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

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

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

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

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

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

Phosphorylation State of Mutant RAF1 Proteins

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

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

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

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

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

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

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

ELK Transactivation in Mutant RAF1 Proteins

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

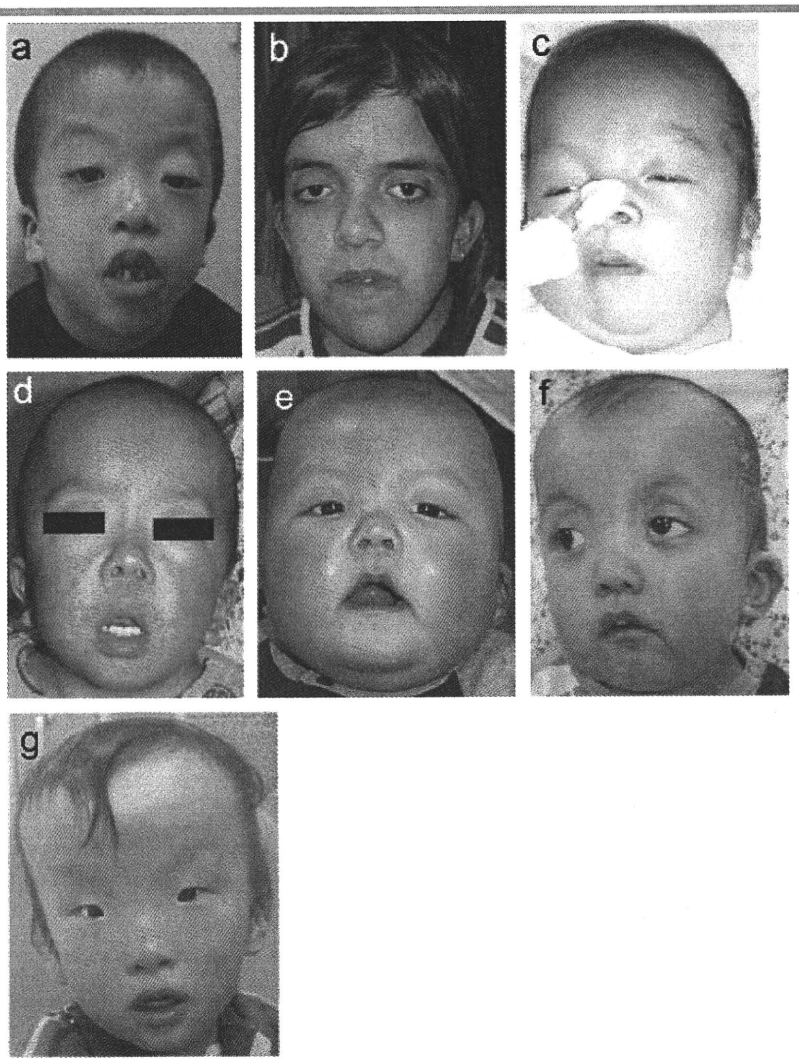

Figure 1. Facial appearance of patients with *RAF1* mutations. **a–f:** patients with p.S257L mutations. **a:** NS135; **b:** NS146; **c:** NS215; **d:** NS256; **e:** NS258 at 6 months; **f:** 2 years and 4 months; **g:** NS222 with p.S427G. [Color figure can be viewed in the online issue, which is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

