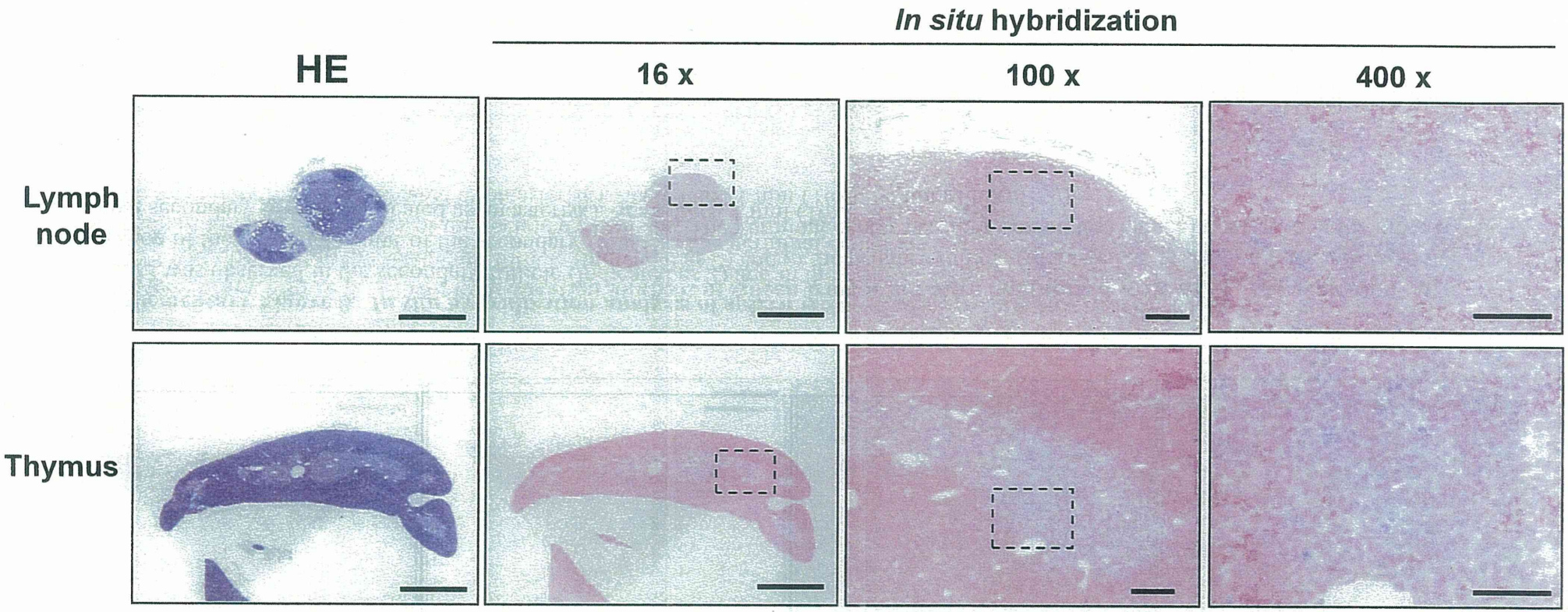
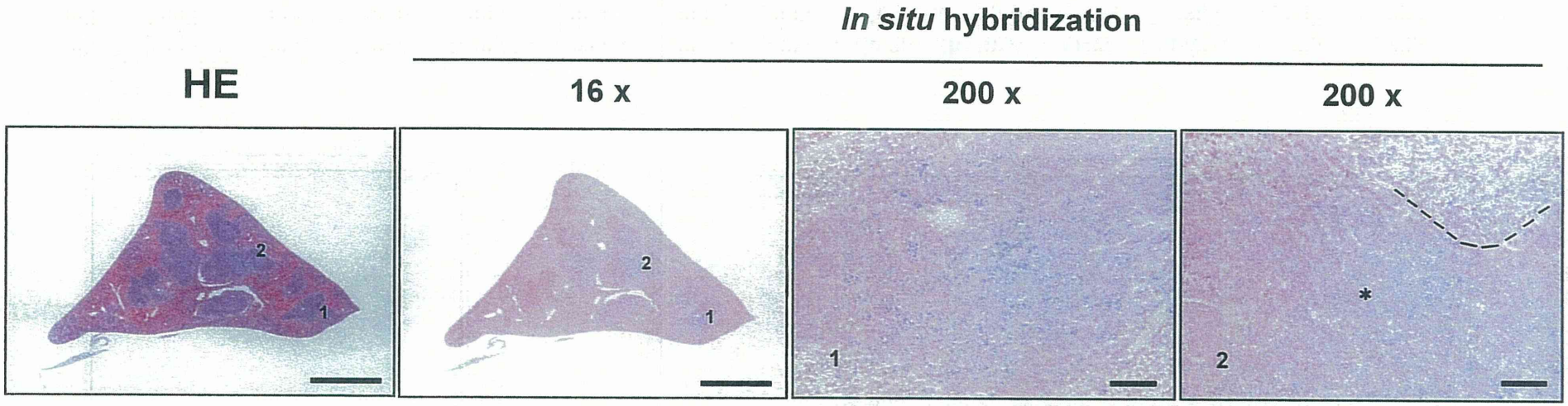
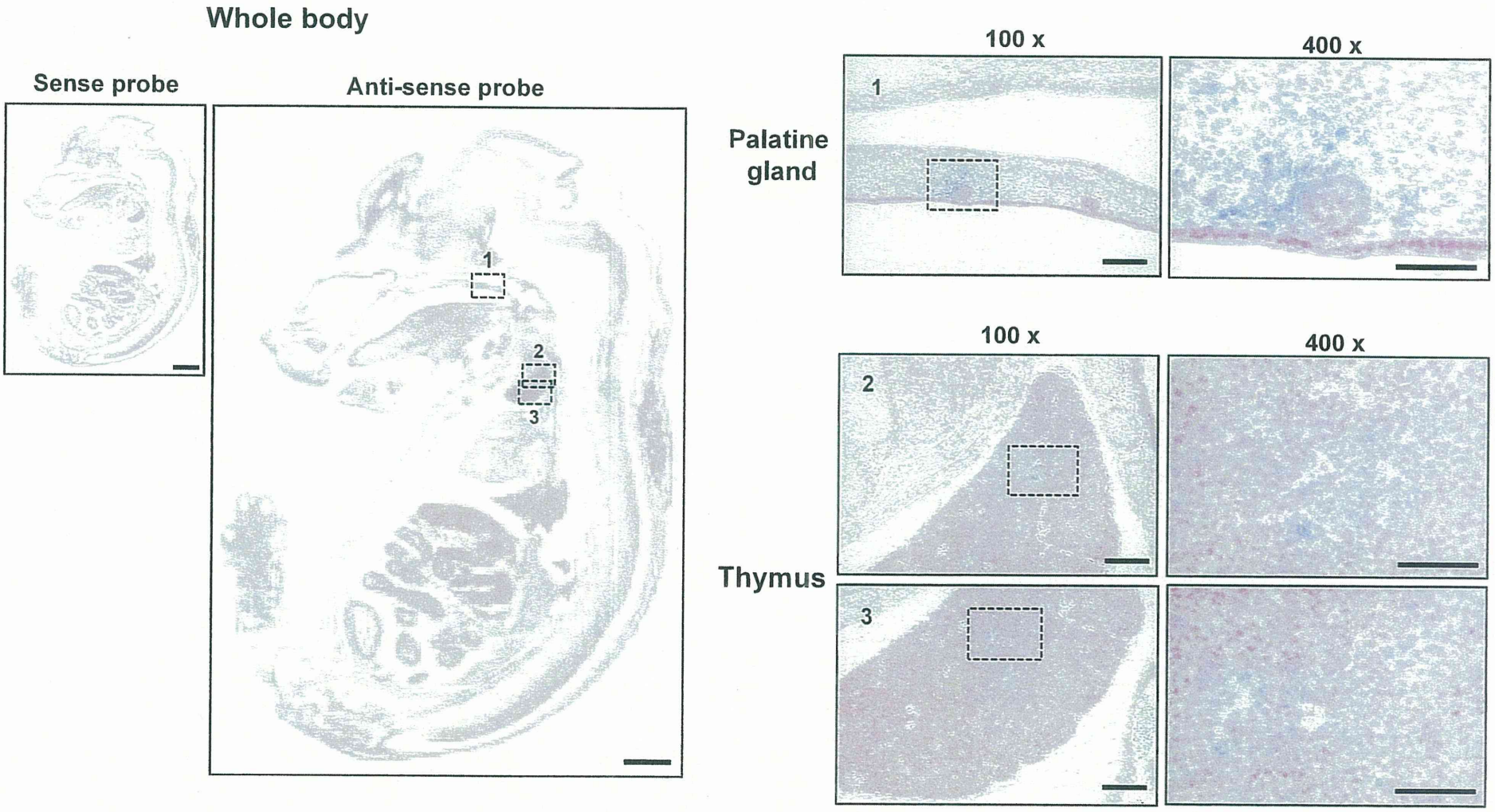


