Nodes plan or have already joined forces with the corresponding rare disorders registries.

Listing of Needs

To successfully maintain the existing HVP Country Nodes, to encourage the establishment of new Nodes, and to expand HVP and thereby deliver improved healthcare, additional steps need to be undertaken (summarized in Box 2)

First, a need exists to strengthen the role of the International Confederation of Countries Advisory Council since the Country Nodes will play a vital role in the efforts of the HVP. At the same time, each HVP Country Node and accompanying NEMDB, should bear an electronic seal of HVP as a quality feature, indicating that all necessary recommendations, standards, and guidelines are followed. A dedicated Committee or Working group from the International Confederation of Countries Advisory Council will be responsible to assign this quality feature and monitor the Country Node to ensure it continues to conform to the standards agreed. Reciprocally, the corresponding Human Genetics Societies should formally endorse their Country Node to be admitted to the International Confederation of Countries.

Also, careful consideration and planning of the long-term financial stability and sustainability of each HVP Country Node is an absolute requirement. Viable financial planning by the local HVP Country Node must accompany the country's application and possibly a detailed Strength/Weakness/Opportunities/Threat (SWOT) analysis to allow the HVP Country Node Council to evaluate the feasibility of the proposed approach and the ability of the applicants to successfully establish and manage the Node. Fund raising should be sought locally, such as from governments, national grants, charities, patients' organizations, or others. Similarly, the HVP could also facilitate fund raising efforts by providing grants for the start-up and the initial operation of the Node. Besides grants for start-up of Nodes, funding could also be provided centrally by HVP, for example, to upgrade the NEMDB software, to organize educational and/or outreaching activities, and so on. Funding assistance is particularly important for developing countries.

Consortium members, particularly those from countries having close ties with international organizations like UNESCO and WHO, indicated the importance of these organizations, particularly when speaking to their own governments through Ministries of Health, and Ministries with mandates for science, research, and education. HVP has the status of an international nongovernmental organization in operational relations with UNESCO, which facilitates cooperation with the United Nations. UNESCO has some 56 field offices throughout the world with headquarters in Paris. In addition, UNESCO has a global network of national cooperating bodies known as National Commissions for UNESCO, set up by their respective governments. Presently, there are 197 National Commissions for UNESCO across the world, which operate for the purpose of associating their governmental and nongovernmental bodies in education, sciences, culture, and communication with the work of the Organization. HVP Country Nodes are encouraged to build relationships with their respective UNESCO National Commissions and field offices in areas of UNESCO's mandate. With regard to WHO, as it has a complex structure of approximately 150 country offices, 6 regional offices, and a headquarter in Geneva, building relationships with it are complex. With WHO having a core mandate focusing on public health and its governance being largely in the hands of Ministers of Health, it is important for HVP Country Nodes to develop good relationships with their national Ministries of Health based on how Country Nodes can contribute to the health and well being of the populations they serve.

Media coverage in the national and international arena can add to highlighting the importance of the local HVP effort and improving public knowledge of genetics, genomics, and health. Careful consideration needs to be given to how Country Nodes can best be supported in managing positive relationships with various types of local media. A major aim for the next 12 months is to begin work toward globally agreed standards for databases as well as invite more countries to establish Nodes.

Box 2. Recommendations for Development of HVP Country Nodes

(a) Political measures
- Empowerment of the HVP International Confederation of Countries Advisory Council.
- Develop quality standards and follow their implementation. HVP Country Nodes would have a permission to show HVP seal as a quality feature, to indicate that all necessary recommendations, standards, and guidelines are followed.
- Formal endorsement of each country's application to become an HVP Country Node by the respective national Human Genetics and Genomics Society.
- Agreement on distribution of anonymized information.
- Advocacy with governments: (1) in particular Ministries of Health for awareness of and support for genetic- and genomic-related health in their national health plans; and (2) through Ministries responsible for education, science, and research and through the National Commissions for UNESCO in each country for awareness and support for capacity building in genomics and bioinformatics research and education.

(b) Financial measures
- Careful consideration and planning of a long-term sustainability upon application to become an HVP Country Node.
- Fund raising from local sources, such as national grants, philanthropic organizations, and patients' organizations.
- Financial support by the HVP International Coordinating Office for the start-up and the initial operation of the Country Node, particularly for developing countries.
- Financial support by the HVP Central office for software upgrade and maintenance, for organizing educational and outreaching activities.

(c) Other measures
- Tackling ethical, legal, and societal issues, particularly where patient registries are involved.
- Adopt a country-specific data gathering and sharing approach.
- Provision of education for the general public and healthcare professionals on issues relating to genetics and genomics.
- Development of standards, recommendations, and data models for NEMDBs and encouragement of their implementation to software products.

Conclusions

The success of existing HVP Country Nodes and progress on multiple other planned national platforms underscores the potential value of the HVP Country Node concept. HVP Country Nodes would best be served by coordinating their efforts promoted by the International Confederation of Countries Advisory Council meeting, as described above. A crucial parameter that would guarantee the successful outcome of these efforts is collaborative work of partners from all over the world, which will save time and effort, improve knowledge generation for all, and will result in better local and global solutions for genomic medicine.

Overall, HVP Country Node operational guidelines will help expand the establishment of centers focusing on genome medicine, which will in turn facilitate the fulfillment of the HVP goals toward a comprehensive worldwide genome variation data collection.

