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 (%)	60 (55)	ND	79 (55)	ND	50 (55)	72 (55)	53 (45)	77 (55)
(mean for each patient's age)								
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.

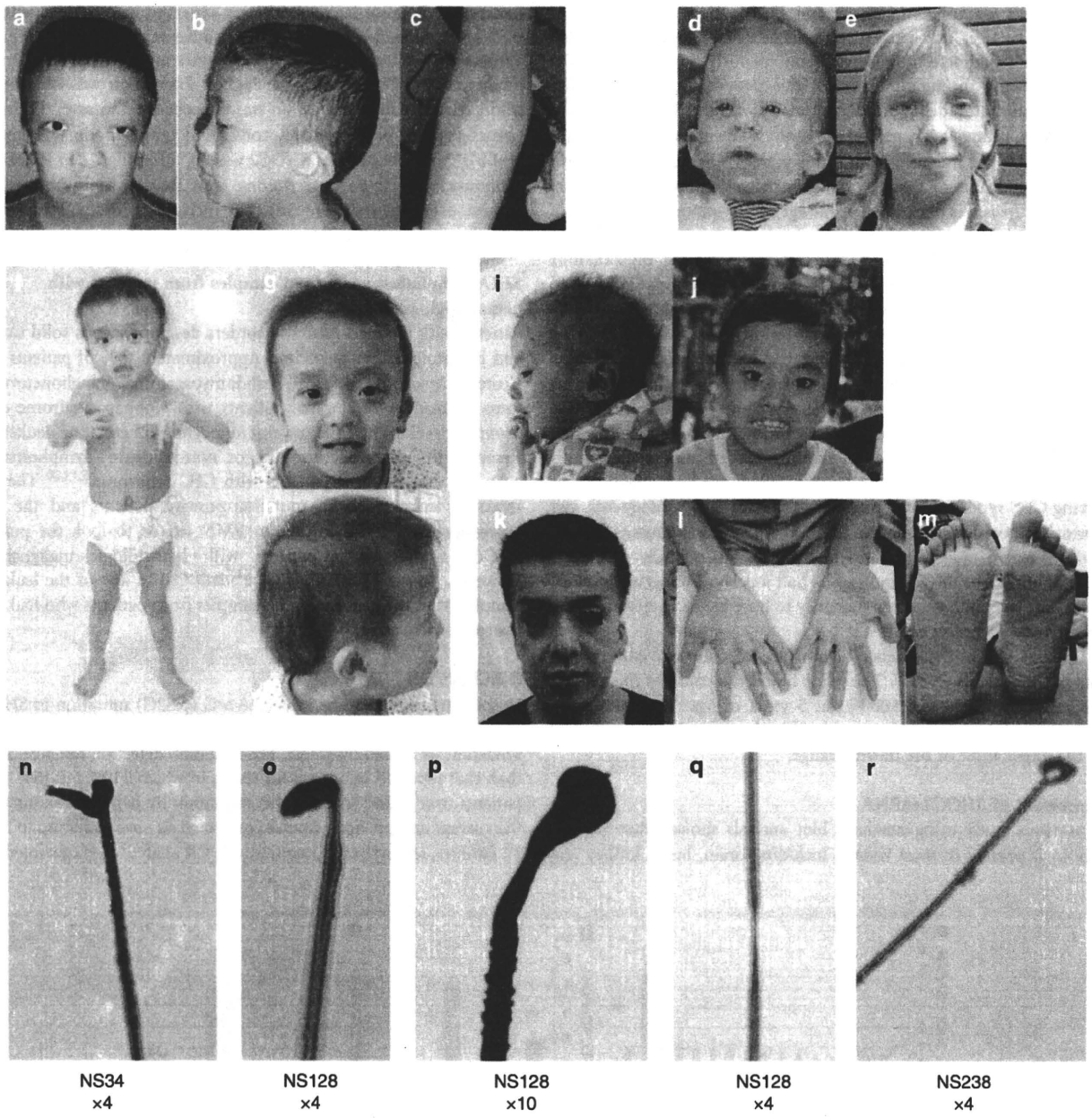

Figure 2 (**a**, **b**) Facial appearance of NS34 at the age of 13 years. (**c**) Dry and atopic skin seen in NS34. (**d**, **e**) NS93. (**f–h**) NS97. (**i**, **j**) NS128. (**k–m**) NS232 at the age of 25 years. (**l**, **m**) Palms and soles of NS232 showing fine wrinkling. Light micrographs of hairs 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 16000 to 23000/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

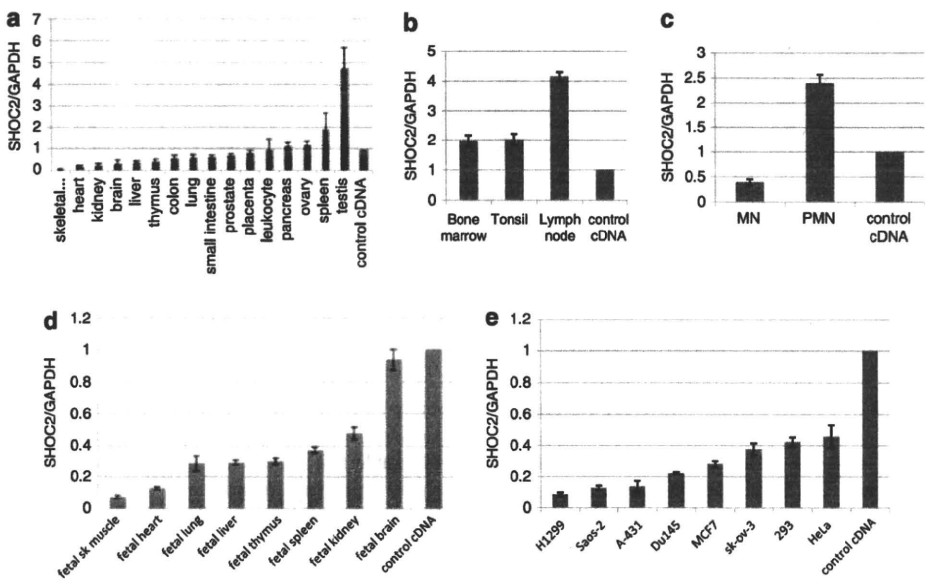

Figure 3 Relative expression of *SHOC2*. Expression levels of *SHOC2* mRNA in various adult human tissues (**a**), adult immune tissues (**b**), human leukocytes (**c**), human fetal tissues (**d**) and human tumor cell lines (**e**) were evaluated by quantitative PCR using *GAPDH* mRNA as the control. 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 12000 to 24600/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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RESEARCH ARTICLE

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Molecular and Clinical Analysis of *RAF1* in Noonan Syndrome and Related Disorders: Dephosphorylation of Serine 259 as the Essential Mechanism for Mutant Activation

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ABSTRACT: Noonan syndrome (NS) and related disorders are autosomal dominant disorders characterized by heart defects, facial dysmorphism, ectodermal abnormalities, and mental retardation. The dysregulation of the RAS/MAPK pathway appears to be a common molecular pathogenesis of these disorders: mutations in *PTPN11*, *KRAS*, and *SOS1* have been identified in patients with NS, those in *KRAS*, *BRAF*, *MAP2K1*, and *MAP2K2* in patients with CFC syndrome, and those in *HRAS* mutations in Costello syndrome patients. Recently, mutations in *RAF1* have been also identified in patients with NS and two patients with LEOPARD (multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness) syndrome. In the current study, we identified eight *RAF1* mutations in 18 of 119 patients with NS and related conditions without mutations in known genes. We summarized clinical manifestations in patients with *RAF1* mutations as well as those in NS patients with *PTPN11*, *SOS1*, or *KRAS* mutations previously reported. Hypertrophic cardiomyopathy and short stature were found to be more frequently observed in patients with *RAF1* mutations. Mutations in *RAF1* were clustered in the conserved region 2 (CR2) domain, which carries an inhibitory phosphorylation site (serine at position 259; S259). Functional studies revealed that the RAF1 mutants located in the CR2 domain resulted in the decreased phosphorylation of S259, and that mutant RAF1 then dissociated from 14-3-3, leading to a partial ERK activation. Our results suggest that the dephosphorylation of S259 is the primary pathogenic mechanism in the activation of RAF1 mutants located in the CR2 domain as well as of downstream ERK.
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KEY WORDS: RAS; MAPK; RAF1; Noonan syndrome; PTPN11; hypertrophic cardiomyopathy

Introduction

Noonan syndrome (NS; MIM# 163950) is an autosomal dominant developmental disorder characterized by facial dysmorphism, including hypertelorism, low-set ears, ptosis, short stature, skeletal abnormalities, and heart defects [Allanson et al., 1985; Mendez and Opitz, 1985]. Frequently observed features in NS patients are pulmonary stenosis (PS), hypertrophic cardiomyopathy, chest deformities, a webbed/short neck, mental

Additional Supporting Information may be found in the online version of this article.

