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Figure Legend

Figure 1 (a) Facial appearance of the patient.

Figure (TIF or EPS only; 300 ppi images and 1200 ppi Line-Art)

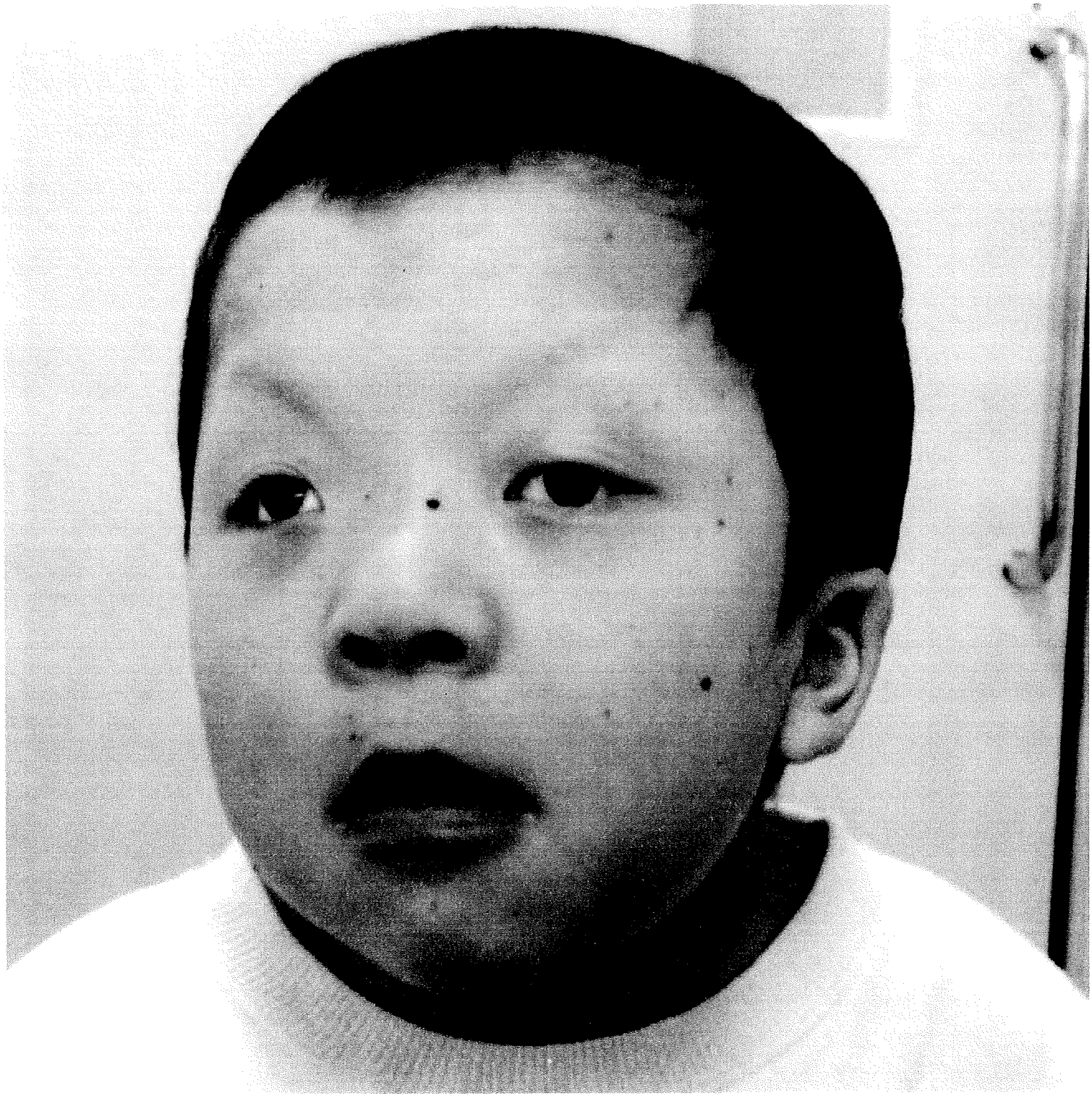

Table 1. Summary of CFC patients who developed malignant tumors

	1 [2, 30]	2 [17]	3 (current study)	4 [18]
Gene	*BRAF*	*BRAF*	*BRAF*	MEK1
Amino acid change	G469E	E501G	A246P	Y130C
Clinical				
Clinical diagnosis	CFC	CFC	Noonan (2 months of age), CFC (9 years)	Costello (6 weeks) CFC
Heart defects	mild PS, ASD and asymmetrical hypertrophy of the interventricular septum	patent ductus arteriosus and asymmetrical hypertrophy of the interventricular septum	No	heart transplantation due to severe hypertrophic cardiomyopathy (8 months of age), a small anterior muscular septal defect
Skin and hair	keratosis pilaris (3 y) cafe-au-lait spots, sparse, friable hair	generalized pigmentation and patchy hyperkeratosis, sparse curly hair	multiple nevi (9 years)	loose plantar and palmer skin with deep creases, sparse thin hair
Mental and growth development	moderate mental retardation	severe mental retardation	moderate mental retardation, short stature (-3.1 SD)	developmental delay
Other		bilateral cryptorchidism		
Hematologic malignancy	ALL	ALL	precursor T lymphoblastic lymphoma	hepatoblastoma
Age at diagnosis	5 y	1 y 9 mo	2 mo	35 mo
Initial symptoms	hepatosplenomegaly	hepatosplenomegaly and right testicular swelling	coughing, rhinorrhea and feeding difficulty	progressive dyspnea, systolic murmur and hepatomegaly