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART 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 result 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 157 (95% confidence interval, 86–229), 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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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

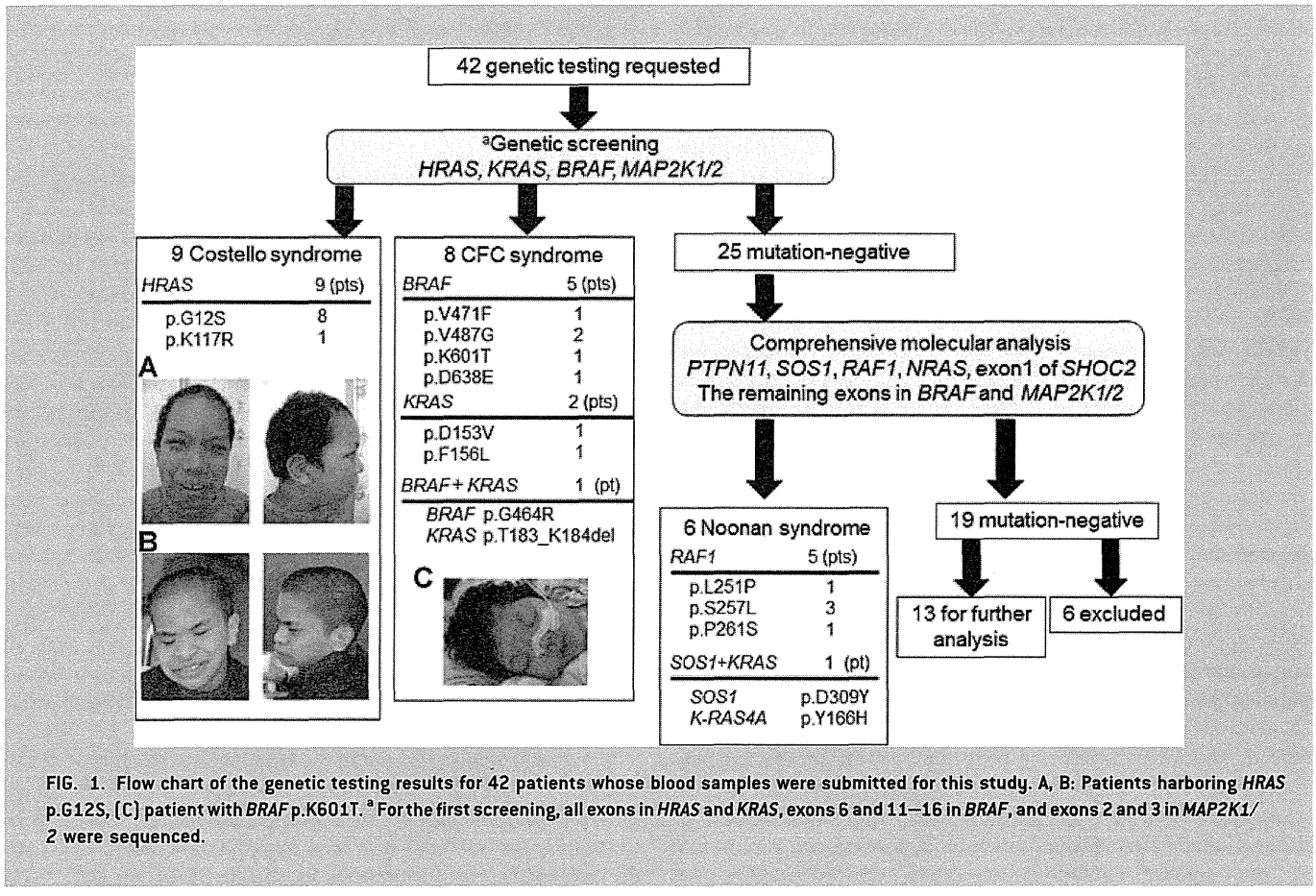

FIG. 1. Flow chart of the genetic testing results for 42 patients whose blood samples were submitted for this study. A, B: Patients harboring *HRAS* p.G12S, (C) patient with *BRAF* p.K601T. [a] For the first screening, all exons in *HRAS* and *KRAS*, exons 6 and 11–16 in *BRAF*, and exons 2 and 3 in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

