TABLE II. Summary of Clinical Manifestations Obtained From the Second-Stage Survey

	Costello syndrome (%)	CFC syndrome (%)
Total number of patients[a]	43	54
Gender		
Male	17/42 (40)	28/52 (54)
Female	25/42 (60)	24/52 (46)
Genes mutated	*HRAS* 38	*BRAF* 38
	HRAS, 5 but type of mutation unknown	*MAP2K1/2* 8
		KRAS 8
Neoplasia		
Papillomata	7/35 (20)	2/24 (8)
Other tumors	6/34 (18)[b]	5/29 (17)[c]
Growth and development		
Postnatal failure to thrive	41/41 (100)	37/38 (97)
Intellectual disability	39/40 (98)	52/52 (100)
Cardiac defect		
Hypertrophic cardiomyopathy	25/39 (64)[d]	13/50 (26)
Pulmonic stenosis	3/38 (8)	16/51 (31)[e]
Congenital heart malformation[f]	6/39 (15)	13/52 (25)
Arrhythmia	18/41 (44)[d]	10/51 (20)
Central nervous system		
Abnormal brain structure[g]	8/28 (29)	7/23 (30)
Seizure	8/25 (32)	16/33 (48)
Craniofacial characteristics		
Relative macrocephaly	33/39 (85)	31/36 (86)
Musculoskeletal characteristics		
Short stature	18/25 (72)	37/45 (82)
Skin characteristics		
Curly and/or sparse hair	39/41 (95)	38/43 (88)
Soft, loose skin	38/41 (93)[d]	27/37 (73)
Deep palmar/plantar creases	39/41 (95)[d]	29/38 (76)
Outcome		
Alive	38/43 (88)	54/54 (100)
Dead	5/43 (12)[h,d]	0/54 (0)

[a]Number of patients for whom detailed clinical manifestations were obtained in the second-stage survey.
[b]Includes one patient with bladder cancer, two with rhabdomyosarcoma, one with ganglioneuroblastoma, and one with subcutaneous cystic lymphangioma, and one with multiple gallbladder polyps and renal angioma.
[c]Includes one patient with acute lymphoblastic leukemia, one with non-Hodgkin lymphoma, one with hemangioma, and one with calcifying epithelioma.
[d]The frequency of manifestations in patients with Costello syndrome was significantly higher compared with that observed in patients with CFC syndrome ($P < 0.05$ by Fisher's exact test).
[e]The frequency of the manifestation in patients with CFC syndrome was significantly higher compared with that observed in patients with Costello syndrome ($P < 0.05$ by Fisher's exact test).
[f]Includes an atrial septal defect, a ventricular septal defect, a patent ductus arteriosis, a persistent left superior vena cava, and a pulmonary arteriovenous fistula.
[g]Includes a type I Arnold–Chiari malformation, a periventricular leukomalacia, a hydrocephalus, a ventricular dilation, cortical atrophy, a thinning of the corpus callosum, and corpus callosum agenesis.
[h]Cause of death included chronic atrial fibrillation, rhabdomyosarcoma and ganglioneuroblastoma. For two patients, the cause of death is unknown.

We compared the clinical manifestations between patients with *KRAS*, *BRAF*, or *MAP2K1/2* mutations (See Supplemental eTable II in supporting information online). The frequencies of curly hair and hyperkeratosis in patients with *BRAF* mutations were significantly higher than in patients with a *KRAS* mutation. The frequency of hypertrophic cardiomyopathy in patients with *KRAS* mutations was significantly higher than that in patients with *MAP2K1/2* mutations.

DISCUSSION

This is the first nationwide epidemiological study of patients with Costello and CFC syndrome. Before our identification of the genes responsible for Costello and CFC syndromes in 2005 and 2006, only a few Japanese patients with these syndromes had been reported. The availability of molecular analysis facilitated diagnosis of both syndromes, and the number of reports of such patients has steadily increased. In this study, we estimated the prevalence of Costello syndrome and CFC syndrome as 1 in 1,290,000 and 1 in 810,000 in the general population, respectively. The second-stage survey clarified the clinical manifestations of both disorders, including the daily activities of 15 adult patients.

The natural history of Costello and CFC syndromes in adulthood has not been fully clarified. A previous report describing 17 adult patients with Costello syndrome ranging in age from 16 to 40 years showed that all eight individuals who had a bone density measurement taken had abnormal results, suggesting osteoporosis or osteopenia; three of the patients had bone pain, vertebral fractures,

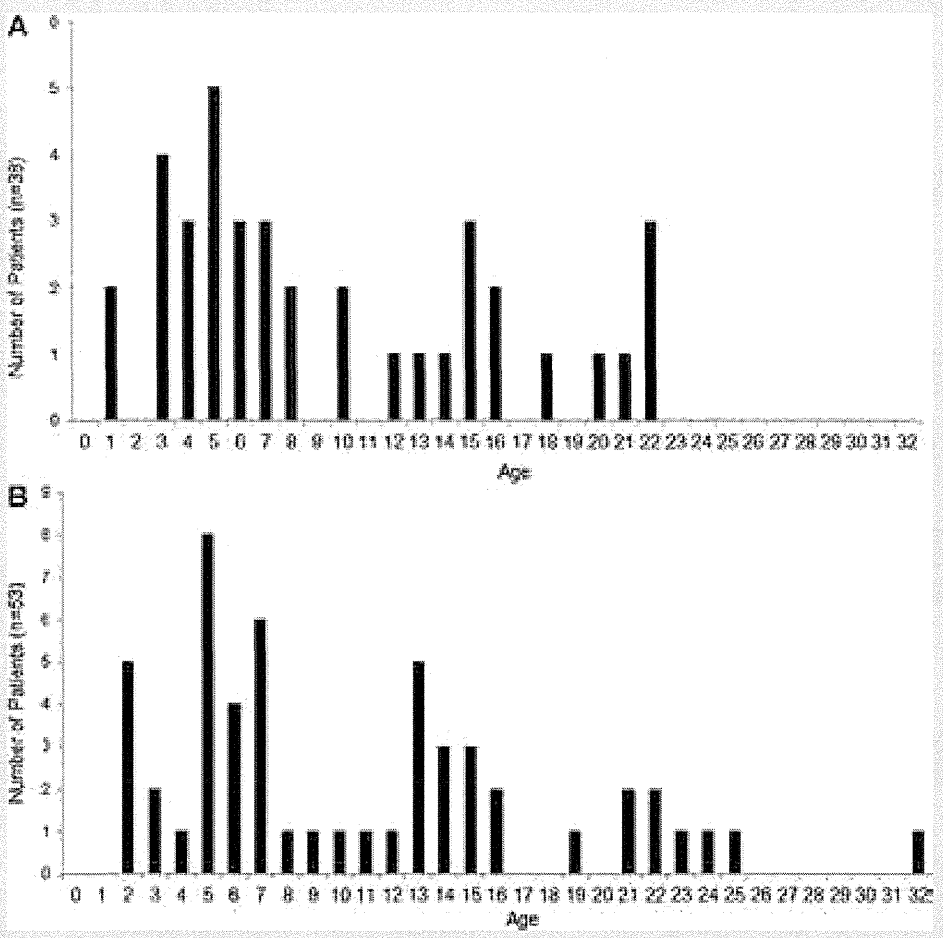

FIG. 2. Age distribution of 38 patients with Costello syndrome (A) and 53 patients with CFC syndrome (B) as of March 31, 2011. Five patients with Costello syndrome were deceased and the age was unknown for one of the 54 patients with CFC syndrome whose clinical manifestations were obtained by the second survey (Table II).

