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ORIGINAL ARTICLE

Mutation analysis of the *SHOC2* gene in Noonan-like syndrome and in hematologic malignancies

Shoko Komatsuzaki[1], Yoko Aoki[1], Tetsuya Niihori[1], Nobuhiko Okamoto[2], Raoul CM Hennekam[3,4], Saskia Hopman[5], Hirofumi Ohashi[6], Seiji Mizuno[7], Yoriko Watanabe[8], Hotaka Kamasaki[9], Ikuko Kondo[10], Nobuko Moriyama[11], Kenji Kurosawa[12], Hiroshi Kawame[13], Ryuhei Okuyama[14], Masue Imaizumi[15], Takeshi Rikiishi[16], Shigeru Tsuchiya[16], Shigeo Kure[1,16] and Yoichi Matsubara[1]

Noonan syndrome is an autosomal dominant disease characterized by dysmorphic features, webbed neck, cardiac anomalies, short stature and cryptorchidism. It shows phenotypic overlap with Costello syndrome and cardio-facio-cutaneous (CFC) syndrome. Noonan syndrome and related disorders are caused by germline mutations in genes encoding molecules in the RAS/MAPK pathway. Recently, a gain-of-function mutation in *SHOC2*, p.S2G, has been identified as causative for a type of Noonan-like syndrome characterized by the presence of loose anagen hair. In order to understand the contribution of *SHOC2* mutations to the clinical manifestations of Noonan syndrome and related disorders, we analyzed *SHOC2* in 92 patients with Noonan syndrome and related disorders who did not exhibit *PTPN11*, *KRAS*, *HRAS*, *BRAF*, *MAP2K1/2*, *SOS1* or *RAF1* mutations. We found the previously identified p.S2G mutation in eight of our patients. We developed a rapid detection system to identify the p.S2G mutation using melting curve analysis, which will be a useful tool to screen for the apparently common mutation. All the patients with the p.S2G mutation showed short stature, sparse hair and atopic skin. Six of the mutation-positive patients showed severe mental retardation and easily pluckable hair, and one showed leukocytosis. No *SHOC2* mutations were identified in leukemia cells from 82 leukemia patients. These results suggest that clinical manifestations in *SHOC2* mutation-positive patients partially overlap with those in patients with typical Noonan or CFC syndrome and show that easily pluckable/loose anagen hair is distinctive in *SHOC2* mutation-positive patients.
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INTRODUCTION

Noonan syndrome (MIM 163950) is an autosomal dominant disorder characterized by short stature, webbed or short neck, characteristic features (hypertelorism, low-set ears and ptosis), pulmonary valve stenosis and hypertrophic cardiomyopathy.[1,2] Noonan syndrome is a heterogeneous disease and overlaps phenotypically with Costello syndrome (MIM 218040) and cardio-facio-cutaneous (CFC) syndrome (MIM 115150). Costello syndrome is characterized by mental retardation, distinctive facial features, neonatal feeding difficulties, curly hair, loose skin, and hypertrophic cardiomyopathy and carries an increased risk of malignancy.[3] CFC syndrome, on the other hand, is characterized by mental retardation, ectodermal abnormalities (sparse hair, hyperkeratotic skin and ichthyosis), distinctive facial features (high forehead, bitemporal constriction, hypoplastic supraorbital ridges, downslanting palpebral fissures and depressed nasal bridge) and congenital heart defects (pulmonic stenosis, atrial septal defect and hypertrophic cardiomyopathy).[4]

Recent studies have shown that all three of these disorders result from dysregulation of the RAS/MAPK cascade. It has been suggested that these syndromes be comprehensively termed the RAS/MAPK syndromes[5] or the neuro-cardio-facial-cutaneous syndrome.[6] Germline mutations in *PTPN11*, *KRAS*, *SOS1* and *RAF1* have been

[1]Department of Medical Genetics, Tohoku University School of Medicine, Sendai, Japan; [2]Department of Medical Genetics, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Osaka, Japan; [3]Clinical and Molecular Genetics Unit, Institute of Child Health, Great Ormond Street Hospital for Children, University College London, London, UK; [4]Department of Pediatrics, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; [5]Department of Pediatric Oncology, Emma Children's Hospital, Academic Medical Center, Amsterdam, The Netherlands; [6]Division of Medical Genetics, Saitama Children's Medical Center, Saitama, Japan; [7]Department of Pediatrics, Central Hospital, Aichi Human Service Center, Aichi, Japan; [8]Department of Pediatrics and Child Health, Kurume University School of Medicine, Kurume, Japan; [9]Department of Pediatrics, Sapporo Medical University, Sapporo, Japan; [10]Division of Pediatrics, Oida Hospital, Kochi, Japan; [11]Department of Pediatrics, Hitachi Ltd, Mito General Hospital, Ibaraki, Japan; [12]Division of Medical Genetics, Kanagawa Children's Medical Center, Yokohama, Japan; [13]Department of Genetic Counseling, Ochanomizu University, Tokyo, Japan; [14]Department of Dermatology, Shinshu University School of Medicine, Matsumoto, Japan; [15]Department of Hematology and Oncology, Miyagi Children's Hospital, Sendai, Japan and [16]Department of Pediatrics, Tohoku University School of Medicine, Sendai, Japan
Correspondence: Dr Y Aoki, Department of Medical Genetics, Tohoku University School of Medicine, 1-1 Seiryo-machi, Sendai, Miyagi 980-8574, Japan.
E-mail: aokiy@med.tohoku.ac.jp
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identified in 60–80% of Noonan syndrome patients.[7–12] In patients with Costello syndrome, germline mutations in *HRAS* have been identified,[13] and mutations in *KRAS*, *BRAF* or *MAP2K1/MAP2K2* have been identified in approximately 70% of patients with CFC syndrome.[14,15] However, in approximately 40% of patients with these disorders, specific mutations have not been identified.

