研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Ⅳ．研究成果の刊行物・別刷

RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics A

Prevalence and Clinical Features of Costello Syndrome and Cardio-Facio-Cutaneous Syndrome in Japan: Findings From a Nationwide Epidemiological Survey

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Costello syndrome and cardio-facio-cutaneous (CFC) syndrome are congenital anomaly syndromes characterized by a distinctive facial appearance, heart defects, and intellectual disability. Germline mutations in *HRAS* cause Costello syndrome, and mutations in *KRAS*, *BRAF*, and *MAP2K1/2* (*MEK1/2*) cause CFC syndrome. Since the discovery of the causative genes, approximately 150 new patients with each syndrome have been reported. However, the clinico-epidemiological features of these disorders remain to be identified. In order to assess the prevalence, natural history, prognosis, and tumor incidence associated with these diseases, we conducted a nationwide prevalence study of patients with Costello and CFC syndromes in Japan. Based on the results of our survey, we estimated a total number of patients with either Costello syndrome or CFC syndrome in Japan of 99 (95% confidence interval, 77–120) and 153 (95% confidence interval, 86–220), respectively. The prevalences of Costello and CFC syndromes are estimated to be 1 in 1,290,000 and 1 in 810,000 individuals, respectively. An evaluation of 15 adult patients 18–32 years of age revealed that 12 had moderate to severe intellectual disability, and most live at home without constant medical care. These results suggested that the number of adult patients is likely underestimated and our results represent a minimum prevalence. This is the first epidemiological study of Costello syndrome and CFC syndrome; identifying patients older than 32 years of age and following up on the patients reported here is important to estimate the precise prevalence and the natural history of these disorders.
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INTRODUCTION

Costello syndrome (OMIM 218040), a rare, multiple congenital anomaly syndrome, was first described by Costello in 1971 [Costello, 1971]. Costello syndrome is characterized by intellectual disability, a high birth weight, neonatal feeding problems, short stature, congenital heart defects, curly hair, distinctive facial features, nasal papillomata, and loose integuments of the back of the hands [Hennekam, 2003]. Cardio-facio-cutaneous (CFC) syndrome (OMIM 115150) was first described in 1986 [Reynolds et al., 1986]. Affected individuals present with heart defects, short stature, frequent intellectual disability, and ectodermal abnormalities such as sparse, fragile hair, hyperkeratotic skin lesions, and a generalized ichthyosis-like condition. These syndromes overlap phenotypically with Noonan syndrome (OMIM 163950). We discovered that *HRAS* mutations of are causative of Costello syndrome [Aoki et al., 2005], and we and other group subsequently identified mutations in *KRAS*, *BRAF*, and *MAP2K1/2* (MEK1/2) in patients with CFC syndrome [Niihori et al., 2006; Rodriguez-Viciana et al., 2006]. Missense mutations in *PTPN11*, *SOS1*, *KRAS*, *RAF1*, and *NRAS* have been identified in individuals affected by Noonan syndrome or Noonan syndrome with multiple lentigines, previously known as LEOPARD syndrome (OMIM 151100, 611554) [Tartaglia et al., 2001; Schubbert et al., 2006; Pandit et al., 2007; Razzaque et al., 2007; Roberts et al., 2007; Tartaglia et al., 2007; Cirstea et al., 2010]. Mutations in *SHOC2* have been identified in patients with Noonan-like disorder with loose anagen hair (OMIM 613563) [Cordeddu et al., 2009]. Because the clinical manifestations of these diseases are similar, a novel disease entity was proposed that consists of a syndrome characterized by a dysregulation of the RAS/MAPK signaling pathway [Aoki et al., 2008; Tidyman and Rauen, 2009].