and height loss [White et al., 2005]. A recent study showed the detailed quality of life issues in individuals with Costello syndrome [Hopkins et al., 2010]. Our survey identified the daily activities of six adults with Costello syndrome and nine with CFC syndrome. Although intellectual disability was severe in most patients, 11 adults lived in their houses and did not need constant medical care. Ten of the 15 patients walked independently, and seven could communicate with other people. Thirteen adult patients, not including the two bedridden patients with CFC syndrome, could feed themselves with some assistance. Especially all six patients with Costello syndrome could feed themselves. One had recurrent bladder papillomata and another patient had multiple gallbladder polyps and a renal angioma. None of the examined patients had developed malignant tumors. This survey was unable to identify patients older than 32 years. The tentative prevalence at ages younger than 32 years was estimated to be 1 in 431,000 for Costello syndrome and 1 in 270,000 for CFC syndrome. A follow-up program is important in order to delineate the natural history of older patients.

Our study method has previously been used to estimate the prevalence of intractable diseases, including moyamoya disease, myasthenia gravis, and idiopathic cardiomyopathy [Miura et al., 2002; Kawamura et al., 2006; Kuriyama et al., 2008; Murai et al., 2011] (See Supplemental eTable III in supporting information online). One of the advantages of this survey is that researchers are able to conduct the postal survey without governmental involvement. Another merit of this method is its usefulness for estimating the prevalence of very rare diseases, because we can effectively collect information all over the country, including small hospitals. The response rate from the departments is key to minimizing the standard errors of the estimation. The response rate for our first-stage survey was 76%, which was the highest among the previous eight prevalence studies using this protocol (See Supplemental eTable III in Supporting Information online). However,

TABLE III. Clinical Manifestations and Daily Living Activities in Adult Patients

Patients	**NS30**[a]	**NS125**[b]	**NS157**[b]	**NS239**[b]	**KCC J-210**	**KCC11**	**NS7**[c]	**NS164**
Diagnosis	CS	CS	CS	CS	CS	CS	CFCS	CFCS
Mutation								
Gene	*HRAS*	*HRAS*	*HRAS*	*HRAS*	*HRAS*	*HRAS*	*BRAF*	*BRAF*
Nucleotide substitution	c.38G>A	c.34G>A	c.34G>A	c.34G>A	ND	c.34G>A	c.769C>A	c.770A>G
Amino acid substitution	p.G13D	p.G12S	p.G12S	p.G12S	ND	p.G12S	p.Q257K	p.Q257R
Sex	F	F	F	M	M	M	F	M
Age	18 yr	22 yr	22 yr	22 yr	21 yr	20 yr	32 yr	19 yr
Neoplasia								
Papillomata	Facial papillomata	Nasal papillomata	Bladder papillomata	Facial and hand papillomata	ND	–	–	–
Other tumors	Multiple gallbladder polyps, Renal angioma	–	–	–	ND	–	+ Hemangioma	–
Cardiac defect								
Hypertrophic cardiomyopathy	+	+	+	+	ND	–	–	–
Pulmonic stenosis	–	–	–	–	ND	–	+	+
Congenital heart malformation	–	–	–	–	ND	–	–	–
Arrhythmia	–	–	–	+ Mobitz type II atrioventricular block	ND	–	–	–
Central nervous system								
Abnormal brain structure	ND	–	–	+ Type I Arnold–Chiari malformation	ND	–	–	+ Cortical atrophy
Seizure	ND	–	–	–	ND	+	+	–
Activities of daily living								
Transferring	Cane-assisted gait	Independent	Independent	Independent	Independent	Wheelchair	Independent	Independent
Mental faculties	Severe ID (IQ = 33) (At 4 yr of age)	Severe ID	Moderate ID (IQ44)	Moderate ID (DQ = 35) (At 2 yr of age)	ID (Severity unknown)	Severe ID	Severe ID	Moderate ID (IQ = 37) (At 2 yr of age)
Verbal skills	2-word sentences	2-word sentences	Daily conversation	Daily conversation	ND	Simple conversation	2-word sentences	Single-word utterances
Residence	ND	Home	Home	ND	ND	Home Sometimes using outpatient facilities	Home	Home
School/workplace	Graduated from a school for disabled children; Vocational training facility	Vocational training facility	Vocational training facility	Vocational training facility	ND	None	Graduated from public school class for disabled children	Graduated from a school for disabled children
Other (Feeding, continence)	Self-feeding	Self-feeding	Self-feeding, toileting, and bathing	Self-feeding	Self-feeding	Self-feeding	Almost self-reliant but sometimes needs assistance	Self-feeding, toileting, and bathing

Patients	**NS184**	**NS228**	**NS233**	**NS283**	**KCC U-10**	**KCC B-1**	**KCC6**	
Diagnosis	CFCS	CFCS	CFCS	CFCS	CFCS	CFCS	CFCS	
Mutation								
Gene	*BRAF*	*BRAF*	*BRAF*	*BRAF*	*BRAF*	*BRAF*	*KRAS*	*BRAF*
Nucleotide substitution	c.770A>G	c.1406G>A	c.770A>G	c.1785T>G	c.770A>G	ND	c.547_552del ACAAAG	c.1390G>A
Amino acid substitution	p.Q257R	p.G469E	p.Q257R	p.F595L	p.Q257R	ND	p.183_184delTK	p.G464R
Sex	F	F	M	F	M	M	F	
Age	22 yr	23 yr	24 yr	21 yr	25 yr	21 yr	22 yr	
Neoplasia								
Papillomata	−	−	−	Cervical papillomata	−	−	ND	
Other tumors	−	−	−	−	−	−	ND	
Cardiac defect								
Hypertrophic cardiomyopathy	−	+	−	−	−	−	+	
Pulmonic stenosis	−	+	−	−	−	+	−	
Congenital heart malformation	−	−	−	−	−	−	−	
Arrhythmia	−	−	−	+ Atrioventricular block	−	−	+ Atrial tachycardia	
Central nervous system								
Abnormal brain structure	+ Periventricular leukomalacia Ventricular dilation	+ Ventricular dilation	−	+ Cortical atrophy White matter volume reduction Thinning of corpus callosum; West syndrome	−	−	ND	
Seizure	+	+	+	+	+	−	ND	
Activities of Daily Living								
Transferring	Independent	Abnormal gait	Independent	Bedridden	Bedridden	Independent	Independent	
Mental faculties	Severe ID	Severe ID	Moderate ID	Very severe ID	Very severe ID	ID (Severity unknown)	ID (Severity unknown)	
Verbal skills	Simple conversation	Daily conversation	Simple conversation	No meaningful word	No meaningful word	Simple conversation	ND	
Residence	Home	Home	Home	Home, Sometimes using outpatient facilities	Home, Sometimes using outpatient facilities	Home	ND	
School/Workplace	Vocational training facility	Vocational training facility	Vocational training facility	None	None	Vocational training facility	ND	
Other (Feeding, Continence)	Self-feeding	Almost self-reliant but sometimes needs assistance	Self-feeding	Full assistance using percutaneous endoscopic gastrostomy	Full assistance	Self-feeding	Self-feeding	