SHOC2 is homologous to *soc2*, a gene that was discovered in *Caenorhabditis elegans*. The *soc2* gene encodes leucine-rich repeats[16] and acts as a positive modulator of the RAS/MAPK pathway.[17] Recently, Cordeddu *et al*.[18] reported a gain-of-function missense mutation, c.4A>G (p.S2G), in *SHOC2* in patients with Noonan-like syndrome with loose anagen hair. However, clinical features of patients with a mutation in *SHOC2* remain unknown. In this study, we analyzed 92 patients with Noonan syndrome and related disorders to characterize mutations in the *SHOC2* gene. We also performed expression analysis of *SHOC2* in adult and fetal human tissues and performed sequence analysis of *SHOC2* in 82 leukemia samples.

MATERIALS AND METHODS

DNA samples from patients with Noonan syndrome and related disorders and from leukemia cells

We analyzed 92 patients with Noonan syndrome and related disorders who did not display *PTPN11*, *KRAS*, *HRAS*, *BRAF*, *MAP2K1/2* (*MEK1/2*), *SOS1* or *RAF1* mutations. At the time at which samples were sent, the primary diagnoses of these patients were as follows: 34 Noonan syndrome, 17 Costello syndrome, 21 CFC syndrome, 4 Noonan/CFC, 2 Costello/CFC and 14 others. Control DNA was obtained from 132 healthy Japanese individuals. Control DNA from 105 healthy Caucasian individuals was purchased from Coriell Cell Repositories (Camden, NJ, USA). Eighty-two leukemia DNA samples were collected from leukemia patients (32 acute myeloid leukemia, 41 acute lymphoblastic leukemia, 1 juvenile chronic myelogenous leukemia, 1 Ki-lymphoma, 2 malignant lymphoma, 1 myelodysplastic syndrome, 1 aplastic anemia, 2 transient abnormal myelopoiesis and 1 unknown). Nine additional genomic DNA samples were collected from patients who had developed leukemia and had achieved complete remission (eight acute lymphoblastic leukemia and one aplastic anemia).

This study was approved by the Ethics Committee of Tohoku University School of Medicine. We obtained informed consent from all subjects involved in the study and specific consent for photographs from seven patients.

Analysis of *SHOC2* mutations

Genomic DNA was extracted from patients' peripheral leukocytes. Exons and flanking intron sequences of *SHOC2* were amplified by PCR with primers based on GenBank sequences (Supplementary Table 1, GenBank accession no. NC_000010.10). The M13 reverse or forward sequence was added to the 5′ end of the PCR primers for use as a sequencing primer. PCR was performed in 15 μl of solution containing 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 17 mM NH_4SO_4, 6.7 μM EDTA, 10 mM β-mercaptoethanol, 1.5 mM dNTPs, 10% (v/v) dimethylsulfoxide (except fragment 7), 1 μM of each primer, 50 ng genomic DNA and 1 unit of Taq DNA polymerase. The reaction consisted of 37 cycles of denaturation at 94 °C for 20 s, annealing at the indicated temperature for 30 s and extension at 72 °C for 30 s. The PCR products of fragment 1a were gel purified; PCR products of the other fragments were purified using MultiScreen PCR plates (Millipore, Billerica, MA, USA). The purified PCR products were sequenced on an ABI PRISM 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Development of a mutation detection system using the light cycler

Real-time PCR and melting curve analysis to detect the c.4A>G mutation was developed using the LightCycler system (Roche Diagnostics, Mannheim,

