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H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

Ⅲ．研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

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(作成上の留意事項)

1．「研究成果の刊行に関する一覧表」に記入した書籍又は雑誌は、その刊行物又は別刷り一部を添付すること。

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3．日本工業規格A列４番の用紙を用いること。各項目の記入量に応じて、適宜、欄を引き伸ばして差し支えない。

Ⅳ. 研究成果の刊行物・別刷

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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.

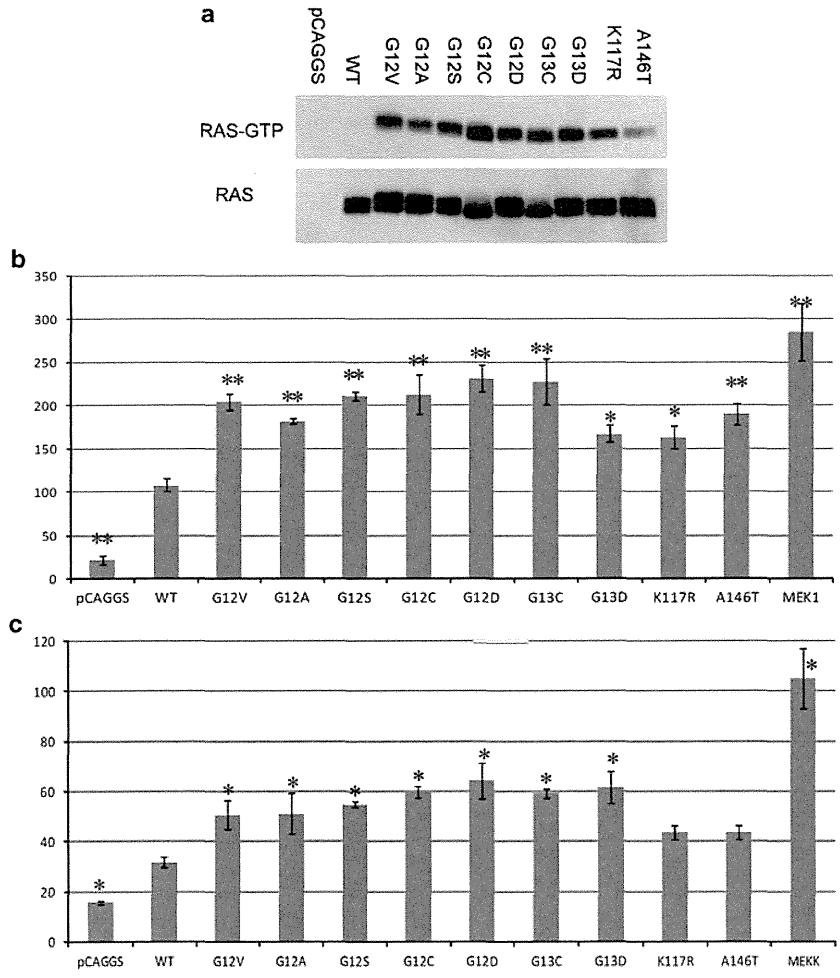

Figure 1 Functional characterization of HRAS mutants. (**a**) Ras-guanosine triphosphate (GTP) in NIH 3T3 cells transfected with wild-type or mutant HRAS constructs. HRAS protein levels were similar in NIH3T3 cells expressing each protein and were subsequently used as a loading control. (**b**, **c**) 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