CS, Costello syndrome; CFCS, cardio-facio-cutaneous syndrome; yr, years of age; ID, intellectual disability; IQ, intelligence quotient; DQ, development quotient; ND, not described.
Mutations and a portion of the clinical manifestations have been reported; [a]Aoki et al. [2005]; [b]Niihori et al. [2011]; [c]Narumi et al. [2007].

there are limitations to our survey method. Most survey slips were sent to pediatric departments in general hospitals, which might have precluded identification of adult patients. Another limitation is the possible diagnostic bias of these disorders. In this study, there were major peaks at 5 years of age in both diseases, suggesting that the diagnosis of both disorders is usually made in a certain age range, and patients are less likely to receive the correct diagnosis at a later age. In addition, individuals with Costello syndrome who are mildly or only borderline affected may not be diagnosed by pediatricians at the sampled hospitals [Axelrad et al., 2007]. These effects could lead to a substantial underestimation of the prevalence.

Costello and CFC syndrome fall into the category of rare diseases. To compare the epidemiological features of Costello and CFC syndromes to other genetic disorders, we summarized the results of epidemiologic studies of other genetic disorders (See Supplemental eTable IVin supporting information online). The prevalence and incidence of Sotos syndrome has been reported to be 1 in 20,000 and 1 in 5,000 newborns, respectively [Kurotaki et al., 2003]. A recent nationwide epidemiological study showed that the prevalence of Alexander disease to be 1 in 2,700,000 [Yoshida et al., 2011]. An earlier report estimated the prevalence of Kabuki syndrome at 1 in 32,000 [Niikawa et al., 1988]. Using the similar method with Kabuki syndrome [Niikawa et al., 1988], the incidence of Costello syndrome was estimated to be 1 in 60,000–100,000 (Kurosawa, personal communication). Given that the annual number of live births in Japan is approximately 1,000,000, 10 to 16 patients with Costello syndrome could be born annually. This estimated incidence was higher than the estimated prevalence in patients younger than 32 years of age in our study.

Two mutations in the RAS/MAPK pathway have been identified in a single patient with Noonan syndrome and related disorders [Brasil et al., 2010; Ekvall et al., 2011]. In our study, variations in two molecules that participate in the RAS/MAPK signaling pathway were identified in two patients. One patient had a *SOS1* p.D309Y mutation, which has previously been identified in Noonan syndrome patients [Narumi et al., 2008], and a *K-RAS4A* p.Y166H mutation (a novel variation, inherited from the father). Another patient with CFC syndrome had a *BRAF* p.G464R mutation (known mutation) and a *K-RAS4B* p.T183_ K184del mutation (novel variant). Further study is required to clarify the variations in the RAS pathway that could modify the effect of the disease-causing mutations and the patient phenotypes.

Approximately 13% of patients with Costello syndrome have developed malignant tumors, including rhabdomyosarcomas, ganglioneuroblastomas, and bladder carcinomas [Aoki et al., 2008]. The frequency of malignant tumors in Costello syndrome in the current study was 9% (4 of 43 patients), lower than that reported recently [Lin et al., 2011]. An association between malignant tumors and CFC syndrome was considered rare. However, we identified three patients with CFC syndrome who developed hematologic malignancies [Niihori et al., 2006; Makita et al., 2007; Ohtake et al., 2011], suggesting the importance of molecular diagnoses and careful observation in patients with Costello and CFC syndrome. A tumor screening protocol for patients with Costello syndrome has been proposed [Gripp et al., 2002] and may be useful for patients with CFC syndrome as well. Long-term follow-up is required to determine the incidence and type of tumors in patients with both disorders.

In conclusion, we conducted a nationwide epidemiological survey of patients with Costello and CFC syndrome and estimated the total number of patients with each disease from the results of the postal survey as well as those of molecular analysis. The prevalences of Costello syndrome and CFC syndrome were estimated as 1 in 1,290,000 and 1 in 810,000, respectively. Evaluation of 15 adult patients showed that they had severe intellectual disability but that most of them live at home without constant medical care, suggesting that the number of adult patients may be underestimated. Further epidemiological studies to identify adult patients and follow-up of the patients reported in this study will help us to better understand the natural history of both disorders.

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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 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

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/planter 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 phRLnull-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 MEKK 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

Table 1 Clinical findings and *HRAS* mutations in our CS patients

Patients	*NS71*	*NS123*	*NS125*	*NS132*	*NS137*	*NS139*	*NS156*	*NS157*	*NS167*	*NS181*	*NS198*	*NS217*
Gender	F	F	F	F	M	F	M	F	M	M	M	M
Age	9 months	11 years	17years	3 years	10 years	7 months	2 years 3 months	17 years	3 months	3 years	1 year 2 months	4 years 6 months
Paternal age at birth (years)	39	29	42	37	30	35	34	34	37	33	31	40
Maternal age at birth (years)	28	26	27	31	28	35	36	36	34	33	31	37
Growth and development												
Postnatal failure to thrive	+	+	+	+	+	+	+	+	+	+	+	+
Mental retardation	+	+	+	+	+	+	+	+	+	+	+	+
Craniofacial characteristics												
Relative macrocephaly	+	+	+	+	+	+	+	+	+	+	+	+
Coarse facial appearance	+	+	+	+	+	+	+	+	+	+	+	+
Muscloskeltal characteristics												
Short neck	+	+	+	+	+	+	+	+	–	+	–	+
Hyperextensive fingers	+	+	+	+	–	+	+	+	+	–	–	–
Tight Achilles tendon	–	+	+	+	+	–	+	–	–	–	–	+
Abnormal foot position	+	+	+	+	NA	+	NA	–	–	+	–	+
Skin characteristics												
Curly, sparse hair	+	+	+	+	+	+	+	+	+	+	Curly	+
Soft, loose skin	+	+	+	+	+	+	+	+	–	+	+	+
Deep palmer/ planter creases	+	+	+	+	+	+	+	+	+	+	+	+
Cardiac defect												
Hypertrophic cardiomyopathy	+	–	+	–	+	+	NA	+	–	+	–	–
Others	PS	–	–	–	–	–	PAC	Anomalous septum in the right atrium	VSD, arrhythmia	Atrial tachycardia	–	ASD, PSVT, PVC, CAR
Neoplasia												
Papillomata	–	–	+	–	–	–	NA	+	+	–	+	–
Other tumors		Bladder cancer							Heart neoplasia			
Others			GH deficiency		GSDIII	Chiari I, syringomyelia	Pyrolic stenosis	Congenial stridor, GH deficiency	Hypoplastic nails	Hypertention		Hydronephrosis, GER, laryngomalacia
HRAS mutation												
Nucleotide substitution	c.34G>A	c.35G>C	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A
Amino acid substitution	p.G12S	p.G12A	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S