Supplementary Figure 7. *In situ* hybridization analysis of lymph node and thymus in mice. Small round cells expressing *Rnf213* mRNA was observed in the primary follicles in the lymph node. Specific signals for *Rnf213* mRNA was also detected in thymocytes in the medulla of the thymus. Dotted squares indicate the fields of the higher magnification images. HE stands for hematoxylin-eosin staining. Scale bars, 1 mm (16x), 100 μm (100x), 50 μm (400x).



Supplementary Figure 8. *In situ* hybridization analysis of spleen from adult mice immunized with KLH. The expression of *Rnf213* was observed in the secondary follicle (indicated as 2) and in the primary follicle (indicated as 1). Dotted lines indicated the location of the germinal center of the secondary follicles. The germinal center was represented less signal intensity than the other region of the secondary follicle (indicated as an asterisk). Scale bars, 1 mm (16x), 50 μm (200x).



Supplementary Figure 9. Expression of *RNF213* mRNA in mouse fetus. In an E16.5 mouse embryo, the *Rnf213* expression was observed in small round cells around mucous the palatine gland (1) and in medulla in thymus (2, 3). Dotted squares indicate the fields of the higher magnification images. No specific signals were detected by the sense probe. Scale bars, 1 mm (whole body), 100 μm (100x), 50 μm (400x).

ELSEVIER

Nonketotic Hyperglycinemia: Proposal of a Diagnostic and Treatment Strategy

Yuka Suzuki, MD*, Shigeo Kure, MD†, Masaaki Oota, MD*, Hitomi Hino, MD*, and Mitsumasa Fukuda, MD*

Early myoclonic encephalopathy presents neonatally with fragmented myoclonus and a suppression-burst electroencephalography pattern. We describe a newborn boy with early myoclonic encephalopathy caused by nonketotic hyperglycinemia. He presented with severe hypotonia, progressive apneic episodes, and erratic myoclonus. Screening of deletions in *GLDC*, using the multiplex ligation-dependent probe amplification method, and a ^{13}C breath test confirmed the diagnosis of nonketotic hyperglycinemia. Treatment with the *N*-methyl-D-aspartate receptor antagonist ketamine exerted dramatic suppressive effects on his seizures, and ameliorated his clinical status.

Suzuki Y, Kure S, Oota M, Hino H, Fukuda M. Nonketotic hyperglycinemia: Proposal of a diagnostic and treatment strategy. Pediatr Neurol 2010;43:221-224.