Laboratory findings/ imagings	8% of 1.4×10^9/l leukocytes in peripheral blood, 98% lymphoblasts in bone marrow: positive for TdT, HLA-DR, CD34, CD13, CD33, CD19, CD10, CD22 and CD79	100% of 8.3×10^{10}/l leukocytes in peripheral blood, 98% lymphoblasts in bone marrow: positive for TdT, HLA-DR, CD19, CD10, CD22 and CD79	right lung pneumonia with pleurisy; cytological examination of pleural fluid showed T-cell lymphoblasts: positive for CD2, CD3, CD5, and CD7.	Intracardiac mass in the right atrium, extending into the inferior vena cava, to a level close to the renal veins; 5.2 cm x 6.4 cm intrahepatic mass infiltrating the posterior branch of the right portal vein and extending into the right hepatic lobe.

Treatment	vincristine, dexamethasone and E. coli asparginase for induction therapy	vincristine, predonsolone, E.coli asparginase and doxorubicin for induction therapy	vincristine, predonisolone, tetrahydropyranyl adriamycin, cyclophosphamide and E. coli asparaginase	Surgical dissection of intra-cardiac mass revealed hepatoblastoma; cisplatin, vincristine and 5-Flurouracil as chemotherapy
Outcome	Healthy at 15 y of age	healthy as of age 9 y 3 mo	healthy as of age 12 y 4 mo	Died at 35 mo

Tabe2. *BRAF* mutations identified in hematologic malignancies

Nucleotide change	Amino acid change	Malignant tumor	References
c. 1402 G>C	p.G468R	Diffuse large B cell lymphoma	21
c. 1403 G>C	p.G468A	Diffuse large B cell lymphoma	21
c. 1403 G>C	p.G468A	Diffuse large B cell lymphoma	21
c. 1403 G>C	p.G468A	B cell ALL	22
c. 1403 G>C	p.G468A	B cell ALL	22
c. 1403 G>C	p.G468A	Bisphenotypic acute leukemia	22
c. 1403 G>C	p.G468A	AML	22
c. 1768G>A	p.V590I	Pre B ALL	23
c. 1778A>G	p.D593G	Diffuse large B cell lymphoma	21
c. 1786G>A	p.G596S	T-ALL	23
c. 1790T>A	p.L597Q	Pre B ALL	23
c. 1790T>A	p.L597Q	Pre B ALL	23
c. 1790T>A	p.L597Q	Pre B ALL	23
-	p.V600E	U266 myeloma cells	31, 32
c.1796T>A	p.V600E	t-AML(M5)	24
c.1796T>A	p.V600E	t-AML(M5)	24
c.1796T>A	p.V600E	t-AML(M5)	24
c.1796T>A	p.V600E	T-ALL	23