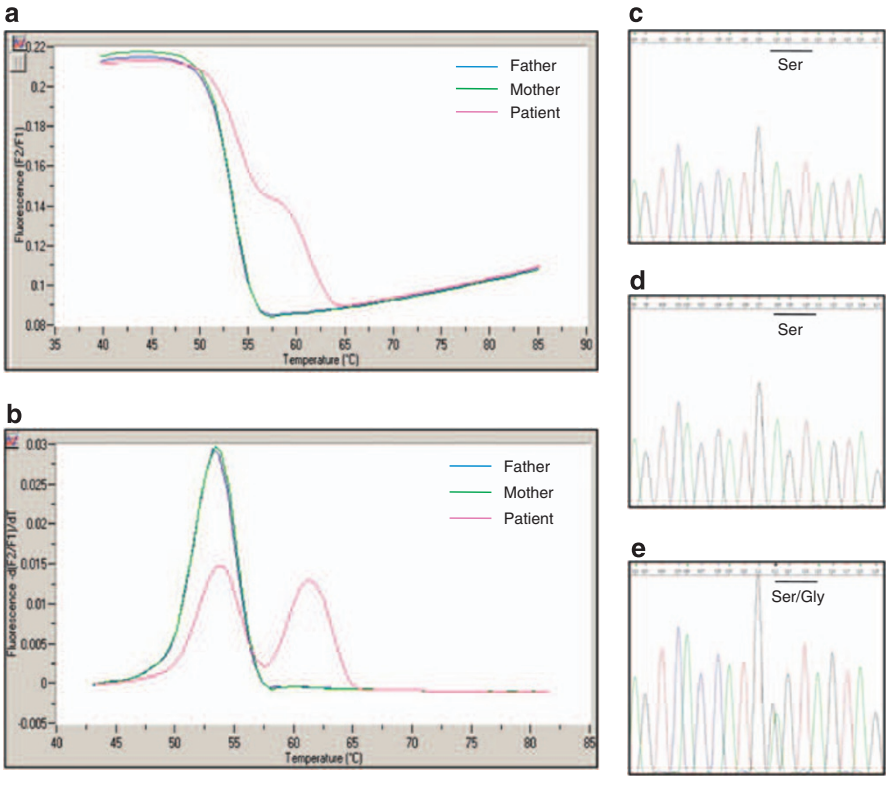

Figure 1 (**a**) PCR followed by melting analysis to detect the c.4A>G mutation. F2 represents the fluorescence emission of the LC Red 640 fluorophore, whereas F1 shows the fluorescence emission of the fluorescein fluorophore. (**b**) Melting curves are automatically converted into melting peaks, which are given as the first negative derivative of the fluorescence (F) versus temperature (T) (-dF/dT) (*y* axis) versus temperature (temp)(*x* axis). The homozygous wild-type allele (parents of NS128) shows a single melting temperature, whereas the heterozygote (NS128) shows two different melting temperatures. (**c**, **d**) Sequencing traces of parents of NS128. (**e**) Sequencing trace of NS128.

Germany). Primer and probe sequences are shown in Supplementary Table 2. The acceptor probe, which matches the mutant allele sequence, was labeled at its 3′ end with fluorescein isothiocyanate. The donor probe was labeled at its 5′ end with LC Red640 and phosphorylated at its 3′ end to prevent probe elongation by the Taq polymerase. Probes were designed by Nihon Gene Research Laboratories (Sendai, Japan). Amplification was performed in a final volume of 20 μl in glass capillaries containing 10 ng of sample DNA, 2 μl of 10× LightCycler-FastStart DNA Master HybProbe (Roche Diagnostics), 12 nM $MgCl_2$, 0.3 μM of each forward and reverse primer and 0.2 μM of each acceptor and donor hybridization probe. PCR was performed under the following conditions: initial denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 7 s with a ramping time of 20 °C s^{-1}. After amplification, melting curve analysis was performed under the following conditions: 95 °C with 0-s hold, cooling to 40 °C for 30 s and slowly heating the sample to 85 °C with a ramp rate of 0.4 °C s^{-1}.

Real-time quantitative PCR

MTC Multiple Tissue cDNA panels Human 1, 2, Human Fetal, Human Immune and Human Cell Line (Clontech, Palo Alto, CA, USA) were used to evaluate the relative expression of *SHOC2* in various tissues. Separation of mononuclear and polymorphonuclear (PMN) leukocytes from whole blood was performed using Polymorphoprep (Nycomed, Oslo, Norway); total RNA was prepared with the RNeasy Mini Kit (Qiagen, Hilden, Germany). One hundred ng of total RNA was used to synthesize complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription kit (ABI). Primers for real-time PCR were designed using software provided by Roche (https://www.roche-applied-science.com) (Supplementary Table 3). Universal ProbeLibrary #42 and #60 (Roche) were used for *SHOC2* and *GAPDH*, respectively. PCR was performed in 20 μl of solution containing 10 μl FastStart Universal Probe Master (Rox) (Roche), 18 pmol of each primer, 5 μl cDNA and 0.25 μM universal HybProbe. The reaction conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 11 min. The real-time PCR program was run by the 7500 Real-Time PCR system (ABI). Diluted control cDNA (1:1, 1:10, 1:100, 1:1000 and 1:10 000) from Multiple Tissue cDNA panels (Clontech) was amplified with each reaction in order to generate a standard curve and calculate relative gene expression of *SHOC2*.

RESULTS

Mutation analysis in patients and development of a rapid mutation detection system

Sequence analysis of all coding regions of *SHOC2* in 92 patients revealed a c.4A>G mutation (p.S2G) in exon1 of *SHOC2* in eight unrelated patients. Parental samples were available in three families; the mutation was not identified in parents, suggesting that the mutation occurred *de novo*.

Our results and the previous report identified a c.4A>G mutation in patients with Noonan-like syndrome. To further characterize the occurrence of this mutation, we developed a rapid mutation detection system using a Lightcycler. Two probes were generated for melting curve analysis, and melting curve analysis was performed after PCR. The PCR products from a patient heterozygous for the c.4A>G mutation differed from those obtained from the patient's parents as well as from those obtained from control subjects (Figures 1a and b). The PCR products were verified by sequencing (Figures 1c–e).

Clinical manifestations of patients with the *SHOC2* mutation

The clinical manifestations of eight patients with the *SHOC2* mutation are shown in Table 1; photographs of five of these patients are shown in Figure 2. The ages of the patients ranged from 4 to 25 years. The primary diagnoses for these patients were Costello, Noonan or CFC syndrome. Three had perinatal abnormalities, including tachypnea, hydramnios, pulmonary hemorrhage and intracranial hemorrhage.