Table 1 Continued

Patients	*NS223*	*NS231*	*NS239*	*NS248*	*NS254*	*NS263*	*NS299*	*NS318*	*NS324*	*Total*
Gender	F,	F	M	M	F	M	F	F	F	
Age	6 months	5 months	18 years	5 years	2 months	1 month	3 years	1 month	1 year 6 months	
Paternal age at birth (years)	34	27	27	NA	37	35	34y	33	33	
Maternal age at birth (years)	36	27	26	30	34	36	35y	32	33	
Growth and development										
Postnatal failure to thrive	+	+	+	+	+	+	+	+	+	21/21
Mental retardation	+	+	+	+	NA	+	+	+	+	20/20
Craniofacial characteristics										
Relative macrocephaly	–	+	+	–	+	+	–	–	+	17/21
Coarse facial appearance	+	+	+	+	+	+	+	+	+	21/21
Muscloskeltal characteristics										
Short neck	–	+	NA	NA	+	+	+	–	–	14/19
Hyperextensive fingers	–	+	–	+	+	–	–	+	+	13/21
Tight Achilles tendon	+	NA	–	+	–	–	–	+	+	10/20
Abnormal foot position	–	–	NA	NA	NA	–	–	+	+	9/16
Skin characteristics										
Curly, sparse hair	+	Curly	Curly	+	+	+	Curly	+	Curly	21/21
Soft, loose skin	–	+	+	+	+	+	–	+	+	18/21
Deep palmer/planter creases	+	–	+	+	+	+	+	+	+	20/21
Cardiac defect										
Hypertrophic cardiomyopathy	+	–	+	+	+	+	+	+	+	14/20
Other	PAC	PVC	–	–	–	–	–	PAC	PAC	
Neoplasia										
Papillomata	+	–	+	–	–	–	–	–	–	6/20
Other tumors										
Others	Prabastatin administration	Laryngomalasia, hydrocephallus	GH deficiency, Arnold Chiari, scoliosis	Empty sella, GH deficiency, hypothyroidism, hypogonadism, syringomyelia		Hyperinsulinemia		Laryngomalasia seizure	Laryngomalasia	
HRAS mutation										
Nucleotide substitution	c.34G>T	c.35G>A	c.34G>A	c.34G>A	c.34G>A	c.35G>C	c.34G>A	c.35G>C	c.34G>A	
Amino acid substitution	p.G12C	p.G12D	p.G12S	p.G12S	p.G12S	p.G12A	p.G12S	p.G12A	p.G12S	

Abbreviations: –, absent; +, present; ASD, atrial septal defect; F, female; GER, gastroesophageal relfux; GH, growth hormone; GSDIII, glycogen storage disease III; M, male; NA, not available; PAC, premature atrial contraction; PS, pulmonic stenosis; PSVT, paroxysmal supraventricular tachycardia; PVC, premature ventricular contraction; VSD, ventricular septal defect.

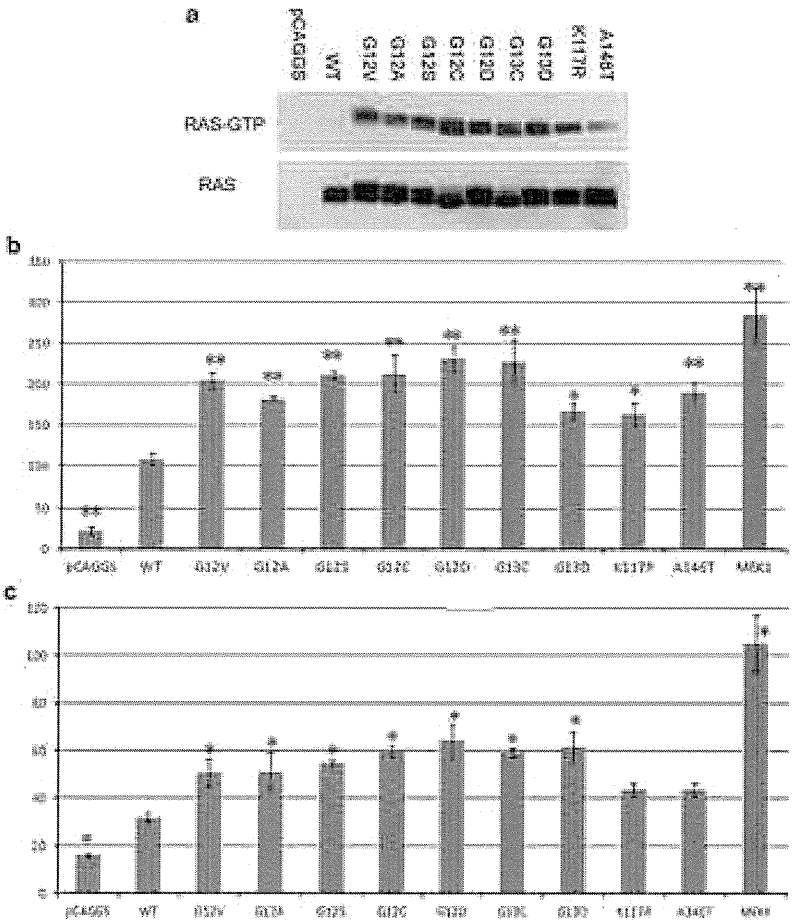

Figure 1 Functional characterization of HRAS mutants. (**a**) Ras-guanosine triphosphate (GTP) in NIH 3T3 cells transfected with wild-type or mutant HRAS constructs. HRAS protein levels were similar in NIH3T3 cells expressing each protein and were subsequently used as a loading control. (**b**, **c**) Stimulation of ELK (**b**) and c-Jun (**c**) transcription by *HRAS* mutants. The ELK-and c-Jun-GAL4 vectors and GAL4-luciferase trans-reporter vector were transiently co-transfected with various *HRAS* constructs into unstimulated NIH 3T3 cells. Relative luciferase activity (RLA) was normalized to the activity of a co-transfected control vector (phRLnull-luc) expressing *Renilla reniformis* luciferase. The results are expressed as the means and s.d. from triplicate samples. MEK1 and MEKK were used as positive controls. WT, wild type. $*P<0.05$; $**P<0.01$ compared with WT.

associated β-galactosidase staining confirmed that these cells showed cellular senescence.