t-AML, therapy related acute myeloid leukemia

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Familial cases of atypical clinical features genetically diagnosed as multiple lentigines syndrome (LEOPARD syndrome)

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Title: Familial cases of atypical clinical features genetically diagnosed as multiple lentigines syndrome (LEOPARD syndrome)

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Abstract

Five familial cases exhibited ephelides-like multiple lentigines, and we examined three of them, a mother and two sons. All three patients presented with small dark-brown maculae on the face and neck and electrocardiographic abnormalities. These findings sufficed to fulfil the criteria for multiple lentigines syndrome (LEOPARD syndrome), though they lacked five of seven major clinical features. However, the family members presented with a webbed neck and pectus excavatum, which are more frequently seen in Turner or Noonan syndrome. Histological examination of the lentigines revealed slightly elongated rete ridges, a hyperpigmented basal layer, and melanophages in the papillary dermis. Direct sequencing of the patients' genomic DNA revealed that all three had a consistent missense mutation [c.1403C>T (p.T468M)] in the *PTPN11* gene, confirming LEOPARD syndrome with an atypical phenotype. It was suggested that LEOPARD syndrome shows a diverse phenotype but its diagnosis can be verified by mutation analysis.

Introduction

In 1936, Zeisler and Becker[1] reported on a 24-year-old female with multiple lentigines scattered on her body, pectus carinatum, ocular hypertelorism, and mandibular prognathism, which was later named LEOPARD syndrome (LS) by Gorlin *et al.*[2] LEOPARD is an acronym for the major features that characterize the syndrome: multiple *L*entigines, *E*lectrocardiographic conduction defects, *O*cular hypertelorism, *P*ulmonary stenosis, genital *A*bnormality, *R*etardation of growth, and sensorineural *D*eafness. LS is an autosomal dominant disorder that has been presented not only by dermatologists, but also by other specialists,[3-8] and is also called multiple lentigines syndrome.[2,9] The life-threatening problems in LS patients are hypertrophic cardiomyopathy and malignant tumors.[10,11]

Missense mutations in exons 7, 12, and 13 of the protein-tyrosine phosphatase, nonreceptor type 11 (PTPN11) gene, which is located on chromosome 12q24.1 and encodes the protein tyrosine phosphatase SHP2, have been found in LS;[10,12,13] all the mutations are located at the catalytic cleft of the *PTPN11* protein.[14] The SHP2 protein plays an important role in several signal transduction pathways involving several cytokines and hormones, with a particular role in the RAS-mitogen activated protein kinase pathway.[15-17] Thus, although genetic testing is not commonly performed, it is helpful for confirming a diagnosis and differentiating LS from similar diseases, such as Peutz-Jeghers syndrome, Carney syndrome, Noonan syndrome, and Turner syndrome.

We describe a family with members exhibiting multiple lentigines with less frequent symptoms, such as a webbed neck (pterygium colli) and pectus excavatum (trichterbrust), who were genetically diagnosed as having LS.

