Key words: Costello syndrome; cardio-facio-cutaneous syndrome; prevalence; RAS-MAPK pathway

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

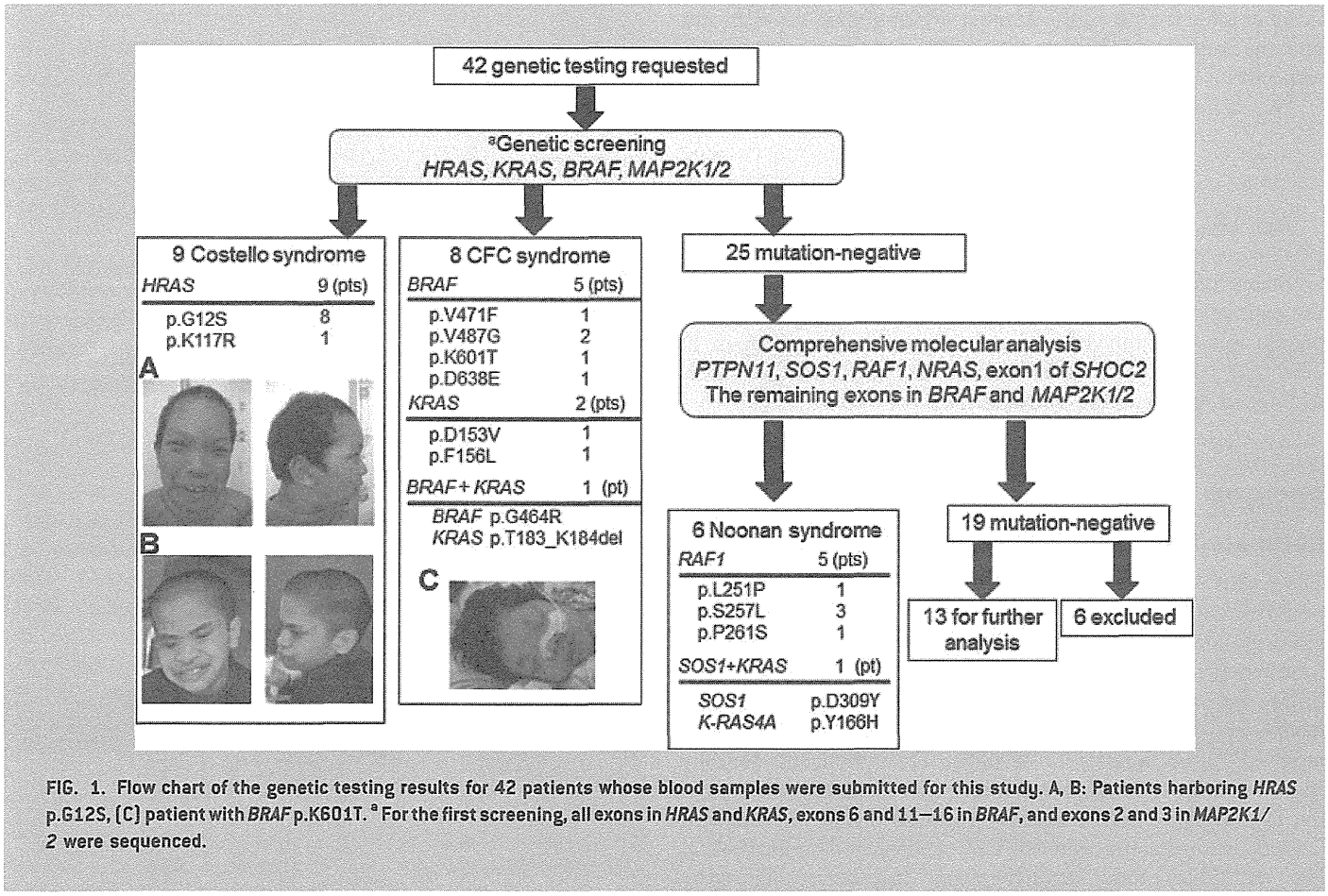

FIG. 1. Flow chart of the genetic testing results for 42 patients whose blood samples were submitted for this study. A, B: Patients harboring *HRAS* p.G12S, (C) patient with *BRAF* p.K601T. [a] For the first screening, all exons in *HRAS* and *KRAS*, exons 6 and 11–16 in *BRAF*, and exons 2 and 3 in *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			Genetic testing performed	Newly identified CS	Newly identified CFCS
						CS[c] (deceased)	CFCS[c]	CS/CFCS suspected			
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 HRAS, 5 but type of mutation unknown	*BRAF* 38 *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,