Two downstream signaling pathways, p53 and Rb-p16, are activated during cellular senescence. To examine oncogene induced cellular senescence at the molecular level, we assessed senescence markers, including phosphorylated extracellular signal-regulated kinase, phosphorylated p53 and p16, in cells expressing HRAS mutant proteins (Figure 2b). As expected, phosphorylated p53 (Ser15) and p16 levels, as well as phospho-extracellular signal-regulated kinase levels, were significantly increased in the cells transfected with HRAS mutants relative to cells transfected with mock vector or wild-type HRAS. These results demonstrate that not only p.G12V, but also the other eight CS-related HRAS mutants, can cause OIS.

DISCUSSION

In this study, we identified four *HRAS* mutations in 21 patients with CS and evaluated their detailed clinical manifestations of the disease in these patients. Biochemical analyses, including a GTP binding assay and luciferase assays to detect ELK and c-Jun trans-activation, showed that there were no significant differences among the analyzed mutations in codon 12/13. 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 mutants in codon 12 or 13. Our results indicated that all HRAS mutants detected in CS patients were able to cause OIS.

Our study is the first to demonstrate that HRAS mutants other than p.G12V can induce senescence when they are overexpressed in human fibroblasts. The symptoms of CS seem to be caused by either hyperproliferation or hypoproliferation, coupled with growth factor resistance, which may be ascribable to DNA damage response or OIS. Postnatal cerebellar tonsillar herniation, Chiari 1 malformation,[42] deep palmar and plantar creases and papillomata may all be caused by hyperproliferation. In contrast, the poor weight gain, short stature and endocrine dysfunction observed in CS patients[43–45] might be caused by hypoproliferation. Adult brain and heart progenitor cells in a zebrafish CS model with a homozygous HRAS p.G12V mutation

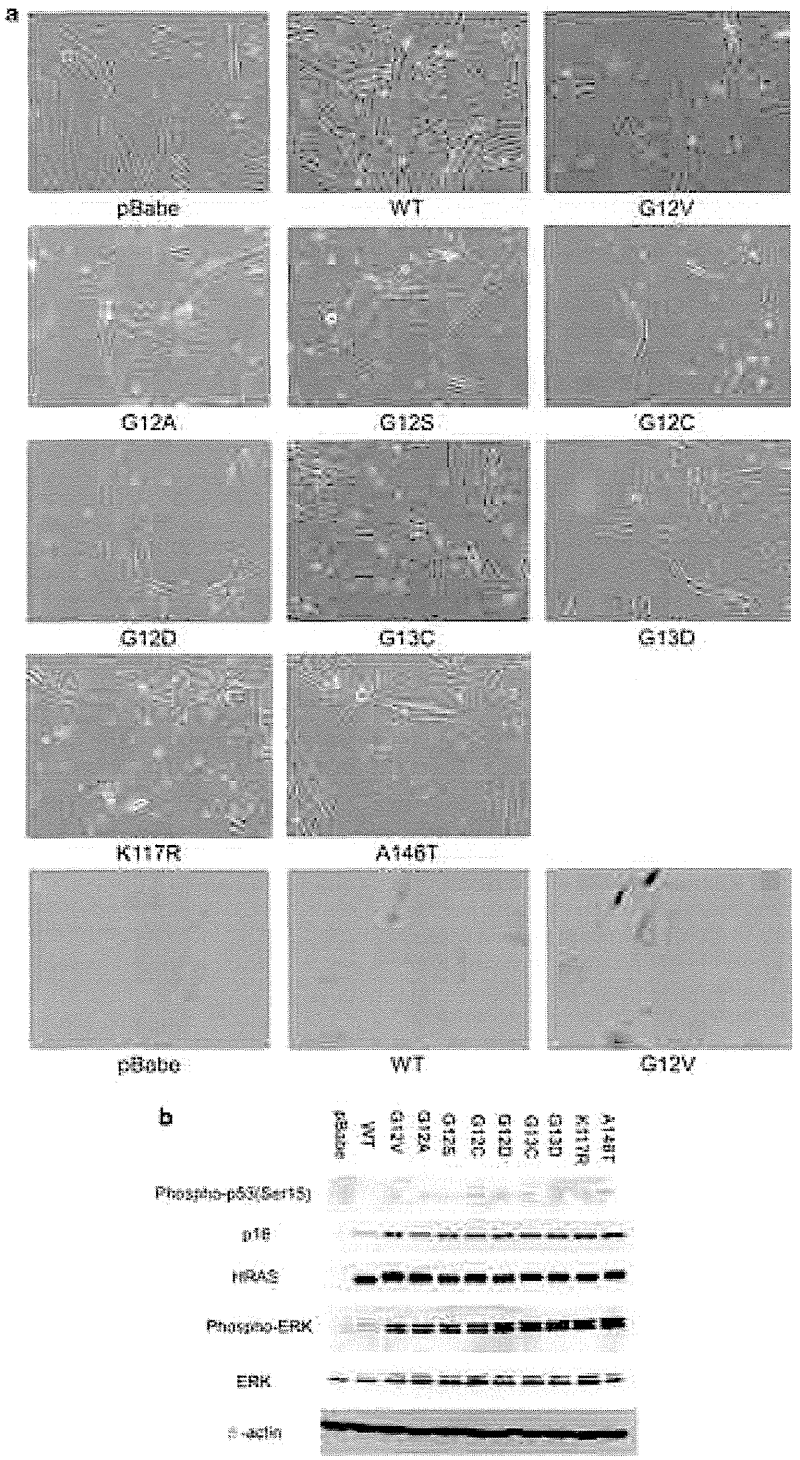

Figure 2 Effect of Costello syndrome (CS)-associated HRAS mutants on primary fibroblasts. (**a**) BJ cells transduced with retroviruses expressing wild-type or mutant HRAS. Images in the lowest tier show senescence-associated β-galactosidase staining. (**b**) Immunoblots of cellular lysates from BJ cells transduced with empty vector (pBabe) or with wild-type or mutant HRAS retroviruses.

exhibited cellular senescence, suggesting that the age-related worsening of the Costello phenotype[46] might occur, because the replicative capability of adult progenitor cells is exhausted. Osteoporosis has frequently been found in adult patients with CS,[47] suggesting that cellular senescence affects osteogenesis. However, further studies will be needed to determine whether OIS indeed contributes to the pathogenesis in patients with CS.

