p.K117R and p.A146T (Figure 1b). The RLA of c-Jun was significantly increased in cells transfected with HRAS p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C and p.G13D (Figure 1c). In these assays with ELK1 and c-Jun, we observed no significant difference among RLAs in the cells transfected with HRAS p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C and p.G13D. These results suggest that HRAS-p.K117R and p.A146T had a weaker effect on the c-Jun N-terminal kinase pathway than the other mutants.

Cellular senescence in human fibroblasts transfected with HRAS mutants

The HRAS p.G12V mutant causes a senescence phenotype when transduced into human diploid fibroblasts. To examine the ability of the various mutants identified in patients with CS to cause senescence, we introduced wild-type or mutated HRAS cDNAs into human fibroblast BJ cells, using retroviral gene transfer. Figure 2a shows these cells six days after infection. Wild-type HRAS-induced cells exhibited a narrow and elongated morphology and they were not flat like senescent cells. They proliferated at levels similar to cells transfected with empty vector. In contrast, the p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C, p.G13D, p.K117R and p.A146T mutants produced cells with a senescence phenotype, exhibiting flat, enlarged and multivacuolated morphology and prominent nucleoli. Senescence