Evaluation of the clinical manifestations of Costello and CFC syndromes revealed the similarities and differences between individuals with the diseases. Individuals with either syndrome have distinctive facial features; full cheeks and a large nose and mouth are characteristic of individuals with Costello syndrome, and a high cranial vault, bitemporal narrowing and a hypoplastic supraorbital ridge are characteristic of individuals with CFC syndrome. Wrinkled palms and soles have been thought to be characteristic features of individuals with Costello syndrome. A recent evaluation showed that 30% of individuals with CFC syndrome also have wrinkled palms and soles [Narumi et al., 2007]. Heart defects have been frequently reported in individuals with Costello and CFC syndromes; 61% of patients with Costello syndrome have hypertrophic cardiomyopathy, while 44 and 56% of Costello syndrome patients have congenital heart defects and arrhythmia, respectively. In contrast, hypertrophic cardiomyopathy, congenital heart defects, and arrhythmia have been observed in 36, 45, and 9%, respectively, of patients with CFC syndrome [Lin et al., 2011].

Approximately 10–15% of individuals with Costello syndrome develop malignant tumors, including transitional carcinomas in the bladder, rhabdomyosarcomas, and neuroblastomas [Aoki et al., 2008; Kratz et al., 2011]. Although association of malignant tumors has been rarely reported in individuals with CFC syndrome, we observed patients with *BRAF* mutations who developed acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma [Niihori et al., 2006; Makita et al., 2007; Ohtake et al., 2011].

The number of patients known to have these diseases is growing due to the identification of the causative genes. At least 150 genotyped patients with Costello syndrome have been reported [Lin et al., 2011]. In addition, more than 100 individuals with CFC syndrome have been reported in the literature [Rauen, 2007]. Till date, however, an epidemiological study has not been conducted. In order to identify the precise number of patients with these diseases, the natural history of the diseases, the prognosis and the rate of tumor development, we performed a nationwide investigation of both Costello syndrome and CFC syndrome.

MATERIALS AND METHODS

First-Stage Survey

The protocol we followed was established by the Research Committee on the Epidemiology of Intractable Diseases funded by the Ministry of Health, Labour and Welfare of Japan [Kawamura et al., 2006]. The prevalence of intractable diseases, including moyamoya disease, pancreatitis and sudden deafness, were all reported using this protocol [Teranishi et al., 2007; Kuriyama et al., 2008; Satoh et al., 2011]. The protocol consists of a two-stage postal survey. The first-stage survey aimed to estimate the number of individuals with Costello syndrome or CFC syndrome, and the second-stage survey aimed to identify the clinico-epidemiological features of the two syndromes.

The pediatric departments of all hospitals were identified based on a listing of hospitals as of 2008 supplied by the R & D Co.LTD (Nagoya, Japan). These hospitals were classified into seven categories according to the type of institution (i.e., university hospital or general hospital) and the number of hospital beds. Hospitals were then randomly selected from each of these categories for sampling. The sampling rate was approximately 5, 10, 20, 40, 80, and 100% of general hospitals with less than 100 beds, 100–199 beds, 200–299 beds, 300–399 beds, 400–499 beds, and 500 or more beds, respectively, and 100% of university hospitals [Kuriyama et al., 2008]. To increase the efficiency of the study, we sent a survey form to 205 pediatricians and 44 clinical geneticists working in the departments of gynecology, genetics, or ophthalmology in university hospitals (See Supplemental eTable I in supporting information online). We also selected 29 physicians who previously sent patient samples to our facility for molecular analysis. These hospitals were separately classified into a "selected hospitals" category, and all hospitals in this category were surveyed. Another 205 institutions that treat the disabled were included in order to identify adult patients.

The survey was mailed out to the targeted departments of health institutes in October 2009 along with cover letters. A simple questionnaire was used to ask about the number of patients with Costello syndrome known to have an *HRAS* mutation, CFC syndrome patients with mutations in *KRAS*, *BRAF*, or *MAP2K1/2*

(*MEK1/2*) and clinically suspected patients. Photographs of patients, obtained with their specific consent, were printed on the brochure describing the disease overview. In December 2009, a second request was sent to departments that had not responded by the earlier deadline (the end of November 2009). Following the first-stage survey, we sent acknowledgement letters to departments that had responded.