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retardation, genitourinary defects including cryptorchidism in males, and bleeding diathesis due to factor XI deficiency. The incidence of this syndrome is estimated to be 1 in 1,000–2,500 live births. LEOPARD (multiple *l*entigines, *e*lectrocardiographic conduction abnormalities, *o*cular hypertelorism, *p*ulmonary stenosis, *a*bnormal genitalia, *r*etardation of growth, and sensorineural *d*eafness) syndrome (MIM# 151100) is known to be a NS-related disorder [Digilio et al., 2002]. The features of NS overlap with those of Costello syndrome and cardio-facio-cutaneous (CFC) syndrome. Patients with Costello syndrome (MIM# 218040) show distinctive facial features, mental retardation, high birth weight, neonatal feeding problems, curly hair, nasal papillomata, deep skin creases at palms and soles, and hypertrophic cardiomyopathy [Hennekam, 2003]. CFC syndrome (MIM# 115150) is characterized by distinctive facial features, mental retardation, heart defects (PS, atrial septal defect [ASD], and hypertrophic cardiomyopathy), and ectodermal abnormalities such as sparse, friable hair, hyperkeratotic skin lesions, and a generalized ichthyosis-like condition [Reynolds et al., 1986].

The molecular pathogenesis of these syndromes has been investigated. Tartaglia et al. [2001] have identified missense mutations in *PTPN11*, a gene encoding protein tyrosine phosphatase (PTP) SHP-2, in 45% of clinically diagnosed NS patients. Specific mutations in *PTPN11* has been identified in patients with LEOPARD syndrome [Digilio et al., 2002]. In 2005, we identified *HRAS* germline mutations in patients with Costello syndrome [Aoki et al., 2005]. Mutations in *KRAS*, *BRAF*, and *MAP2K1/2* have been identified in those with CFC syndrome [Niihori et al., 2006; Rodriguez-Viciana et al., 2006]. Mutations in *KRAS* and *SOS1* have also been identified in patients with NS [Roberts et al., 2007; Schubbert et al., 2006; Tartaglia et al., 2007]. Mutations in *NF1* and *SPRED1* have been identified in patients with neurofibromatosis type I (MIM# 162200) [Brems et al., 2007]. These findings suggest that dysregulation of the RAS/RAF/MEK/ERK pathway causes NS and related disorders, and thus it has been suggested that these syndromes be comprehensively termed the RAS/MAPK syndromes [Aoki et al., 2008] or the neuro-cardio-facial-cutaneous syndrome [Bentires-Alj et al., 2006].

In 2007, gain-of-function mutations in *RAF1* were identified in 3–17% of patients with NS and two patients with LEOPARD syndrome [Pandit et al., 2007; Razzaque et al., 2007]. RAF1 is a member of the RAF serine–threonine kinase family and transmits the upstream RAS signaling to downstream MEK and ERK. RAF1, ARAF, and BRAF share three conserved regions, CR1, CR2, and CR3 [Mercer and Pritchard, 2003]. Mutations in *BRAF* identified in patients with CFC syndrome are clustered in CR1 and CR3 domains [Aoki et al., 2008]. In contrast, reported *RAF1* mutations in NS and LEOPARD syndrome were located in the CR2 domain and some mutations were located in CR3 domain. These mutants had enhanced RAF1 kinase activities and most mutations, but not all, showed enhanced phosphorylation of ERK1/2 [Pandit et al., 2007; Razzaque et al., 2007]. Pandit et al. [2007] suggested that RAF1 mutations might interfere with RAF1 phosphorylation at serine 259 as well as with 14-3-3 interaction, and reported that p.P261S did not bind to 14-3-3. However, the mechanisms of RAF1 activation in mutants remain unexplained.

In the present study, we analyzed the *RAF1* gene in 119 patients with NS and related phenotypes without mutations in *PTPN11*, *HRAS*, *KRAS*, *BRAF*, *MAP2K1/2*, and *SOS1*. Detailed clinical manifestations in our new patients with *RAF1* mutations were evaluated, and those in patients with *RAF1 KRAS*, *PTPN11*, and *SOS1* mutations previously reported by us and others were examined. Furthermore, we explored the molecular mechanisms by which RAF1 mutants are activated.

Materials and Methods

Patients

One hundred nineteen patients with NS or related phenotypes were recruited. The primary diagnoses made by clinical dysmorphologists and general pediatricians were as follows: 44 patients with NS, 46 patients with CFC syndrome, 25 patients with Costello syndrome, and 4 patients with atypical phenotypes. No mutations in *PTPN11*, *HRAS*, *KRAS*, *BRAF*, *MAP2K1*, *MAP2K2*, or *SOS1* were identified in these patients. Control DNA was obtained from 105 healthy Japanese individuals. Control DNA from 105 healthy Caucasian individuals was purchased from Coriell Cell Repositories (Camden, NJ). 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 six patients.

Mutation Analysis in *RAF1*

Genomic DNA was isolated from the peripheral blood leukocytes of the patients. Each exon with flanking intronic sequences in *RAF1* was amplified with primers based on GenBank sequences (Supp. Table S1; GenBank accession no. NC_000003.10). The M13 reverse or forward sequence was added to the 5′ end of the polymerase chain reaction (PCR) primers for use as a sequencing primer. PCR was performed in 30 μl of a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10% (v/v) DMSO, 24 pmol of each primer, 100 ng genomic DNA, and 1.5 units of Taq DNA polymerase. The reaction conditions consisted of 35 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec, and extension at 72°C for 40 sec. The products were gel-purified and sequenced on an ABI PRISM 310 or 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Determination of the RAF1 phosphorylation status

The expression construct, including a *RAF1* cDNA (pUSEamp-*RAF1*), was purchased from Millipore (Billerica, MA). A Myc-tag was introduced at the 5′ terminus of the cDNA by PCR and the PCR product was subcloned into pCR4-TOPO (Invitrogen, Carlsbad, CA). The entire cDNA was verified by sequencing. A single-base substitution resulting in p.H103Q, p.R191I, p.S257L, p.S259F, p.P261A, p.N262K, or p.S427G was introduced using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All mutant constructs were verified by sequencing. The Myc-tagged wild-type *RAF1* cDNA and mutant cDNAs were digested with *Eco*RI and *Eco*RV and subcloned into the *Eco*RI–*Eco*RV site of the pUSEamp-*RAF1*.

COS7 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in DMEM containing 10% fetal calf serum (FCS), 50 U/ml penicillin, and 50 μg/ml streptomycin. COS7 cells were seeded at 1×10^5 cells per 6-cm dish, and 24 hr later, 2.0 μg of pUSE vectors encoding one of the wild-type (WT) or mutant RAF1 cDNAs were transfected using 8 μl of PLUS Reagent and 12 μl of Lipofectamine Reagent (Invitrogen). After 3 hr, the medium was replaced to complete medium. After 48-hr culture, cells were scraped and collected by centrifugation after two washes with phosphate-buffered saline

(PBS). Lysates were prepared in 100-μl lysis buffer (10 mM Tris-HCl pH 8.0 and 1% SDS) and boiled for 3 min. The DNA was sheared with a syringe. The lysates were centrifuged at 14,000 × *g* for 15 min at 4°C and protein concentration was determined by Bradford assay. Thirty micrograms of protein was subjected to SDS-polyacrylamide gel electrophoresis (5–20% gradient gel) (ATTO, Tokyo, Japan), transferred to nitrocellulose membrane, and probed with anti-Myc antibody and phospho-specific RAF1 antibodies (Cell Signaling, Danvers, MA). All the membranes were visualized using a Western Lightning ECL-Plus Kit (Perkin-Elmer, Norwalk, CT). The following antibodies were used for Western blotting: anti-Myc (9E10, Santa Cruz Biotech, Santa Cruz, CA), antiphospho-c-Raf (S259) (Cell Signaling), antiphospho-c-Raf (S338) (Millipore), antiphospho-c-Raf (S289/296/301) (Cell Signaling), antiphospho-c-Raf (S621) (Millipore), and antineomycin phosphotransferase II (Millipore).

For immunoprecipitation, lysates were prepared in 1 ml of ice-cold RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1:100 protease inhibitor (Sigma, St. Louis, MO), 1:1000 phosphatase inhibitor (Sigma), and 1% Triton X) and incubated on ice for 15 min. Four hundred micrograms of protein was incubated with anti-Myc (9E10) antibody for 1 hr at 4°C. Immune complexes were collected by adding 50 μl of 50% protein G-Sepharose bead slurry (GE Healthcare, Milwaukee, WI) for 1 hr at 4°C, washed three times with RIPA buffer, and then boiled in 2 × SDS buffer. The samples were resolved in 5–20% gradient polyacrylamide gels, transferred to nitrocellulose membranes and probed with antiphospho-c-Raf (S259) and anti-Myc (9E10) antibodies.

Reporter Assay

NIH 3T3 cells (ATCC) were maintained in DMEM containing 10% newborn calf serum, 50 U/ml penicillin, and 50 μg/ml of streptomycin. One day prior to the transfection, the NIH 3T3 cells were plated in 12-well plates with a density of 1×10^5 cells per well. Cells were transiently transfected using Lipofectamine and PLUS Reagents with 700 ng of pFR-luc, 15 ng of pFA2-Elk1, 7 ng of phRLnull-luc, and 35 ng of WT or mutant expression constructs of *RAF1*. Eighteen hours after transfection, the cells were cultured in DMEM without serum for 24 hr. Cells were harvested in passive lysis buffer, and luciferase activity was assayed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Renilla luciferase expressed by phRLnull-luc was used to normalize the transfection efficiency. The experiments were performed in triplicate. Data are shown as mean ± SD. Statistical analysis was performed using Excel.