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

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

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

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

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

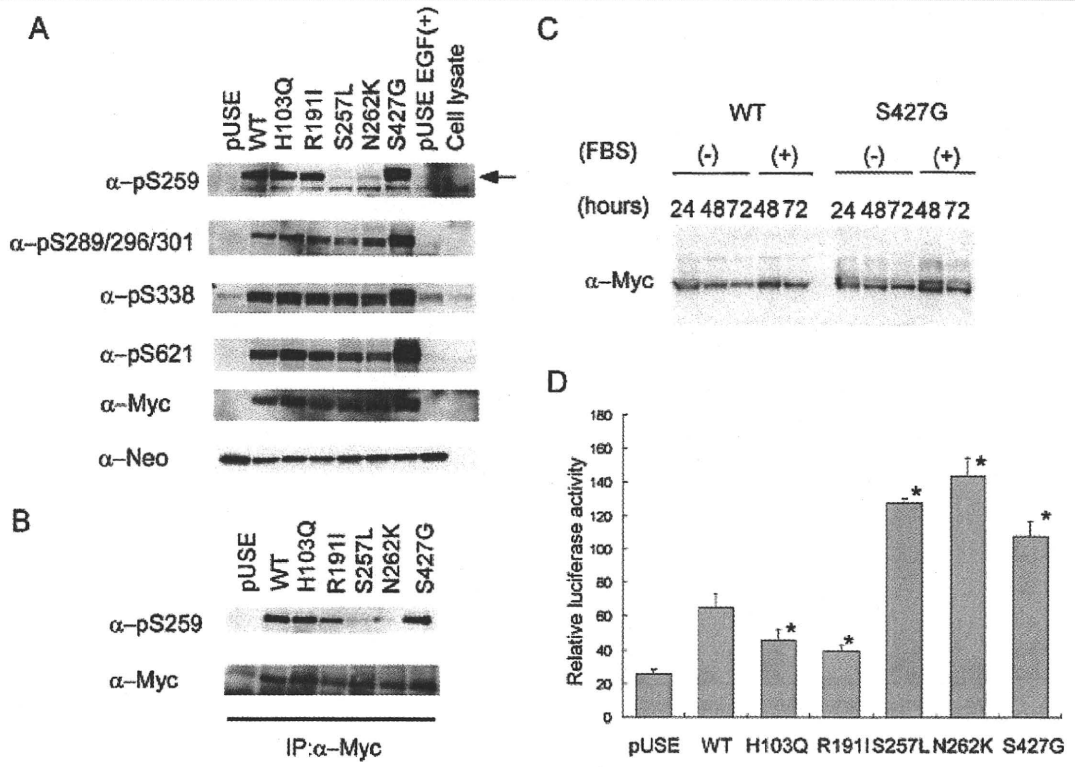

Figure 2. Analysis of phosphorylation status, degradation, and effect on downstream signaling in RAF1 mutants identified in this study. **A:** Phosphorylation status of wild-type (WT) RAF1 and mutants. Expression levels of RAF1 proteins and their phosphorylation levels were detected with different antibodies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