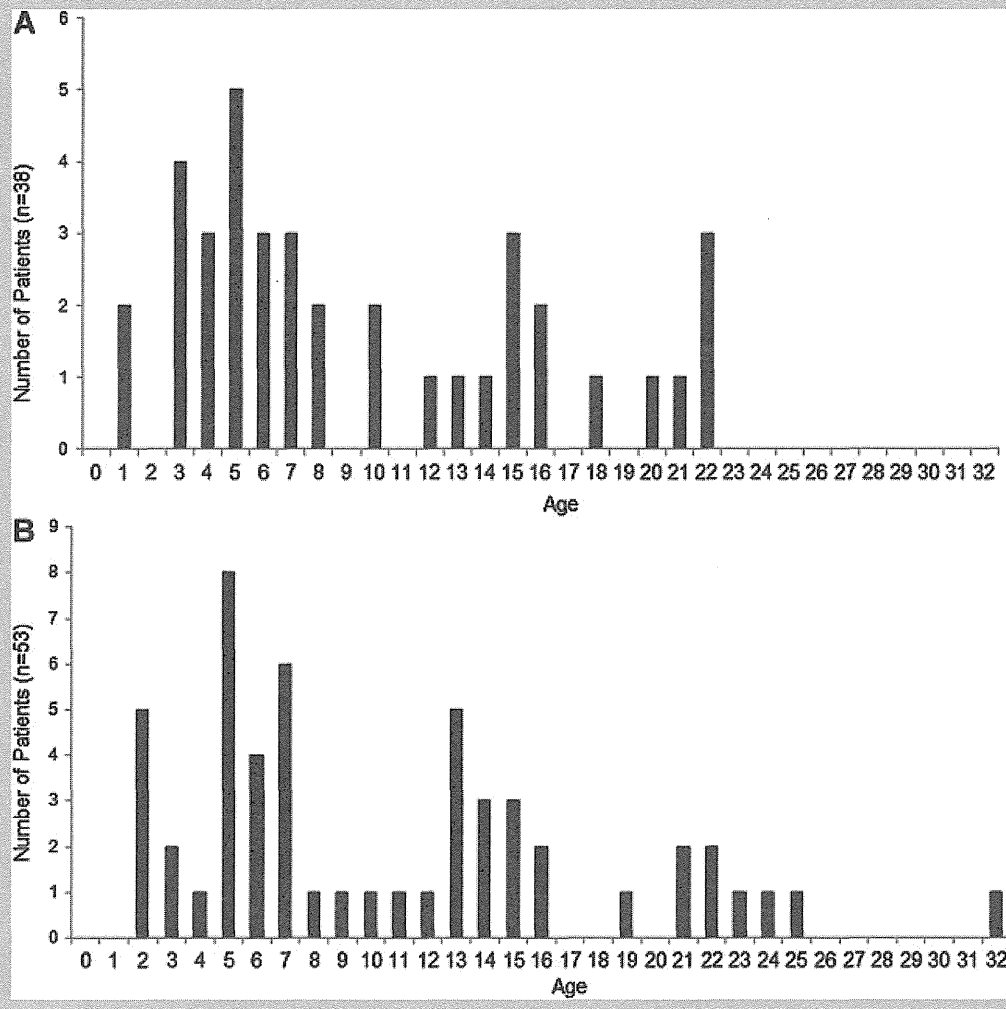

FIG. 2. Age distribution of 38 patients with Costello syndrome (A) and 53 patients with CFC syndrome (B) as of March 31, 2011. Five patients with Costello syndrome were deceased and the age was unknown for one of the 54 patients with CFC syndrome whose clinical manifestations were obtained by the second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

ACKNOWLEDGMENTS

The authors thank the patients and their families who participated in this study. We are also grateful to the physicians who responded to the first and second surveys. We thank Kumi Kato, Riyo Takahashi, and Yoko Tateda for technical assistance. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for the Promotion of Science, and the Ministry of Health, Labour and Welfare of Japan to Y.M. and Y.A.