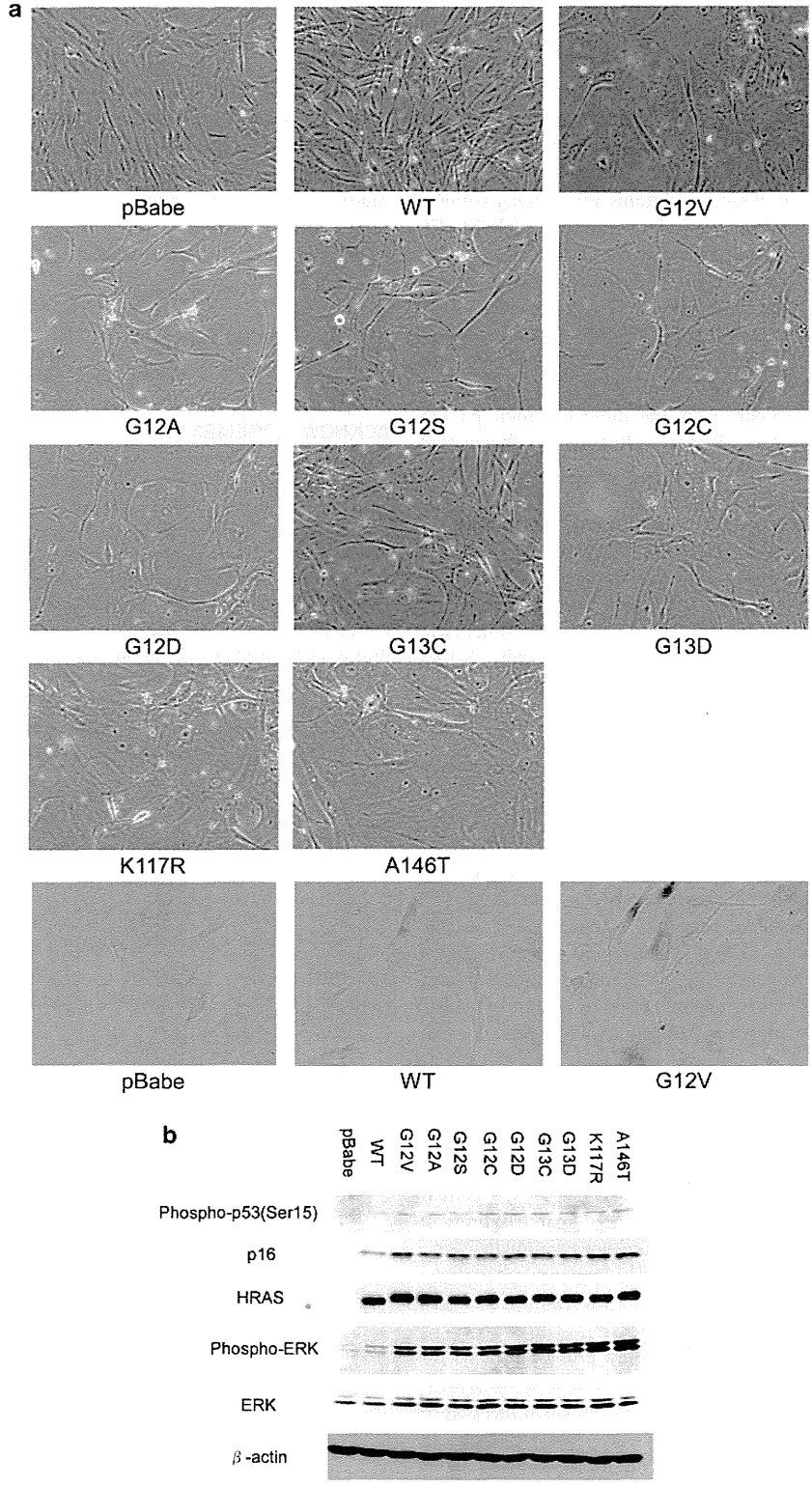

Figure 2 Effect of Costello syndrome (CS)-associated HRAS mutants on primary fibroblasts. (**a**) BJ cells transduced with retroviruses expressing wild-type or mutant HRAS. Images in the lowest tier show senescence-associated β-galactosidase staining. (**b**) Immunoblots of cellular lysates from BJ cells 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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Casitas B-cell lymphoma mutation in childhood T-cell acute lymphoblastic leukemia

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ABSTRACT

Somatic *CBL* mutations have been reported in a variety of myeloid neoplasms but are rare in acute lymphoblastic leukemia (ALL). We analyzed 77 samples from hematologic malignancies, identifying a somatic mutation in *CBL* (p.C381R) in one patient with T-ALL that was associated with a uniparental disomy at the *CBL* locus and a germline heterozygous mutation in one patient with JMML. Two *NOTCH1* mutations and homozygous deletions in *LEF1* and *CDKN2A* were identified in T-ALL cells. The activation of the RAS pathway was enhanced, and activation of the NOTCH1 pathway was inhibited in NIH 3T3 cells that expressed p.C381R. This study appears to be the first to identify a *CBL* mutation in T-ALL.