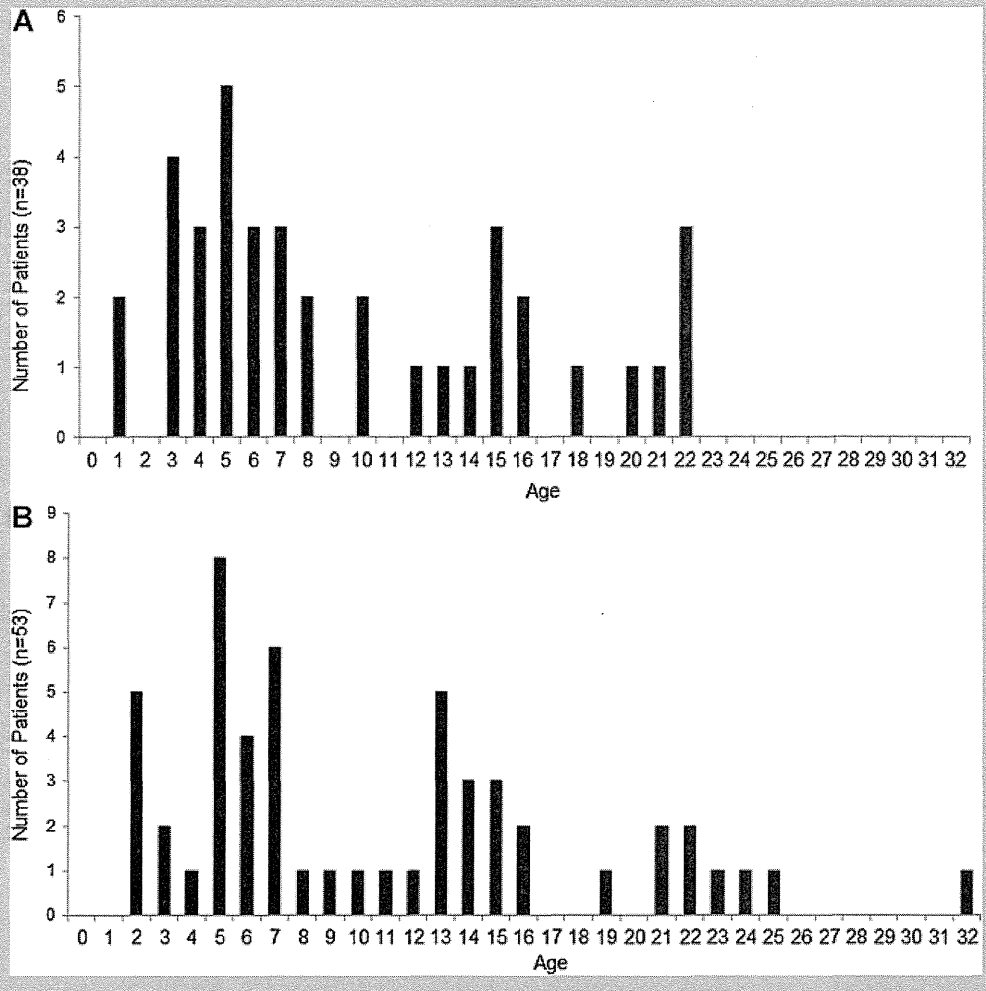

FIG. 2. Age distribution of 38 patients with Costello syndrome (A) and 53 patients with CFC syndrome (B) as of March 31, 2011. Five patients with Costello syndrome were deceased and the age was unknown for one of the 54 patients with CFC syndrome whose clinical manifestations were obtained by the second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

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A Case of Almost Unilateral Focal Dermal Hypoplasia Resulting From a Novel Mutation in the *PORCN* Gene

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Focal dermal hypoplasia (FDH), also known as Goltz syndrome, is an X-linked dominant disorder of ecto-mesenchymal development. In general, FDH presents with characteristic linear streaks of hypoplastic dermis and various abnormalities in some organs (1). Although this disorder is normally lethal in male patients, approximately 10% of cases of FDH are male, and most represent post-zygotic mosaicism (2–4).

The molecular basis of FDH involves mutations in the *PORCN* (human porcupine locus MG61/PORC) gene (5). *PORCN* is a member of the porcupine (porc) gene family and is located on chromosome Xp11. 23 (1, 6). The gene is thought to encode an O-acyltransferase that catalyses cysteine N-palmitoylation and serine O-acylation in the endoplasmic reticulum that allows membrane targeting and secretion of several Wnt proteins that have key roles in embryonic tissue development, notably for fibroblast proliferation and osteogenesis (1, 7).

We describe here a case of FDH with a novel deletion mutation at exon 14 (c.1179_1193 del) detected by PCR-sequencing analysis of the entire coding sequences of the *PORCN* gene. Interestingly, in this case, the characteristic symptom was prominent in the patient's left hemibody. To the best of our knowledge, this is the second case report that reveals a mutation of the PORCN gene in a patient with almost unilateral FDH.

CASE REPORT

A 2-year-old Japanese girl visited our outpatient clinic for hypopigmented and atrophic linear skin lesions on the trunk and extremities. She had been surgically treated one year before for syndactyly of the left middle and ring finger. On her first visit to our hospital, physical examination disclosed hypo-pigmented patches of dermal hypoplasia distributed along Blaschko's lines on the arms, legs and trunk (Fig. 1). Interestingly, these symptoms in lesions were prominent on her left hemibody. Her hair was sparse and brittle and she had non-scarring alopecia. In addition, extensive dental caries was noted. From the above information, we diagnosed this patient as having almost unilateral FDH.

To confirm the diagnosis, we examined the *PORCN* gene for mutations. After receiving informed consent, DNA was extracted from the peripheral blood sample taken from affected individual using standard methods. Primers were designed to amplify individual exons and the flanking intron of the *PORCN* gene, as described previously (1). PCR-sequencing analysis of the entire coding sequences of *PORCN* revealed c.1179_1193 del mutation at exon 14 (Fig. 2). This variant was predicted to result in altered residues 394–412 and to produce a truncated protein (p.G394 fs X20). The sequence chromatograms showed low signal intensities at the site of mutation, indicating post-zygotic mutation. To verify the mosaic state, the PCR products were cloned into the pCR4 TOPO TA Cloning Vector (Invitrogen, Karlsruhe, Germany), transfected. *E. coli* clones were chosen and subjected to colony PCR, and PCR products from individual clones were sequenced. By DNA sequence analysis of selected clones we could assign the wild-type and the mutant sequence to either of the 2 banding patterns and identified a ratio of 5/26 (~19%; mutant vs. wild-type sequence).