Genetic Testing of Clinically Suspected Patients

Blood samples from 42 individuals clinically suspected to have Costello or CFC syndrome were sent to our facility. After DNA was extracted by a standard protocol, we performed genetic screening for all four exons of *HRAS* and 14 exons of *BRAF*, *MAP2K1*, *MAP2K2*, and *KRAS* in which mutations have been previously identified (*BRAF* exons 6 and 11–16, *MAP2K1* exons 2 and 3, *MAP2K2* exons 2 and 3 and *KRAS* exons 1, 2, and 5) (Fig. 1). In samples negative for the first screening, we further analyzed all of the known causative genes for Noonan syndrome and related disorders (including the remaining exons in *BRAF*, *KRAS*, *MAP2K1*, and *MAP2K2*, all 17 exons in *RAF1*, all 23 exons in *SOS1*, all 4 exons in *NRAS*, and exon 1 of *SHOC2*). The clinical manifestations of the patients were evaluated by clinical dysmorphologists (K.K., H.O., H.K., N.O., S.M.).

Second-Stage Survey

The second questionnaires were forwarded to the departments that reported patients with Costello or CFC syndrome on the first questionnaires. Detailed clinical information was collected, including the age, gender, growth and development pattern, cardiac defects, central nervous system defects, craniofacial characteristics, musculoskeletal characteristics, skin characteristics, tumors, identified mutations, and the facility where the genetic analysis had been performed. Duplicate results were excluded using the information regarding the patient's age, gender, and the type of mutations, if available. The Ethics Committee of Tohoku University School of Medicine approved this study. We obtained informed consent from all subjects involved in the genetic testing and specific consent for the photographs from three patients shown in Figure 1.

Estimation of Prevalence

We first estimated the number of patients in departments who responded the first survey, using the number of mutation-positive patients from the first-stage postal survey and the number of newly identified patients by mutational analysis in the current study. PR_k denotes the number of mutation-positive patients reported in the first-stage survey. The estimate was made based on the assumption that mutation-positive patients equally existed in the clinically