Introduction

Nonketotic hyperglycinemia, also known as glycine encephalopathy (Mendelian Inheritance in Man 605899), is an inborn metabolic disorder caused by a glycine cleavage system deficiency. The glycine cleavage system is a mitochondrial enzymatic complex consisting of four distinctive proteins (P, H, T, and L). The majority of confirmed mutations were discovered in genes encoding the P and T proteins. The typical neonatal form of nonketotic hyperglycinemia is characterized by lethargy, apnea, and myoclonic jerks in the early neonatal period. Some patients die during the newborn period, and survivors exhibit severe mental retardation and intractable seizures.

In patients with nonketotic hyperglycinemia, the dysfunction in the glycine cleavage system leads to glycine accumulation in all body compartments. Glycine acts as an inhibitory neurotransmitter in the brainstem and spinal cord, and as an excitatory neurotransmitter via the *N*-methyl-D-aspartate receptor in the cerebral cortex. The neurologic damage associated with nonketotic hyperglycinemia is mostly attributed to *N*-methyl-D-aspartate receptor overstimulation. The standard treatment strategies for nonketotic hyperglycinemia include a reduction of glycine by sodium benzoate [1], and a blockade of the *N*-methyl-D-aspartate receptor by the *N*-methyl-D-aspartate receptor antagonists dextromethorphan and ketamine [2,3]. However, no treatments have been proven to prevent neurologic sequelae. Here, we describe the clinical and electroencephalographic features of a patient with nonketotic hyperglycinemia whose seizures demonstrated dramatic responses to treatment with *N*-methyl-D-aspartate receptor antagonists. He was genetically confirmed to be a compound heterozygote diagnosed by the method of multiplex ligation-dependent probe amplification.

Case Report

A 3-day-old Japanese boy was transferred to our neonatal intensive care unit because of repetitive seizure-like movements and frequent apnea. Three days before admission, the boy was born at full term after a normal pregnancy and delivery. His birth weight was 2885 g, and his Apgar scores were 9 at 1 minute and 10 at 5 minutes. Immediately after birth, he appeared well, and manifested no signs of distress. Over the next several hours, poor sucking and apneic episodes became apparent, and assisted ventilation was initiated on the next day. On admission, he was markedly hypotonic, without spontaneous breathing or movements. Deep tendon reflexes were not elicited, but repetitive myoclonic responses were induced. Magnetic resonance imaging revealed hypoplasia of the corpus callosum and cerebellar vermis. Over the next week, he remained unresponsive and flaccid, without any sedative medications. His erratic myoclonus progressively worsened, and tonic posturing often appeared in quick succession after myoclonus. An electroencephalographic study demonstrated a suppression-burst pattern. At times, the paroxysmal bursts were roughly synchronous with myoclonus, whereas at other times, fragmented myoclonus appeared without electroencephalographic associations. The electroencephalographic and clinical features were consistent with a diagnosis of

From the *Department of Pediatrics, Ehime University Graduate School of Medicine, Toon, Ehime, Japan; and †Department of Pediatrics, Tohoku University School of Medicine, Miyagi, Japan.

Communications should be addressed to:
Dr. Suzuki; Department of Pediatrics, Ehime University Graduate School of Medicine; Shitsukawa; Toon, Ehime 791-0295, Japan.
E-mail: yusuzuki@m.ehime-u.ac.jp
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early myoclonic encephalopathy [4]. Analyses of blood and cerebrospinal fluid amino acids performed on day 21 revealed elevated glycine levels and ratios of cerebrospinal fluid to plasma glycine (Table 1). We rendered a clinical diagnosis of nonketotic hyperglycinemia, and treatment with sodium benzoate (150 mg/kg) orally and ketamine (0.8 mg/kg) intramuscularly was initiated at 30 days of age. His muscle tone gradually improved, and he was successfully extubated at age 45 days. Subsequently, a [1-^{13}C]glycine breath test performed at age 30 days indicated a decreased $^{13}CO_2$ excretion of 7.9% (normal range, 24.1% ± 4.0%) during 5 hours, which implied reduced glycine cleavage system activity [5]. Mutation analyses of *GLDC* collected on day 34 demonstrated compound heterozygosity, with a missense mutation of c.1382G>A, which resulted in an amino acid change of p.R461Q, and a large deletion involving all *GLDC* exons 1-25 (Fig 1). Based on these findings, we made a definitive diagnosis of nonketotic hyperglycinemia. Ketamine was then replaced with dextromethorphan (9 mg/kg). He manifested almost no seizures (including myoclonus and tonic seizures). His electroencephalogram indicated a continuous pattern consisting of medium-voltage θ and δ range activities, without clear epileptiform discharges.

At age 3 months, the patient was able to respond to sounds and follow objects horizontally with his eyes, and he was discharged from the hospital. At age 4.5 months, he began to manifest frequent seizures, and appeared to lose interest in feeding. At age 5 months, his erratic myoclonic seizures became nearly continuous, and repetitive truncal myoclonus was often followed by tonic seizures. His electroencephalogram revealed an alternating pattern without complete suppression periods. Based on high levels of glycine in his blood and cerebrospinal fluid (Table 1), ketamine was reintroduced. On the next day, the frequencies of his seizures were greatly reduced and he became more alert. Although his consciousness did not return to baseline level, he became relatively alert and could respond to external stimulations. He is now 10 months old, with developmental milestones appropriate to 5-6 months of age, and his seizures have been relatively well controlled with an increased dose of dextromethorphan.

Discussion

Early myoclonic encephalopathy is characterized by a very early onset of erratic myoclonus in the neonatal period. The main ictal phenomena include partial or fragmentary massive myoclonus, partial motor seizures, and the frequent late occurrence of repetitive tonic spasms. Early myoclonic encephalopathy is typically associated with underlying metabolic disorders, such as nonketotic hyperglycinemia, but most early myoclonic encephalopathy cases are cryptogenic [4]. Although both early myoclonic encephalopathy and nonketotic hyperglycinemia cases were previously reported, particular treatment strategies, depending on the underlying disorders, are not yet fully understood.