DISCUSSION

FDH is characterized by linear and whorled lesions of hypoplastic dermis and variable abnormalities of organs including the bone, nails, hair, limbs and teeth (1–3). Cutaneous features that predominate at birth are red atrophic, linear lesions following Blaschko's lines

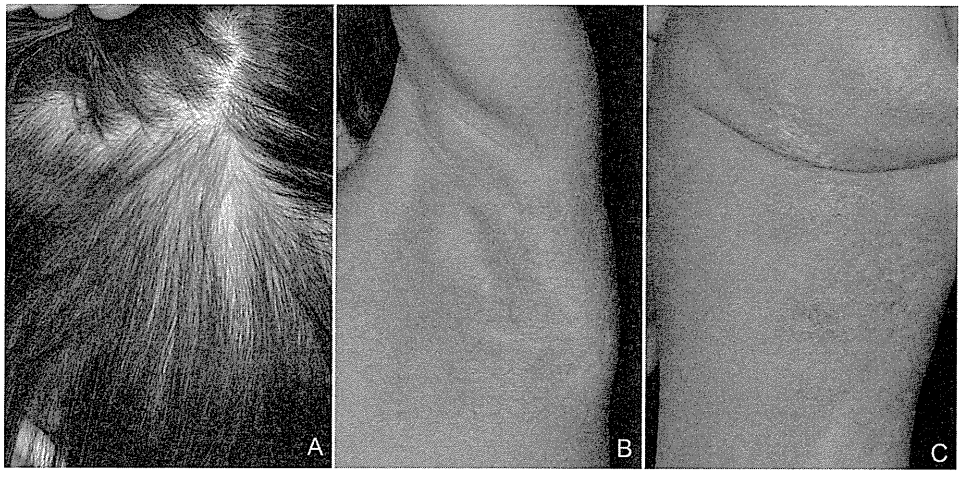

Fig. 1. Skin manifestation in the patient. (A) Hair loss on the left temporal scalp. (B, C) Small whitish depigmented spots, slightly depressed from the skin surface, distributed linearly on the left side of the armpit (B) buttocks and lower limbs (C).

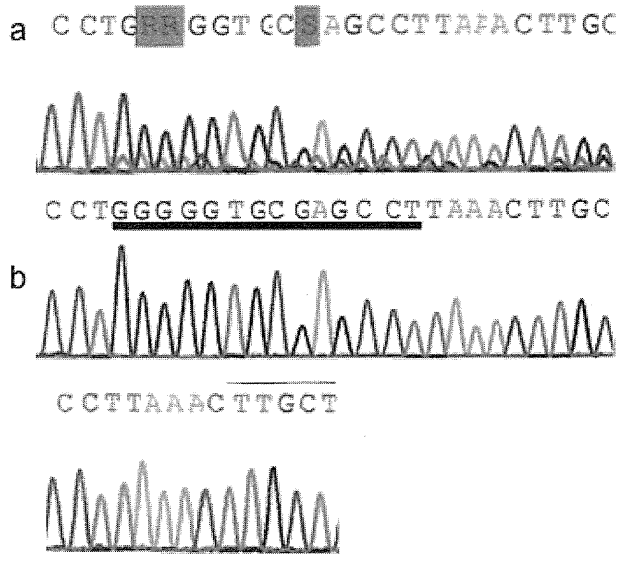

Fig. 2. Molecular basis of focal dermal hypoplasia (FDH). (a) Sequencing of exon 14 of the *PORCN* gene in the patient's DNA reveals c.1179_1193 del mutation. (b) Sequencing of control DNA shows the wild-type and mutant sequences.

and telangiectasias. It is presumed to be lethal in men who are fully hemizygous for a mutation in *PORCN* (4). FDH generally involves both sides of the body. Only 6 cases of unilateral FDH have been published (8–10). Recently, Maalouf et al. (8) reviewed all cases of unilateral FDH and found no side predeliction. One case was male (17%) and the other 5 were females (83%). Moreover, they described that gene sequence analysis on the *PORCN* gene detected a novel heterogeneous mutation in exon 10, c.854-855insACCTGAC [p.T285fsX316]. In addition, a substantial majority of cases of FDH have previously been reported to result from post-zygotic mutations (11). In the present case, sequencing analysis of the entire coding sequences of *PORCN* gene revealed a novel deletion mutation at exon 14 (c.1179_1193 del). As a ratio of 5/26 mutant vs. wild-type alleles was found, a post-zygotic mutation is the likely cause of the syndrome in our case. A germline mutation would have resulted in a 1:1 distribution of both alleles in functional X-chromosome-mosaicism as described previously by Bornholdt et al. (11). Although we did not perform the X-chromosome inactivation analysis using DNA extracted from the affected and non-affected skin, the present case suggests that the deletion mutation at exon 14 might be connected with the unilateral involvement of FDH. To confirm our hypothesis, further case reports and molecular studies of patients with unilateral FDH are necessary.