Binding of RAF1 with 14-3-3

An expression construct containing Myc- and Flag-tagged 14-3-3 ζ (pCMV6-14-3-3 ζ) was purchased from Origene (Rockville, MD). In order to remove the Myc-tag from the construct, the 3′ half of the cDNA and the Myc-tag were removed by digestion with *Eco*RV and the 3′ half of cDNA was filled using PCR. An S621A mutation, which impairs phosphorylation of S621 to bind 14-3-3, was introduced into pUSE RAF1 harboring WT, p.S257L, or p.N262K cDNA by a Quickchange Site-Directed Mutagenesis Kit. HEK293 cells (ATCC) were transfected with 2 μg RAF1 constructs and 2 μg pCMV6-14-3-3 ζ construct using Lipofectamine and PLUS Reagents. After 48 hr, cells were scraped and collected by centrifugation after two washes with PBS. Lysates were prepared as described above. The Myc-tagged RAF1 was immunoprecipitated with anti-Myc antibody (clone4A6, Millipore) for 1 hr at 4°C. Immune complexes were collected by adding 50 μl of 50% protein G-Sepharose bead slurry (GE Healthcare) for 1 hr at 4°C, washed three times with RIPA buffer, and then boiled in 2 × SDS buffer. The samples were resolved in 5–20% gradient polyacrylamide gels, transferred to nitrocellulose membranes, and probed with anti-FLAG M2 (Sigma) and anti-Myc antibodies. For immunoprecipitation of 14-3-3, anti-FLAG M2 antibody was used and immunoblotting was performed using anti-FLAG M2 and anti-c-Raf (Cell Signaling) antibodies.

Results

Mutation Analysis in Patients

We identified eight amino acid changes in 18 patients (Table 1). A C-to-T nucleotide change, resulting in an amino acid change p.S257L, was identified in 11 patients. Novel p.R191I (c.572G>T) and p.N262K (c.786T>A) were identified in one each patient. Previously reported mutations, including p.S259F (c.776C>T), p.P261A (c.781C>G), p.P261L (c.782C>T), p.S427G (c.1279A>G), and p.L613V (c.1837C>G), were identified in a single patient. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in Genbank NM_002880.3, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. The mutation p.S427G, which has been reported in a patient with therapy-related acute myeloid leukemia [Zebisch et al., 2006], was identified in one patient. None of the newly identified mutations were observed in the control DNA of 105 ethnically matched healthy subjects. Parental samples were obtained from six patients (NS86, 92, 209, 210, 222, and 258). The analysis showed that p.S257L, p.P261A, and p.N262K occurred de novo. p.S427G was also identified as well in his 32-year-old mother, who also exhibited a Noonan phenotype with distinctive facial appearance, sparse hair in infancy, and multiple lentigines. The p.H103Q (c.309C>G) was identified in patient NS86, in whom p.S257L was also identified. This amino acid change was identified in one of his parents without any clinical features, suggesting that this amino acid change was polymorphic.

Clinical Manifestations of Patients with *RAF1* Mutations

Initial diagnoses of patients with *RAF1* mutations were as follows: NS in 11 patients, CFC syndrome in 4 patients, and Costello in 3 patients (Supp. Table S2). Four patients who were first diagnosed as having CFC syndrome were reclassified as NS because of facial features and normal mental development after identification of *RAF1* mutations. Three patients were diagnosed as having Costello syndrome. One patient was rediagnosed as having NS (NS135) and the other patient died at 1 month (NS209). Detailed information on clinical manifestations of NS205 was not available.

Detailed clinical manifestations in 18 patients with *RAF1* mutations were evaluated (Table 2 and Fig. 1). Nine of 15 patients had prenatal abnormality, including cystic hygroma, polyhydramnions, and asphyxia. Most patients had characteristic craniofacial abnormalities frequently observed in NS: relative macrocephaly (94%), hypertelorism (93%), downslanting palpebral fissures (63%), epicanthal folds (86%), and low-set ears (93%). Mental retardation was observed in 6 of 11 (55%) patients. Short stature (73%), short neck (93%), and webbing of neck (81%) were also observed. As for cardiac abnormalities, hypertrophic cardiomyopathy was observed in 10 of 16 patients (63%), followed by pulmonic stenosis (47%),

Table 1. *RAF1* Mutations Identified in This Study*

Patient ID	Country of origin	Final diagnosis	Exon	Nucleotide change	Amino acid change	Domain	Genotype of father/mother
NS213	France	atypical NS	5	c. 572G>T	p.R191I[a]	CR1	NA
NS39	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS86	France	NS	3, 7	c.309C>G	p.H103Q	CR1,	H103Q/WT
				c.770C>T	p. S257L	CR2	WT/WT
NS92	Germany	NS	7	c.770C>T	p.S257L	CR2	WT/WT
NS135	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS146	Spain	NS	7	c.770C>T	p.S257L	CR2	NA
NS199	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS200	France	NS	7	c.770C>T	p.S257L	CR2	NA
NS215	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS227	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS256	Japan	NS	7	c.770C>T	p.S257L	CR2	NA
NS258	Japan	NS	7	c.770C>T	p.S257L	CR2	WT/WT
NS279	Japan	NS	7	c.776C>T	p.S259F	CR2	NA
NS210	France	NS	7	c.781C>G	p.P261A	CR2	WT/WT
NS205	France	CS[b]	7	c.782C>T	p.P261L	CR2	NA
NS209	France	CS[c]	7	c.786T>A	p.N262K[a]	CR2	WT/WT
NS222	Japan	NS	12	c.1279A>G	p.S427G[d]	CR3	WT/p.S427G
NS285	Japan	NS	17	c.1837C>G	p.L613V	CR3	NA

NS, Noonan syndrome; CS, Costello syndrome; WT, wild type; CR, conserved region; NA, not available.
*GenBank RefSeq: NM_002880.3 Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.
[a]Novel mutation.
[b]Detailed clinical manifestations were not obtained.
[c]The patient died at 1 month.
[d]The mutation was previously identified in a patient with a therapy-related acute leukemia.

ASD (31%), arrhythmia (38%), and mitral valve anomaly (29%). Other observed clinical features were hyperelastic skin (58%), curly hair (47%), and cryptorchidism in males (50%). Coagulation defects were observed in two patients.

Four patients with *RAF1* mutations died before 5 years of age (Supp. Table S2). Patient NS39 were diagnosed as having cystic hygroma in the prenatal period and had suffered from neonatal hypertrophic cardiomyopathy. At 1 year of age, she contracted acute respiratory distress syndrome after having pneumonia and died of respiratory failure. Patient NS199 had been suspected to have achondroplasia because of short limbs. He was diagnosed as having NS at 3 years of age because of distinct facial features, growth failure, short stature, and hypertrophic cardiomyopathy. He had pneumonia without fever for a week and died suddenly at 5 years of age. Patient NS227 suffered from feeding difficulties, ectopic atrial tachycardia, as well as VSD and pulmonary hypertension. The patient died at 2 months of tachycardia (>200/min) and laryngeal edema.

Clinical manifestations in our patients with *RAF1* mutations were compared with those previously reported (Table 2). The high frequency of hypertrophic cardiomyopathy in our study (63%) was consistent with that observed in patients with *RAF1* mutations previously reported (77%). The frequency of ASD and that of mitral valve anomaly were similar to those of the previous studies. However, the frequency of PS in our study (47%) was higher than that previously reported (11%). Arrhythmia was less frequently observed in our patients with *RAF1* mutations (38 vs. 89%). The frequency of mental retardation (55%) was almost same as that of the previous studies (56%). Hyperelastic skin (58%) and coagulation defects (two cases) were also described in previously reported patients with *RAF1* mutations (24% and one case, respectively).

Phosphorylation State of Mutant RAF1 Proteins

RAF1 is a ubiquitously expressed RAF serine/threonine kinase, which regulates the RAS pathway. It has been shown that phosphorylation of serine, threonine, and tyrosine residues contributes to a conformational change of RAF1 protein and activation in growth factor stimulation [Mercer and Pritchard, 2003]. In the inactive state, phosphorylated S259 and S621 serve as binding sites for 14-3-3, leading to a closed confirmation [Dhillon et al., 2007]. Phosphorylation of S621 seems essential for RAF1 activation. In contrast, phosphorylation of serine 259 has been shown to have an inhibitory role in RAF1 activation. When cells are stimulated with growth factors, dephosphorylation of S259 by protein phosphatase 1 (PP1) and/or protein phosphatase 2A (PP2A) promotes the dissociation of 14-3-3 from RAF1, resulting in an activated conformation of RAF1 protein. For full activation, multiple residues, including S338, are phosphorylated and substrate of RAF1 enters the catalytic cleft in the CR3 kinase domain. Negative feedback from activated ERK results in the phosphorylation of S289, 296, and 301 [Dhillon et al., 2007].

To examine the phosphoryalation status of mutants observed in NS patients, we transfected constructs harboring WT *RAF1* cDNA and five mutants identified in NS patients. Immunoblotting was performed using four phospho-specific antibodies of RAF1 (Fig. 2A). We first analyzed the phosphorylation status of two phosphorylation sites, S259 and S621, using antibodies that recognize each site. Immunoblotting showed that phosphorylation of S259 was scarcely observed in cell lysates expressing p.S257L and p.N262K. In contrast, phosphorylation of S259 of p.H103Q, p.R191I, and p.S427G was similar to that in WT RAF1. To confirm this observation, immunoprecipitation was performed using an anti-Myc antibody, and phosphorylation levels at S259 were examined (Fig. 2B). Immunoprecipitated RAF1 mutants (p.S257L and p.N262K) were not phosphorylated at S259, confirming that these mutants had impaired phosphorylation of S259. The phosphorylation level of S621 in four mutants (p.H103Q, p.R191I, p.S257L, and p.N262K) was similar to that in WT (Fig. 2A), whereas that in cells expressing p.S427G was enhanced. Phosphorylation levels at S338 and S289/296/301 were similar to that in WT except for p.S427G (Fig. 2A).