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

Discussion

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

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

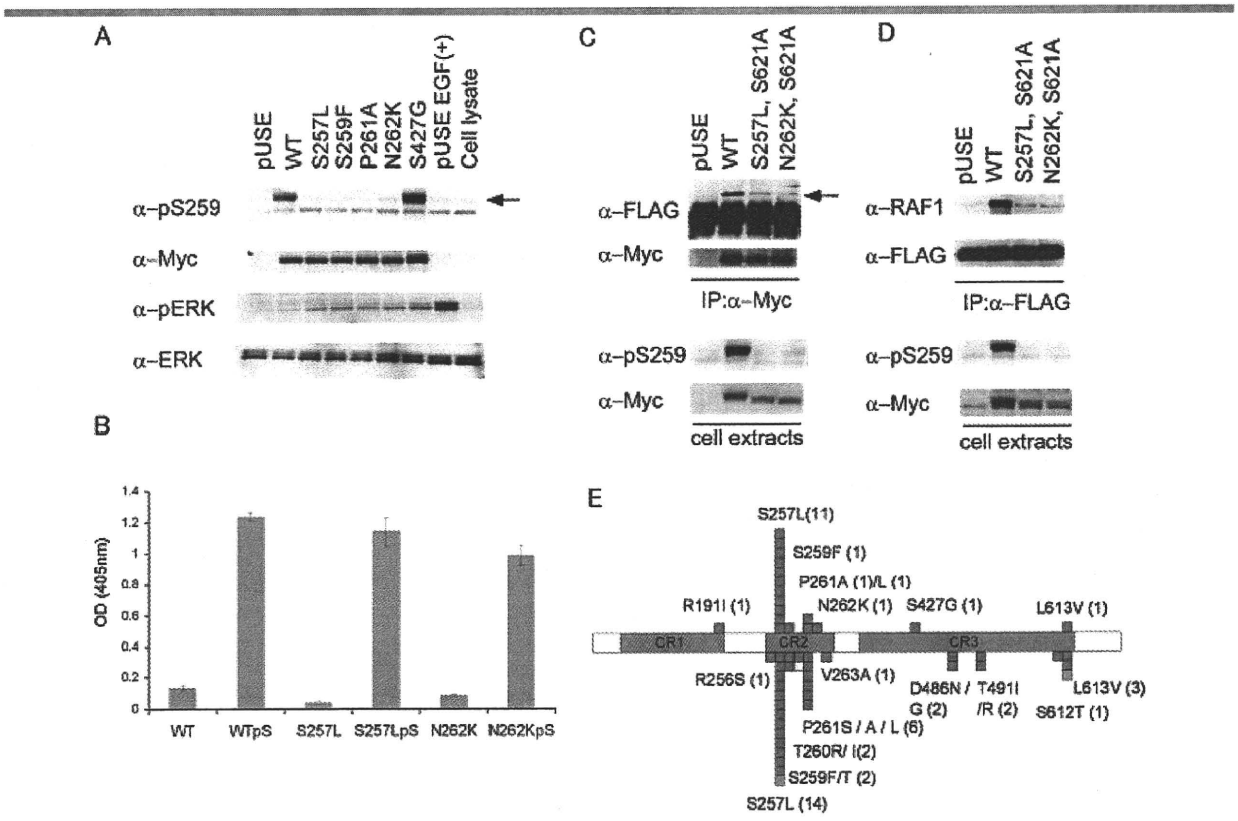

Figure 3. Phosphorylation of S259, binding to 14-3-3 and ERK activation of mutants located in the CR2 domain. **A:** Phosphorylation status of WT and mutants located in the CR2 domain. Phosphorylation of S259 was not observed in cells expressing p.S257L, p.S259F, p.P261A, and p.N262K. In order to examine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