The clinical signs and seizures of our patient were dramatically ameliorated by ketamine, an *N*-methyl-D-aspartate receptor antagonist, during the newborn period and at 5 months of age. All his seizures (including complex partial seizures, tonic seizures, and myoclonus) were controlled with ketamine, independent of cerebrospinal fluid glycine levels. Therapy with the *N*-methyl-D-aspartate receptor antagonist was reported to exert beneficial effects on glycine inhibitory signs such as hypotonia and apnea [2,3]. However, little attention has been paid to the therapeutic benefits of administering the *N*-methyl-D-aspartate receptor antagonist for glycine excitatory signs such as seizures [6]. Ketamine is a noncompetitive *N*-methyl-D-aspartate receptor antagonist, whereas dextromethorphan is a competitive *N*-methyl-D-aspartate receptor antagonist. Changing the treatment from dextromethorphan to high dose ketamine successfully suppressed all of the patient's seizures, including partial as well as myoclonic and tonic seizures. The *N*-methyl-D-aspartate receptor plays an important role in the pathophysiology of seizures in various neurologic disorders. The inhibition of *N*-methyl-D-aspartate receptor overstimulation can constitute an efficient treatment for other types of intractable seizures [7]. The mechanism by which glutamatergic *N*-methyl-D-aspartate receptor hyperexcitability contributes to the pathophysiology of early myoclonic encephalopathy remains to be elucidated.

Previously, the clinical diagnoses of nonketotic hyperglycinemia in a limited number of patients were confirmed by genetic testing [8]. With the exception of a Finnish population, no common mutations were evident, and the full sequencing of the 25 exons of the *GLDC* gene is laborious. A nonconsanguineous patient is likely to constitute a compound heterozygote because the mutation spectrum of nonketotic hyperglycinemia is highly heterogeneous. The reported *GLDC* mutations include not only many base substitutions, but also various lengths of deletions involving multiple exons. These conditions complicate the genotyping of *GLDC*. Recently, a screening system for genomic deletions within *GLDC* was established, using the multiplex ligation-dependent probe amplification method [9]. With this screening method, the deletion of all 25 *GLDC* exons was detected in our patient. A missense mutation, p.R461Q, was also revealed by sequencing the other allele. The patient was revealed to be a heterozygote for the deletion of all 25 *GLDC* exons and a missense mutation.

Our patient manifested initial clinical signs within a few days of birth, which is consistent with findings in the classic neonatal form of nonketotic hyperglycinemia. Although we confirmed the elevation of his glycine cerebrospinal fluid/plasma ratio at age 22 days, increased glycine levels are

Table 1. Concentration of glycine in CSF and plasma

	Day 22	Day 38	Day 74	Day 168	Day 220
CSF glycine concentration (reference range: 2.9-10.4) μM	150.9	76.2	58.7	69.8	150.4
Plasma glycine concentration (reference range: 56-308) μM	1274	310	411.5	581.8	911.9
CSF/serum glycine ratio (reference range: <0.04) μM	0.11	0.24	0.14	0.12	0.16

Abbreviation:
CSF = Cerebrospinal fluid

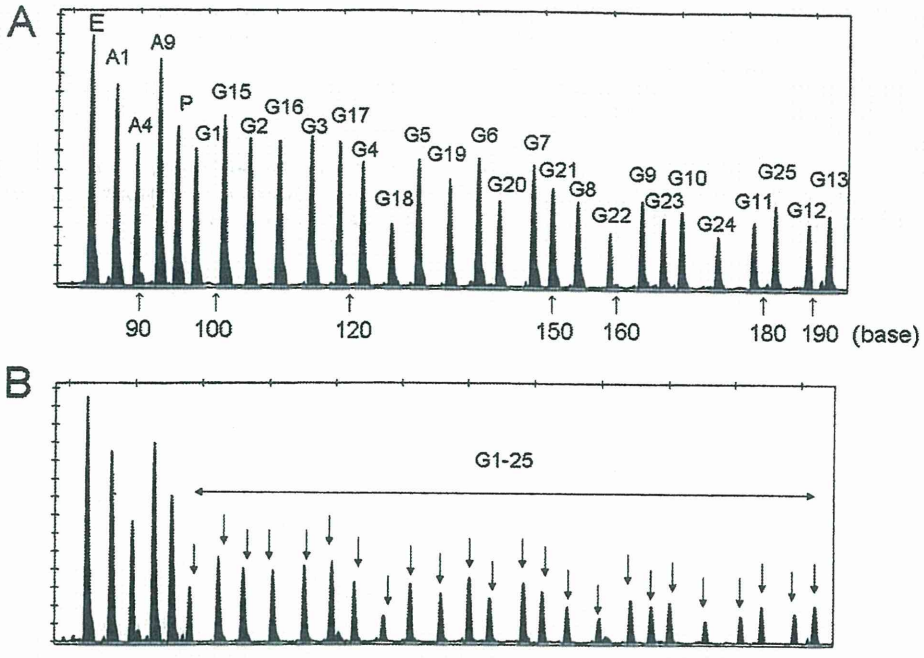

Figure 1. Multiplex ligation-dependent probe amplification analysis. (A) Multiplex ligation-dependent probe amplification analysis of a control subject. Each peak represents fluorescence signal of exons of GLDC, AMT, GLDCP, *and* EXT2 *with chromatogram (E,* EXT2*; A1, A4, and A9, exons 1, 4, and 9, respectively, of* AMT*; P,* GLDCP*; G1-G25, exons 1-25 of* GLDC*). Abscissa represent amplified fragment length polymorphism. (B) Multiplex ligation-dependent probe amplification analysis of this patient. The G1-G25 peaks reach approximately half the ordinate values of those of a control subject, indicating heterozygotic deletion of all 25* GLDC *exons.*