Case report

A 41-year-old man (hereafter referred to as the second brother) presented with small, dark brown, irregularly pigmented maculae 1 to 4 mm in size on the face and neck, including the vermillion, but not involving the oral and orbital mucosa (Fig. 1). The maculae had been present since birth, and new lesions gradually developed until his 20s and darkened with age. The second brother also presented with other features, such as a webbed neck with a lower hairline and pectus excavatum. Electrocardiography indicated arterial fibrillation, ventricular extrasystole, tachycardia, and left anterior hemiblock. Echocardiography showed mild mitral valve regurgitation, tricuspid valve regurgitation, aorta dilation, and left ventricular dilation. Pulmonary stenosis was not found. Gastrointestinal and colon fibrescopy did not detect

polyposis or any other abnormalities. Levels of thyroid stimulating hormone, free thyroxin, and free triiodothyronine were normal. Chromosome analysis showed a normal 46,XY karyotype in all the 50 peripheral lymphocytes examined.

The second brother informed us that his family members presented with similar symptoms, and we examined his 70-year-old mother and 44-year-old brother (hereafter referred to as the first brother) (Fig. 1). Physical examinations of the mother and brothers revealed that all of them had multiple dark-brown lentigines, mainly on the face (similar appearance to ephelides), a webbed neck, and pectus excavatum without a short stature (Fig. 1). Only the second brother had nevus spilus-like maculae on the back and left arm, but neurofibroma did not present in any of the family members. Bilateral blepharoptosis was noted also only by the second brother, though there was no accompanying exophthalmus or ocular hyperterolism.

We also collected information on other family members we were unable to see in person. The father and mother did not marry consanguineously, and the father had already died of lung cancer at the age of 64. The mother's younger sister (65-years-old) had multiple lentigines and no children before she died. The first brother has two sons, aged 6 and 5 years, with no symptoms suggesting LS. The second brother and a sister (39-years-old) do not have any children. There was no abnormality of the external genitalia or urinary organs in any family members. Intelligence, mental development, and hearing were also normal. The clinical data are summarized in Table 1 and Fig. 2.

Materials and Methods

Human tissue analyses were performed in compliance with the Declaration of Helsinki Principles. A skin biopsy of a pigmented facial lesion was taken from the second brother. Peripheral blood samples were taken from the mother and both brothers using an ethics committee-approved protocol for genomic DNA analyses after each patient provided informed consent. Photo release consent was also obtained from each patient. The biopsied sample was processed for HE staining and Fontana-Masson ammoniac silver staining. Leukocyte genomic DNA was amplified by PCR for the 15 exons and flanking introns of *PTPN11* and was subjected to direct sequencing from both directions using a CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA). The primer sequences and PCR conditions were described previously.[18] To confirm any mutations, three independent PCR products were examined.

Results

Mutation analysis in the second brother indicated a heterozygous C>T substitution at position c.1403 in *PTNP11* exon 12, resulting in the missense mutation Thr468Met (Fig. 3), which is one of the known mutations for LS. Both the mother and first brother had this mutation as well. This mutation is located at the catalytic cleft of the PTP domain and impairs phosphatase activity of SHP2.[19]

Histological examination of the lentigine specimen (Fig. 4) revealed that epidermal rete ridges were slightly elongated and basal layer of the epidermis were hyperpigmented with increased numbers of melanocytes. No nevus cells were observed. Deposition of melanophages was slightly detected in the top region of the dermal papillae, and we observed moderate infiltration of lymphocytes into the epidermis and hair follicle epithelium.

Discussion

There are many reports in the literature of multiple lentigines associated with other symptoms, including Neurofibromatosis–Noonan syndrome,[20] Watson syndrome,[21] centrofacial lentiginosis,[22] inherited patterned lentiginosis,[23] Carney complex,[24] Peutz-Jeghers syndrome,[25] Laugier-Hunziker-Baran syndrome, and Cronkhite-Canada syndrome. In our cases, ephelides-like lentigines were spread predominantly on the face and neck without eruptions on the oral mucosa, and neither neurofibroma nor schwannoma were seen. Intestinal polyposis, myxoma, or endocrine dysfunction was not noted. However, our cases also lacked many major manifestations associated with LS; none of the patients exhibited ocular hypertelorism, pulmonary stenosis, abnormal genitalia, growth retardation, or sensorineural deafness. On the other hand, a webbed neck and pectus excavatum, which are less frequent in LS[9,26] and frequently seen in Noonan syndrome and Turner syndrome,[27] were noted.