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ORIGINAL ARTICLE

Exome sequencing identifies a novel *TTN* mutation in a family with hereditary myopathy with early respiratory failure

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Myofibrillar myopathy (MFM) is a group of chronic muscular disorders that show the focal dissolution of myofibrils and accumulation of degradation products. The major genetic basis of MFMs is unknown. In 1993, our group reported a Japanese family with dominantly inherited cytoplasmic body myopathy, which is now included in MFM, characterized by late-onset chronic progressive distal muscle weakness and early respiratory failure. In this study, we performed linkage analysis and exome sequencing on these patients and identified a novel c.90263G>T mutation in the *TTN* gene (NM_001256850). During the course of our study, another groups reported three mutations in *TTN* in patients with hereditary myopathy with early respiratory failure (HMERF, MIM #603689), which is characterized by overlapping pathologic findings with MFMs. Our patients were clinically compatible with HMERF. The mutation identified in this study and the three mutations in patients with HMERF were located on the A-band domain of titin, suggesting a strong relationship between mutations in the A-band domain of titin and HMERF. Mutation screening of *TTN* has been rarely carried out because of its huge size, consisting of 363 exons. It is possible that focused analysis of *TTN* may detect more mutations in patients with MFMs, especially in those with early respiratory failure.
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Keywords: A-band; cytoplasmic body; Fn3 domain; hereditary myopathy with early respiratory failure; HMERF; myofibrillar myopathy; titin; *TTN*

INTRODUCTION

Myofibrillar myopathies (MFMs) were proposed in 1996 as a group of chronic muscular disorders characterized by common morphologic features observed on muscle histology, which showed the focal dissolution of myofibrils followed by the accumulation of products of the degradative process.[1] The clinical phenotype of MFM is characterized by slowly progressive muscle weakness that can involve proximal or distal muscles, with onset in adulthood in most cases. However, other phenotypes are highly variable. Although 20% of patients with MFMs have been revealed to have mutations in *DES*, *CRYAB*, *MYOT*, *LDB* (*ZASP*), *FLNC* or *BAG3*, the major genetic basis of MFMs remains to be elucidated.

Respiratory weakness is one of the symptoms of MFMs. The early or initial presentation of respiratory failure is not a common manifestation of MFMs as a whole, and there are limited reports regarding a fraction of patients with *DES*,[2] *MYOT*[3] or *CRYAB*[4] mutation. In 1993, our group reported a Japanese family with dominantly inherited cytoplasmic body (CB) myopathy,[5] which is now included in MFM. Currently, this family includes 20 patients in five successive generations who show almost homogeneous clinical features characterized by chronic progressive distal muscle weakness and early respiratory failure. However, the underlying genetic etiology in this family was unknown. The aim of this study was to determine the genetic cause in this family. To identify the responsible genetic mutation, we performed linkage analysis and whole-exome sequencing.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Tohoku University School of Medicine, and all individuals gave their informed consent before their inclusion in the study.

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Clinical information on the family

This family includes 20 patients (13 males and 7 females) in five successive generations (Figure 1). The family is of Japanese ancestry, and no consanguineous or international mating was found. Of all patients, seven underwent a muscle biopsy, and two were autopsied. All of the histological findings were compatible with MFM (see clinical data).

The age of onset ranged from 27–45 years. The most common presenting symptom was foot drop. At the initial evaluations, muscle weakness was primarily distributed in the ankle dorsiflexors and finger extensors. The patients were generally built and showed no other extramuscular abnormalities. In addition to this chronic progressive distal muscle weakness, respiratory distress occurred between 0 and 7 years from the initial onset (average 3.8 years) in seven patients (IV-9, V-2, A, B, E, H, and J) with adequate clinical information. Two patients who had not had any respiratory care died of respiratory failure approximately a decade from the initial onset. The other patients have been alive for more than 10 years (maximum 18 years) but require nocturnal non-invasive positive pressure ventilation. They were 37–58 years of age as of 2012 and able to walk independently with or without a simple walking aid. Although the time at which patients recognized dysphagia or dysarthria varied between 1 to more than 10 years from the initial onset, decreased bulbar functions had been noted at the initial evaluation in most cases. Cardiac function was normally maintained in all patients of the family.

Clinical data

The level of serum creatine kinase was normal or mildly elevated. Electromyography of affected muscles showed a chronic myogenic pattern, and the nerve conduction study did not suggest any neuropathic involvement. Muscle imaging showed focal atrophy in the tibialis anterior, tibialis posterior, extensor hallucis and digitorum longus, peroneal and semitendinosus muscle on initial assessment (Figure 2A), and atrophy became clear in cervical muscles, shoulder girdles, intercostals and proximal limb muscles in the following several years. Upon muscle biopsy, the most common finding was numerous cytoplasmic bodies (CBs), which were found on 7.3% of myofibers in the tibialis anterior of individual E (Figure 2B (a–c)) and 50–80% of intercostals in other cases.[5] Other nonspecific findings were increased variability in the size of myofibers, central nuclei and rimmed vacuoles observed on a few fibers. No strong immunoreaction of desmin was seen in the CBs (Figure 2B (d, e)). An electron microscope examination showed that the regular sarcoplasmic pattern was replaced by abnormal fine filamentous structures, which seemed to attach to the Z-band. CBs were also found in almost all skeletal muscles and some smooth muscles in autopsied cases.[5] Cardiac myofibers also contained numerous CBs in one of the autopsied cases (V-2),[5] although the patient did not present any cardiac complication. The sequence analysis of the coding regions and flanking introns of *DES* and *MYOT* showed no pathogenic mutation in individual E. An array comparative genomic hybridization performed with the Agilent SurePrint G3 Human CGH 1M microarray format in individual A did not reveal any aberrations of genomic copy number.