It has been suggested that clinical symptoms vary among patients with mutations in codon 12 or 13. In previous studies, a total of 19 CS patients have been reported to die from severe cardiomyopathy, cardiac arrhysmia, rhabdomyosarcoma, respiratory failure, multi-organ failure or sepsis. The number of fatal cases was 5/138 patients with p.G12S, 4/6 with p.G12C, 3/17 with p.G12A, 3/4 with p.G12D, 2/2 with p.G12V, 1/1 with p.G12E and 1/1 with p.E63K.[3,5–23] The mortality of patients with p.G12C or p.G12D was significantly higher than that of the patients with the more common p.G12S (P=0.026 by Fisher's exact test). Previous studies have shown that the p.G12V substitution has the highest transformative potential (p.G12V>p.G12A, p.G12S, p.G12C, p.G12D>p.G13D) and is the most frequently found mutation in human tumors.[48,49] However, our Ras activity assays and luciferase assays did not show any differences among *HRAS* codon 12/13 mutants. This may be due to the extremely high expression level of HRAS protein in our transient transfection study, which could make it difficult to detect subtle differences between mutants. Further studies will be necessary to clarify whether the high mortality in patients with p.G12C or p.G12D is due to functional differences in these mutants or due to bias because of our small sample size of patients.

Mutations at codons 117 and 146 are rare in CS and somatic cancers. Meanwhile, mutations at codons G12, G13 and Q61 have been shown to impair intrinsic and GTPase activating protein-mediated GTP hydrolysis, leading to elevated levels of cellular RAS-GTP. It has been reported that the nucleotide exchange rate of both p.K117R and p.A146V HRAS is increased, relative to wild type.[13,27,28] However, the transformational potential of p.A146V HRAS is partially activated,[27] whereas that of p.K117R-HRAS is not; its transformational activity is instead similar to that of GTPase impaired mutants.[28] Our results and those of other reports suggest that p.K117R and p.A146T have milder effects on downstream effectors than do mutations in codon 12/13.

The clinical manifestations of CS in patients with p.K117R or p.A146V mutations suggest that these alleles have distinct effects, compared with mutations in codon 12/13. Of two CS patients with a p.K117R mutation, one patient had an atypical phenotype such as microretrognatism and slightly less-pronounced plantar and palmar creases.[7] The other patient had mild craniofacial manifestations of CS.[13] One patient with the p.A146V mutation showed a mildly coarse face and did not have deep palmar creases.[6] These atypical phenotypes might be attributed to the mild effects of p.K117R or p.A146V compared with codon 12/13 mutants.

Inhibitors of the RAS/MAPK pathway could provide benefits for patients with RAS/MAPK syndromes. Statins are 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors that result in decreased isoprenylation of RAS[50] and are now widely used for the treatment of hyperlipidemia. Statins have been used to modify the clinical manifestation of neurofibromatosis type I, which is caused by a genetic defect in a negative regulator of the RAS/MAPK pathway. Studies using mouse models of NF1 (Nf1 mice) have shown that treatment with a statin reverses the cognitive deficits of these mice.[51] A randomized control trial for neurofibromatosis type I treatment with simvastatin had a negative outcome.[52] Furthermore, statins have displayed antitumor activity in experimental tumor models, though clinical antitumor effects of statins have not been established.[53] Well-designed clinical studies will be needed to determine the effects of statins or other RAS inhibitors on manifestations of CS.

In conclusion, we identified *HRAS* mutations in 21 patients and examined the clinical manifestations of mutation-positive patients. Functional analysis revealed that CS-causing mutant HRAS proteins caused OIS in human fibroblasts. These findings may help enable more accurate prognoses for patients with *HRAS* mutations and contribute to our understanding of the mechanism underlying CS pathogenesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Craniofacial and dental malformations in Costello syndrome: A detailed evaluation using multi-detector row computed tomography

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ABSTRACT **Costello syndrome is a rare multiple congenital anomaly syndrome caused by heterozygous germline *HRAS* mutations, which is characterized by intellectual disability, growth retardation, distinctive facies, loose skin, cardiomyopathy and a preposition to malignancies. Although teeth abnormalities have been encountered in nearly two-thirds of the patients in literature, the evaluation tended to be limited to the extent which can be obtained from physical examination. We investigated detailed craniofacial, oral and dental findings in four patients with Costello syndrome. In this study, images reconstructed by multi-detector row computed tomography (MDCT) were used as substitutes for dental cast study and panoramic and lateral cephalometric radiograph studies to evaluate dental arches, tooth size, relationships between craniofacial and dental structures, and hypodontia. All four patients showed true/relative macrocephaly with facial bone hypoplasia and gingival hypertrophy. Occlusal attrition, malocclusion, small dental arches, microdontia, and convex face were noted in three patients. In addition, one patient showed dental caries, conic tooth and gingivitis, and another patient showed hypodontia. Our study suggests that craniofacial and dental abnormalities are common in Costello syndrome patients and comprehensive dental care should be provided from early infancy. To our knowledge, this is the first study of thorough craniofacial and dental evaluation by using MDCT in Costello syndrome. MDCT is a useful tool for precise evaluation of craniofacial and oral manifestations in patients with congenital anomaly/intellectual disability syndromes.**

Key Words: cephalometric analysis, Costello syndrome, multi-detector row computed tomography, small dental arch, malocclusion

INTRODUCTION

Costello syndrome is a rare multiple congenital anomaly syndrome characterized by intellectual disability, growth retardation, distinctive facies, loose skin, cardiomyopathy and a preposition to malignancies (Hennekam 2003), the prevalence of which is estimated to be 1 in 1 290 000 (Abe et al. 2012). Costello syndrome is caused by heterozygous germline *HRAS* mutations (Aoki et al. 2005) and is listed as one of the RASopathies, a group of related disorders caused by germline mutations in the Ras/mitogen-activated protein kinase pathway, which includes Noonan syndrome, cardio-facio-cutaneous (CFC) syndrome, and Costello syndrome, with considerable phenotypic overlap among these disorders (Rauen et al. 2010).

Craniofacial and oral features previously reported in Costello syndrome include macrocephaly, prominent forehead, high-arched palate, macroglossia, gingival hypertrophy, malocclusion, enamel hypoplasia and caries (Der Kaloustian et al. 1991; Di Rocco et al. 1993; Teebi and Shaabani 1993; Zampino et al. 1993; Johnson et al. 1998; van Eeghen et al. 1999; Delrue et al. 2003; Hennekam 2003; Kawame et al. 2003). Although teeth abnormalities were encountered in nearly two-thirds of the patients in the comprehensive review by Hennekam et al. (2003), the evaluation tended to be limited to the extent that can be obtained from physical examination. We here report on the result of our investigation of detailed craniofacial, oral and dental findings in four patients with Costello syndrome by using multi-detector row computed tomography (MDCT).

MATERIALS AND METHODS

Patients

A total of four patients with Costello syndrome, two male and two female, ranging in age between 5 and 7 years, were included in this study. All four patients were identified as having a missense mutation in the *HRAS* gene; c.34G>A (p.Gly12Ser) in patients 1, 2 and 3, and c.38G>A (p.Gly13Asp) in patient 4, either in exon 2. Clinical manifestations of the four patients are shown in Table 1. This study protocol was approved by the Ethics Committee of Saitama Children's Medical Center and proper informed consent was obtained from the legal guardians of patients.