LEOPARD syndrome has been reported to present with extremely variable phenotypes. Voron *et al.*[9] grouped the LS features into the following nine categories: cutaneous abnormalities, cardiac abnormalities, genitourinary abnormalities, endocrine findings, neurogenic defects, cephalofacial dysmorphism, short stature, skeletal anomalies, and familial history consistent with an autosomal dominant mode of inheritance. Voron also proposed minimal diagnostic criteria for LS: at least two other features must be present in cases with multiple lentigines, whereas a diagnosis of LS may be made in cases with family history and three other major features despite an absence of multiple lentigines.[9] In our cases, three other features (cardiac and skeletal abnormalities and family history) were present in addition to multiple lentigines, but only two (multiple lentigines and ECG

abnormality) of the seven major clinical manifestations advocated by Gorlin *et al.*[2] were noted. Therefore, careful differentiation from Noonan syndrome is needed because most of the clinical features of LS, such as heart defects, growth retardation, and facial dysmorphism, overlap with those of Noonan syndrome, which presents as a Turner-like phenotype, such as short stature, cephalofacial dysmorphism, webbed neck, skeletal anomalies, and genitourinary and cardiac abnormalities, particularly pulmonary valve stenosis, although Noonan syndrome has a normal karyotype.[28]

Both LS and Noonan syndrome are known to be caused by heterozygous germline missense mutations in the PTPN11 gene. Approximately 85% of the patients with a definite diagnosis of LS have a missense mutation in the PTPN11 gene,[10] and mutations in the PTPN11 gene are also seen in roughly 50% of Noonan syndrome cases.[27,29] However, it was recently established by analyzing accumulated genetic data of LS and Noonan syndrome that the mutations in LS and Noonan syndrome are almost mutually exclusive.[14,30,31] In Noonan syndrome, PTPN mutations are detected at 33-60%,[27,30] and are recurrent and clustered mostly in exons 3, 7, 8 and 13.[12,27] Noonan syndrome mutations are recognized as gain-of-function mutations, while LS mutations were identified as having dominant negative, not activating, effects.[32] The most frequently (approximately 90%) reported PTPN11 mutations in LS are located in exons 7 (Tyr279Cys) and 12 (Thr468Met),[30] the latter of which were detected in all three family members examined here. In addition, to our knowledge, Thr468Met has never been detected in NS syndrome.[27,33] Taken together with the clinical finding that the three familial patients sufficed Voron's minimal diagnostic criteria for LS, we diagnosed them as LS.

It has been reported that there are typically two histological types of lentigines seen in LS patients:[9,26] melanocytic navi and lentigo simplex. The biopsy specimen from our case exhibited histological features compatible with the latter, a lack of nevus cells and the presence of epidermal hypermelanosis.

In conclusion, three familial cases presented with ECG abnormalities and multiple lentigines on the face and neck, lacked most of other major features of LS, and exhibited a webbed neck and pectus excavatum. Genetic testing revealed that all of the patients carry a consistent germline missense mutation (Thr468Met, 1403C→T) in the exon 12 of PTPN11 gene, which suggested the diagnoses of LS.