Linkage analysis

DNA was extracted by standard methods. Linkage analysis was performed on nine family members (A–I in Figure 1; four of them were affected, and the others were unaffected) through genotyping using an Illumina Human Omni 2.5 BeadChip (Illumina, San Diego, CA, USA). We chose single-nucleotide polymorphisms (SNPs) that satisfied all of the following criteria: (1) autosomal SNPs whose allele frequencies were available from the HapMap project (http://hapmap.ncbi.nlm.nih.gov/), (2) SNPs that were not monomorphic among members and (3) SNPs that were not in strong linkage disequilibrium with neighboring SNPs (r2 values <0.9). Then, we selected the first five SNPs from each position of integer genetic distance from SNPs that met the above criteria for the initial analysis. The details were as follows; we chose a SNP closest to 0 cM and the neighboring four SNPs. If the genetic distance of a SNP was the same as that of the next SNP, we considered the genomic position to determine their order. We repeated this process at 1 cM, 2 cM and so on.

We performed a multipoint linkage analysis of the data set (17 613 SNPs) using MERLIN[6] 1.1.2 under the autosomal dominant mode with the following parameters: 0.0001 for disease allele frequency, 1.00 for individuals heterozygous and homozygous for the disease allele and 0.00 for individuals

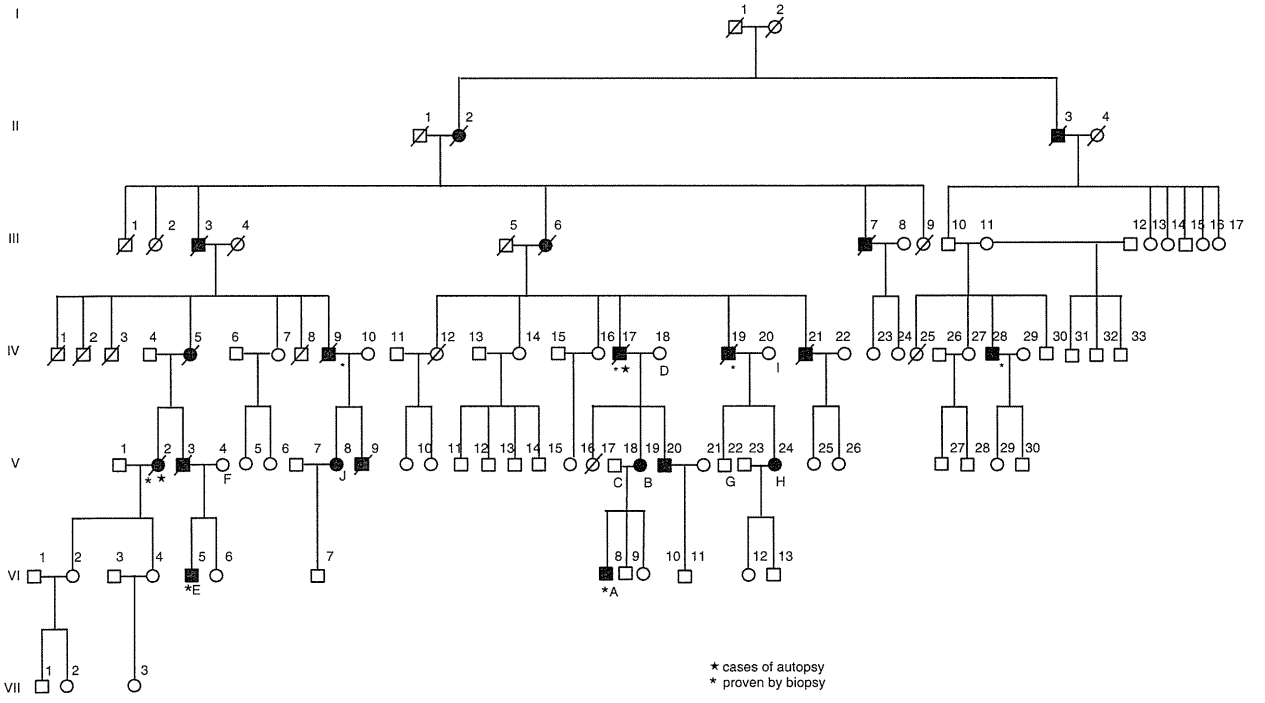


Figure 1 Family pedigree. Filled-in symbols indicate individuals with MFM. Empty symbols indicate unaffected individuals. A star and asterisk indicate autopsy-proven and muscle biopsy-proven cases, respectively. (A–J) indicates individuals whose DNA was used for this study.