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Clinical and Experimental Hypertension, 2012; 34(4): 290–296

ISSN 1064-1963 print/1525-6006 online
DOI: 10.3109/10641963.2012.681086

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Daily Serial Hemodynamic Data During Pregnancy and Seasonal Variation: The BOSHI Study

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Abstract

Although there are some reports that low plasma volume or increased cardiac output is associated with developing preeclampsia, there are few reports of daily serial hemodynamic data during pregnancy. A total of 37 092 home blood pressure (BP) and heart rate (HR) measurements were obtained from 425 normal pregnant women. Heart rate and shock index (SI) gradually increased by gestational week 32 and then decreased, whereas double product (DP) increased linearly during pregnancy. Although systolic BP and DP were consistently and negatively correlated with daily minimum outside temperature, HR and SI were positively correlated with minimum outside temperature in summer.

Keywords: clinical science, blood pressure measurement/monitoring, preeclampsia/pregnancy, self-monitoring of blood pressure

INTRODUCTION

Gestational hypertension and preeclampsia are common disorders during pregnancy, with the majority of cases developing at or near term (1). Plasma volume is significantly lower in preeclampsia than in normal pregnancy at a gestational age of 14–17 weeks (2). Recently, it has been reported that cardiac output is increased in the first trimester in women who develop preeclampsia (3,4). Although hemodynamic changes during pregnancy appear to be important, there are few reports dealing with daily serial hemodynamic changes during pregnancy.

Heart rate (HR), double product (DP), which is calculated from systolic blood pressure (SBP) multiplied by HR, and shock index (SI), which is calculated from HR divided by SBP, are parameters that are easy to obtain from blood pressure (BP) measurements. Double product is a surrogate measure of myocardial oxygen demand and cardiac workload, which has recently become widely used in cardiovascular medicine (5). Shock index is an index to determine hypovolemia, which is accompanied by hypotension and tachycardia (6,7), and it predicts the quantity of hemorrhage from a ruptured ectopic pregnancy better than HR or SBP alone (6). Birkhahn et al. reported that acute blood loss of 450 mL significantly increased SI from 0.61 to 0.65 bpm/mm Hg (7).

Although home BP measurement has been recognized as an important tool among pregnant women (8,9), data derived from home BP measurements are rare. The guidelines for hypertension in pregnancy do not mention home BP measurements (10–12). We have previously reported the associations among home BP, gestational age, and seasonal variation (13). The aim of this study was to collect daily serial hemodynamic data (HR, DP, and SI) during pregnancy with adjustment for gestational age and seasonal variation using home measurements of BP and HR.

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Received 31 August 2011; revised 4 October 2011; accepted 25 October 2011.

METHODS

The present report is a part of the Babies and their Parents' Longitudinal Observation in Suzuki Memorial Hospital on Intrauterine Period (BOSHI) study (13). The study was conducted at Suzuki Memorial Hospital, which is the only hospital specializing in obstetrics, gynecology, and in vitro fertilization in the Sendai City area of Miyagi Prefecture, Japan. Sendai is the central city of northeastern Japan. There were 1098 births in Suzuki Memorial Hospital in 2006. All study protocols were approved by the Institutional Review Board of Tohoku University School of Medicine and by the Hospital Review Board of Suzuki Memorial Hospital.

In Japan, the interval for medical checkups during pregnancy is once every 4 weeks until week 23, once every 2 weeks until week 35, and once a week after 36 weeks.

Only healthy pregnant women before gestational week 20 with no history of hypertension and who could measure their home BP during pregnancy were included after obtaining their written informed consent. Gestational age was calculated by last menstrual period with correction for crown-lump length before 12 weeks of gestation. After delivery, the obstetrician and physician verified that the pregnancy had been normal without hypertension or proteinuria.