Table 1 Clinical manifestations in *SHOC2* mutation-positive patients

Patient ID	*NS34*	*NS93*	*NS97*	*NS121*	*NS128*	*NS180*	*NS220*	*NS232*
SHOC2 mutation	p.S2G	p.S2G	p.S2G	p.S2G	p.S2G	p.S2G	p.S2G	p.S2G
Genotype of father/mother	WT/WT	ND	ND	ND	WT/WT	ND	ND	WT/WT
Gender	M	F	F	M	F	M	F	M
Age (years)	13.8	21	10	5.7	8	9	4	25
Country	Japan	The Netherlands	Japan	Japan	Japan	Japan	Japan	Japan
Primary diagnosis	NS/CFC	CFC	CFC	CFC	CFC	NS	CS	CS
Perinatal abnormality	+	ND	–	–	–	+	+	–
Polyhydramnios	–	ND	–	–	–	+	–	–
Birth weight	3118 g	3360 g	3068 g	2865 g	2308 g	3258 g	3160 g	3090 g
Others	Tachypnea					Pulmonary hemorrhage	Intracranial hemorrhage	
Growth and development								
Failure to thrive	+	+	+	+	+	+	+	+
Mental retardation	+ WISC III at 9 years 3 months VIQ 81, PIQ 87, FIQ 82	–	+ (DQ44)	+ (DQ48)	+ (DQ 66)	+ WISC III at 9 years 4 months VIQ 61, PIQ <40 FIQ 45	+ (DQ53)	+ (IQ65)
Hyperactivity	–	–	+	–	–	–	–	– (irritability in infancy)
Delayed independent walking (age)	+ (3.6 years)	–	+ (1.8 years)	+ (2.8 years)	+ (4 years)	+ (5 years)	+ (4 years)	+ (3.6 years)
Craniofacial characteristics								
Relative macrocephaly	+	+	+	+	+	+	+	+
Hypertelorism	+	–	–	–	–	+	–	+

Table 1 Continued

Patient ID	*NS34*	*NS93*	*NS97*	*NS121*	*NS128*	*NS180*	*NS220*	*NS232*
Downslanting palpebral fissures	+	+	+	–	–	+	–	–
Ptosis	–	+	–	+	–	–	–	–
Epicanthal folds	–	–	+	+	–	+	+	±
Low-set ears	+	+	+	+	+	+	+	+
Highly arched palate	+	+	–	+	+	–	+	+
Prominent forehead	ND	–	+	+	ND	ND	+	ND
Broad forehead	+	+	+	+	+	+	+	ND
Skeletal characteristics								
Short stature	−3.4 s.d. at 13 years	−3 s.d. at 21 years	−4 s.d. at 6 years	−3 s.d. at 1 year 9 months	−5 s.d. at 8 years	−6 s.d. at 9 years	−4.5 s.d. at 3 years 3 months	−2 s.d. at 23 years
Short neck	+	+	–	+	+	+	–	+
Webbing of neck	+	+	–	–	–	–	–	±
Cubitus valgus	+	+	–	–	–	–	–	–
Pectus anomalies	ND	–	+	+	–	+	–	–
Cardiac defects								
Hypertrophic cardiomyopathy	–	–	+	–	+	+	±	–
Atrial septal defect	–	–	+	–	–	–	+	+
Ventricular septal defect	–	–	–	–	–	–	–	+
Pulmonary stenosis	+	–	+	–	+	–	+	–
Mitral valve anomaly	+	–	–	–	–	–	–	+
Others	Pulmonary regurgitation	Arrhythmia						Hypoplasia of papillary muscle
Hair anomalies								
Curly hair	–	–	+	+	+	+	+	+
Sparse hair	+	+	+	+	+	+	+	+
Easily pluckable hair	+	+	+	+	+	ND	+	+
Skin anomalies								
Dark skin	+	+	+	+	+	+	+	+
Hyperkeratosis	ND	+	+	+	+	–	–	+
Hyperelastic skin	+	–	+	+	+	–	–	+
Café-au-lait spots	+	–	–	–	–	–	–	–
Lentigines	+	–	–	–	–	+	–	–
Atopic skin/eczema	+	+	+	+	+	+	+	+
Others					Deep palmar/ planter creases			Facial erythema, nummular eczema
Genital abnormalities	+ (Cryptorchidism)	–	–	–	–	+(Cryptorchidism)	–	–
Blood test abnormality								
Coagulation defect (normal range)	+[a]	ND	–	+[b]	ND	+[c]	–	–
Number of white blood cells(/μl)	7200	8400	16 000	5300	10 900	9900	10 300	9900
(normal range for patient's age)	(5000–10 000)	(5000–10 000)	(4500–13 500)	(6000–15 000)	(4500–13 500)	(4500–13 500)	(6000–15 000)	(5000–10 000)
Polymorph nuclear cell (%) (mean for each patient's age)	60 (55)	ND	79 (55)	ND	50 (55)	72 (55)	53 (45)	77 (55)
IgE (U ml^{-1})	ND	ND	2300	94	ND	1800	ND	820
Hypernasal/hoarse voice	ND	–	+	+	–	ND	+	+
Miscellaneous	GH deficiency	Delayed puberty, EEG abnormalities, easy bruising	GH deficiency	GH deficiency	Adenoid hypertrophy, GH deficiency		Dilatation of cerebral ventricles, epilepsy	Congenital hydro-nephrosis, frostbite in winter