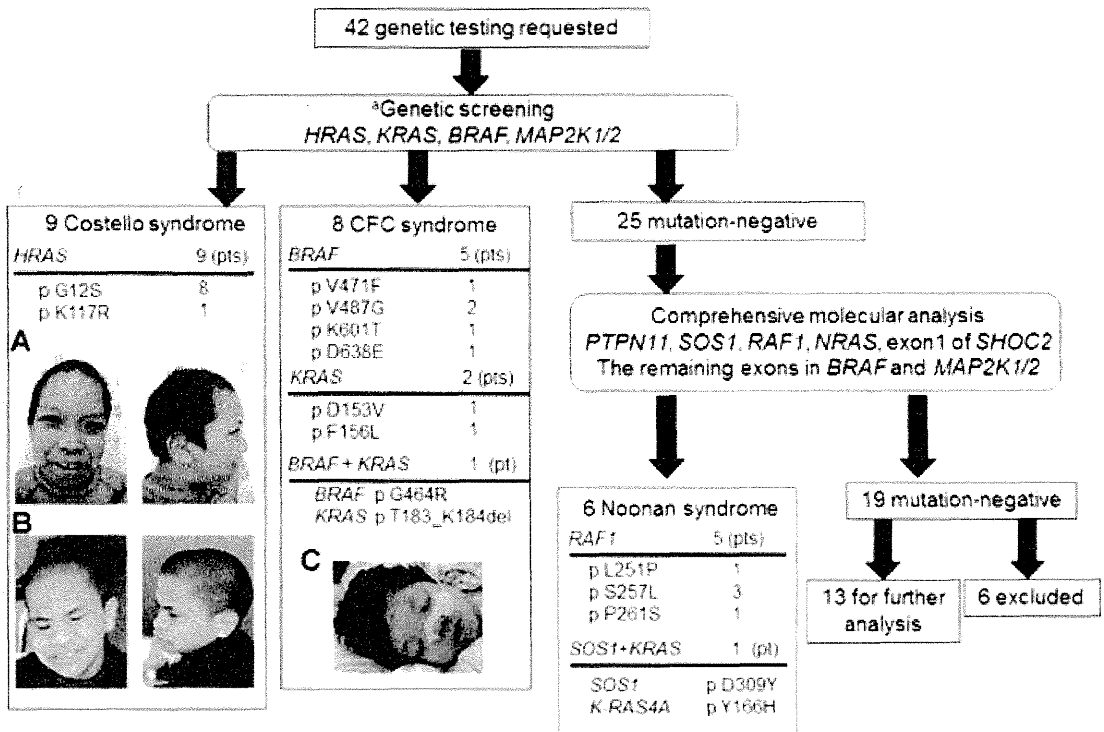

FIG. 1. Flow chart of the genetic testing results for 42 patients whose blood samples were submitted for this study. A, B: Patients harboring *HRAS* p.G12S, (C) patient with *BRAF* p.K601T. [a] For the first screening, all exons in *HRAS* and *KRAS*, exons 6 and 11–16 in *BRAF*, and exons 2 and 3 in *MAP2K1/2* were sequenced.

suspected patients who did not receive the genetic testing. The number of mutation-positive patients estimated by the mutation analysis was calculated using the number of the clinically suspected patients reported in the first-stage survey (PS_k), the ratio of the number of newly identified mutation-positive patients (PD_k), and the total number of patients examined (PA_k). Therefore, the total estimated number of patients in hospitals in stratum k $\sum iN_{ki}$, which responded to the first survey, was calculated as follows:

$$\sum_i iN_{ki} = PR_k + PS_k \frac{PD_k}{PA_k}$$

To calculate the total number of patients in all hospitals listed, we estimated that the mean number of patients among the departments that responded to the survey was equal to that of those departments that did not respond.

The number of patients in stratum k was therefore estimated as

$$\begin{aligned} \hat{\alpha}_k &= \frac{1}{SRT_k RRT_k} \sum_i iN_{ki} \\ &= \frac{1}{\frac{NS_k}{n_k} \frac{N_k}{NS_k}} \sum_i iN_{ki} \\ &= \frac{n_k}{N_k} \sum_i iN_{ki} \end{aligned}$$

where SRT_k, RRT_k, NS_k, n_k, N_k, and N_{ki} denote the sampling rate, the response rate, the number of sampled departments, the total number of departments, the number of responding departments, and the number of departments with i patients in stratum k, respectively.

The total number of patients, $\hat{\alpha}$, was computed as follows:

$$\hat{\alpha} = \sum_k \hat{\alpha}_k$$

The 95% CI of $\hat{\alpha}_k$ was calculated as previously described [Kuriyama et al., 2008]. Five deceased patients with Costello syndrome reported in the first survey (Table I) were excluded in the estimation of prevalence. The prevalence rate per 100,000 people was determined based on the population of Japan in 2009 (127,510,000) with data from the Statistics Bureau, Ministry of Internal Affairs and Communications.