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

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

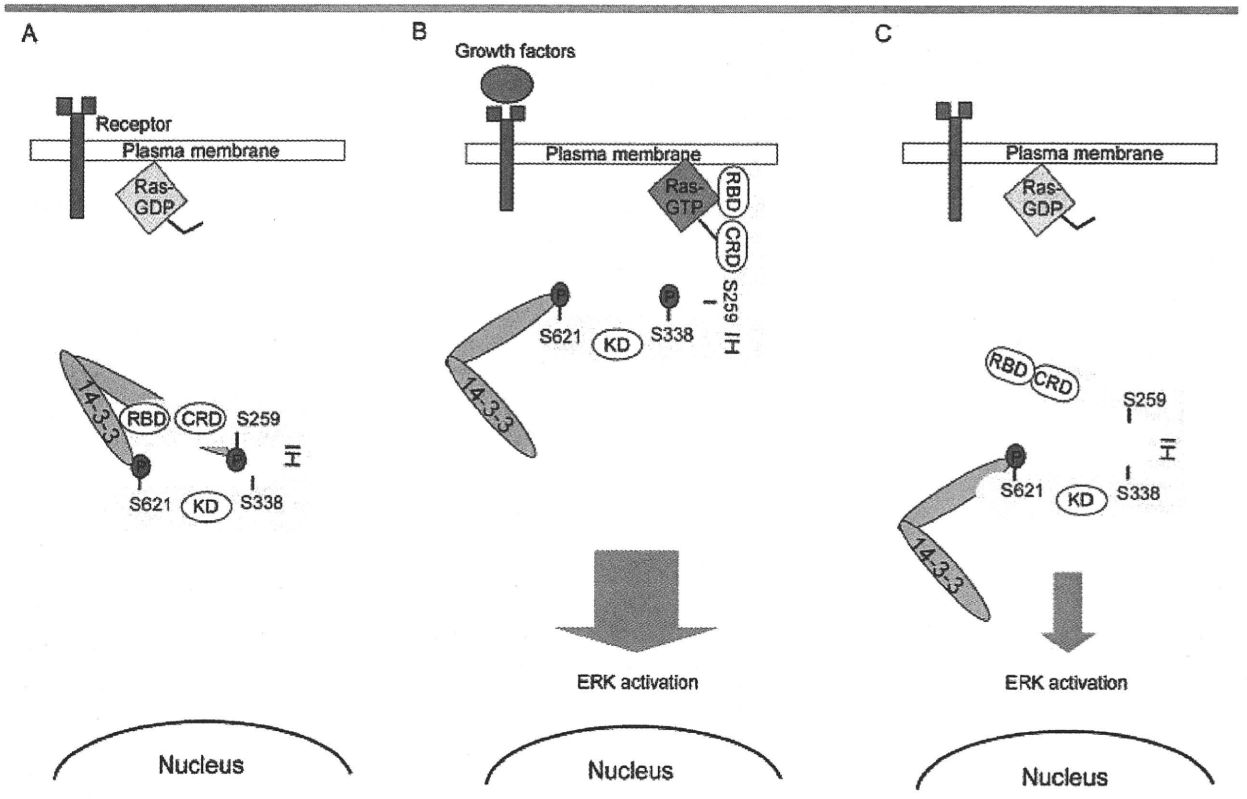

Figure 4. Schematic model of WT and mutant activation. **A:** In an inactive state, RAF1 is phosphorylated on S259 and S621 and is bound to 14-3-3. **B:** In growth-factor stimulation, the GTP-bound RAS binds to the CR1 domain of RAF1, which displaces 14-3-3. S259 is dephosphorylated by protein phosphatase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

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

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

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

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

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

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

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

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

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Reduced expression by *SETBP1* haploinsufficiency causes developmental and expressive language delay indicating a phenotype distinct from Schinzel –Giedion syndrome

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Reduced expression by *SETBP1* haploinsufficiency causes developmental and expressive language delay indicating a phenotype distinct from Schinzel–Giedion syndrome

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ABSTRACT

Background Mutations of the SET binding protein 1 gene (*SETBP1*) on 18q12.3 have recently been reported to cause Schinzel–Giedion syndrome (SGS). As rare 18q interstitial deletions affecting multiple genes including *SETBP1* correlate with a milder phenotype, including minor physical anomalies and developmental and expressive speech delay, mutations in *SETBP1* are thought to result in a gain-of-function or a dominant-negative effect. However, the consequence of the *SETBP1* loss-of-function has not yet been well described.

Methods Microarray-based comparative genomic hybridisation (aCGH) analyses were performed to identify genetic causes for developmental and expressive speech delay in two patients. SETBP1 expression in fibroblasts obtained from one of the patients was analysed by real-time RT-PCR and western blotting. A cohort study to identify nucleotide changes in *SETBP1* was performed in 142 Japanese patients with developmental delay.

Results aCGH analyses identified submicroscopic deletions of less than 1 Mb exclusively containing *SETBP1*. Both patients show global developmental, expressive language delay and minor facial anomalies. Decreased expression of SETBP1 was identified in the patient's skin fibroblasts. No pathogenic mutation of *SETBP1* was identified in the cohort study.

Conclusion SETBP1 expression was reduced in a patient with *SETBP1* haploinsufficiency, indicating that the *SETBP1* deletion phenotype is allele dose sensitive. In correlation with the exclusive deletion of *SETBP1*, this study delimits a milder phenotype distinct from SGS overlapping with the previously described phenotype of del(18)(q12.2q21.1) syndrome including global developmental, expressive language delay and distinctive facial features. These findings support the hypothesis that mutations in *SETBP1* causing SGS may have a gain-of-function or a dominant-negative effect, whereas haploinsufficiency or loss-of-function mutations in *SETBP1* cause a milder phenotype.

Mutations in the SET binding protein 1 gene (*SETBP1*) have recently been shown to cause Schinzel–Giedion syndrome (SGS, MIM #269150).[1] Whole-exome sequencing for four patients with SGS identified nucleotide alterations in the conserved region of *SETBP1*. Further analyses by standard Sanger sequencing for nine patients with SGS were performed, and eight of the nine patients showed *SETBP1* mutations. All five identified mutations were missense mutations, rather than nonsense mutations or truncations. As previously reported, rare chromosomal deletions in 18q including *SETBP1* correlate with a milder phenotype, and the severe SGS phenotype was proposed to be the consequence of a gain-of-function or dominant-negative effect of the mutations. However, the exact function of the gene is not known, and the consequences of an exclusive *SETBP1* loss-of-function or haploinsufficiency are not well described.

We identified de novo heterozygous microdeletions containing exclusively *SETBP1* in two patients with developmental, expressive language delay and distinctive facial features. The phenotypes are milder and differ significantly from the severe clinical appearance of SGS. Genotype–phenotype correlations of *SETBP1* haploinsufficiency are demonstrated in this study and discussed.