Abbreviations: APTT, activated partial thrombin time; AT, antithorombin; BT, bleeding time; CFC, cardio-facio-cutaneous; CS, Costello syndrome; DQ, developmental quotient; EEG, electroencephalogram; FIQ, Full Scale intelligence quotient; GH, growth hormone; ND, not described; NS, Noonan syndrome; PIQ, Performance intelligence quotient; PT, prothrombin time; VIQ, verbal intelligence quotient; WISC, Wechsler Intelligence Scale for Children; WT, wild type.
[a]The test was performed when bloody stool was observed at 7 years of age. BT 180 sec (2.5–13), PT 11.5 sec (10.1–12.0) APTT 62.5 sec (26–37), Factor VIII 53% (52–120). Parenthesis represents normal range for the patient's age.
[b]APTT 54 sec (26–37), Factor IX 22% (47–104), Factor XII 34% (64–129), Factor XIII 51 (72–143). Parenthesis represents normal range for the patient's age.
[c]APTT 57 sec (26–37). Parenthesis represents normal range for the patient's age.

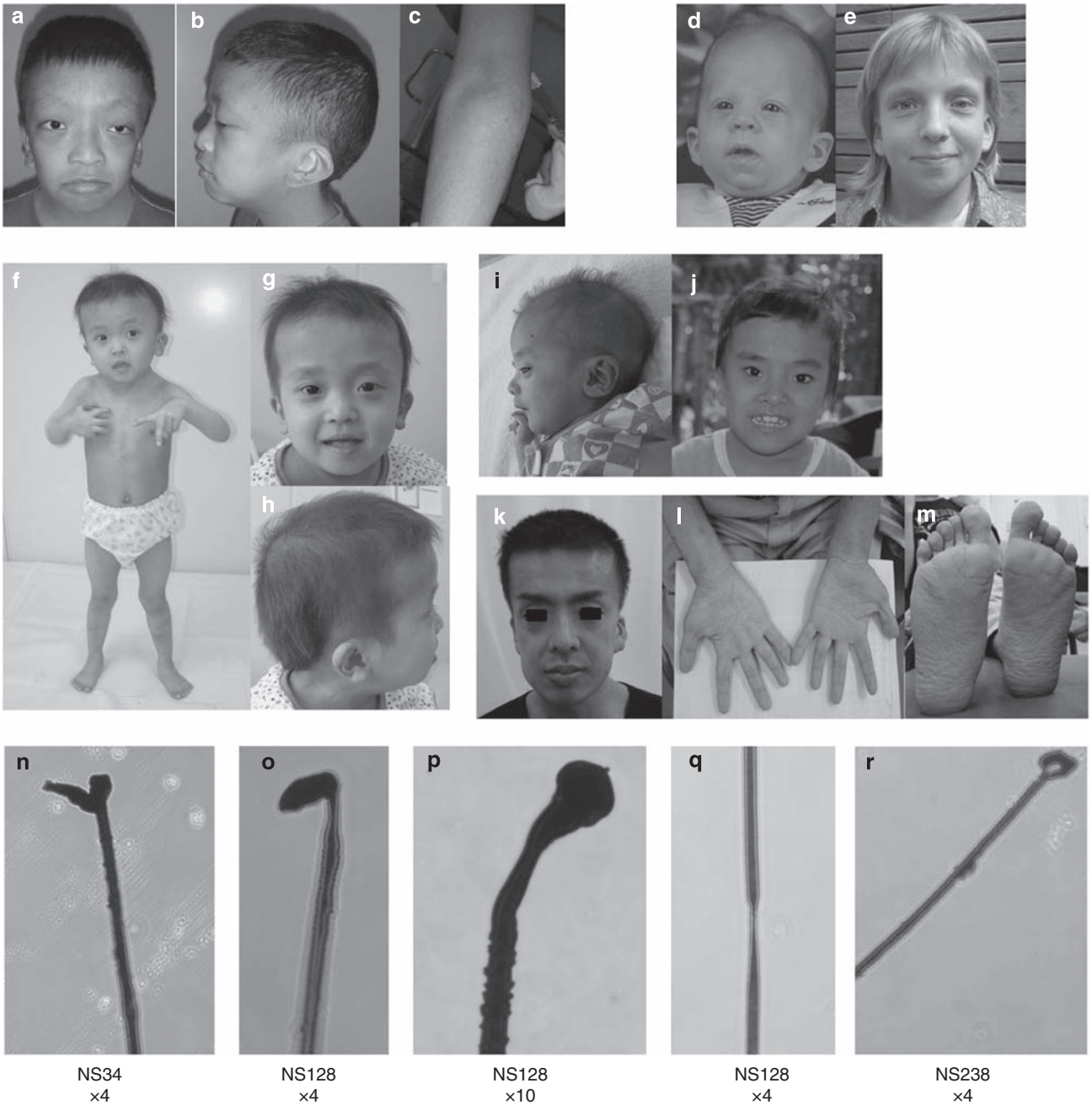

Figure 2 (**a**, **b**) Facial appearance of NS34 at the age of 13 years. (**c**) Dry and atopic skin seen in NS34. (**d**, **e**) NS93. (**f**–**h**) NS97. (**i**, **j**) NS128. (**k**–**m**) NS232 at the age of 25 years. (**l**, **m**) Palms and soles of NS232 showing fine wrinkling. Light micrographs of hairs from patients NS34 (**n**), NS128 (**o**–**q**) and NS238 (**r**). The hair bulb is distorted at an acute angle to the hair shaft, a characteristic described as 'mousetail deformity.' The hair shaft is twisted and longitudinally grooved.