1. Introduction

Casitas B-cell lymphoma (CBL) is the cellular homologue of the v-Cbl transforming gene of the Cas NS-1 murine leukemia virus [1]. CBL primarily functions as an E3 ubiquitin ligase and is responsible for the intracellular transport and degradation of a large number of receptor tyrosine kinases. CBL also retains important adaptor functions; approximately 150 proteins associate with or are regulated by CBL [2]. The majority of *CBL* somatic mutations have been reported in myelodysplastic syndrome/myeloproliferative disorder (MDS/MPD), including chronic myelomonocytic leukemia (CMML; approximately 15%), juvenile myelomonocytic leukemia (JMML; approximately 17%) and atypical chronic myeloid leukemia (approximately 5%) [3–9]. *CBL* mutations are primarily associated with an 11q-acquired uniparental disomy (aUPD) that involves the *CBL* locus and converts *CBL* mutations into a homozygous state [3]. However, *CBL* mutations have been rarely reported in acute lymphoblastic leukemia (ALL).

Germline mutations in *CBL* have been identified in three JMML patients who displayed a variable combination of dysmorphic features reminiscent of the facial gestalt of Noonan syndrome [10], as well as in 17 children with JMML [11] and two patients with sporadic Noonan syndrome [12]. Noonan syndrome and related disorders are autosomal dominant congenital anomaly syndromes, and patients with these disorders have distinctive faces, heart defects, mental retardation and tumor predisposition [13]. *CBL* mutations have been shown to activate the downstream RAS pathway, and patients with germline *CBL* mutations have been grouped with those with Noonan syndrome and related disorders, i.e., RAS/mitogen-activated protein kinase (MAPK) pathway syndromes or RASopathies [13,14].

In this study, we analyzed somatic and germline *CBL* mutations in leukemia cells from 77 patients with hematopoietic malignancies and identified a somatic *CBL* mutation in a T-ALL sample. The functional properties of the mutant CBL protein were further analyzed.

2. Materials and methods

2.1. Patients with hematopoietic malignancies

A total of 77 children with hematopoietic malignancies (40 ALL, including 29 B cell ALL, 6 T-ALL, 1 mixed lineage ALL and 4 unknown; 28

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acute myeloid leukemia (AML); 3 malignant lymphoma; 2 transient abnormal myelopoiesis (TAM) associated with Down syndrome; 2 MDS; 1 JMML; and 1 CML) were studied (Supplementary Table 1). The AML subtypes, according to the French–American–British (FAB) classification, were as follows: M0 ($n = 6$), M1 ($n = 3$), M2 ($n = 8$), M4 ($n = 3$), M5 ($n = 4$), M7 ($n = 3$) and unknown subtype ($n = 1$). Bone marrow (BM) and/or peripheral blood (PB) cells were obtained from these patients at the time of diagnosis, and pleural effusions were obtained from the malignant lymphoma patients. Using a standard protocol, genomic DNA was prepared from the BM, PB and pleural effusion samples that contained tumor cells. The Ethics Committee of the Tohoku University School of Medicine approved this study.

2.2. Mutation analysis

Sequencing was conducted for exons 8 and 9 of *CBL*, exons 4–12 of *FBW7* and exons 26, 27 and 34 of *NOTCH1*, which correspond to the heterodimerization [HD] and proline-, glutamic acid-, serine- and threonine-rich [PEST] domains of NOTCH1. If a *CBL* mutation was detected in a sample, then the remainder of the coding exons of *CBL* were also sequenced (Supplementary Table 2). The PCR products were purified using a MultiScreen PCR plate (Millipore, Billerica, MA, USA) and sequenced on an Applied Biosystems 3500xL genetic analyzer (Applied Biosystems, Foster City, CA, USA).

2.3. SNP array karyotyping analysis

DNA from the T-ALL sample and the paired DNA from remission leukocytes were analyzed on a high-density Affymetrix single-nucleotide polymorphism array (SNP-A; 250 K) to identify loss of heterozygosity (LOH), microamplification and microdeletion, as described previously [15].