Oral, dental, and craniofacial studies

Intraoral features such as palatal morphology, tooth calcification, occlusion and tooth eruption status were evaluated on physical examination. In addition, images reconstructed by MDCT were used as substitutes for dental cast study and panoramic and lateral cephalometric radiograph studies to evaluate the dental arches, tooth size, relationships between craniofacial and dental structures, and hypodontia. The following MDCT imaging conditions were used: window width, 1500; and window level, 450 (Hirai et al. 2010, 2011; Yamauchi et al. 2010). Crown and dental arch sizes were measured using Image J with a resolution accuracy of 0.1 mm. Lateral cephalometric analysis was performed based on the method developed by Iizuka and Ishikawa (1957). To perform cephalometric measurements, we made adjustments by rotating the mandibular bone image toward the expected actual intercuspal position. All measured data in this study were compared with standard values for

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Table 1 Clinical manifestations of four patients with Costello syndrome

Patients	1	2	3	4
Gender	M	M	F	F
Age (years)	5	6	6	7
Intellectual disability	+ (Severe)	+	+	+
Height (SD)	−6.8	−5.9	−4.9	−2.7
Head circumference (SD)	−2.5	+0.27	+0.29	+2.9
Distinctive facies	+	+	+	+
Cardiac defect	HCM, AT	HCM, AT, VSD, MR	−	HCM, AT
Skeletal abnormality	HD, FD	HD, FD	HD, FD	FD
Neoplasia	−	−	−	−
Other	Tracheomalacia	Inguinal hernia	Exotropia	GHD
HRAS mutation	c.34G>A (p.G12S)	c.34G>A (p.G12S)	c.34G>A (p.G12S)	c.38G>A (p.13G>D)

+, present; −, absent; AT, atrial tachycardia; F, female; FD, foot deformity; GHD, growth hormone deficiency; HCM, hypertrophic cardiomyopathy; HD, hip dislocation; M, male; MR, mitral regurgitation; VSD, ventricular septal defect.

Japanese individuals (Iizuka and Ishikawa 1957; Otsubo 1964; Kato 1979; Fukawa 2008).

RESULTS

Oral and dental features noted in four patients are summarized in Table 2. On physical examination, all four patients had open mouth, thick lips and gingival hypertrophy. Other common features noted in all but one patient were occlusal attrition (patients 1, 2, 3), high-arched palate (patients 2,3,4) and malocclusion (patients 1 and 4 exhibited open bite, and patient 3 cross bite). In addition, one patient (patient 4) exhibited dental caries in a single tooth, a single conic tooth, and gingivitis. Enamel hypoplasia, an occasionally reported feature in patients with Costello syndrome, was not apparent in any of our patients (Fig. 1). Crowding of teeth was also not observed in the four patients. Panolamic images reconstructed with MDCT revealed a congenital tooth defect (mandibular left second premolar) in one patient (patient 3; Fig. 2).

Small dental arch was present in all patients except patient 1 (Table 3). Morphological categorization of small dental arches in the three patients were as follows: one patient (patient 2) exhibited U-shaped dental arch in the maxilla and narrow dental arch in the mandible; and two patients (patients 3 and 4) showed narrow dental arch in the maxilla and rectangular dental arch in the mandible (Fig. 3).

In terms of tooth size, maxillary teeth, especially lateral incisors and first and second molars in primary teeth and first molar in permanent teeth, tend to be small in these patients studied (Table 4). The degree of smallness of the maxillary teeth was most marked in the second molars in primary teeth of the two male patients (patients 1 and 2).

Cephalometric analysis (Table 5) revealed that, among the four patients studied, a convex face (increased facial convexity) was present in three patients (patients 1, 2 and 3), associated with maxillary overhang (increased SNA angle) observed in one patient (Patient 1), and with mandibular retrusion (decreased SNB angle) in two patients (patients 2 and 3).

Three-dimensional reconstructed images by MDCT also demonstrated the craniofacial manifestations shared by all four patients such as true/relative macrocephaly with maxillofacial hypoplasia, dolichocephaly, and mandibular anomalies characterized by thick and flat head of the condylar process, short condylar neck, narrow mandibular notch, and antegonial notching (Fig. 4). Calcified falx cerebri were also noted in all patients.

Table 2 Oral and dental features in four patients with Costello syndrome

	Patient				
	1	2	3	4	Total
Open mouth	+	+	+	+	4/4
Thick lips	+	+	+	+	4/4
Gingival hypertrophy	+	+	+	+	4/4
Gingivitis	−	−	−	+	1/4
Dental caries	−	−	−	+	1/4
Occlusal attrition	+	+	+	−	3/4
Relative macrocephaly with facial bone hypoplasia	+	+	+	+	4/4
High-arched palate	−	+	+	+	3/4
Convex face	+	+	+	−	3/4
Maxillary overhang	+	−	−	−	1/4
Mandibular retrusion	−	+	+	−	2/4
Malocclusion	O	−	C	O	3/4
Small dental arch	−	+	+	+	3/4
Maxilla	U	U	N	N	
Mandible	U	N	R	R	
Hypodontia	−	−	+	−	1/4
Conic teeth	−	−	−	+	1/4

+, present; −, absent; C, cross bite; O, open bite; N, narrow dental arch; R, rectangle dental arch; U, U-shaped dental arch.

DISCUSSION

We performed thorough evaluation of craniofacial and dental features in four patients with Costello syndrome. As previously

Table 3 Dental arch measurements in four patients

Patient	1		2		3		4	
	L1	L2	L1	L2	L1	L2	L1	L2
Maxilla	1.25	−0.67	−2.06	−2.90	−1.61	−2.52	−0.91	−1.04
Mandibular	0.79	−0.38	2.50	−0.06	−3.30	−1.98	−3.86	−2.42
	WC	WE	WC	WE	WC	WE	WC	WE
Maxilla	0.97	−0.02	−1.31	−2.88	−4.14	−2.80	−1.45	−1.52
Mandibular	0.97	0.51	−2.98	−1.49	−1.66	−0.24	−3.02	−2.53

WC and WE represent the distance between the primary cuspids (the cuspids), and the primary second molars, respectively. L1 represents the distance between the central point of the incisors and the line connecting the primary cuspids of both sides, and L2 represents the distance between the central point of the incisors and the line connecting the primary second molars of both sides. Unit, S.D.