Phosphorylation levels at S259, S289/296/301, S338, and S621 were shown to be enhanced in cells expressing p.S427G. The expression of p.S427G appeared enhanced and the band was

Table 2. Clinical Manifestations in *RAF1*-Positive Patients in This Study and Past Studies

	Present cohort (%)	NS with *RAF1* mutations (%)	LS with *RAF1* mutations (%)
Number of patients in total	17	35[a]	2
Perinatal abnormality			
Polyhydramnios	6/15 (40)	6/19 (32)	ND
Fetal macrosomia	5/11 (45)	6/20 (30)	ND
Growth and development			
Failure to thrive in infancy	10/12(83)	3	ND
Mental retardation	6/11 (55)	19/34 (56)	1
Outcome			
Died	4/17 (24)	2/11 (18)	ND
Craniofacial characteristics			
Relative macrocephaly	16/17 (94)	16/21 (76)	ND
Hypertelorism	14/15 (93)	20/21 (95)	2
Downslanting palpebral fissures	10/16 (63)	19/21 (90)	2
Ptosis	9/16 (56)	19/21 (90)	1
Epicanthal folds	12/14 (86)	12/21 (57)	1
Low-set ears	14/15 (93)	18/21 (86)	2
Skeletal characteristics			
Short stature	11/15 (73)	30/35 (86)	2
Short neck	14/15 (93)	21/31 (68)	2
Webbing of neck	13/16 (81)	25/30 (83)	2
Cardiac defects			
Hypertrophic cardiomyopathy	10/16 (63)	27/35 (77)	2
Atrial septal defect	5/16 (31)	11/35 (31)	0
Ventricular septal defect	3/17 (18)	3/35 (9)	0
Pulmonic stenosis	7/15 (47)	4/35 (11)	1
Patent ductus arteriosus	2/17 (12)	ND	ND
Mitral valve anomaly	5/17 (29)	8/32 (25)	2
Arrhythmia	6/16 (38)	8/9 (89)	ND
Others	TR 1, PH 1, atrioventricular valve dysplasia 1, valvular AS 1	polyvalvular dysplasia 2 pulmonary valve dysplasia 1, PFO 1, TOF 2, AS 1, right shaft deflection 1	
Skeletal/extremity deformity			
Cubitus valgus	2/9 (22)	7/22 (32)	2
Pectus deformity	5/13 (38)	20/31 (65)	2
Others		prominent finger pads 2	prominent finger pads 1
Skin/hair anomaly			
Curly hair	8/17 (47)	6/24 (25)	2
Hyperelastic skin	7/12 (58)	5/21 (24)	2
Café au lait spots	1/14 (7)	2/20 (10)	2
Lentigines	1/14 (7)	2/21 (10)	2
Naevus	3/15 (20)	9/22 (41)	0
Others	low posterior implantation 4, hyperpigmentation 3, redundant skin 3, sparse hair 2, sparse eyebrows 2, hemangioma 2	dry skin 3, sparse hair 3, sparse eyebrows 2, keratosis pilaris 2	
Genitalia	6/11 (55)	11/16 (69)	
Cryptorchidism	5/10 (50)	8/13 (62)	ND
Blood test abnormality			
Coagulation defects	2/11 (18)	1/4 (25)	ND

NS, Noonan syndrome; LS, LEOPARD syndrome; ND, not described; TR, tricuspid regurgitation; PH, pulmonary hypertension; AS, aortic stenosis; PFO, patent foramen ovale; TOF, tetralogy of Fallot.
[a]Includes affected family members. Clinical manifestations in 3, 21, and 11 NS patients with *RAF1* mutations were summarized from three reports [Ko et al., 2008; Pandit et al., 2007; Razzaque et al., 2007], respectively.

rather broad. However, Western blotting using antineomycin phosphoacetyltransferase antibody that recognizes the amount of plasmids introduced in cells showed that the transfection efficiency in cells expressing p.S427G was similar to that in cells expressing other mutants (Fig. 2A). These findings were consistently observed in three independent experiments. Recent studies have shown that autophosphorylation of S621 is required to prevent proteasome-mediated degradation [Noble et al., 2008]. To explore the possibility that p.S427G mutant is resistant to proteasome-mediated degradation, we examined the amount of WT RAF1 and p.S427G at 24, 48, and 72 hr after transfection in serum-starved or complete medium (Fig. 2C). The results showed that the expression of Myc-tagged RAF1 in cells expressing p.S427G was similar to that in WT RAF1, although multiple bands were observed, suggesting the hyperphosphorylation of the p.S427G mutant.

ELK Transactivation in Mutant RAF1 Proteins

To examine the effect on the downstream pathway of mutant RAF1, we introduced five RAF1 mutants into NIH3T3 cells and examined ELK transactivation (Fig. 2D). ELK is a transcription factor, which is phosphorylated by activated ERK and then binds the serum response element in the promoter of the immediate-early genes, including *C-FOS*. ELK transactivation was enhanced in cells expressing p.S257L, p.N262K, and p.S427G without any stimulation, suggesting that these mutants were gain-of-function

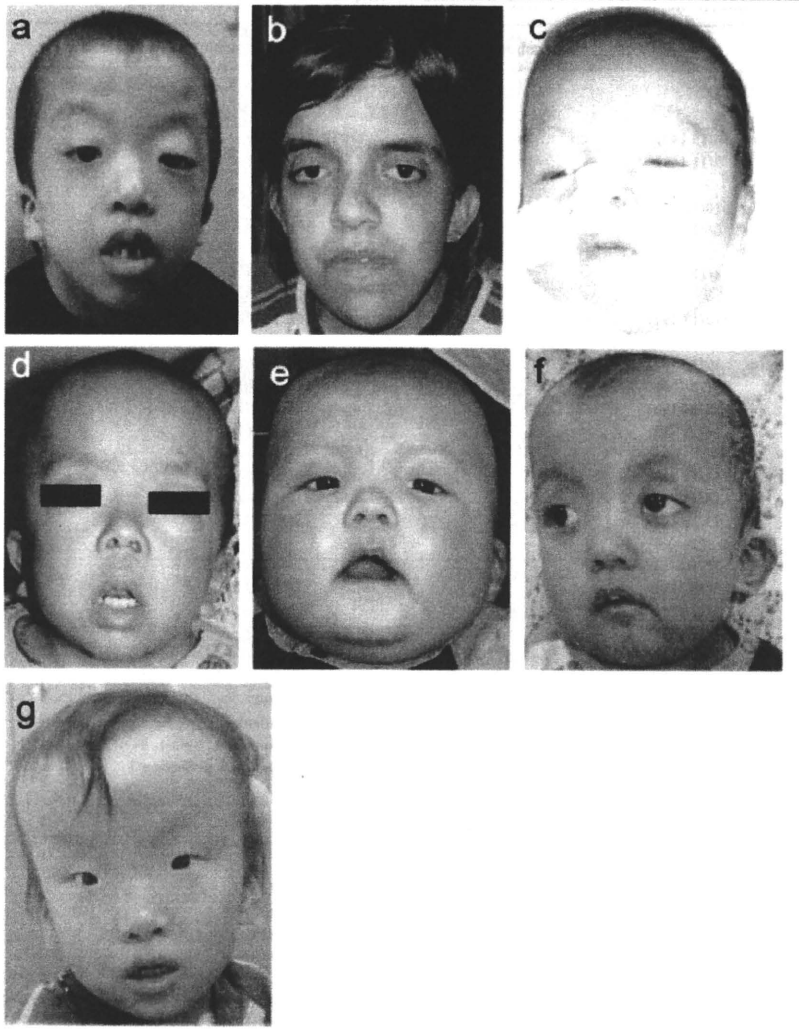

Figure 1. Facial appearance of patients with *RAF1* mutations. **a–f:** patients with p.S257L mutations. **a:** NS135; **b:** NS146; **c:** NS215; **d:** NS256; **e:** NS258 at 6 months; **f:** 2 years and 4 months; **g:** NS222 with p.S427G. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mutations. ELK transactivation in cells expressing p.H103G and p.R191I was not enhanced.

Phosphorylation State, ERK Activation, and Binding to the Scaffolding Protein 14-3-3 in Mutations in the CR2 Domain

Previous studies as well as the present study showed that mutations in NS-associated *RAF1* mutations were clustered in the CR2 domain. We hypothesized that amino acid changes in the CR2 domain impaired phosphorylation of serine at 259. We additionally generated expression construct harboring p.S259F and p.P261A substitutions, and their phosphorylation status was examined using anti-pRAF1 (S259) antibody together with RAF1 WT, p.S257L, p.N262K, and p.S427G (Fig. 3A). The results showed that phosphorylated proteins were scarcely observed in p.S257L, p.S259F, p.P261A, and p.N262K. Phosphorylation of ERK p44/42 was determined using anti-p-ERK (p44/42) antibody. All mutations activated the downstream ERK without any stimulation. The level of ERK phosphorylation in cells expressing mutants was lower than that in those treated with epidermal growth factor (EGF), suggesting that the expression of p.S257L, p.S259F, p.P261A, and p.N262K resulted in a partial activation of ERK.