RESULTS

Estimated Number of Patients

The results of the first postal survey and the molecular analysis performed in this study are shown in Table I. Of 1,127 departments, 856 responded to the first-stage survey questionnaire (76%). Fifty-four patients, including five deceased patients, with Costello syndrome with mutations in *HRAS* and 54 patients with CFC syndrome who had mutations in *KRAS*, *BRAF*, or *MAP2K1/2* were reported. Blood samples for 42 of the 114 individuals clinically suspected to have Costello syndrome or CFC syndrome were sent to our laboratory. Molecular screening identified nine patients with Costello syndrome and eight with CFC syndrome (described below, Fig. 1 and Table I). Results from the second-stage survey followed by

TABLE I. Results of the First Postal Survey and the Number of Newly Identified Patients

	Total departments	Surveyed departments	Sampling rate (%)	Departments that responded	Response rate (%)	Reported in the first-stage postal survey: CS[c] (deceased)	CFCS[c]	CS/CFCS suspected	Genetic testing performed	Newly identified CS	Newly identified CFCS
University hospitals	166[b]	163	98.2	158	96.9	11(2)	13	44	15	5	1
Selected hospitals[a]	29	29	100	18	62.1	28(2)	33	16	1	0	1
Institutions for the mentally and physically disabled	208	205	98.6	142	69.3	10(1)	5	16	5	2	1
General hospitals with ≥500 beds	261	254	97.3	205	80.7	5	1	25	12	0	5
General hospitals with 400–499 beds	212	151	71.2	124	82.1	0	0	5	6	2	0
General hospitals with 300–399 beds	402	150	37.3	106	70.7	0	0	5	1	0	0
General hospitals with 200–299 beds	362	70	19.3	43	61.4	0	0	1	1	0	0
General hospitals with 100–199 beds	740	67	9.1	42	62.7	0	2	2	1	0	0
General hospitals with ≤99 beds	830	38	4.6	18	47.4	0	0	0	0	0	0
Total	3210	1127	35.1	856	76	54(5)	54	114	42	9	8

CS, Costello syndrome; CFCS, CFC syndrome.
[a]Hospitals that had asked for genetic testing of Costello/CFC syndrome to our laboratory prior to the survey.
[b]131 university hospitals were listed, and we sent survey forms to 249 physicians in 166 departments.
[c]Possible duplications among patients were excluded.

exclusion of duplicates showed that in total, 63 patients with Costello syndrome and 62 patients with CFC syndrome were identified. Taking into consideration the sampling rates in each stratum of the general hospitals and the number of undiagnosed patients in the clinically suspected patients, we estimated the total numbers of patients in Japan with Costello syndrome and CFC syndrome to be 99 (95% confidence interval, 77 to 120) and 157 (95% confidence interval, 86 to 229), respectively. Therefore, the prevalence of Costello syndrome and CFC syndrome was estimated to be 1 in 1,290,000 (95% confidence interval, 1 in 1,061,000 to 1 in 1,660,000), and 1 in 810,000 (95% confidence interval, 1 in 556,000 to 1 in 1,490,000) individuals, respectively.

Results of the Molecular Analysis

Screening of 42 clinically diagnosed patients identified nine patients with Costello syndrome and eight patients with CFC syndrome (Fig. 1). Eight of the nine patients with *HRAS* mutations had a p.G12S mutation, and the remaining one had a p.K117R mutation. Six of the eight patients with CFC syndrome had *BRAF* mutations (p.G464R, p.V471F, p.K601T, and p.D638E in a single patient, and p.V487G in two patients), and two patients had *KRAS* mutations (p.D153V and p.F156L). One patient had *BRAF* p.G464R, which has previously been reported in a patient with CFC syndrome [Nava et al., 2007], and a novel *KRAS* variation, c.547_552delACCAAG (p.T183_K184del). Parental samples were not available for this patient, and it is unknown if this variation was pathogenic or not. A subsequent, comprehensive mutation analysis showed that *RAF1* mutations, including p.L251P, p.S257L, and p.P261S, were identified in five patients. Four of the five patients had severe perinatal problems, including polyhydramnios, fetal distress, pleural effusion, and hypertrophic cardiomyopathy. An *SOS1* p.D309Y mutation was identified in a single patient diagnosed with Noonan syndrome. The patient also had another novel variation (p.Y166H) in *K-RAS4A*. Her asymptomatic father had the same variation, suggesting that this variation is a benign polymorphism. The five patients with *RAF* mutations and one patient with the *SOS1* mutation were diagnosed as having Noonan syndrome. In the remaining 19 patients who had no mutations, six patients were excluded based on the review of dysmorphologists because of non-matching facial features and clinical manifestations. The remaining 13 patients will be further analyzed.