evident in other pathologic conditions. The confirmation of a deficiency in glycine cleavage system activity is therefore required for a definitive diagnosis of nonketotic hyperglycinemia, and calls for a relatively large amount of liver tissue obtained through a biopsy. Such invasive procedures can be difficult to perform for patients in poor general condition. Furthermore, the glycine cleavage system enzymatic activity assay can only be performed in a few laboratories worldwide. The metabolism of glycine by the glycine cleavage system leads to CO_2 production, and CO_2 production can therefore reflect glycine cleavage system activity [5]. Consequently, we used the novel ^{13}C-glycine breath test to evaluate glycine cleavage system activity, and this procedure required only 14 days for analysis. Subsequently, we could proceed confidently with targeted treatments after confirming the clinical diagnosis of nonketotic hyperglycinemia via this rapid, noninvasive ^{13}C-glycine breath test. A $^{13}CO_2$ analyzer, involving infrared spectrophotometry, has been widely distributed for the diagnosis of *Helicobacter pylori* infection. Therefore, this ^{13}C-glycine breath test can be readily performed in many hospitals [5]. When nonketotic hyperglycinemia is suspected, we suggest that this novel ^{13}C-glycine breath test should be performed for confirmation, after the analysis of glycine levels in the blood and cerebrospinal fluid.

To date, no reliable genotype-phenotype correlations have been published in cases of nonketotic hyperglycemia [10]. However, a lack of genotype-phenotype correlations was derived from a limited number of genetically diagnosed cases. More patients can be genetically diagnosed using the multiplex ligation-dependent probe amplification analysis [8,9]. Our patient was a compound heterozygote of a missense mutation, p.R451Q, with the deletion of the entire *GLDC* gene. These findings suggest that he may manifest either null or very low residual glycine cleavage system activity, which is in agreement with his low cumulative recovery rate of 7.9% in the novel ^{13}C-glycine breath test. Definitive genetic and enzymatic diagnoses using these novel methods can be important in genetic counseling. Therefore, we encourage performing multiplex ligation-dependent probe amplification analysis for genetic confirmation after clinical and enzymatic diagnosis. To provide a longer term and more accurate prospect of lifelong prognosis, we need to accumulate further information for genotype-phenotype correlations, using fast and reliable testing.

We acknowledge and greatly appreciate the genetic study performed by Junnko Kanno, MD.

References

[1] Van Hove JL, Vande Kerckhouve K, Hennermann JB, et al. Benzoate treatment and the glycine index in nonketotic hyperglycinaemia. J Inherit Metab Dis 2005;28:651-63.

[2] Hamosh A, Maher JF, Bellus GA, Rasmussen SA, Johnston MV. Long-term use of high-dose benzoate and dextromethorphan for the treatment of nonketotic hyperglycinemia. J Pediatr 1998;132:709-13.

[3] Boneh A, Degani Y, Harari M. Prognostic clues and outcome of early treatment of nonketotic hyperglycinemia. Pediatr Neurol 1996;15:137-41.

[4] Aicardi J, Ohtahara S. Severe neonatal epilepsies with suppression-burst pattern. Epileptic syndromes in infancy, childhood and adolescence. 4th ed. Montrouge, France: John Libbey Eurotext, 2005; 39-72.

[5] **Kure** S, Korman SH, Kanno J, et al. Rapid diagnosis of glycine encephalopathy by ^{13}C-glycine breath test. Ann Neurol 2006;59:862-7.

[6] **Schmitt** B, Steinmann B, Gitzelmann R, Thun-Hohenstein L, Mascher H, Dumermuth G. Nonketotic hyperglycinemia: clinical and electrophysiologic effects of dextromethorphan, an antagonist of the NMDA receptor. Neurology 1993;43:421-4.

[7] **Schmitt** B, Netzer R, Fanconi S, Baumann P, Boltshauser E. Drug refractory epilepsy in brain damage: Effect of dextromethorphan on EEG in four patients. J Neurol Neurosurg Psychiatry 1994;57:333-9.

[8] **Kanno** J, Hutchin T, Kamada F, et al. Genomic deletion within *GLDC* is a major cause of non-ketotic hyperglycinaemia. J Med Genet 2007;44: e69.

[9] **Kure** S, Kato K, Dinopoulos A, et al. Comprehensive mutation analysis of *GLDC*, *AMT*, and *GCSH* in nonketotic hyperglycinemia. Hum Mutat 2006;27:343-52.

[10] **Hoover-Fong** JE, Shah S, Van Hove JL, Applegarth D, Toone J, Hamosh A. Natural history of nonketotic hyperglycinemia in 65 patients. Neurology 2004;63:1847-53.

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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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Keywords: cardio-facio-cutaneous syndrome; costello syndrome; hematologic malignancy; loose anagen hair; melting curve analysis; noonan syndrome