Subjects

A total of 3362 women were diagnosed as being pregnant between October 1, 2006 and September 30, 2009 and reserved delivery in the hospital. All of these women were invited to participate by a poster and a letter from the investigating staff; 1032 women received an explanation of the research from a physician, pharmacist, or midwife.

Daily Serial Hemodynamic Data Using Home BP Measurements

Home BP was measured using an HEM-747IC or HEM-7080IC semiautomatic device (Omron Healthcare, Kyoto, Japan) based on the cuff-oscillometric method, which generates a digital display of not only both SBP and diastolic BP (14) but also HR. Double product was calculated from SBP multiplied by HR, and SI was calculated from HR divided by SBP.

Physicians, pharmacists, and midwives instructed subjects on how to perform home BP measurements. On the basis of the Japanese Society of Hypertension guidelines for self-monitoring of BP at home (15), the subjects were asked to measure their home BP every morning within 1 hour of waking, after micturition, before breakfast, while seated, and after resting for more than 1 minute and to keep recording their home BP until 1 month after delivery.

Meteorological Data

Meteorological data measured at Sendai Meteorological Observatory for the period during which home BP measurements were included: daily minimum, maximum, and mean outside temperatures; daily mean atmospheric pressure; relative humidity; and duration of sunshine. Normalized data were also obtained from Sendai Meteorological Observatory as averaged meteorological data from 1970 to 2000.

Statistical Analysis

Daily serial hemodynamic data (SBP, HR, DP, and SI) were examined using a mixed linear model with gestational age as the fixed effect and subjects as the random effect. When we adjusted for seasonal effect, meteorological data were also regarded as fixed effect. We further examined yearly variation of daily serial hemodynamic data; we examined weekly serial hemodynamic data in a year without adjustment for meteorological data. The measurement week of the year was regarded as a fixed effect in a mixed model.

We analyzed data using the SAS package (version 9.2, SAS Institute Inc., Cary, NC, USA). Values are expressed as mean ± SD and least square means were calculated by the mixed linear model and expressed as mean with their 95% confidence intervals (CI; Figures 1 and 2).

A sample size of 387 women was required to estimate the distribution of mean home BP values within a ±0.8 mm Hg range at a 95% CI, assuming that the SD of home BP values in pregnant women is 8 mm Hg based on the previous report (13).

RESULTS

Subjects

A total of 518 women finally entered the study. Nine women were excluded due to fetal death in the first trimester. Another four women were transferred to other hospitals because of threatened premature delivery (two women), premature rupture of the membranes (one woman), and diabetes (one woman). One woman was excluded because she transferred to the nearest midwifery clinic. During the follow-up period, 51 women developed gestational hypertension or preeclampsia and were excluded. Among the remaining 452 healthy pregnant women, home BP monitoring was not available for 27 women during pregnancy. Data of the remaining 425 healthy pregnant women were analyzed.

The mean age of the 425 healthy pregnant women analyzed in this study was 31.3 ± 4.6 years at entry. Their mean height, weight, and BMI were 158.4 ± 5.3 cm, 54.2 ± 9.0 kg, and 21.6 ± 3.4 kg/m^2, respectively. The frequency of ever smokers was 16.8% and that of ever drinkers was 50.7%. Among them, 71% of ever smokers and 95% of ever drinkers stopped during pregnancy. The mean birth weight of their children was 3054 ± 394 g.