2.4. Construction of expression vectors

The expression construct pCMV6-CBL, which included the *CBL* cDNA, was purchased from OriGene (Rockville, MD, USA). One of two single-base substitutions, either c.1141T>C, resulting in p.C381R, or c.1259G>A, resulting in p.R420Q, was introduced using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). All of the mutant constructs were verified by sequencing. An HES-Luc expression construct in the pGV-B vector [16] and a mouse intracellular NOTCH1 (ICN1) region expression construct in the pEF-BOSneo vector [17] were obtained from Riken BRC DNA Bank (Tsukuba, Ibaraki, Japan).

2.5. Reporter assay for ELK and c-Jun

NIH 3T3 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The NIH 3T3 cells were maintained in DMEM containing 10% newborn calf serum (NCS), 100 U/ml penicillin and 100 μg/ml streptomycin. The NIH 3T3 cells were plated in 24-well plates at a density of 5×10^4 cells per well one day prior to the transfection. The cells were transiently transfected using Lipofectamine and PLUS Reagents with 350 ng pFR-luc, 25 ng pFA2-ELK1 or pFA2 c-Jun, 3.5 ng phRLnull-luc and 200 ng wild-type (WT) or mutant expression constructs of CBL for ELK or c-Jun transactivation. The luciferase activity was assayed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Renilla luciferase, expressed by phRLnull-luc, was used to normalize the transfection efficiency. All of the experiments were performed in triplicate.

2.6. HES1 reporter assay

The NIH 3T3 cells were plated in 24-well plates at a density of 5×10^4 cells per well one day prior to the transfection. The cells were transiently transfected using Lipofectamine and PLUS Reagents with 100 ng HES-Luc, 5 ng phRLnull-luc, 120 ng ICN region expression construct and 60 ng, 120 ng or 240 ng WT or mutant expression constructs of CBL. The luciferase assays were performed as described above.

3. Results

3.1. Mutation analysis

We sequenced exons 8 and 9 in *CBL* in 77 children with hematopoietic malignancies. *CBL* mutations were detected in 2 patients. A T-to-C substitution at nucleotide 1141 (c.1141T>C) in *CBL*, which resulted in a p.C381R homozygous mutation, was detected in Patient PL1, who was diagnosed with T-ALL (Fig. 1A). DNA isolated from the buccal mucosa and peripheral blood during complete remission revealed no mutation of *CBL*, suggesting that the p.C381R mutation occurred somatically. Additionally, c.1222T>C, which resulted in a p.W408R homozygous mutation, was identified in JMML cells from Patient PL52 (Fig. 1B). An analysis of a DNA sample from the buccal mucosa revealed a heterozygous mutation in c.1222T>C, suggesting a heterozygous germline mutation. No mutations were identified in any of the coding exons in *PTPN11*, *HRAS*, *KRAS* or *SOS1*, exons 6, 11–16 in *BRAF*, exons 7, 14 or 17 in *RAF1* or exon 1 in *SHOC2* [13,18] in Patient PL52.

3.2. Clinical course of PL1 and PL52

Patient PL1 was the first son of unrelated healthy parents. He developed a swelling of the cervical lymph glands at 10 years of age, and he was admitted to our hospital following a laboratory finding of leukocytosis and thrombocytopenia. The laboratory findings were hemoglobin 12.3 g/dl, white blood cells 403.4×10^9/l and platelets 83×10^9/l. Bone marrow aspiration revealed a hypercellular marrow with 93.4% lymphoblasts with a T-cell phenotype: the cells were positive for CD2, CD3, CD5, CD7, CD4, CD8, cytoplasmic CD3 and TdT and negative for CD10, CD13, CD19, CD20 and CD33 according to immunophenotyping using flow cytometry. Chromosomal testing demonstrated 46, XY. T-ALL was diagnosed, and the cerebrospinal fluid was negative for leukemia. Induction therapy, which consisted of vincristine, prednisolone, tetrahydropyranyl adriamycin, cyclophosphamide and *Escherichia coli* asparaginase, was performed. Although this patient underwent leukapheresis before induction therapy, he developed tumor lysis syndrome that required dialysis therapy. Complete remission was achieved at Day 15, and he has remained in complete remission.