PATIENTS AND METHODS

Patients

After informed consent based on permission from the ethics committee of the institutions or individual written consent had been obtained, peripheral blood samples were taken from patients with developmental delay of unidentified aetiology to investigate potential genomic copy number aberrations.

Patients' reports

Patient 1 (DECIPHER #TWM253969) is a 7-year old boy, the second child of non-consanguineous parents (https://decipher.sanger.ac.uk/). His 10-year-old sister is healthy and normally developed. He was born with a birth weight of 2504 g (3–10th centile), length of 47 cm (10–25th centile), and occipitofrontal circumference (OFC) of 33.5 cm (=50th centile). At the time of his birth, his father and mother were 34 and 40 years old, respectively. His development was moderately delayed with crawling at 1 year, free walking at 2 years, and the first word at 5 years. He suffered febrile seizures several times, but EEG and brain MRI showed no abnormal findings. At 7 years, his height was 115 cm (25–50th centile), weight was 15.0 kg (<3rd centile), and OFC was 49.3 cm (3–10th centile). He showed distinctive facial features with an inverted triangle face, prominent forehead, ptosis with periorbital fullness, epicanthus and

pointed chin (figure 1A). He can walk by himself and can speak only a few words. The Kyoto developmental scale measured his developmental quotient as 40, which indicated moderate developmental delay. Visual acuity examination showed a refractive error of +8D in both eyes, indicating hyperopia. Previously performed conventional chromosomal analysis showed a normal male karyotype of 46,XY.

Patient 2, the 3rd child of non-consanguineous healthy parents, was born at 38 weeks by caesarean section for breech presentation after an uneventful pregnancy. In the neonatal period, the boy was hypotonic, sleepy and passive and rarely cried. He showed significantly delayed motor development, with sitting at 14 months and walking at 2 years, as well as delayed pincer grip. Initially, a discrete hemiparesis of the left part of his body manifested only while running with a slight spastic posture of his left hand and gait asymmetry suggested a perinatal or prenatal stroke. Cerebral MRI at the age of 4 years was normal except an unspecific T2 hyperintense infratentorial lesion in the right cranial paramedian cerebellum. The patient still exhibits coordination deficits in fine motoricity. His growth parameters are in the normal range (75th–90th centile), and OFC is within the 10th–25th centile. Hearing was found to be normal. Interestingly, the boy has not developed any expressive speech at all to date, whereas receptive language abilities are intact. He actively communicates using gestures illustrating his demands and ideas, but well understands his interlocutor, permitting a bidirectional exchange. He exhibits kind and social behaviour but at the same time a restless search for interactive communication. He has difficulty concentrating and has no sense of danger or pain. Facial dysmorphisms include frontal upsweep, a lighter blond hair corona in the front, hypertelorism, ptosis of eyelids predominantly on the left, periorbital fullness, straight and sparse eyebrows, flat nasal bridge, short nose, thin upper lip, short fingers and broad distal phalanges (figure 1B–D). No major malformations have been found. Microcytic hypochrome anaemia remains unexplained; the search for HbH inclusion bodies which would indicate X-linked α-thalassaemia/mental retardation syndrome was negative.

Microarray-based comparative genomic hybridisation (aCGH)

aCGH analyses were performed using the Human Genome CGH Microarray 44K (Agilent Technologies, Santa Clara, California, USA) and the whole genome tiling NimbleGen CGH array (Human CGH 2.1M WG-T v2.0; NimbleGen; Roche NimbleGen Inc, Madison, Wisconsin, USA) for patient 1 and patient 2, respectively, according to the manufacturer's protocols.

Fluorescence in situ hybridisation

Identified aberrations were confirmed by fluorescence in situ hybridisation (FISH) using locus-specific BAC clones as probes. In patient 1, two clones, CTD-3236P11 on 18q12.3 (chr18:40 779 351–40 864 576) as a target and RP11-105C15 on 18p11.31 (chr18:5 910 725–60 63 460) as a marker, were selected from the UCSC genome browser (http://www.genome.ucsc.edu). In patient 2, the locus-specific probe RP11-24L5 (BlueGnome, Cambridge, UK) in the region 18q12.3 (chr18:40 588 784–40 776 858) was used on metaphase spreads. Physical positions refer to the March 2006 human reference sequence (NCBI Build 36.1).