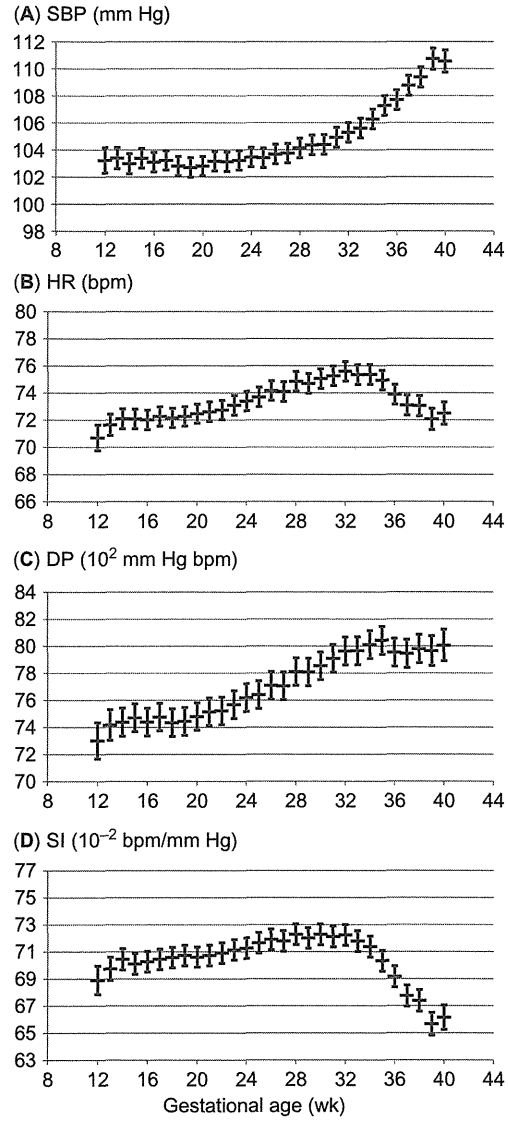

Figure 1. (A) Systolic blood pressure (SBP), (B) heart rate (HR), (C) double product (DP), and (D) shock index (SI) values and their 95% confidence intervals for each week of gestational age, calculated on the basis of a mixed linear model.

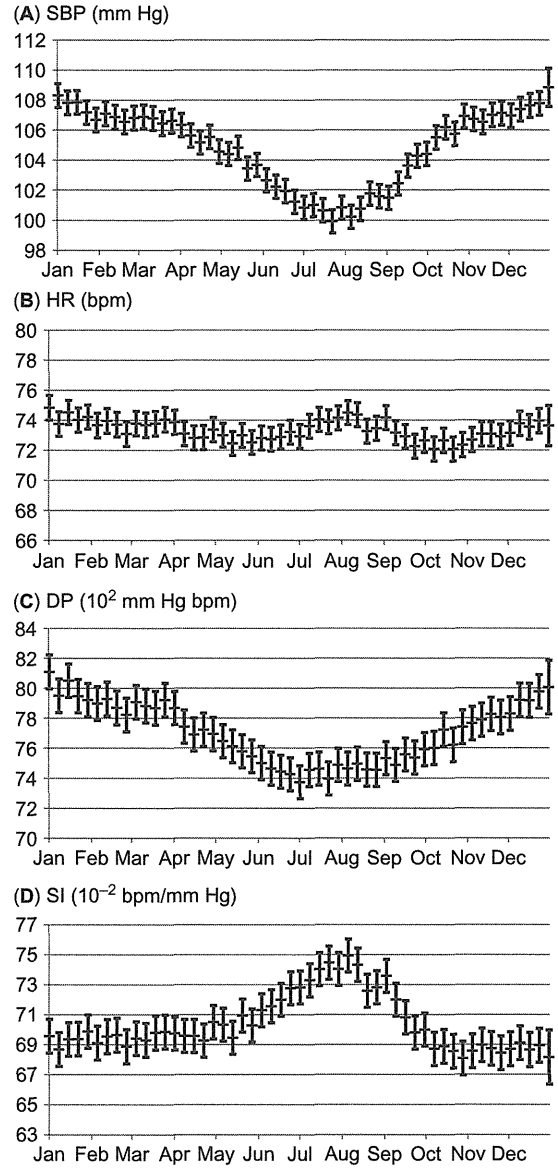

Figure 2. (A) Systolic blood pressure (SBP), (B) heart rate (HR), (C) double product (DP), and (D) shock index (SI) values and their 95% confidence intervals for each week for a year, calculated on the basis of a mixed linear model without adjusting for seasonal variation.