All showed short stature $\geqslant -2$ s.d.) despite normal growth during the fetal period. Mild-to-moderate mental retardation was observed in seven patients. It is of note that delayed independent walking was observed in seven patients. The facial appearances of these patients changed with age. Features frequently observed were relative macrocephaly (8/8 patients), low-set ears (8/8), highly arched palate (6/8) and broad forehead (7/7). Cardiac abnormalities included hypertrophic cardiomyopathy in four patients, atrial septal defect in three patients, pulmonic stenosis in four patients and mitral valve anomaly in two patients. Atopic skin and eczema were observed in all eight patients (Figure 2c), and serum immunoglobulin E level was elevated in three patients. Seven patients had sparse and easily pluckable hair. The hair bulb was bent at an acute angle to the hair shaft, which was irregular and twisted (Figures 2n–r). Four patients had hyponasal/hoarse voice as previously described[18] and three patients showed coagulation defects with prolonged activated partial thrombin time.

The clinical history of two adult patients, NS232 and NS93, differed from those of patients typical for Noonan syndrome. NS232 was a 25-year-old patient, the first son of unrelated healthy parents. Delivery

at 40 weeks was uncomplicated, and birth weight was 3090 g. At 1 month of age, this patient was diagnosed as having an atrioventricular septal defect; the defect spontaneously closed at 5 months of age. During the infantile period, this patient showed irritability and mental/motor delay: head control was achieved at 1 year and 10 months, sitting at 2 years and 4 months and walking at 3 years and 6 months. At his infantile period, this patient was suspected to have Noonan syndrome or Costello syndrome. Pyelostomy for congenital hydronephrosis was performed at the age of 10 months. At 23 years of age, mitral valve replacement was performed because of mitral valve prolapse (III–IV). The dissected mitral valve showed myxomatous change. At 25 years, this patient shows mild mental retardation and displays a gentle personality. Other characteristics include hypertelorism, a highly arched palate and posteriorly rotated ears. During infancy, his hair was pluckable, but the hair abnormality is now subtle. He possesses variable skin abnormalities including fine wrinkles on the palm and soles as well as erythematous rash on the face and eczematous skin changes on the trunks and extremities together with xerotic skin, which are reminiscent of atopic dermatitis (Figures 2k–m). Another adult patient, NS93, has been diagnosed as having CFC syndrome at 1 year of age (Figure 2d). Subsequently her normal motor development and her cognitive development that fell within normal ranges (but was lower than other family members) shed doubt about this diagnosis. She had a delayed pubertal development. She has quite a marked tendency to have bleeding episodes after surgery and to bruise easily.

Leukocytosis in the absence of obvious infection was observed in one of the patients (NS97). The white blood cell count of this patient ranged from 16 000 to 23 000/μl at 5 years of age. The number of leukocytes of the other patients was within the normal range, but close to the upper limit of the normal range.

Expression of *SHOC2* mRNA

A previous study using northern blot analysis showed that *SHOC2* mRNA is present in most tissues, including brain, heart, kidney and pancreas.[16] Because leukocytosis was observed in a patient with the p.S2G mutation, we examined the relative expression of *SHOC2* in various tissues including blood leukocytes and lymphocytes. In the adult human cDNA panel, the highest expression was observed in testis; relatively high expression was also observed in several immune tissues (spleen, bone marrow, tonsil and lymph node) (Figures 3a and b). The expression of *SHOC2* was six times higher in PMN than mononuclear (Figure 3c). Among fetal tissues, brain showed the highest expression (Figure 3d). No increase in *SHOC2* expression was observed in cultured tumor cells (Figure 3e).

SHOC2 mutation analysis in samples from patients with hematologic malignancies

Patients with Noonan-related disorders develop various solid tumors and hematologic malignancies.[5] Approximately 10% of patients with Costello syndrome develop rhabdomyosarcoma, ganglioneuroblastoma or bladder carcinoma. Patients with Noonan syndrome occasionally develop juvenile myelomonocytic leukemia or leukemia.[2] Recently, the occurrence of ALL or non-Hodgkin's lymphoma has been reported in three patients with CFC syndrome.[5,19,20] The presence of leukocytosis in mutation-positive patients and the high expression of *SHOC2* mRNA in PMN led us to look for possible *SHOC2* mutations in patients with hematologic malignancies. However, no such mutations were identified in any of the leukemia samples or in the genomic DNA samples from patients who had been treated for leukemia.