Anti-pRAF1 (S259) antibody was produced by immunizing rabbits with a synthetic phospho-peptide corresponding to residues surrounding Ser259 of human RAF1. To examine if this antibody was able to recognize phosphorylation at S259 when mutations such as S257L and N262K were introduced, we performed a solid-phase immunoassay using biotinylated peptides as per the manufacturer's recommendation (Mimotopes, Victoria, Australia; Supp. Methods). The result showed that at least in peptides, this antibody could recognize serine phosphorylation in amino acid 259 when mutations S257L and N262K were introduced (Fig. 3B). These results support the data in Figure 3A, suggesting that S259 was not phosphorylated in mutants in the CR2 domain.

To examine if the RAF1 mutants without S259 phosphorylation were able to bind to 14-3-3, we cotransfected three double mutants (WT/S621A, S257L/S621A, and N262K/S621A) with FLAG-tagged 14-3-3, and coimmunoprecipitation was performed using anti-Myc antibody (Fig. 3C). The result showed that the WT/S621A mutant bound 14-3-3. In contrast, p.S257L/S621A and

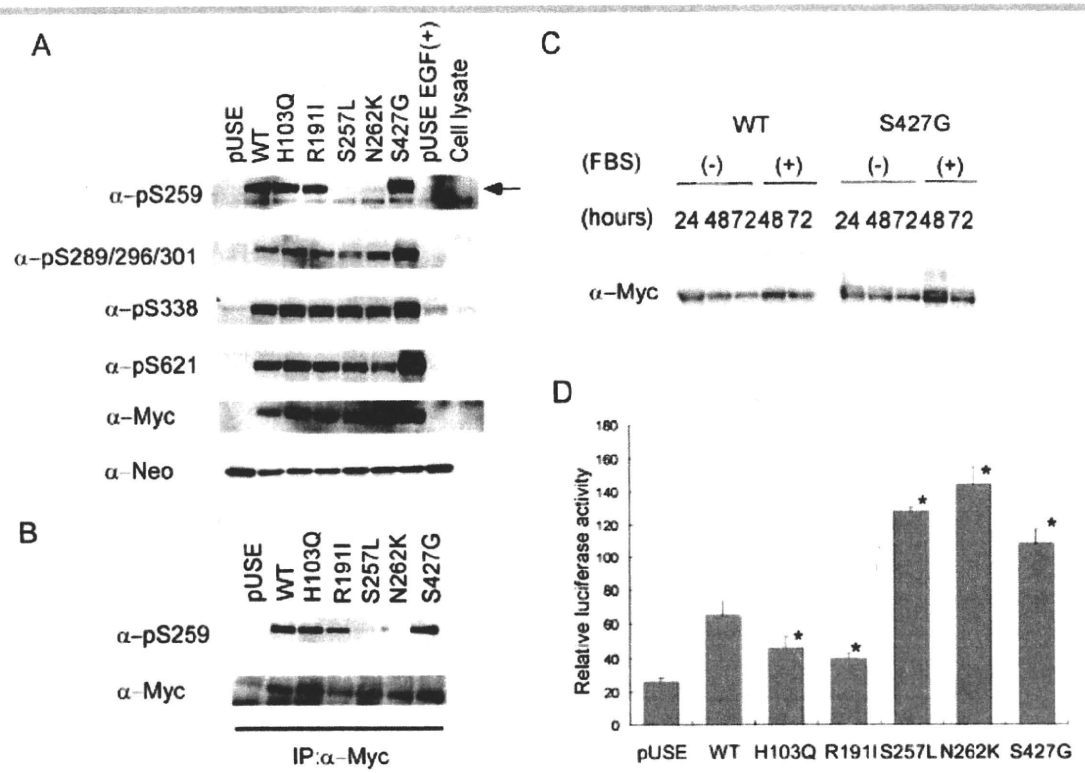

Figure 2. Analysis of phosphorylation status, degradation, and effect on downstream signaling in RAF1 mutants identified in this study. **A:** Phosphorylation status of wild-type (WT) RAF1 and mutants. Expression levels of RAF1 proteins and their phosphorylation levels were detected with different antibodies indicated in the figure. Transfection efficiency was measured using antineomycin phosphotransferase II (α-Neo) antibody. The arrow indicates the serine-phosphorylated expressed RAF1. **B:** Phosphorylation of S259 was confirmed by immunoprecipitation. Myc-tagged RAF1 was immunoprecipitated using anti-Myc antibody and the phosphorylation of S259 was determined. **C:** Time course experiments of WT RAF1 and p.S427G. The RAF1 protein was detected using anti-Myc antibody (clone 4A6; Millipore). FBS, fetal bovine serum. **D:** ELK transactivation in WT and mutants. Results are expressed as the means and standard deviations of mean values from triplicate samples. A significant increase in relative luciferase activity (RLA) was observed in cells transfected with p.S257L, p. N262K, and p.S427G, but not in cells transfected with p.H103Q or p.R191I. WT, wild-type; $^{*}P<0.01$ by Student's *t*-test.

p.N262K/S621A mutants did not bind 14-3-3, suggesting that the decreased phosphorylation of S259 prevented 14-3-3 binding. A similar result was obtained in the coimmunoprecipitation study using anti-FLAG antibody (Fig. 3D). These results showed that mutants in the CR2 domain impaired phosphorylation of S259, abrogated the binding to 14-3-3 and resulted in a partial activation of ERK.

Discussion

In this study, we identified eight different *RAF1* mutations in 18 patients: p.S257L in 11 patients and p.R191I, p.S259F, p.P261A, p.P261L, p.N262K, p.S427G, and p.L613V in one patient each. Sixteen patients were diagnosed as having NS, although we were not able to reevaluate 2 patients with Costello syndrome. Examination of detailed clinical manifestations in the present study and past studies showed that patients with *RAF1* mutations were associated with hypertrophic cardiomyopathy, arrhythmia, and mental retardation. Results from previous studies and the present study showed 41/52 (79%) mutations to be located in the CR2 domain (Fig. 3E). We first demonstrated that mutations in the CR2 domain had impaired phosphorylation of S259. This caused the impaired binding of RAF1 to 14-3-3, resulting in a partial activation of downstream ERK. These results suggest that dephosphorylation of S259 is the primary mechanism of activation of mutant RAF1 located in the CR2 domain.

Phosphorylation of S259 and subsequent binding to 14-3-3 have been shown to be important for suppression of RAF1 activity [Dhillon et al., 2007]. Light et al. [2002] examined the phosphorylation status at S259 in the p.S257L mutant. Their experiment showed that phosphorylation of S259 still existed in the p.S257L mutant. The mutant was not able to bind 14-3-3 [Light et al., 2002]. In contrast, our functional studies demonstrated that all four mutants located in the CR2 domain (p.S257L, p.S259F, p.P261A, and p.N262K) impaired phosphorylation of S259 and that two of them impaired binding of 14-3-3. Impaired binding to 14-3-3 was also shown in p.P261S mutant [Pandit et al., 2007]. The reason for the difference on S259 phosphorylation between the result by Light et al. [2002] and ours is unclear. Enhanced kinase activities of mutants, including p.S257L, p.P261S, p.P261A, and p.V263A, were demonstrated in a previous study [Razzaque et al., 2007]. Phosphorylation levels at S338 in p.S257L and p.N262K were not enhanced compared to that in WT RAF1 (Fig. 2A), suggesting that the activation mechanism in these mutants is different from that of the normal state upon RAS-GTP binding. Indeed, ERK activation was partial compared with that in cells after EGF treatment (Fig. 3A). These results suggest that the conformational change around S259 due to amino acid changes results in the decreased phosphorylation of S259 and that mutant