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Table 1 Summarized clinical manifestations of five family members

Manifestations			Fa	Mo	OB	YB	Si
Genome	Missense mutation in the PTPN11 gene		N/A	+	+	+	N/A
L	Multiple Lentigines		–	+	+	+	+
E	ECG abnormalities		N/A	+	+	+	+
O	Ocular hypertelorism		–	–	–	–	–
P	Pulmonary stenosis		N/A	N/A	N/A	–	N/A
A	Abnormal genetalia	Cryptorchidism	–	–	–	–	–
R	Retardation of growth		–	–	–	–	–
D	Sensorineural Deafness		–	–	–	–	–
Skin	Cafè-au-lait spots		–	+	+	+	N/A
	Neurofibromatosis		–	–	–	–	–
	Curly, coarse hair		–	–	–	–	–
Ear	Low-set ear		+	+	+	+	N/A
Eye (Eyelids)	Light-colored irises		–	–	–	–	N/A
	Blepharoptosis		+	+	+	+	+
	Epicanthal folds		–	–	+	+	N/A
Cardiovascular	Congenital heart defects		N/A	N/A	N/A	+	N/A
	Hypertrophic cardiomyopathy		N/A	N/A	N/A	–	N/A
Skeletal	Short stature		–	–	–	–	–
	Pectus excavatum and/or carinatum		–	+	+	+	+
	Vertebral anomalies	Scoliosis	–	–	–	–	–
	Cubitus valgus		–	–	–	–	–
Hematological	Bleeding diathesis (von Willebrand disease, factors XI and XII deficiency)		–	–	–	–	–
	Thrombocytopenia		–	–	–	–	–
	Leukemia		–	–	–	–	–
Others	Webbed neck with low posterior hairline		–	+	+	+	+
	Malocclusion		–	+	+	+	N/A
	Lymphatic disorder	Lymphedema	–	–	–	–	–
	Triangular facies		–	–	–	–	N/A
	Feeding difficulties		–	–	–	–	–
	Cryptorchidism		–	–	–	–	–
	Mental retardation		–	–	–	–	–
	Sexual infantilism		–	–	–	–	–

Fa, father; Mo, mother; FB, first brother; SB, second brother; Si, sister.

Figure legends

Fig 1 Photographs of three family members

All three members presented with multiple small brown maculae on the face and neck, a webbed neck, and pectus excavatum.

Fig 2. Family pedigree

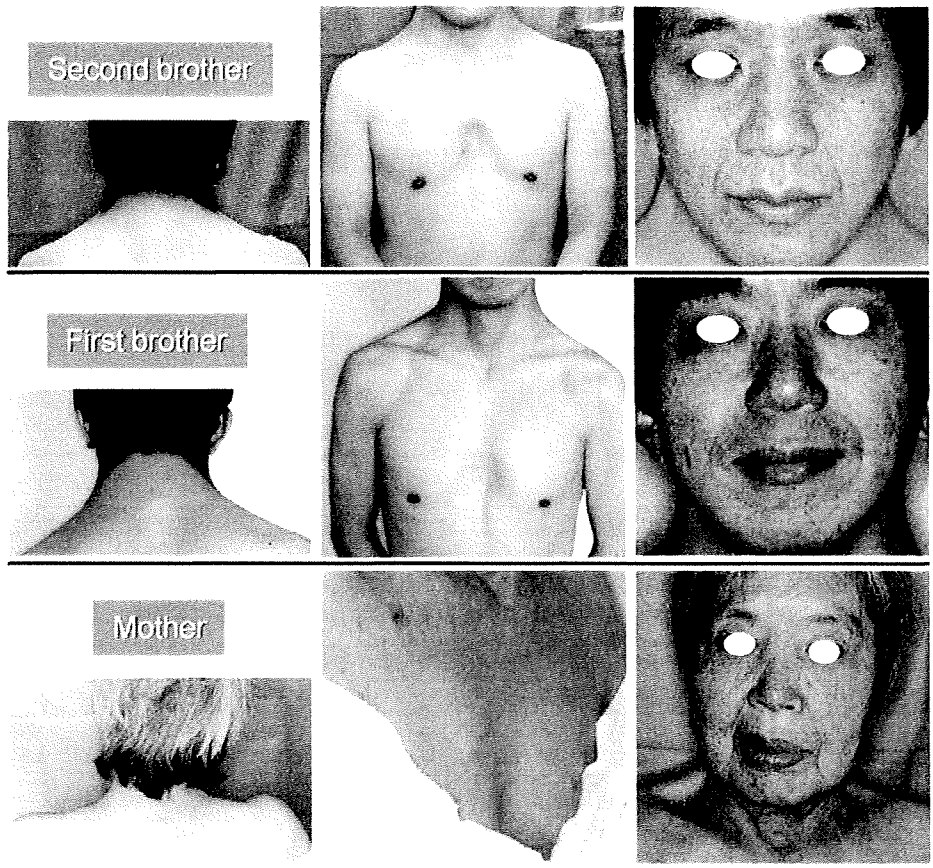
Five family members presented with multiple lentigines (red): the mother, mother's sister, two sons, and one daughter. Multiple lentigines were not noted in the father and first brother's sons. Fa, father; Mo, mother; FB, first brother; SB, second brother; Si, sister.