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,

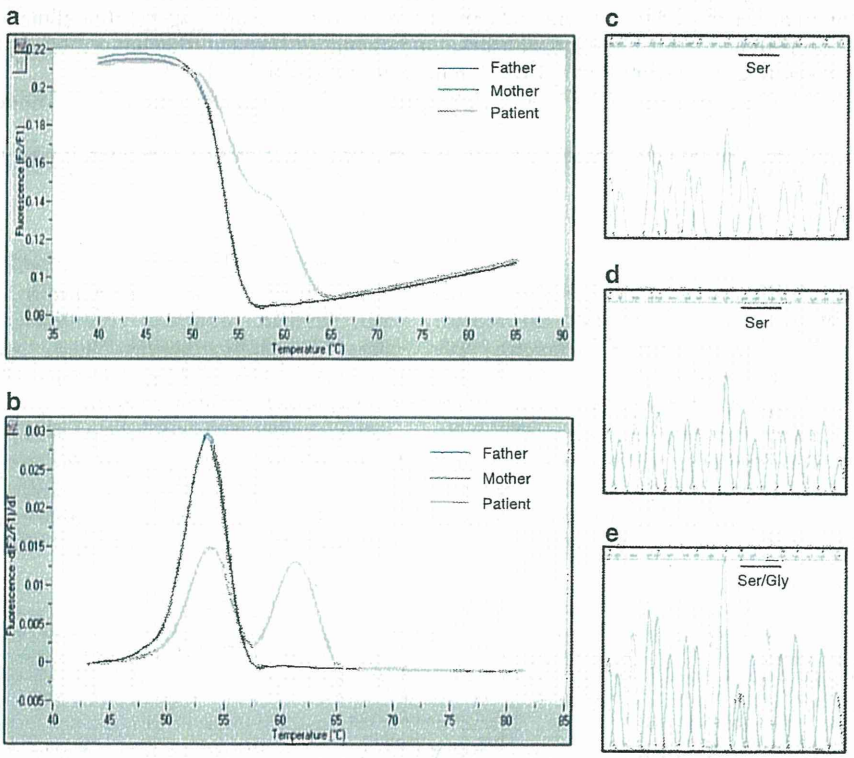

Figure 1 (**a**) PCR followed by melting analysis to detect the c.4A>G mutation. F2 represents the fluorescence emission of the LC Red 640 fluorophore, whereas F1 shows the fluorescence emission of the fluorescein fluorophore. (**b**) Melting curves are automatically converted into melting peaks, which 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 (%)	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.

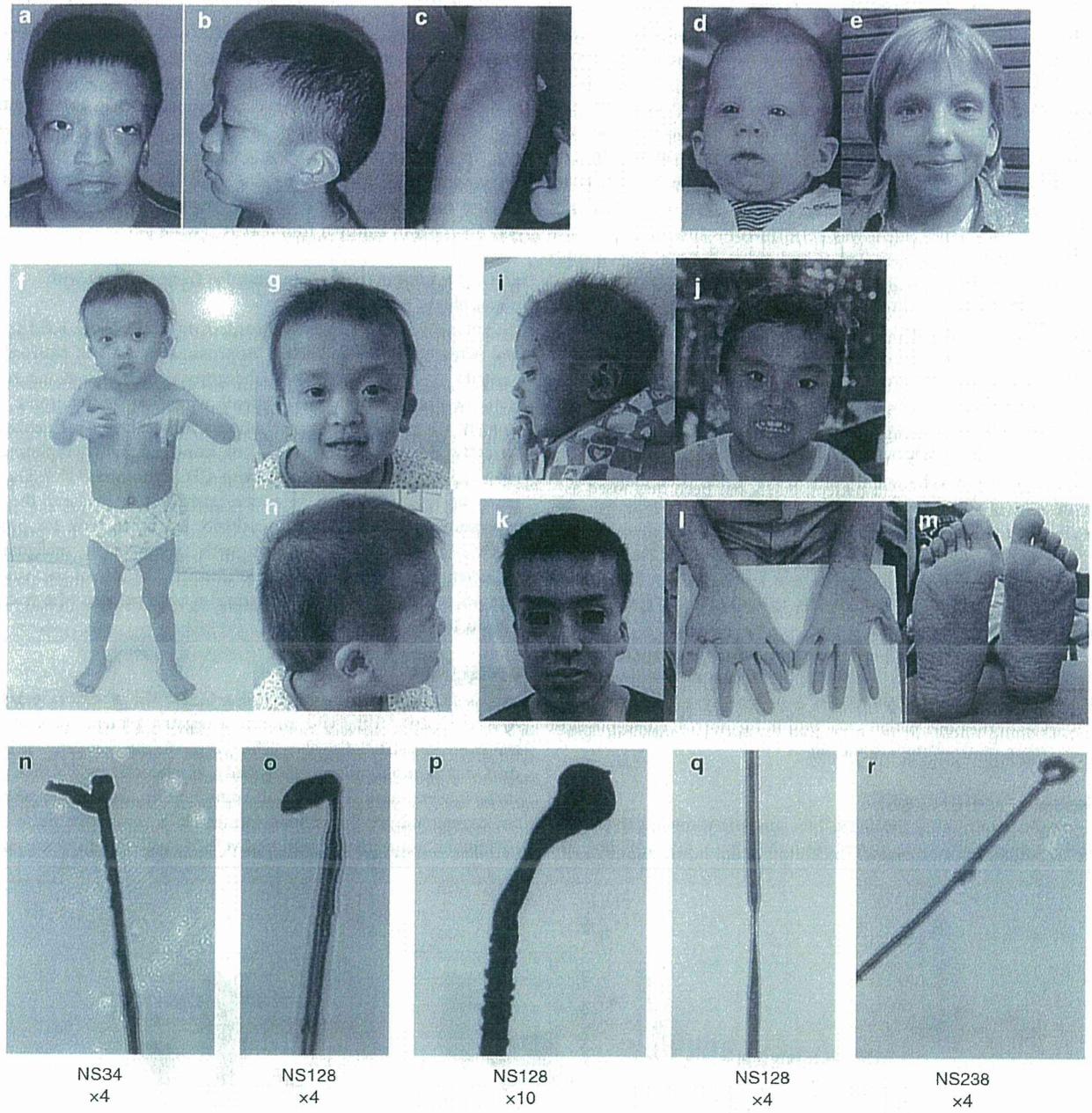

Figure 2 (**a**, **b**) Facial appearance of NS34 at the age of 13 years. (**c**) Dry and atopic skin seen in NS34. (**d**, **e**) NS93. (**f–h**) NS97. (**i**, **j**) NS128. (**k–m**) NS232 at the age of 25 years. (**l**, **m**) Palms and soles of NS232 showing fine wrinkling. Light micrographs of hairs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