Patient PL52 was a three-month-old girl. She developed a fever and was hospitalized for leukocytosis and thrombocytopenia. The laboratory data were hemoglobin 8.8 g/dl, white blood cells 32.5×10^9/l (2.0% myelocytes, 4.0% stab neutrophils, 16% segment neutrophils, 11% monocytes and 67% lymphocytes) and platelets 23×10^9/l. Bone marrow aspiration revealed hypercellular marrow. Spontaneous growth and hypersensitivity to granulocyte/macrophage colony-stimulating factor (GM-CSF) were observed in the colony assay. This patient was diagnosed with JMML. Her brain CT was normal at 3 months of age. She was developmentally normal with no obvious dysmorphic features. At 1 year and 3 months of age, her stature was 79.1 cm (+0.9 SD), body weight was 10.6 kg (+1.3 SD) and no heart murmur was observed. The laboratory data were hemoglobin 8.8 g/dl, white blood cells 17×10^9/l (2.0% myelocytes, 4.0% stab neutrophils, 16% segment neutrophils, 10.3% monocytes and 67% lymphocytes) and platelets 23×10^9/l. She has been observed in outpatient care and will obtain hematopoietic stem cell transplantation if her blood features deteriorate.

3.3. The analysis of the NOTCH1 and FBXW7 genes and of the copy number in the T-ALL sample

Activating mutations of the *NOTCH1* gene that involve the extracellular HD domain and/or the C-terminal PEST domain have been identified in more than half of all T-ALL cases [19]. FBXW7 is a ubiquitin ligase of NOTCH1, and mutations in *FBXW7* are observed in almost 10% of T-ALL cases [20–22]. Exons 26, 27 and 34 in *NOTCH1* and exons 4–12 in *FBXW7* were analyzed in a sample from Patient PL1 to confirm that the leukemia cells had the properties of T-ALL. *NOTCH1* sequencing revealed two mutations in the HD and PEST domains. One mutation, a missense mutation (c.4724T>C) that results in a p.L1575P in the HD domain, has previously been identified in a sample from T-ALL patients [19]. Another mutation, a novel c.7416-7417insGA that causes a frame shift in the amino acid in Position 2478 (p L2473fs(2478*)), has been predicted to result in a partial deletion of the PEST domain. No mutations in *FBXW7* were identified. These results and the analysis of T cell markers confirmed that the sample from Patient PL1 had properties of T cell leukemia.