Fig 3. Electrochromatograms for the three family members

The PTPN11 mutation (Thr468Pro, 1403AfiC) was detected in genomic DNA from the leukocytes of the three patients.

Fig 4. Histological examination of the biopsy specimen from the face of the second brother

Top: Histological examination of a pigmented macule demonstrated slightly elongated rete ridges and epidermal hypermelanosis using Hematoxirin-Eosin staining. Scale bar = 200 μm. Bottom: Higher magnification of the section revealed a hyperpigmented basal layer, incresed numbers of melanocytes without nest formation, and melanophages in the papillary dermis. Masson-fontana ammoniac silver staining. Scale bar = 100 μm.



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All three members presented with multiple small brown maculae on the face and neck, a webbed neck, and pectus excavatum.

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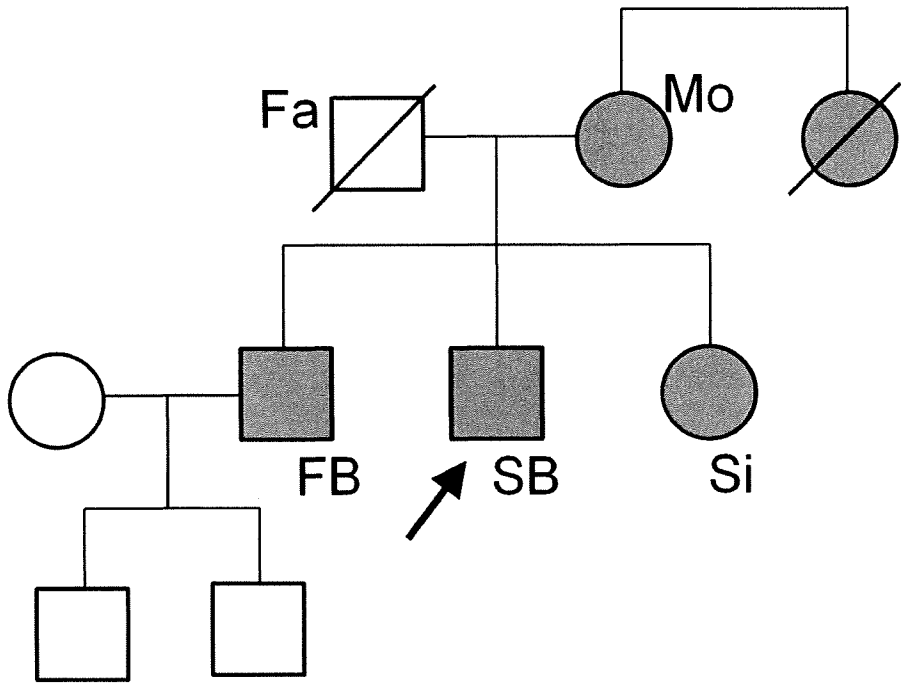

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499x375mm (72 x 72 DPI)