Daily Serial Hemodynamic Data and Gestational Age

The association between SBP, HR, DP, SI, and gestational age using a mixed linear model without adjusting for meteorological data is shown in Figure 1. Heart rate and SI increased gradually, reaching peak values at gestational week 33, while DP increased linearly from the first trimester to the third trimester.

After adjusting for meteorological data, the associations between these daily hemodynamic data and gestational age showed the same tendency (data not shown).

Daily Serial Hemodynamic Data and Seasonal Variation

The yearly variation in SBP, HR, DP, and SI calculated using a mixed linear model adjusting for gestational age is shown in Figure 2. Systolic blood pressure decreased gradually from January to August and gradually increased from August to December. Heart rate decreased gradually from January to June, after which it increased and reached its peak value in August. Double product was the highest in January and decreased to its lowest value in June. From June to December, DP increased gradually. Although SI was stable from January to June and from October to December, it increased from June to August, reached its peak in August, and then decreased to October.

When summer season was defined as June to September based on a minimum outside temperature ≥15°C, HR, DP, and SI showed significant inverse associations with the daily minimum outside temperature in summer when compared with the other seasons. Systolic blood pressure and DP were consistently and significantly correlated with daily minimum outside temperature throughout the year (summer: β (SBP) = −0.3055 and β (DP) = −0.2172, and in the other seasons: β (SBP) = −0.1999 and β (DP) = −0.2051, all $P < .0001$). The correlations between HR and daily minimum outside temperature were significant but weak in each season (summer: $\beta = 0.0095$, in the other seasons: $\beta = -0.0560$, all $P < .0001$). Although SI was positively correlated with daily minimum outside temperature in summer ($\beta = 0.2235$), in the remaining seasons weak correlations were observed ($\beta = 0.0811$; $P = <.0001$). The interactions between daily minimum outside temperature and seasonality (summer vs. the other seasons) for SBP, HR, DP, and SI were significant (Table 1, all interactions $P < .0001$).

Hemodynamic Parameters, Gestational Age, and Seasonal Variation

The associations among hemodynamic parameters (SBP, HR, DP, and SI), gestational age, and seasonal variation are shown in Figure 3. Systolic blood pressure increased gradually and achieved its peak values (≥110 mm Hg) at gestational week 40. Heart rate increased gradually and reached its peak (≥75 bpm) at gestational week 32. Double product increased gradually as gestational age increased and reached its peak (≥8000 mm Hg bpm) at gestational week 40. The effect of expected date of birth on HR and DP was smaller than the effect of gestational age. On the other hand, women who gave birth in winter had a high SI (≥0.73 bpm/mm Hg) in their first or second trimester, and women who gave birth in autumn had a high SI (≥0.73 bpm/mm Hg) in their third trimester.

DISCUSSION

This is the first study to describe the relationships of HR, DP, and SI with a combination of gestational age and seasonality in a cohort of normal pregnant women. This study collected daily serial hemodynamic data during pregnancy based on the self-measurement of BP and HR at home.

Heart Rate

As in a previous study that used ambulatory BP measurement (16), home HR showed the highest value in gestational week 32 in this study. Seasonal variations exist in home BP values (17); however, there are few studies that have observed seasonal variation of HR. Some articles reported that there is no significant relationship between seasonality and HR (18,19), whereas Izzo et al. (20) reported wintertime HR increased by 7% ($P < .017$), with larger parallel increases in systemic vascular resistance (+24%, $P < .0017$) and plasma norepinephrine (+26%, $P < .017$). In this study, the association between HR and temperature was weak, but the association was significant.

Double Product

The DP of SBP and HR indicates cardiovascular load. It is a surrogate measure of myocardial oxygen demand and cardiac workload, which has recently become widely used in cardiovascular medicine (5). There is a report using ambulatory BP monitoring that showed that the DP increased from summer to winter (daytime) by 1053 mm Hg bpm for smokers (21). In this study, DP was also higher in winter than in summer. The interaction between seasonality and temperature on the effect on DP was significant.