DISCUSSION

In this study, we identified the c.4A>G (p.S2G) mutation in *SHOC2* in 8 of 92 (9%) otherwise mutation-negative patients with Noonan syndrome or related disorders. The mutation detection rate was higher than that reported in a previous study, in which 21 of 410 (5%) such patients were found to carry this mutation. By parental examination, the current and previous studies confirmed *de novo* mutation in 3 and 12 families, respectively. Quantitative PCR analysis demonstrated that

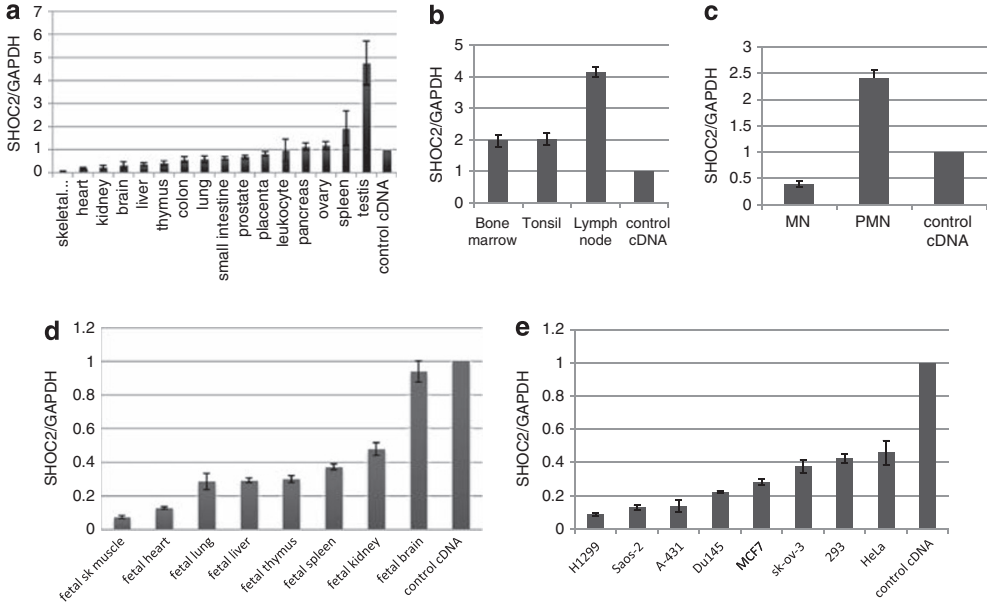

Figure 3 Relative expression of *SHOC2*. Expression levels of *SHOC2* mRNA in various adult human tissues (**a**), adult immune tissues (**b**), human leukocytes (**c**), human fetal tissues (**d**) and human tumor cell lines (**e**) were evaluated by quantitative PCR using *GAPDH* mRNA as the control. Results are expressed as the means and s.d.s of mean values from triplicate samples. Control DNA supplied with Clontech cDNA panels was used as a control.

Table 2 Summary of clinical manifestations in patients with CFC syndrome, Noonan-like syndrome and Noonan syndrome

	CFC syndrome (%)	*Noonan-like syndrome (%)*	*Noonan syndrome (%)*
References	[20,21][a]	Cordeddu *et al.*[18] and this study	[22]
Gene mutations	*KRAS*, *BRAF* and *MAP2K1/2*	*SHOC2*	*PTPN11*, *KRAS*, *SOS1* and *RAF1*
Total patients	63	33	315
Perinatal abnormality			
Polyhydramnios	23/30 (77)	1/7 (14)	21/50 (42)
Fetal macrosomia	ND	ND	20/46 (43)
Growth and development			
Failure to thrive	24/36 (67)	8/8 (100)	51/74 (69)
Mental retardation	25/25 (100)	27/32 (84)	124/293 (42)
Craniofacial characteristics			
Relative macrocephaly	40/58 (69)	31/33 (94)	50/70 (71)
Hypertelorism	17/25 (68)	26/33 (79)	66/82 (80)
Downslanting palpebral fissures fissures fissures	20/25 (80)	4/8 (50)	77/99 (78)
Ptosis	12/25 (48)	24/33 (73)	75/105 (71)
Epicanthal folds	13/25 (52)	5/8 (63)	41/72 (57)
Low-set ears	20/25 (80)	30/33 (91)	115/132 (87)
Skeletal characteristics			
Short stature	46/63 (73)[b]	32/32 (100)	172/297 (58)
Short neck	22/25 (88)	23/33(70)	76/107 (71)
Webbing of neck	6/25(24)	20/33 (61)	84/188 (45)
Cardiac defects			
Hypertrophic cardiomyopathy	24/58 (41)	9/33 (27)	57/277 (21)
Atrial septal defect	11/57 (19)	11/33 (33)	20/69 (29)
Ventricular septal defect	7/57 (12)	3/33(9)	7/70 (10)
Septal defect total	18/57 (32)	14/33 (42)	85/313 (27)
Pulmonic stenosis	23/58 (40)	13/33 (39)	196/312 (63)
Patent ductus arteriosus	ND	0/33 (0)	3/38 (8)
Mitral valve anomaly	10/63 (16)[a]	10/32 (31)	16/67 (24)
Arrhythmia	4/63 (6)	1/33 (3)	14/25 (56)
Skeletal/extremity deformity			
Cubitus valgus	6/25 (24)[a]	2/8 (25)	26/100 (26)
Pectus deformity	27/54 (50)	23/32 (72)	184/287 (64)
Skin/hair anomaly			
Curly hair	58/63 (92)	6/8 (75)	30/75 (40)
Sparse hair	56/63 (89)	33/33 (100)	ND
Loose anagen hair/easily pluckable hair	ND	19/19(100)	ND
Hyperelastic skin	7/25 (28)[a]	5/8 (63)	16/51 (31)
Café-au-lait spots	13/58 (22)[a]	1/8 (13)	5/49 (10)
Lentigines	ND	2/8 (25)	3/49 (6)
Nevus	37/62 (60)[a]	ND	12/46 (26)
Genitalia			
Cryptorchidism	11/41(27)[a]	8/25 (32)	114/211 (54)
Blood test abnormality			
Coagulation defects	1[c]	9/29 (31)	65/180 (36)

Abbreviations: CFC, cardio-facio-cutaneous; ND, not described.
[a]Includes our unpublished data.
[b]Includes short stature (height below the 3rd centile).
[c]A patient with von Willebrand disease was reported.