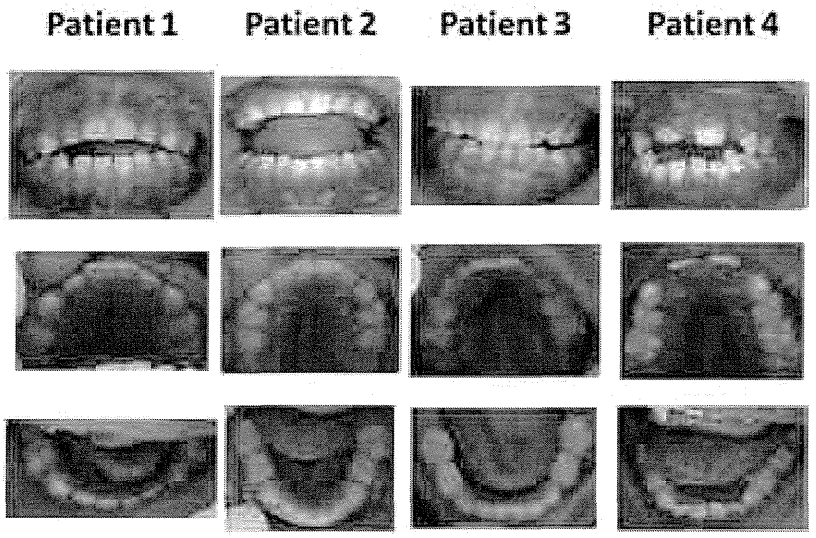

Fig. 1 Oral photographs of four patients. Patients 2, 3 and 4 showed occlusal attrition, high-arched palate and small dental arch. Patient 4 showed dental caries, gingivitis and conic teeth.

Fig. 2 Multi-detector row computed tomography (MDCT)-synthesized panoramic radiograph of Patient 3 at 6 years of age. Note the missing lower second premolar on left side (arrow).

reported, our patients showed true/relative macrocephaly and gingival hypertrophy (all patients). In addition, they exhibited malocclusion, occlusal attrition, small dental arches, microdontia, and convex face (three patients). Dental caries, conic tooth and gingivitis were noted in one patient, and hypodontia was noted in another patient. Enamel hypoplasia, an occasionally reported feature in patients with Costello syndrome, was not apparent in any of our patients.

True/relative macrocephaly is a well-known feature of patients with Costello syndrome. In this study, MDCT images showed macrocephalic skull in all four patients. In addition, facial bone hypoplasia was also evident which was associated with malformed mandible characterized by thick and flat head of the condylar process, short condylar neck, narrow mandibular notch, and antegonial notching on MDCT (Fig. 4). Antegonial notching is a feature seen in several congenital malformation syndromes associated with facial bone dysplasia, such as Treacher Collins syndrome, Nager syndrome and Pierre-Robin syndrome (Becker et al. 1976). Facial skeletal maldevelopment should be considered a feature of Costello syndrome.

Occlusal attrition is a finding that was frequently observed in our patients (patients 2, 3, 4). While the causes of attrition are diverse, malocclusion and habits such as bruxism and clenching are main possible causes. In view of behavioral characteristics of irritability of patients with Costello syndrome, habits such as teeth clenching might be a major cause for attrition.

It is of interest to know whether there are phenotypic similarities in craniofacial and dental features among Noonan-related disorders

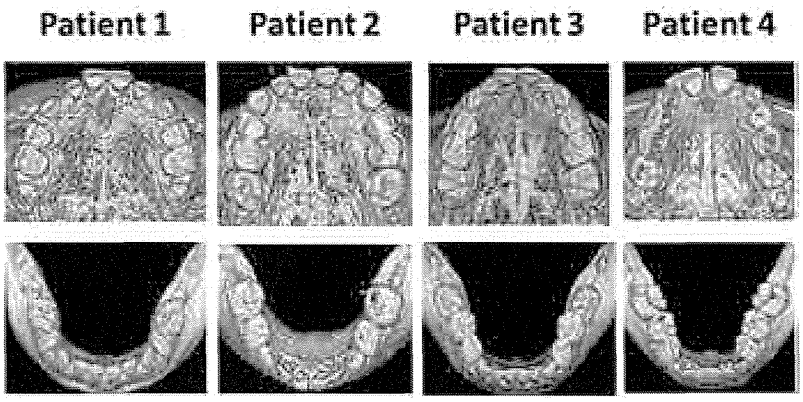

Fig. 3 Multi-detector row computed tomography (MDCT)-synthesized upper and lower dental arches in all four patients. Small dental arches are noted in three patients (patients 2, 3 and 4). Patient 2 exhibited a U-shaped dental arch in the maxilla and narrow dental arch in the mandible; Patients 3 and 4 exhibited narrow dental arches in the maxilla and rectangle arches in the mandible.

Table 4 Tooth size measurements in four patients

Patient	1		2		3		4	
	Right	Left	Right	Left	Right	Left	Right	Left
Maxillary								
Primary teeth								
Central incisor	0.54	0.54	−1.08	−0.54	−0.45	−0.45		
Lateral incisor	−2.00	−1.78	−1.56	−1.56	−2.44	−2.44	−0.49	
Cuspid	−1.22	−0.98	−1.22	−1.71	−0.73	−0.73	−0.98	−0.24
First molar	−2.39	−2.61	−2.17	−1.96	−1.09	−0.87	−0.43	−0.43
Second molar	−3.33	−3.16	−3.68	−3.51	−1.93	−1.58	−1.05	−0.53
Permanent teeth								
Central incisor							0.24	0.98
First molar							−1.95	−1.95
Mandibular								
Primary teeth								
Central incisor	0.31	0.00	−0.31	0.00	−1.00	−0.67		
Lateral incisor	1.21	1.52	0.00	−0.30	−0.83	−1.39		
Cuspid	0.31	0.31	−0.31	−0.63	−0.33	0.00	1.00	1.33
First molar	−1.30	−1.52	−1.30	−1.30	−0.60	−0.40	0.20	0.00
Second molar	1.04	1.04	−1.88	−1.46	0.00	−0.20	0.98	1.18
Permanent teeth								
Central incisor							−0.56	−0.83
Lateral incisor							−0.51	0.00
First molar							1.50	1.83

Tooth size represents the distance from the medial to the distal. Unit, S.D.

(RASopathies), including Noonan syndrome, CFC syndrome and Costello syndrome. However, a detailed investigation about craniofacial and dental findings has not yet been done. Recently, Goodwin et al. (2012) studied craniofacial and dental development in CFC syndrome based on 32 patients as the first large cohort study and found characteristic findings of the syndrome, including macrocephaly, convex facial profile, malocclusion with open bite, posterior cross bite, dental crowding, and high-arched palate. Among these features of CFC syndrome, macrocephaly, convex facial profile, malocclusion and high-arched palate were features noted in our patients with Costello syndrome as well. Unfortunately, as the evaluations of CFC syndrome by Goodwin et al. were mainly based on physical examination, more detailed evaluation regarding, such as dental arches, tooth size, and relationships between craniofacial, dental and skeletal structures have not been fully provided.

We used MDCT as a substitute for cephalometric, panoramic and dental cast studies. To our knowledge, this is the first study of thorough craniofacial and dental evaluation by using MDCT in Costello syndrome. As intellectual disability is common in Costello syndrome, these conventional cephalometric, panoramic and dental