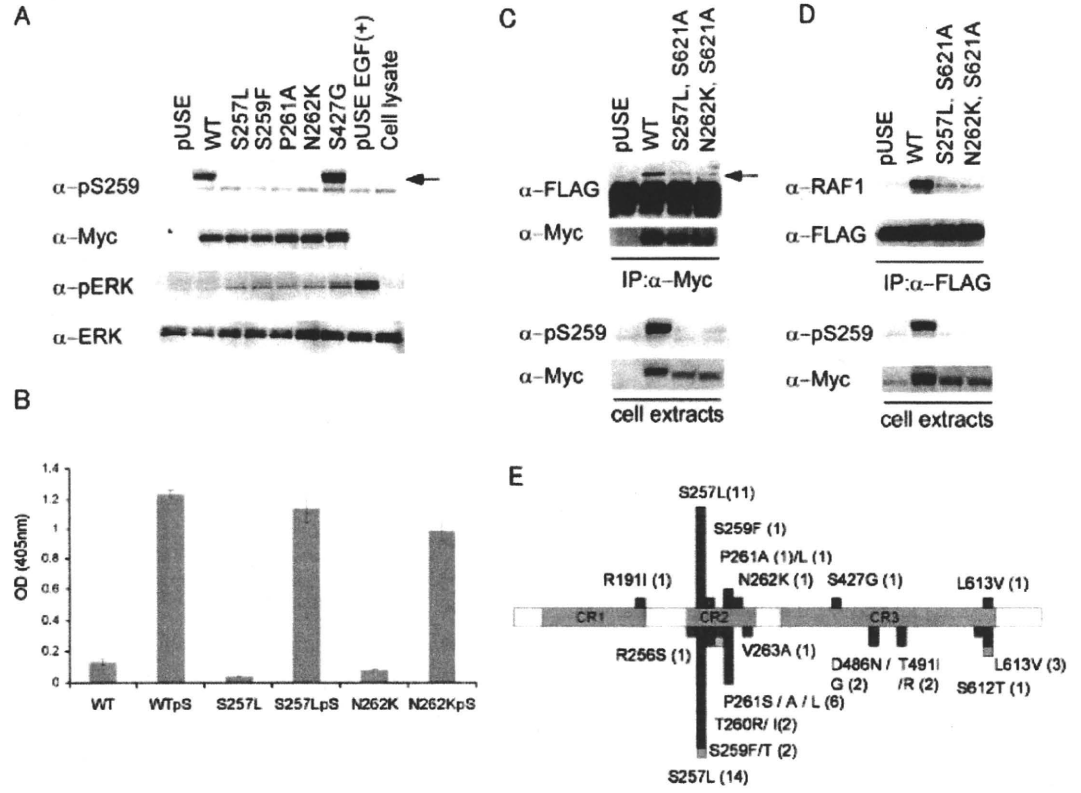

Figure 3. Phosphorylation of S259, binding to 14-3-3 and ERK activation of mutants located in the CR2 domain. **A:** Phosphorylation status of WT and mutants located in the CR2 domain. Phosphorylation of S259 was not observed in cells expressing p.S257L, p.S259F, p.P261A, and p.N262K. In order to examine the level of full activation of ERK, mock-transfected cells were treated with 10 ng/ml EGF. ERK activation was observed in cells expressing p.S257L, p.S259F, p.P261A, and p.N262K, but was weaker than those in cells expressing p.S427G and EGF-treated cells. The arrow indicates the serine-phosphorylated expressed RAF1. **B:** Epitope mapping of the anti-pRAF1 (S259) antibody using a solid-phase immunoassay. The antibody was able to recognize peptides with S257L or N262K mutations when S259 was phosphorylated, but was not able to recognize peptides without Ser259 phosphorylation. Results are expressed as the means and standard deviations of mean values from triplicate samples. **C:** Binding of RAF-1 to 14-3-3ζ. HEK293 cells were transfected with constructs harboring FLAG-tagged 14-3-3 and one construct of pUSE WT, p.S257L/p.S621A, or p.N262K/ p.S621A. Immunoprecipitation was performed using anti-Myc antibody, and 14-3-3 binding was determined by anti-FLAG antibody (upper panel). Phosphorylation of S259 and RAF1 expression were determined in cell lysates used for the immunoprecipitation (lower panel). The arrow indicates the band for 14-3-3. **D:** Binding of 14-3-3ζ to RAF-1. Immunoprecipitation was performed using anti-FLAG antibody and RAF1 binding was examined using anti-RAF1 antibody (upper panel). The binding of 14-3-3 to endogenous RAF1 was scarcely observed (lane 1, pUSE). Phosphorylation of S259 and RAF1 expression were determined using cell lysates used for the immunoprecipitation (lower panel). **E:** Domain organization and the distribution of mutations in RAF1 protein. The three regions conserved in all RAF proteins (conserved region [CR] 1, CR2, and CR3) are shown in pink. Mutations identified in this study are shown above the bar and those reported before [Ko et al. 2008; Pandit et al. 2007; Razzaque et al. 2007] are shown below the bar. Green squares indicate families with NS. Orange squares indicate patients with LEOPARD syndrome and the yellow square indicates a patient with hypertrophic cardiomyopathy.

RAF-1 then dissociates from 14-3-3; the substrate would thus be targeted to the catalytic domain in the CR3 domain (Fig. 4).

To highlight the clinical pictures of patients with *RAF1* mutations, clinical manifestations in 52 patients with *RAF1* mutations [Ko et al., 2008; Pandit et al., 2007; Razzaque et al., 2007], 172 patients with *PTPN11* mutations [Jongmans et al., 2005; Musante et al., 2003; Tartaglia et al., 2002; Zenker et al., 2004], 73 patients with *SOS1* mutations [Ferrero et al., 2008; Narumi et al., 2008; Roberts et al., 2007; Tartaglia et al., 2007; Zenker et al., 2007a] and 18 patients with *KRAS* mutations [Carta et al., 2006; Ko et al., 2008; Lo et al., 2008; Schubbert et al., 2006; Zenker et al., 2007b] are summarized in Table 3. The frequency of perinatal abnormalities was similar between patients with *RAF1* and *SOS1*. In contrast, the description of perinatal abnormalities was rare in patients with *PTPN11* and *KRAS* mutations. Growth failure and mental retardation were observed in 100 and 94% of NS with *KRAS* mutations, respectively. Growth failure and mental retardation were observed in 87 and 56% of patients with *RAF1* mutations, respectively. In contrast, those manifestations were less frequent (63 and 43%) in patients with *PTPN11* mutations. The frequency of mental retardation was lowest in patients with *SOS1* mutations (18%). We were unable to compare gene-specific features in craniofacial characteristics because such details were not described in the previous reports. As for skeletal characteristics, short stature was frequently manifested in patients with *RAF1* mutations (82%) followed by *KRAS* mutation-positive patients (71%). The association of short stature was lower in *PTPN11* mutation-positive and *SOS1* mutation-positive patients (56 and 38%, respectively). It is noteworthy that the association of hypertrophic cardiomyopathy was specifically high (73%) in *RAF1* mutation-positive patients. In contrast, hypertrophic cardiomyopathy was observed in 20% of clinically diagnosed Noonan

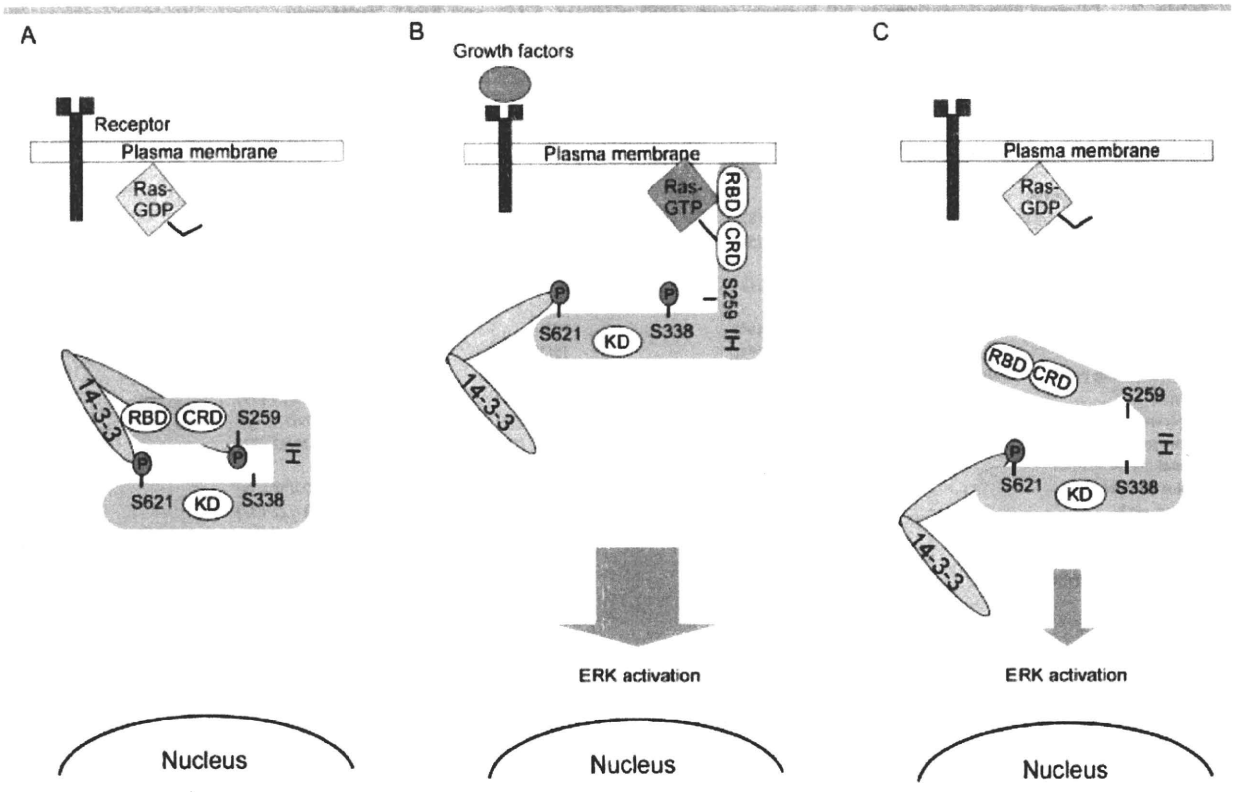

Figure 4. Schematic model of WT and mutant activation. **A:** In an inactive state, RAF1 is phosphorylated on S259 and S621 and is bound to 14-3-3. **B:** In growth-factor stimulation, the GTP-bound RAS binds to the CR1 domain of RAF1, which displaces 14-3-3. S259 is dephosphorylated by protein phosphatase 1 (PP1) and/or protein phosphatase 2A (PP2A). After RAF1 is recruited to the plasma membrane, phosphorylation of S338, Y341, T491, and S494 occurs. The phosphorylation of these residues is thought to be important for the full activation of RAF1. **C:** Mutants whose amino acid changes are located in the CR2 domain. It has been reported that S259 was phosphorylated by Akt and dephosphorylated by PP1 and/or PP2A. Amino acid changes in the CR2 domain would cause structural changes in the CR2 domain, leading to the access of PP2A to S259. Alternatively, Akt kinase would not be able to phosphorylate S259. S259 is dephosphorylated without stimulation and substrate(s) would be able to enter the kinase domain, leading to a partial activation. RBD, RAS-binding domain; CRD, cysteine-rich domain; KD, kinase domain; IH, isoform-specific hinge segment region. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

patients [van der Burgt 2007] and in 7, 10, and 17% of patients with *PTPN11*, *SOS1*, and *KRAS* mutations, respectively. These results strongly suggest that patients with *RAF1* mutations have a significantly higher risk of hypertrophic cardiomyopathy. Mitral valve abnormality and arrhythmia were also frequently observed in patients with *RAF1* mutations (27 and 56%, respectively). In summary, these results highlight specific manifestations of patients with *RAF1* mutations: high frequency of hypertrophic cardiomyopathy, septal defects of the heart, short stature, and less frequent PS (Supp. Fig. S1). The high frequency of heart defects would be associated with a high risk of sudden death in *RAF1* mutation-positive patients.

