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* and *MAP2K1/2* 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 *l*entigines, *e*lectrocardiographic conduction abnormalities, *o*cular hypertelorism, *p*ulmonary stenosis, *a*bnormal genitalia, *r*etardation of growth and sensorineural *d*eafness) 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.

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 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. have identified missense mutations in *PTPN11*, a gene encoding protein tyrosine phosphatase (PTP) SHP-2, in 45% of clinically diagnosed NS patients [Tartaglia et al. 2001]. 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 [Schubbert et al. 2006; Roberts et al. 2007; 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 conservative 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 and 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 four patients with atypical phenotypes. No mutations in *PTPN11*, *HRAS*, *KRAS*, *BRAF*, *MAP2K1/2* 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 s, annealing at 55 °C for 15 s and extension at 72 °C for 40 s. 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 hrs, 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 (PerkinElmer). The following antibodies were used for Western blotting: anti-Myc (9E10, Santa Cruz Biotech, Santa Cruz, CA), anti-phospho-c-Raf (S259) (Cell signaling), anti- phospho-c-Raf (S338) (Millipore), anti- phospho-c-Raf (S289/296/301) (Cell signaling), anti- phospho-c-Raf (S621) (Millipore) and anti-neomycin 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 2X SDS buffer. The samples were resolved in 5-20% gradient polyacrylamide gels,

transferred to nitrocellulose membranes and probed with anti-phospho-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. 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 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 phosphate buffered saline (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 h at 4 °C, washed three times with RIPA buffer, and then boiled in 2X 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 (Table1). A C to T nucleotide change, resulting in an amino acid change p.S257L, was identified in eleven 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 eleven patients, CFC syndrome in four patients and Costello in three patients (Supp. Table S2). Four patients who were firstly 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 6/11 (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%), 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 two 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 (2 cases) were also described in previously reported patients with *RAF1* mutations (24% and 1 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 firstly analyzed the phosphorylation status of two phosphorylation sites, S259 and S621, using antibodies which 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), while 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 rather broad. However, western blotting using anti-neomycin 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 hrs 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 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.3 B). These results support the data in Fig. 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 co-transfected three double mutants (WT/S621A, S257L/S621A and N262K/S621A) with FLAG-tagged 14-3-3, and co-immunoprecipitation 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 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 co-immunoprecipitation 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 firstly 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 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 [Pandit et al. 2007; Razzaque et al. 2007; Ko et al. 2008], 172 patients with *PTPN11* mutations [Tartaglia et al. 2002; Musante et al. 2003; Zenker et al. 2004; Jongmans et al. 2005], 73 patients with *SOS1* mutations [Roberts et al. 2007; Tartaglia et al. 2007; Zenker et al. 2007a; Ferrero et al. 2008; Narumi et al. 2008] and 18 patients with *KRAS* mutations [Carta et al. 2006; Schubbert et al. 2006; Zenker et al. 2007b; Lo et al. 2008; Ko et al. 2008] 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 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] firstly 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 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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