Rang et al. (4) reported that cardiac output was higher in preeclampsia or gestational hypertension without fetal growth restriction but not in preeclampsia or gestational hypertension with fetal growth restriction. Recently, De Paco et al. (3) reported that cardiac output between 11^{+0} and 13^{+6} weeks of gestation was increased in women who developed preeclampsia. In this study, DP increased gradually as gestational age increased, and the effect of seasonality and expected date of birth on DP was less marked than that of gestational age. Double product might be a good way of evaluating cardiac workload in pregnancy due to its linear association with gestational age.

Shock Index

Shock index, which is calculated from HR divided by SBP, is an effective way to diagnose hemorrhagic shock

Table 1. Associations between hemodynamic parameters and daily minimum outside temperature in summer and in other seasons

		Summer (from June to September)			Other seasons (from October to May)			Interaction[a]
		β	SE	P	β	SE	P	P
SBP	(mm Hg)	−0.3055	0.0049	<.0001	−0.1999	0.0074	<.0001	<.0001
HR	(bpm)	0.0095	0.0052	<.0001	−0.0560	0.0080	<.0001	<.0001
DP	(10^2 mm Hg bpm)	−0.2172	0.0069	<.0001	−0.2051	0.0104	<.0001	<.0001
SI	(10^{-2} bpm/mm Hg)	0.2235	0.0058	<.0001	0.0811	0.0088	<.0001	<.0001

Abbreviations: SBP – systolic blood pressure, HR – heart rate, DP – double product, SI – shock index.
[a]Interaction between daily minimum outside temperature and seasonality and hemodynamic parameters.

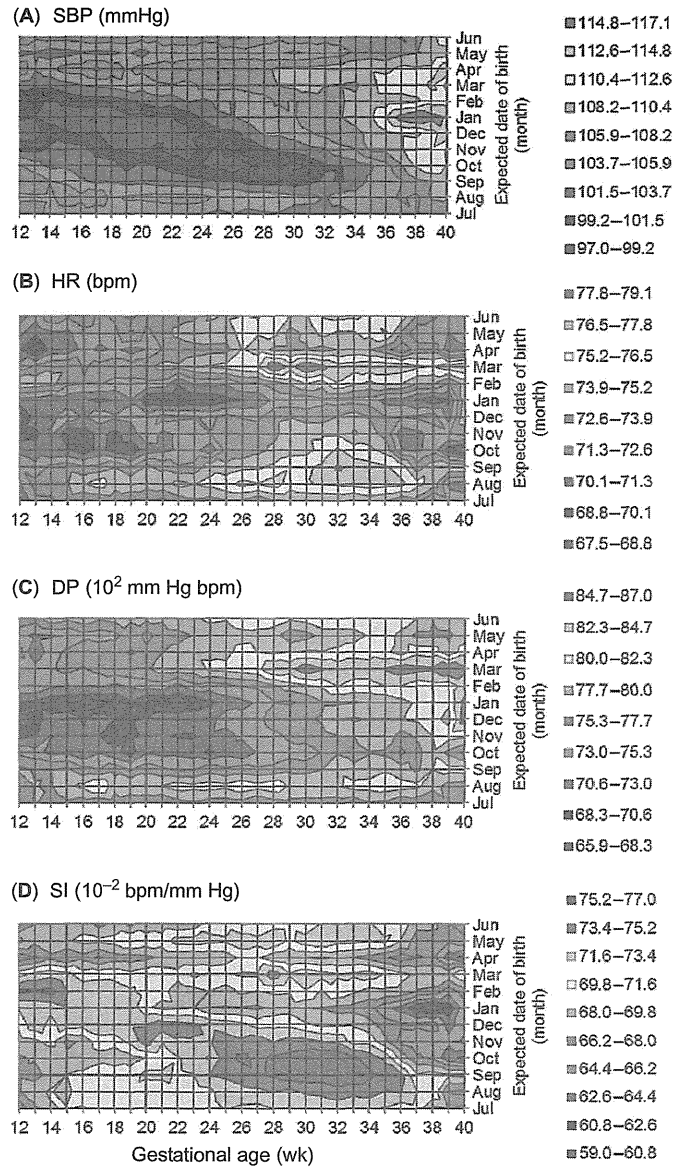

Figure 3. (A) Systolic blood pressure (SBP), (B) heart rate (HR), (C) double product (DP), and (D) shock index (SI) values for the combination of gestational age and expected date of birth, calculated on the basis of a mixed linear model. The horizontal axis shows gestational age, and the vertical axis shows the expected date of birth.