Clinical-Epidemiological Features of the Patients

We collected detailed clinical-epidemiological information on 43 of 63 Costello syndrome patients and 54 of 62 CFC syndrome patients who were reported in the first postal survey and newly diagnosed by the current study (Table II). Seventeen male and 25 female patients with Costello syndrome and 28 male and 24 female patients with CFC syndrome were reported. Twenty-six of the patients with Costello syndrome [Aoki et al., 2005; Niihori et al., 2011] and 10 of the patients with CFC syndrome [Niihori et al., 2006; Narumi et al., 2008] had been previously studied. Of the Costello syndrome patients, 27 of the 43 patients had *HRAS* p.G12S, five had p.G12A and two had p.G13D, p.G12C, p.G12V, p.G12D, and p.K117R were identified in a single patient. In the patients with CFC syndrome, 38 (70%), eight (15%) and eight (15%) of the 54 patients had *BRAF*, *MAP2K1/2*, and *KRAS* mutations, respectively.

Evaluation of clinical manifestations showed that postnatal failure to thrive and intellectual disability were reported at a rate of more than 95% in both disorders (Table II). Short stature was reported in 72 and 82% of patients with Costello syndrome and CFC syndrome, respectively. The frequency of hypertrophic cardiomyopathy and arrhythmia was significantly higher in patients with Costello syndrome compared to CFC syndrome. In contrast, the frequency of pulmonic stenosis was significantly higher in patients with CFC syndrome compared to Costello syndrome. Abnormal brain structure as detected by CT and/or MRI was reported in eight Costello syndrome patients. Of these eight patients, two were reported as having Arnold–Chiari type I, two had hydrocephalus, one had cortical atrophy, one had hydrocephalus and cortical atrophy, one had tonsillar descent, and one had ventricular dilation and a thinning of the corpus callosum. Abnormal brain structure was also observed in seven CFC patients; two had thinning of the corpus callosum, one had cortical atrophy, one had cortical atrophy, thinning of the corpus callosum and a reduction in white matter volume, one had ventricular dilatation, and one had ventricular dilatation and vermis hypoplasia. Regarding the skin characteristics, the frequency of soft, loose skin and deep palmer/plantar creases was significantly higher in patients with Costello syndrome than in CFC syndrome. Four patients with Costello syndrome developed malignant tumors, including bladder carcinomas, ganglioneuroblastomas and rhabdomyosarcomas. Two patients with CFC syndrome were previously reported as developing ALL and non-Hodgkin lymphoma [Makita et al., 2007; Ohtake et al., 2011]. Five patients with Costello syndrome were deceased. Two patients died from ganglioneuroblastoma and rhabdomyosarcoma. One patient died from tachycardia-induced cardiomyopathy at age 18 months.

The age distribution of the 38 patients with Costello syndrome and the 53 CFC syndrome patients whose ages were reported in the second-stage survey is shown in Figure 2. There were major peaks at 5 years of age in both diseases. The oldest patient diagnosed with Costello syndrome was 22 years of age, while the oldest patient with CFC syndrome was 32 years. Six patients with Costello syndrome and nine patients with CFC syndrome age 18–32 years were identified (Table III). Analysis of their daily living activities showed that 10 individuals could walk independently, one had an abnormal gait, one had a cane-assisted gait, and one used a wheelchair. Two patients with *BRAF* mutations were bedridden. All patients showed intellectual disability, and eight (severe in three patients with Costello syndrome and three patients with CFC syndrome, very severe in two patients with CFC syndrome) were severely disabled. Daily conversation was possible for three individuals. Simple conversations and two-word sentences were possible for four and three patients, respectively. Eleven patients lived at home. Three individuals had graduated from a school or public school for disabled children. Eight adults worked in vocational training facilities. Thirteen patients were able to feed themselves, but two of them sometimes needed assistance with feeding. Two patients with CFC syndrome were bedridden and needed full assistance with feeding and toileting.