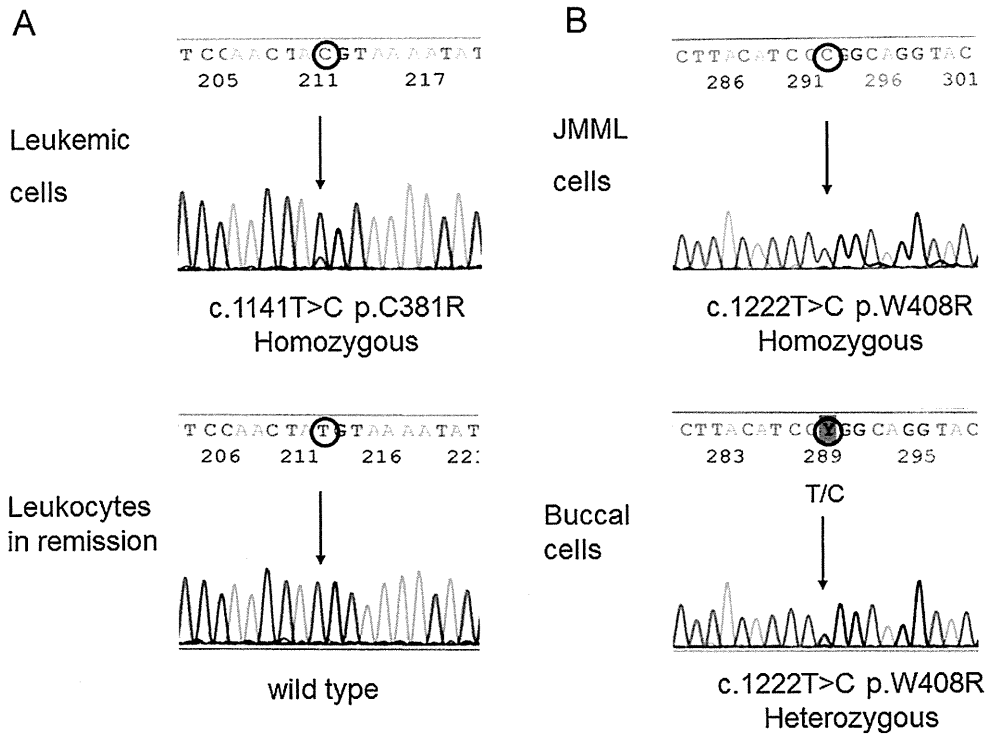

Fig. 1. *CBL* mutations identified in patients. (A) Sequencing charts of leukemic cells and peripheral blood at complete remission from Patient PL1. (B) Sequencing charts of JMML cells and buccal mucosa from Patient PL52.

CBL mutations are associated with an 11q-acquired uniparental disomy (aUPD) involving the *CBL* locus, which converts these mutations into a homozygous state [3]. An SNP array analyzed the difference in DNA between PB samples at disease onset (leukemia cells) and leukocytes in remission. The analysis revealed a UPD 11q13.1qter that contained the *CBL* locus only in the T-ALL sample (Table 1). In addition, homozygous deletions of 4q25, which encodes the *LEF1* gene, and of 9p21.3, including the region that encodes *CDKN2A*, were detected. A UPD at 9pter-p13.3 was also observed. The deletion of 14q11.2, which encodes *TCRA*, and the one-copy deletion in 7q34 (including *TCRB*) that were observed in the DNA from the T-ALL cells may be due to a TCR rearrangement. The effects of the gain of the immunoglobulin light chain at 2p11.2 and the gain at 17q12, which contains *CCL3L3*, *CCL4L2* and *TBC1D3*, are unknown.

3.4. *ELK and c-Jun transactivation in cells expressing mutant CBL proteins*

The *CBL* p.C381R mutation that was identified in one T-ALL patient has also been identified in a JMML patient and a single patient with MDS [5,23]. However, a functional analysis of p.C381R has not been performed.

A WT allele and the two *CBL* mutants, p.C381R and p.R420Q, were introduced in NIH 3T3 cells, and ELK transactivation was examined to elucidate the activation of the ERK pathway. The allele p.R420Q was used as a positive control because this mutant activates ERK [12]. ELK is a transcription factor that is phosphorylated by activated ERK and that binds the serum response element in the promoters of the immediate early genes, including c-FOS [24]. ELK transactivation was remarkably enhanced in cells expressing

Table 1
Genetic abnormalities of T-ALL at diagnosis.

Chromosomal sites	Copy number state (leukemia)	Copy number state (germline)	Loss/gain	Size (kb)	[a]Start_Linear_Position	[a]End_Linear_Position	Genes included in the region
11q13.1qter		2	UPD	69575.00	64877380	134452384	*CBL* and others
14q11.2	1	2	Loss	369.94	21660717	22030660	*TCRA, TCRD, TCR*
17q12	3	2	Gain	192.98	31460821	31653797	*CCL3L3, CCL4L2, TBC1D3*
2p11.2	4	3	Gain	460.63	88914227	89374858	*IGK@*
2p11.2	3	2	Gain	109.16	89753412	89862571	*IGK@*
4q25	0	2	Loss	104.82	109199454	109304271	*LEF1*
7q34	0	2	Loss	491.97	141711730	142203700	*TCRB*
9p11.2	3	2	Gain	127.88	44667843	44795721	
9p21.3	0	2	Loss	117.67	21864256	21981923	*CDKN2A*
9pterp13.3		2	UPD	33701.54	1	33701540	*CDKN2A* and others

Abbreviations: CBL, Cas-Br-M (murine) ecotropic retroviral transforming sequence; TCRA, T cell receptor alpha; TCRD, T cell receptor delta; CCL3L3, chemokine ligand 3-like 3; CCL4L2, chemokine ligand 4-like 2; TBC1D3, TBC1 domain family, member 3; IGK@, immunoglobulin kappa locus; LEF1, lymphoid enhancer binding factor 1; TCRB, T cell receptor beta; CDKN2A, cyclin-dependent kinase inhibitor 2A.

[a] Denoted by NCBI 36 reference human genome (hg18).