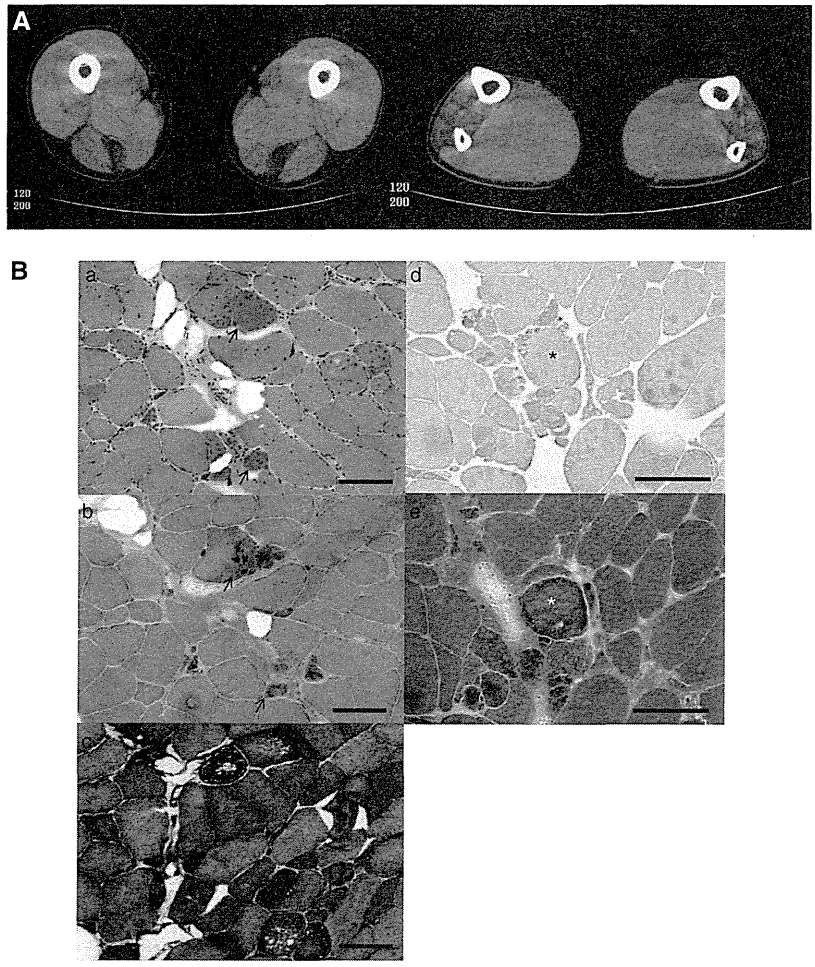


Figure 2 Family clinical data. (**A**) Muscle computed tomography of affected lower extremity. The imaging in the initial assessment of individual A showed symmetrical atrophy and fatty replacement of the semitendinosus in the proximal lower extremities (left) and the tibialis anterior, tibialis posterior, extensor hallucis and digitorum longus, and peroneal muscle in the distal (right) lower extremities. (**B**) Pathology of muscle biopsy. Hematoxylin-eosin (a), Gomori-trichrome (b) and NADH (nicotinamide adenine dinucleotide)-tetrazolium reductase (c) staining of the muscle biopsy sample from the tibialis anterior of individual E are shown. CBs are indicated by arrows. CBs were round or oval, 5–10 μm in diameter and predominantly located in the periphery of type 1 fibers, which stained eosinophilic with hematoxylin-eosin and blue–purple with Gomori-trichrome. NADH-tetrazolium reductase staining showed disorganization of the myofibrillar network. Immunostaining for desmin (d) and Gomori-trichrome staining (e) are serial sections of the muscle biopsy from individual E. Stars indicate corresponding fibers. No strong immunoreaction of desmin was seen in the CBs. Scale bars = 100 μm

homozygous for the alternative allele. After this first analysis, a second analysis was performed with all SNPs fulfilling the above criteria around the peaks identified in the first analysis.

Exome sequencing

Exome sequencing was performed on seven family members in three generations (A–E, H and I in Figure 1), four of whom were affected. Exon capture was performed with the SureSelect Human All Exon kit v2 (individuals E, H and I) or v4 (A–D) (Agilent Technologies, Santa Clara, CA, USA). Exon libraries were sequenced with the Illumina Hiseq 2000 platform according to the manufacturer's instructions (Illumina). Paired 101-base pair reads were aligned to the reference human genome (UCSChg19) using the Burrows-Wheeler Alignment tool.[7] Likely PCR duplicates were removed with the Picard program (http://picard.sourceforge.net/). Single-nucleotide variants and indels were identified using the Genome Analysis Tool Kit (GATK) v1.5 software.[8] SNVs and indels were annotated against the RefSeq database and dbSNP135 with the ANNOVAR program.[9] We used the PolyPhen2 polymorphism phenotyping software tool[10] to predict the functional effects of mutations.