following injury, which is accompanied with hypotension and tachycardia (6,7). In this study, women who gave birth in winter had a high SI in their first or second trimester. Women who gave birth in winter spent their first trimester or second trimester during the summer season. The first trimester SI of the women who gave birth in winter was 0.72–0.77 bpm/mm Hg, while the first trimester SI of the women who were to give birth in other seasons was 0.66–0.70 bpm/mm Hg. Birkhahn et al. reported that acute blood loss of 450 mL significantly increased the SI from 0.61 to 0.65 bpm/mm Hg (7). Shock index might also represent hypovolemia. Women who are to give birth in winter might have hypovolemia in their first to second trimester. Similarly, women who are to give birth in autumn might have hypovolemia in their last trimester.

Plasma volume in pregnancies complicated by preeclampsia is reported to be significantly lower than in normal pregnancies in the first trimester (2). In this study, plasma volume is reported to be the first parameter to show significant intergroup difference among the parameters of progesterone, aldosterone, estradiol, and their combination. Although further studies are necessary to investigate which factors changed before and after hypovolemia, there might be some association between hypovolemia in the first trimester in summer and the

incidence of preeclampsia in winter. Shock index might be a good marker of dehydration in summer, since a similar trend was observed for HR (Figure 2).

LIMITATIONS

There are some limitations in this study. First, SI seems to be a good way to identify hypovolemia within one subject; however, it is impossible to compare SIs among individuals because the SI is low with high BP. Another method may be necessary to identify hypovolemia. There are no previous reports showing that SI reflects chronic hypovolemia, in the same way as acute hypovolemia. Further study is needed to evaluate the amplitude of hypovolemia in chronic conditions. Second, in our study, we did not perform echocardiography or electrocardiography; therefore, we cannot evaluate the real clinical meaning of the DP using such physiological examinations. Another approach might be necessary to evaluate the real clinical meaning of DP. Third, these data are limited to normotensive pregnant women, because we did not perform a similar analysis in preeclamptic women, since few subjects developed preeclampsia. Serial changes of indirect indices might be modified by hospitalization, medication, and termination in subjects with preeclampsia.

CONCLUSION

This study collected daily serial hemodynamic data during pregnancy using home BP monitoring. Double product increased gradually as gestational age increased, and the effect of seasonality and expected date of birth on Double product was less marked than that of gestational age. Shock index might be useful for identifying hypovolemia within individuals. Such data might be useful for examining hemodynamic changes during normal pregnancy, as well as identifying hemodynamic changes during abnormal pregnancy.

ACKNOWLEDGMENTS

The authors thank the women and their families who participated in the BOSHI study. Supercomputing resources at the Cyberscience Center, Tohoku University, were used to analyze some of the results. This work was supported by Grants for Scientific Research (Nos. 18590587, 18390192, 21390201, 22890017, and 23590771) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; Grant-in-Aid (H21-Junkankitou[Seishuu]-Ippan-004) from the Ministry of Health, Labor and Welfare, Health and Labor Sciences Research Grants, Japan; Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) fellows (19.7152, 20.7198, 20.7477, and 20.54043); Grants from Takeda Science Foundation.

Declaration of interest: H.M. is conducting a collaborative research with Omron Healthcare Ltd.

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Leukemia Research 36 (2012) 1009–1015

Contents lists available at SciVerse ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Casitas B-cell lymphoma mutation in childhood T-cell acute lymphoblastic leukemia

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ARTICLE INFO

Article history:
Received 20 December 2011
Received in revised form 1 April 2012
Accepted 16 April 2012
Available online 14 May 2012

Keywords:
CBL
Acute lymphoblastic leukemia
Noonan syndrome
RAS
NOTCH

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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http://dx.doi.org/10.1016/j.leukres.2012.04.018

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.