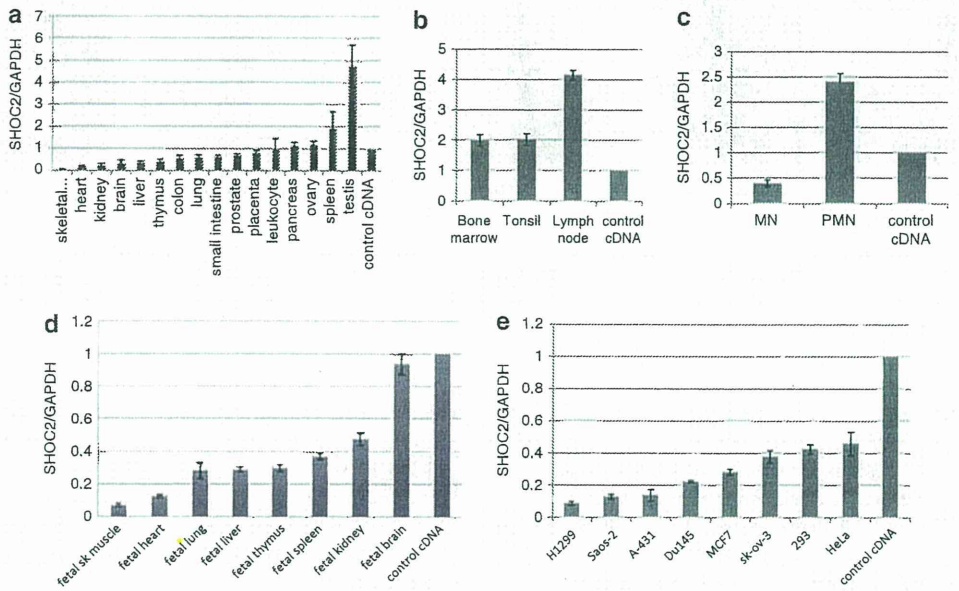

Figure 3 Relative expression of *SHOC2*. Expression levels of *SHOC2* mRNA in various adult human tissues (**a**), adult immune tissues (**b**), human leukocytes (**c**), human fetal tissues (**d**) and human tumor cell lines (**e**) were evaluated by quantitative PCR using *GAPDH* mRNA as the control. 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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1 Allanson, J. E., Hall, J. G., Hughes, H. E., Preus, M. & Witt, R. D. Noonan syndrome: the changing phenotype. *Am. J. Med. Genet.* **21**, 507–514 (1985).
2 van der Burgt, I. Noonan syndrome. *Orphanet. J. Rare Dis.* **2**, 4 (2007).
3 Hennekam, R. C. Costello syndrome: an overview. *Am. J. Med. Genet. C Semin. Med. Genet.* **117**, 42–48 (2003).
4 Reynolds, J. F., Neri, G., Herrmann, J. P., Blumberg, B., Coldwell, J. G., Miles, P. V. *et al.* New multiple congenital anomalies/mental retardation syndrome with cardio-facio-cutaneous involvement–the CFC syndrome. *Am. J. Med. Genet.* **25**, 413–427 (1986).
5 Aoki, Y., Niihori, T., Narumi, Y., Kure, S. & Matsubara, Y. The RAS/MAPK syndromes: novel roles of the RAS pathway in human genetic disorders. *Hum. Mutat.* **29**, 992–1006 (2008).
6 Bentires-Alj, M., Kontaridis, M. I. & Neel, B. G. Stops along the RAS pathway in human genetic disease. *Nat. Med.* **12**, 283–285 (2006).
7 Pandit, B., Sarkozy, A., Pennacchio, L. A., Carta, C., Oishi, K., Martinelli, S. *et al.* Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. *Nat. Genet.* **39**, 1007–1012 (2007).
8 Razzaque, M. A., Nishizawa, T., Komoike, Y., Yagi, H., Furutani, M., Amo, R. *et al.* Germline gain-of-function mutations in RAF1 cause Noonan syndrome. *Nat. Genet.* **39**, 1013–1017 (2007).
9 Roberts, A. E., Araki, T., Swanson, K. D., Montgomery, K. T., Schiripo, T. A., Joshi, V. A. *et al.* Germline gain-of-function mutations in SOS1 cause Noonan syndrome. *Nat. Genet.* **39**, 70–74 (2007).
10 Schubbert, S., Zenker, M., Rowe, S. L., Boll, S., Klein, C., Bollag, G. *et al.* Germline KRAS mutations cause Noonan syndrome. *Nat. Genet.* **38**, 331–336 (2006).
11 Tartaglia, M., Mehler, E. L., Goldberg, R., Zampino, G., Brunner, H. G., Kremer, H. *et al.* Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. *Nat. Genet.* **29**, 465–468 (2001).
12 Tartaglia, M., Pennacchio, L. A., Zhao, C., Yadav, K. K., Fodale, V., Sarkozy, A. *et al.* Gain-of-function SOS1 mutations cause a distinctive form of Noonan syndrome. *Nat. Genet.* **39**, 75–79 (2007).
13 Aoki, Y., Niihori, T., Kawame, H., Kurosawa, K., Ohashi, H., Tanaka, Y. *et al.* Germline mutations in HRAS proto-oncogene cause Costello syndrome. *Nat. Genet.* **37**, 1038–1040 (2005).
14 Niihori, T., Aoki, Y., Narumi, Y., Neri, G., Cave, H., Verloes, A. *et al.* Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. *Nat. Genet.* **38**, 294–296 (2006).
15 Rodriguez-Viciana, P., Tetsu, O., Tidyman, W. E., Estep, A. L., Conger, B. A., Cruz, M. S. *et al.* Germline mutations in genes within the MAPK pathway cause cardio-facio-cutaneous syndrome. *Science.* **311**, 1287–1290 (2006).