The present study is the first to identify p.S427G in a patient with NS. The same mutation has been reported in a patient with therapy-related acute myeloid leukemia [Zebisch et al., 2006]. The patient reported by Zebisch et al. [2006] first developed immature teratoma, yolk sack tumor, and embryonal testicular carcinoma. Thirty-five months after tumor resection and chemotherapy, the patient developed acute myeloid leukemia. Molecular analysis of *RAF1* revealed the de novo p.S427G mutation in leukemia cells and DNA from buccal epithelial cells [Zebisch et al., 2006]. Whether or not the patient had an NS phenotype was not mentioned. *RAF1* mutations have been rarely reported in malignant tumors. As far as we could determine, only six mutations, including p.P207S, p.V226I, p.Q335H, p.S427G, p.I448V, and p.E478K, have been identified in tumors and therapy-related leukemias [Pandit et al., 2007; Razzaque et al., 2007]. A previous study as well as our results showed that p.S427G mutant has transformation capacity [Zebisch et al., 2009], is resistant to apoptosis when introduced into NIH3T3 cells [Zebisch et al., 2009] and activates ERK and ELK transcription, suggesting that p.S427G is a gain-of-function mutation. We identified p.S427G in a familial case of NS. The mother and boy have not yet developed malignant tumors. Although no NS patients with *RAF1* mutations have developed malignant tumors, careful observation might be prudent in *RAF1* mutation-positive children.

We identified two novel mutations, p.R191I and p.N262K. p.R191I is located in the CR1, and arginine at amino acid position 191 is evolutionally conserved [Mercer and Pritchard, 2003]. Activation of ERK was not observed in cells expressing p.R191I. ELK transactivation was rather decreased; parental samples were not available. There is a possibility that this change is a polymorphism.

In conclusion, we identified *RAF1* mutations in 18 patients and detailed clinical manifestations in mutation-positive patients were examined. Our analysis of patients with mutations in *RAF1*, *PTPN11*, *SOS1*, and *KRAS* showed hypertrophic cardiomyopathy and short stature to be frequently observed in patients with *RAF1* mutations. Functional analysis revealed that dephosphorylation of S259 would be the essential mechanism for ERK activation in RAF1 mutations. Despite recent progress in molecular characterization of NS and related disorders, genetic causes in

Table 3. Clinical Manifestations in NS Patients with *RAF1*, *PTPN11*, *SOS1*, and *KRAS* Mutations

	RAF1[a] (%)	PTPN11[b] (%)	SOS1[c] (%)	KRAS[d] (%)
Total patients	52	172	73	18
Perinatal abnormality				
Polyhydramnios	12/34 (35)	ND	9/16 (56)	2
Fetal macrosomia	11/31 (35)	ND	9/15 (60)	ND
Growth and development				
Failure to thrive in infancy	13/15 (87)	35/56 (63)	ND	3/3 (100)
Mental retardation	25/45 (56)	71/164 (43)	12/67 (18)[e]	16/17 (94)[f]
Outcome				
Died	6/28 (21)	ND	ND	ND
Craniofacial characteristics				
Relative macrocephaly	32/38 (84)	ND	9/21 (43)[e]	9/11 (82)
Hypertelorism	34/36 (94)	15/28 (54)[e]	5/6 (83)	12/12 (100)
Downslanting palpebral fissures	29/37 (78)	19/28 (68)	20/22 (91)	9/12 (75)
Ptosis	28/37 (76)	18/29 (62)	19/24 (79)	10/15 (67)
Epicanthal folds	24/35 (69)	15/28 (54)	ND	2/9 (22)[e]
Low set ears	32/36 (89)	56/64 (88)	20/22 (91)	7/10 (70)
Skeletal characteristics				
Short stature	41/50 (82)	97/172 (56)[e]	22/58 (38)[e]	12/17 (71)
Short neck	35/46 (76)	15/29 (52)[e]	17/22 (77)	9/10 (90)
Webbing of neck	38/46 (83)	36/122 (30)[e]	3/6 (50)	7/14 (50)[e]
Cardiac defects				
Hypertrophic cardiomyopathy	37/51 (73)	10/135 (7)[e]	7/73 (10)[e]	3/18 (17)[e]
Septal defect	22/52 (42)	41/170 (24)[e]	17/73 (23)[e]	5/18 (28)
Atrial septal defect	16/51 (31)			4/18 (22)
Ventricular septal defect	6/52 (12)			1/18 (6)
Pulmonic stenosis	11/50 (22)	125/171 (73)[f]	53/73 (73)[f]	7/18 (39)
Patent ductus arteriosus	2/20 (10)	ND	ND	1/18 (6)
Mitral valve anomaly	13/49 (27)	ND	ND	3/18 (17)
Arrhythmia	14/25 (56)	ND	ND	ND
Skeletal/extremity deformity				
Cubitus valgus	9/31(29)	14/61 (23)	1/6 (17)	2/2 (100)
Pectus deformity	25/44 (57)	108/171 (63)	38/56 (68)	13/16 (81)
Skin/hair anomaly				
Curly hair	14/41 (34)	ND	15/22 (68)[f]	1/12 (8)
Hyperelastic skin	12/33 (36)	ND	1/6 (17)	3/12 (25)
Café au lait spots	3/34 (9)	ND	1/6 (17)	1/9 (11)
Lentigines	3/35 (9)	ND	ND	ND
Naevus	12/37 (32)	ND	ND	ND
Genitalia				
Cryptorchidism	13/23 (57)	75/138 (54)	22/39 (56)	4/11 (36)
Blood test abnormality				
Coagulation defects	3/15 (20)	46/90 (51)	14/66 (21)	2/9 (22)

ND, not described.
[a][Ko et al., 2008; Pandit et al., 2007; Razzaque et al., 2007]; and this study.
[b][Jongmans et al., 2005; Musante et al., 2003; Tartaglia et al., 2002; Zenker et al., 2004].
[c][Ferrero et al., 2008; Ko et al., 2008; Narumi et al., 2008; Roberts et al., 2007; Tartaglia et al., 2007; Zenker et al., 2007a].
[d][Carta et al., 2006; Ko et al., 2008; Lo et al., 2008; Schubbert et al., 2006; Zenker et al., 2007b].
[e]The frequency of the manifestation in patients with the gene was significantly lower compared with that observed in *RAF1*-positive patients ($P<0.05$ by Fisher's exact test).
[f]The frequency of the manifestation in patients with the gene was significantly higher compared with that observed in *RAF1*-positive patients ($P<0.05$ by Fisher's exact test).

approximately 30% of NS and related disorders remain unknown. Presently unknown genetic causes for mutation-negative NS and related disorders remain to be identified in molecules in future studies.

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Original article

A familial case of LEOPARD syndrome associated with a high-functioning autism spectrum disorder

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Abstract

A connection between LEOPARD syndrome (a rare autosomal dominant disorder) and autism spectrum disorders (ASDs) may exist. Of four related individuals (father and three sons) with LEOPARD syndrome, all patients exhibited clinical symptoms consistent with ASDs. Findings included aggressive behavior and impairment of social interaction, communication, and range of interests. The coexistence of LEOPARD syndrome and ASDs in the related individuals may be an incidental familial event or indicative that ASDs is associated with LEOPARD syndrome. There have been no other independent reports of the association of LEOPARD syndrome and ASDs. Molecular and biochemical mechanisms that may suggest a connection between LEOPARD syndrome and ASDs are discussed.

Keywords: LEOPARD syndrome, Noonan syndrome; Autism spectrum disorders (ASDs); RAS/MAPK signal transduction pathway

1. Introduction

LEOPARD syndrome (OMIM#151100) is a rare autosomal dominant disorder characterized by **L**entigines, **E**lectrocardiogram abnormalities, **O**cular hypertelorism, **P**ulmonic valvular stenosis, **A**bnormalities of genitalia, **R**etardation of growth, and **D**eafness. This syndrome is caused by germline missense mutations in the *PTPN11* gene that encodes Src homology 2 domain-containing tyrosine phosphatase 2 (Shp2): non-receptor protein-tyrosine phosphatase comprising two N-terminal SH2 domains, a catalytic domain, and a C terminus with tyrosylphosphorylation sites and a proline-rich stretch. The mutations induce catalytically impaired Shp2 by a "dominant negative effect" [1–2].

In the more common Noonan syndrome, approximately 50% of patients have *PTPN11* mutations scattered over the entire Shp2, including the catalytic domain. The mutations resulting in the Noonan phenotype are the "gain-of-function" mutations, and they exhibit substantially increased catalytic ability. Although LEOPARD syndrome and Noonan syndrome are caused by *PTPN11* mutations resulting in opposite effects, they share many common clinical features, including physical dysmorphic findings and intellectual disability [1].