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,

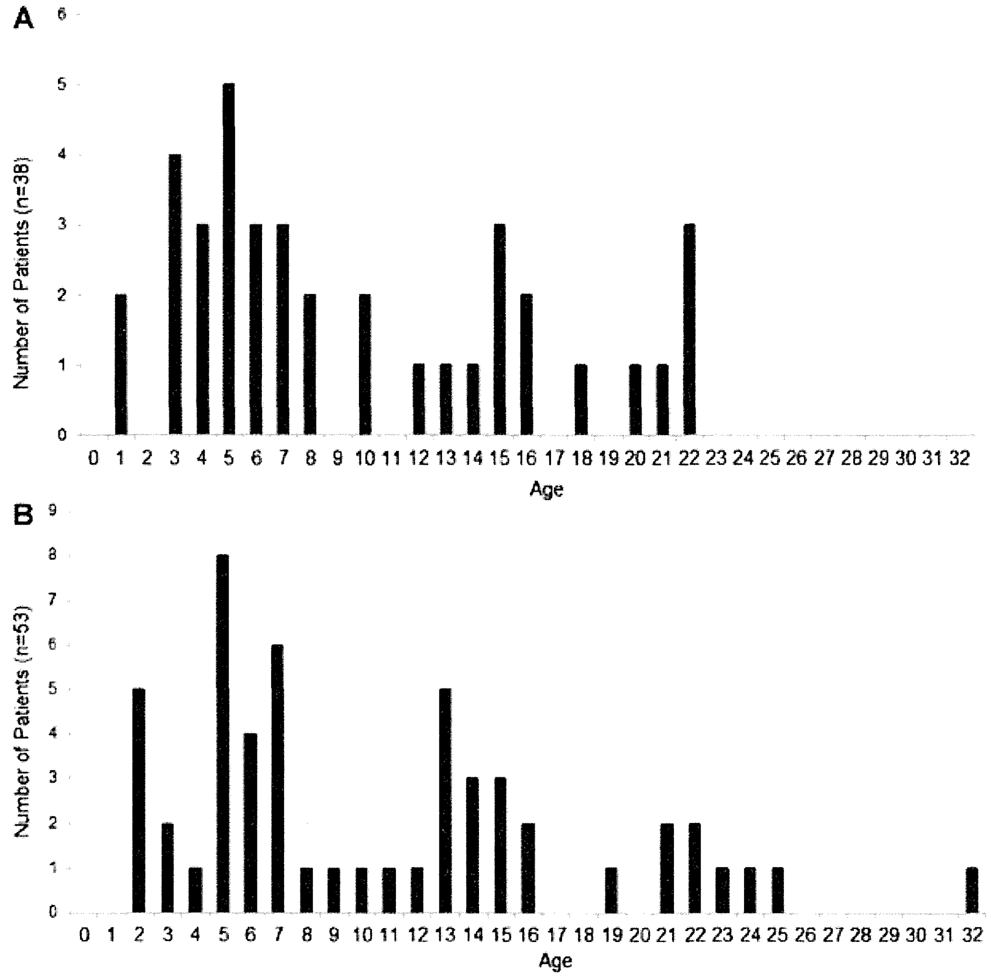

FIG. 2. Age distribution of 38 patients with Costello syndrome (A) and 53 patients with CFC syndrome (B) as of March 31, 2011. Five patients with Costello syndrome were deceased and the age was unknown for one of the 54 patients with CFC syndrome whose clinical manifestations were obtained by the second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