SHOC2 mRNA is abundant in adult testis and immune tissues as well as in fetal brain. The c.4A>G (p.S2G) mutation was not detected in 82 samples from patients with leukemia.

Clinical manifestations in *SHOC2* mutation-positive patients often vary, even among patients who have a common p.S2G mutation (Table 2 and Supplementary Table 4). In this study and in a previous study, relative macrocephaly (94%), hypertelorism (79%), low-set ears (91%) and short stature (100%) were frequently observed in patients with the *SHOC2* p.S2G mutation.[18] Growth hormone deficiency was observed in 70% of patients. With respect to cardiac abnormalities, pulmonic stenosis was observed in 13 of 33 patients (39%), followed by atrial septal defect (33%), mitral valve anomaly (31%) and hypertrophic cardiomyopathy (27%). Dark skin and atopic dermatitis were seen in 75 and 48% of patients, respectively. Hair abnormalities, including sparse hair (100%) and loose anagen hair/easily pluckable hair (100%), were the most characteristic clinical features of *SHOC2* mutation-positive patients.

The symptomatology of patients with the *SHOC2* mutation does not fit existing disorders, including Noonan, Costello and CFC syndrome. In this paper, we summarize the clinical manifestations of patients with CFC syndrome[21,22] or Noonan syndrome,[23] as described in previous reports, as well as *SHOC2* mutation-positive patients (Table 2). The high frequencies of mental retardation (84%) and sparse hair (100%) observed in *SHOC2* mutation-positive patients are similar to those observed in CFC patients (100 and 89%, respectively); the frequency of mental retardation was higher than that in patients with Noonan syndrome (42%). With respect to cardiac abnormalities, the frequencies of hypertrophic cardiomyopathy, atrial septal defect and mitral valve anomaly are similar to those among patients with Noonan syndrome. However, pulmonic stenosis (39%) was less frequent in *SHOC2* mutation-positive patients than in patients with Noonan syndrome (63%). It is of note that short stature (100%) and pectus deformity (72%) were found to be most frequent in patients with the *SHOC2* mutation. Furthermore, loose anagen/easily pluckable hair has not been reported in mutation-positive patients with Noonan, CFC or Costello syndrome. Taken together, these results suggest that clinical manifestations in patients with *SHOC2* partially overlap with those of Noonan syndrome and CFC syndrome. The presence of easily pluckable/loose anagen hair is distinctive in *SHOC2* mutation-positive patients.

Loose anagen hair has been observed in an isolated loose anagen hair syndrome (OMIM 600628)[24] and has been found to be associated with Noonan syndrome.[25,26] The pathogenesis of loose anagen hair remains unknown. A scalp biopsy in a patient with loose anagen hair showed marked cleft formation between the inner root and the irregularly shaped hair shafts. Abnormalities in the keratin gene have been suggested.[24] Functional analysis of the *SHOC2* p.S2G mutant showed that the mutant protein was aberrantly localized in the membrane fraction after stimulation with epidermal growth factor and induced extracellular signal-regulated kinase signaling in a cell-specific manner.[18] It is possible that dysregulated proliferation or cell-to-cell attachment causes the detachment between inner sheaths and hair shafts.

One of our mutation-positive patients exhibited a remarkable leukocytosis ranging from 12 000 to 24 600/mm^3. Other patients also showed mild leukocytosis, which is near the upper range of the normal levels for their age. This observation led us to examine the tissue and cellular expression of *SHOC2*. In adult tissues, the highest *SHOC2* expression was observed in testis; relatively high expression was also observed in several immune tissues, including spleen, bone marrow, tonsil and lymph node. Among leukocytes, the expression of *SHOC2* was six times higher in PMN than in mononuclear, suggesting that *SHOC2* might be important to the proliferation or survival of PMN leukocytes. We did not identify the p.S2G mutation in 82 samples from patients with hematologic malignancies. A recent study reported that no *SHOC2* mutations were identified in 22 patients with juvenile myelomonocytic leukemia.[27] It is possible that the absence of mutation was due to the relatively small sample size. Alternatively, the gain of function of *SHOC2* might not have leukemogenic potential, and other factors such as aberrant cytokine production may be associated with leukocytosis.

In summary, we identified the *SHOC2* p.S2G mutation in eight patients with Noonan-like syndrome. Analysis of the detailed clinical manifestations of these patients showed that relative macrocephaly, hypertelorism, low-set ears, short stature, sparse/easily pluckable hair and a variety of skin abnormalities, including dark skin and atopic dermatitis, are frequently observed in patients positive for this mutation. A previous study and this study show that only one mutation (p.S2G) is causative for the phenotype. The rapid detection system for the *SHOC2* p.S2G mutation using the Lightcycler will be a useful tool to screen for this mutation in patient samples.

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