The term "autism spectrum disorders (ASDs)" was first used by Lorna Wing [3] and then widely used as a category comprised of autistic disorder, Asperger's

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disorder, and other related conditions [4]. These conditions are very common neurobehavioral disorders that are characterized by impairments in three behavioral domains, including social interaction, language/communication/imaginative play, and a range of interests and activities [3–5].

At least ten genes have been reported to be associated with ASDs [6]. Except for Rett syndrome, the other pervasive developmental disorder (PDD) subtypes including autistic disorder, Asperger's disorder, disintegrative disorder, and PDD Not Otherwise Specified (PDDNOS) are not tightly linked to any particular gene mutations. Several common genetic syndromes are known to be associated with ASDs. Autism is frequent in patients with tuberous sclerosis (TSC) [7], with neurofibromatosis type 1 [8,9] and with Fragile X syndrome [10]. Studies of psychological profiles of adults with Noonan syndrome did not suggest a specific behavioral phenotype, but difficulties with social competence and emotional perceptions were noted [11]. A case of Noonan syndrome who was also diagnosed with autism was reported [12]. The present study of neuropsychiatric evaluation in a familial case of LEOPARD syndrome indicates all patients satisfied the criteria of ASDs. An association of LEOPARD syndrome and ASDs has not been reported previously. The familial case presented in this report may suggest such an association.

2. Patients and methods

After obtaining written informed consent, fifteen coding exons in *PTPN11* were sequenced in each patient following the methods described somewhere else [13].

Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV-TR) [5] and The high-functioning Autism Spectrum Screening Questionnaire (ASSQ) [14] were used in neuropsychiatric evaluation of the subjects.

Patient 1 is a 20-year-old male who was born as the second child to a non-consanguineous Japanese couple. His early developmental milestones were reportedly unremarkable. He was clinically diagnosed with LEOPARD syndrome at age 7 years based on findings that included lentigines, multiple café-au-lait spots, electrocardiogram (ECG) abnormalities, ventricular septal defect, ocular hypertelorism, short stature, and unilateral renal hypoplasia. *PTPN11* mutation analysis revealed a heterozygous mutation of 1403C > T (T468 M). The patient was diagnosed as having Asperger's disorder based on ASSQ and DSM-IV-TR, at age 12 years. His intelligence quotient (IQ) by the Wechsler Intelligence Scale for Children-third edition (WISC-III) was 85 (verbal: 77, performance 98). His ASSQ score by mother's rating was 41. He met the DSM-IV-TR diagnostic criteria of Asperger's disorder with all subcategories in the category of Qualitative impairment in social interaction (Category 1), three subcategories (1,2, and 4) in the category of Restricted repetitive and stereotyped patterns of behavior, interests and activities (Category 2), and the rest of the four categories (Table 1).

Patient 2 is a 15-year-old younger brother of Patient 1. His early infantile developmental milestones were unremarkable. He was diagnosed with growth retardation at age 2½ years. At age 12 years his clinical findings of a few café-au-lait spots, ocular hypertelorism, and undescended testes led us to obtain *PTPN11* mutation analysis, which showed the same heterozygous mutation of 1403C > T. At age 9 years, a diagnosis of Asperger's disorder was made based on ASSQ and DSM-IV-TR. His full-scale IQ by WISC-III at age 9 years was 99 (verbal 104, performance 92). His ASSQ score by parental rating was 32 at age 15 years. He also met the Asperger's disorder diagnostic criteria with all subcategories of Category 1, three of Category 2 (1, 2, and 4), and the rest of the categories (Table 1).

Patient 3 is the 22-year-old eldest brother of Patients 1 and 2. His developmental milestones were normal, although his ritualistic behavior and difficulties in relating to peers were noted in his childhood. He had a surgical repair of bilateral undescended testes and inguinal hernia. He was diagnosed with Wolff-Parkinson-White syndrome at age nine years. He has ocular hypertelorism and short stature. The same *PTPN11* heterozygous mutation found in the two younger siblings was identified in this patient. He attends college, and was diagnosed as having PDDNOS, because he also had impaired development of reciprocal social interaction associated with communication skills, repetitive routine, and ritualistic behavior. His ASSQ score was 7 at age 22 years (Table 1).

Patient 4 is a 55-year-old male who is the father of the siblings. He has prominent lentigines, bilateral mild hearing loss, cardiac anomalies, ECG abnormalities, short stature, and apparent ocular hypertelorism. His early developmental milestones are not well known. He has been noted to have obsession with a specific topic, repetitive routine and rituals, and clumsy movements. At age 50 years, his social skills and aggressive behavior were noted to be deteriorating, and consequently he was suspected of having Asperger's disorder based on DSM-IV-TR. He met the diagnostic criteria of Asperger's disorder with Category 1 (1 and 3), Category 2 (1 and 2), and the rest of the four categories. His ASSQ score was 20 at age 55 years by his wife's evaluation. He has the same heterozygous *PTPN11* mutation (Table 1).

3. Discussion

The presented familial case of LEOPARD syndrome included individuals (patients 1, 2, and 4) diagnosed with or suspected of having Asperger's disorder, and

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Table 1
Summary of clinical findings and *PTPN11* mutation.

	Pt. 1 Male	Pt. 2 Male	Pt. 3 Male	Pt. 4Male
Age	20 y	15 y	22 y	55 y
Physical findings				
Skin: café-au-lait spots	multiple	a few	a few	a few
Lentigines	+++	+++	–	+++
Cardiac defects	VSD	No	No	No
EKG abnormalities	+	No	WPW	No
Ocular hypertelorism	+	+	+	+
Pulmonary stenosis	No	No	No	No
Abnormal genitalia	No	Und. Testes*	Und. Testes*	No
Renal anomalies	R-hypoplasia	No	No	No
Retardation of growth	Yes	+	+	No
Deafness	No	No	No	Yes
Miscellaneous:				
Rocker bottom feet	Yes	Yes	Yes	No
Macrocephaly	Yes	Yes	Yes	No
PTPN11 mutation	T468 M	T468 M	T468 M	T468 M
Neuropsychological				
Diagnosis	AD**	AD**	PDDNOS***	AD**
ASSQ score[1] (age)	41 (12 y)	32 (15 y)	7 (22 y)	20 (50 y)
WISC-III[2] (age)	85 (12 y)	99 (9 y)	n/a	n/a
-Verbal/performance	77/98	104/92	n/a	n/a

* Und. Testes, undescended testes.
** AD, Asperger's disorder.
*** PDDNOS, Pervasive developmental disorder not otherwise specified.
[1] ASSQ score, Autism Spectrum Screening Questionnaire Score. The cutoff score of 3 predicts 91% of the true positive rate of Autistic spectrum disorders.
[2] WISC-III, Wechsler Intelligence Scale for Children-third edition.

patient 3 was diagnosed as having PDDNOS, which may lead to the diagnosis of ASD. ASDs were first introduced by Lorna Wing, who suggested that Asperger's disorder is a type of ASD and described in detail its various manifestations in speech, nonverbal communication, social interaction, motor coordination, motor clumsiness, and idiosyncratic interests [3]. Patient 3 did not have enough clinical symptoms to meet the diagnostic criteria for Asperger's disorder; however, he had some symptoms suggestive of ASD in his childhood that led to a diagnosis of PDDNOS.

The ASSQ is a 27-item checklist for completion by lay informants when assessing characteristic symptoms of Asperger's disorder and high-functioning autism in children and adolescents with normal intelligence or mild mental retardation. The ASSQ allows for rating on a 3-point scale (0, 1, or 2; 0 indicating normality, 1 some abnormality, and 2 definite abnormality). The range of possible scores is 0–54. The mean ASSQ parent scores in the Asperger's disorder validation sample were 25.1 (SD, 7.3) [14]. The cutoff score of 13 is 91% of the true positive rate of ASDs. The ASSQ score was established as a screening tool primarily for children between 6 and 17 years of age by parents and/or teachers. The delayed evaluation of patient 3 may account for the difference in diagnosis between this patient and his siblings.

ASDs are known to be associated with particular genetic disorders such as fragile X syndrome [10,15, 16], tuberous sclerosis (TSC) [7], and neurofibromatosis type 1 [8,9]. Fifty percent of children with TSC have behavioral problems in the form of ASDs. Gene mutations in either *TSC1* or *TSC2* influence neural precursors, resulting in abnormal cell differentiation and dysregulated control of cell size. These cells migrate to the cortex to generate an abnormal collection of inappropriately positioned neurons, causing widespread cortical disorganization and structural abnormalities [7]. Mutations in *PTPN11* causing LEOPARD syndrome induce catalytically impaired Shp2. In situ hybridization detected Shp2 expression in the neural ectoderm and nervous system in mouse embryos, suggesting an involvement of Shp2 in neural development. Shp2 is a critical signaling molecule in the coordinated regulation of progenitor cell proliferation and neuronal/astroglial cell differentiation. The studies with mutant mouse strains with Shp2 selectively deleted in neural precursor cells showed a dramatic phenotype of growth retardation, early postnatal lethality, and multiple defects in proliferation and cell fate specification in neural stem/progenitor cells [17]. The product of the TSC2 gene tuberin is known to up-regulate the B-RAF/MEK/MAPK signal transduction pathway. B-RAF is required for neuronal differentiation, suggesting another possible link between B-RAF signaling and the clinical manifestations of TSC including ASDs [18]. Disturbed neuronal cell differentiation and development due to mutations in

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