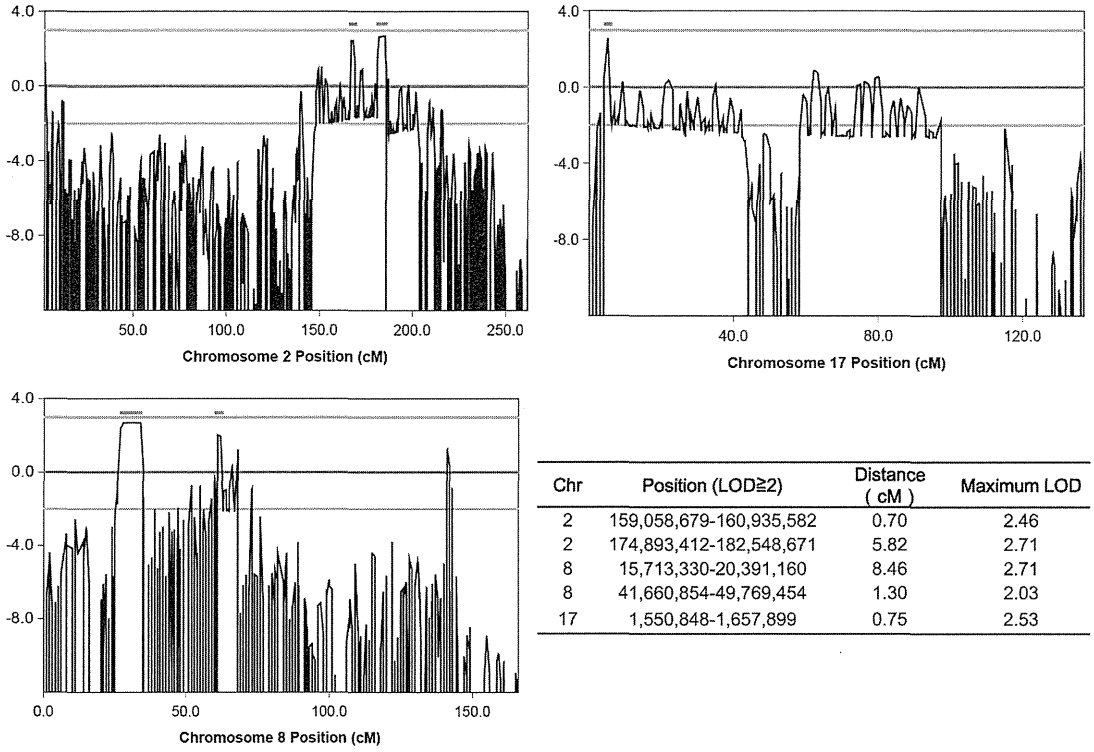
Sanger sequencing

To confirm that mutations identified by exome sequencing segregated with the disease, we performed direct sequencing. PCR was performed with the primers shown in Supplementary Table 1. PCR products were purified with a MultiScreen PCR plate (Millipore, Billerica, MA, USA) and sequenced using BigDye terminator v1.1 and a 3500xL genetic analyzer (Applied Biosystems, Carlsbad, CA, USA).

RESULTS

Linkage analysis

The first linkage analysis identified five regions across autosomes with a logarithm of odds (LOD) score greater than 2 (Figure 3). Of the five regions, two were on chromosome 2 (from 167 cM to 168 cM, with a maximum LOD score of 2.46 and from 182 cM to 185 cM, with a maximum LOD score of 2.71), the other two were on chromosome 8 (from 27 cM to 34 cM, with a maximum LOD score of 2.71 and at 61 cM, with a maximum LOD score of 2.03), and one was on



Chr	Position (LOD≧2)	Distance (cM)	Maximum LOD
2	159,058,679-160,935,582	0.70	2.46
2	174,893,412-182,548,671	5.82	2.71
8	15,713,330-20,391,160	8.46	2.71
8	41,660,854-49,769,454	1.30	2.03
17	1,550,848-1,657,899	0.75	2.53

Figure 3 Linkage analysis. Linkage analysis was performed on nine family members (four of them were affected, the others were unaffected) using an Illumina Human Omni 2.5 BeadChip. Five regions with an LOD score greater than 2 (indicated by bar) were identified. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 1 Summary of detected variants by exome sequencing

Individual *Morbidity*	*A* *Affected*	*B* *Affected*	*C* *Unaffected*	*D* *Unaffected*	*E* *Affected*	*H* *Affected*	*I* *Unaffected*	*Segregated in seven family members*
Exonic, splicing	10089	10064	10079	10065	10230	10194	10216	64
Nonsynonymous, splicing, indel, nonsense	4987	5020	5055	5038	5143	5234	5200	32
Allele frequency not available	577	600	536	555	671	794	786	2

chromosome 17 (at 5 cM, with a maximum LOD score of 2.53). In the second detailed linkage analysis, these peaks were determined to range from 167.49 cM at rs4233674 at position 159 058 679 to 168.19 cM at rs7598162 at position 160 935 582, and from 181.23 cM at rs4402725 at position 174 893 412 to 187.05 cM at rs7420169 at position 182 548 671 on chromosome 2; from 26.42 cM at rs2736043 at position 15 713 330 to 34.88 cM at rs9325871 at position 20 391 160, and from 61.02 cM at rs6999814 at position 41 660 854 to 62.32 cM at rs10957281 at position 49 769 454 on chromosome 8; and from 4.7 cM at rs11078552 at position 1 550 848 to 5.45 cM at rs1057355 at position 1 657 899 on chromosome 17. Haplotypes shared by affected individuals in these regions were confirmed by visual inspection. There were a few incompatible SNPs in these regions, presumably due to genotyping error.

Exome sequencing and segregation analysis

In exome sequencing, an average of 215 million reads enriched by SureSelect v4 (SSv4) and 319 million reads enriched by SureSelect v2 (SSv2) were generated, and 99% of reads were mapped to the reference genome by Burrows-Wheeler Alignment tool. An average of 57% (SSv4) and 61% (SSv2) of those reads were duplicated and removed, and an average of 80% (SSv4) and 66% (SSv2) of mapped reads without duplicates were in target regions. The average coverage of each exome was 163-fold (SSv4) and 130-fold (SSv2). An average of 85% (SSv4) and 69% (SSv2) of target regions were covered at least 50-fold (Supplementary Table 2). On average, 10 133 SNVs or indels, which are located within coding exons or splice sites, were identified per individual (Table 1). A total of 64 variants were common among patients and not present in unaffected individuals, and 32 of those were left after excluding synonymous SNVs. In these variants, only the heterozygous mutation c.90263G>T (NM_001256850) at position 179 410 777 of chromosome 2, which was predicted to p.W30088L in *TTN*, was novel (that is, not present in dbSNP v135 or 1000 genomes). Polyphen2 predicted this mutation as probably damaging. This mutation was located in a candidate region suggested by the linkage analysis in the present study. The other variants were registered with dbSNP135, and the allele frequencies, except for one SNV, rs138183879, in *IKBKB*, ranged from 0.0023 to 0.62.