16 Selfors, L. M., Schutzman, J. L., Borland, C. Z. & Stern, M. J. soc-2 encodes a leucine-rich repeat protein implicated in fibroblast growth factor receptor signaling. *Proc. Natl Acad. Sci. USA* **95**, 6903–6908 (1998).
17 Rodriguez-Viciana, P., Oses-Prieto, J., Burlingame, A., Fried, M. & McCormick, F. A phosphatase holoenzyme comprised of Shoc2/Sur8 and the catalytic subunit of PP1 functions as an M-Ras effector to modulate Raf activity. *Mol. Cell.* **22**, 217–230 (2006).
18 Cordeddu, V., Di Schiavi, E., Pennacchio, L. A., Ma'ayan, A., Sarkozy, A., Fodale, V. *et al.* Mutation of SHOC2 promotes aberrant protein N-myristoylation and causes Noonan-like syndrome with loose anagen hair. *Nat. Genet.* **41**, 1022–1026 (2009).
19 Makita, Y., Narumi, Y., Yoshida, M., Niihori, T., Kure, S., Fujieda, K. *et al.* Leukemia in cardio-facio-cutaneous (CFC) syndrome: a patient with a germline mutation in BRAF proto-oncogene. *J. Pediatr. Hematol. Oncol.* **29**, 287–290 (2007).
20 Ohtake, A., Aoki, Y., Saito, Y., Niihori, T., Shibuya, A., Kure, S. *et al.* Non-Hodgkin lymphoma in a patient with cardio-facio-cutaneous syndrome. *J. Pediatr. Hematol. Oncol.* (e-pub ahead of print 2 June 2010).
21 Armour, C. M. & Allanson, J. E. Further delineation of cardio-facio-cutaneous syndrome: clinical features of 38 individuals with proven mutations. *J. Med. Genet.* **45**, 249–254 (2008).
22 Narumi, Y., Aoki, Y., Niihori, T., Neri, G., Cave, H., Verloes, A. *et al.* Molecular and clinical characterization of cardio-facio-cutaneous (CFC) syndrome: overlapping clinical manifestations with Costello syndrome. *Am. J. Med. Genet. A.* **143A**, 799–807 (2007).
23 Kobayashi, T., Aoki, Y., Niihori, T., Cave, H., Verloes, A., Okamoto, N. *et al.* Molecular and clinical analysis of RAF1 in Noonan syndrome and related disorders: dephosphorylation of serine 259 as the essential mechanism for mutant activation. *Hum. Mutat.* **31**, 284–294 (2010).
24 Tosti, A. & Piraccini, B. M. Loose anagen hair syndrome and loose anagen hair. *Arch. Dermatol.* **138**, 521–522 (2002).
25 Mazzanti, L., Cacciari, E., Cicognani, A., Bergamaschi, R., Scarano, E. & Forabosco, A. Noonan-like syndrome with loose anagen hair: a new syndrome? *Am. J. Med. Genet. A.* **118A**, 279–286 (2003).
26 Tosti, A., Misciali, C., Borrello, P., Fanti, P. A., Bardazzi, F. & Patrizi, A. Loose anagen hair in a child with Noonan's syndrome. *Dermatologica.* **182**, 247–249 (1991).
27 Flotho, C., Batz, C., Hasle, H., Bergstrasser, E., van den Heuvel-Eibrink, M. M., Zecca, M. *et al.* Mutational analysis of SHOC2, a novel gene for Noonan-like syndrome, in JMML. *Blood.* **115**, 913 (2010).

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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

Tomoko Kobayashi,[1] Yoko Aoki,[1]* Tetsuya Niihori,[1] Hélène Cavé,[2] Alain Verloes,[2] Nobuhiko Okamoto,[3] Hiroshi Kawame,[4,5] Ikuma Fujiwara,[6] Fumio Takada,[7] Takako Ohata,[7] Satoru Sakazume,[8] Tatsuya Ando,[9] Noriko Nakagawa,[10] Pablo Lapunzina,[11] Antonio G. Meneses,[11] Gabriele Gillessen-Kaesbach,[12] Dagmar Wieczorek,[13] Kenji Kurosawa,[14] Seiji Mizuno,[15] Hirofumi Ohashi,[16] Albert David,[17] Nicole Philip,[18] Afag Guliyeva,[1] Yoko Narumi,[1] Shigeo Kure,[1,6] Shigeru Tsuchiya,[6] and Yoichi Matsubara[1]

[1]Department of Medical Genetics, Tohoku University School of Medicine, Sendai, Japan; [2]APHP, Hôpital Robert Debré, Département de Génétique; Université Paris 7-Denis Diderot, Paris, France; [3]Department of Medical Genetics, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Osaka, Japan; [4]Division of Medical Genetics, Nagano Children's Hospital, Nagano, Japan; [5]Department of Genetic Counseling, Ochanomizu University, Tokyo, Japan; [6]Department of Pediatrics, Tohoku University School of Medicine, Sendai, Japan; [7]Department of Medical Genetics, Kitasato University Graduate School of Medical Sciences, Sagamihara, Japan; [8]Division of Medical Genetics, Gunma Children's Medical Center, Gunma, Japan; [9]Department of Pediatrics, Jikei University School of Medicine, Tokyo, Japan; [10]Department of Pediatrics, National Defense Medical College, Tokorozawa, Saitama, Japan; [11]Servicio de Genetica Medica, Hospital Universitario La Paz, Madrid, Spain; [12]Institut für Humangenetik Lübeck, Universitätsklinikum Schleswig-Holstein, Lübeck, Germany; [13]Institut für Humangenetik, Universitaetsklinikum Essen Universitaet Duisburg-Essen, Essen, Germany; [14]Division of Medical Genetics, Kanagawa Children's Medical Center, Yokohama, Japan; [15]Department of Pediatrics, Central Hospital, Aichi Human Service Center, Aichi, Japan; [16]Division of Medical Genetics, Saitama Children's Medical Center, Saitama, Japan; [17]CHU Nantes, Nantes, France; [18]Hôpital de la Timone, Marseille, France

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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.

Present address of Yoko Narumi: Department of Medical Genetics, Shinshu University School of Medicine, Matsumoto, Japan.

*Correspondence to: Yoko Aoki, Department of Medical Genetics, Tohoku University School of Medicine, 1-1 Seiryo-machi, Sendai 980-8574, Japan.
E-mail: aokiy@mail.tains.tohoku.ac.jp

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