Expression analysis of SETBP1

Total RNAs were extracted from cultured skin fibroblasts from patient 1 and the control individual using the ISOGEN RNA extraction kit (Wako, Osaka, Japan), reverse-transcribed to complementary DNA (cDNA) using the SuperScript VILO cDNA Synthesis Kit (Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions, then used as templates for real-time PCR using Power SYBR Green PCR master mix (Life Technologies). Primers for SETBP1 mRNA were designed in the coding region (SETBP1 nt374F; 5′-GTCCA CCTGAGATCAAGATC-3′ and SETBP1 nt663R; 5′-GTCCATGT GGTTCTGGCTGC-3′). Beta actin primers (5′-GGCACCCAGCA CAATGAAGATC-3′ and 5′-AAGTCATAGTCCGCCTAGAAGC-3′)

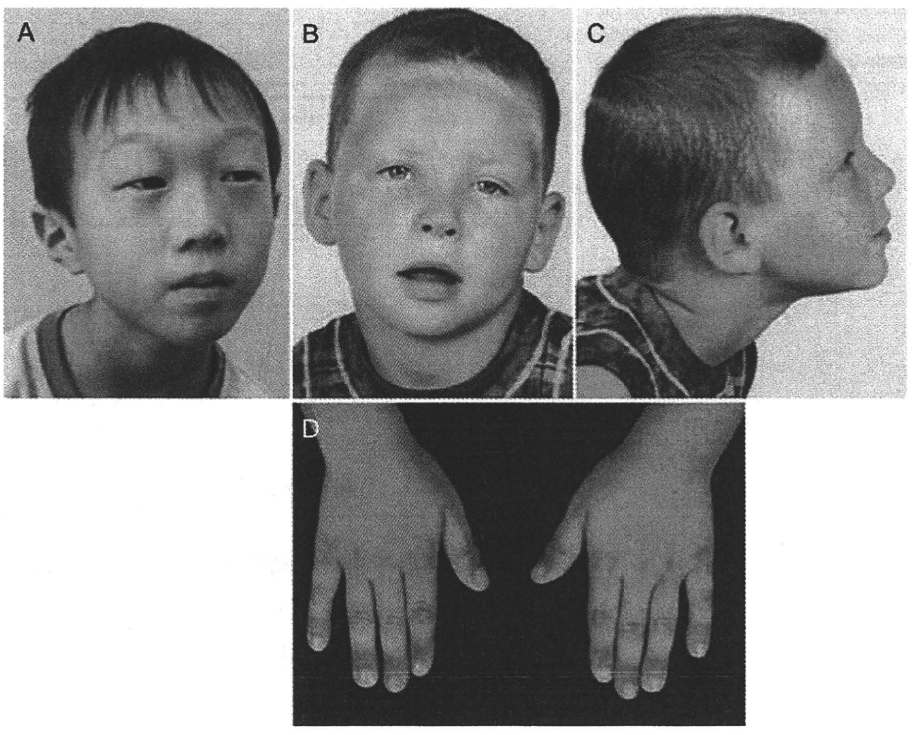

Figure 1 Phenotypes of the patients. (A) Patient 1; (B,C) frontal and lateral views of patient 2; (D) both hands of patient 2.

were used for the internal control. Real-time PCR amplifications were performed in three independent replicates on an ABI7500 (Life Technologies), and the data were evaluated by the Delta Delta Ct method.[2] The SETBP1 expression ratio (patient versus normal control) was calculated in each of the three examinations.

Concentrations of SETBP1 in the cell lysates of skin fibroblasts from patient 1 and the control were also analysed by western blotting using the SETBP1 MaxPab mouse polyclonal antibody (B01), catalogue number H00026040-B01 (Abnova, Taipei City, Taiwan) as described previously.[3]

Cohort study of *SETBP1*

A total of 142 Japanese patients with developmental delay, without genomic copy number aberrations as determined by aCGH, participated in the cohort study.[4] *SETBP1* sequences were analysed by the standard PCR-direct sequencing method. The primers used for PCR and the big-dye sequencing reaction (Life Technologies) were designed using Primer3 (http://primer3.sourceforge.net/) (supplemental online table 1). When we identified nucleotide changes in samples for which parental samples were available, trio analyses were performed to check whether the changes were de novo or familial. The nucleotide sequences of *SETBP1*, in which nucleotide alterations were found in the cohort study, were compared with homologues in species including *Callithrix jacchus*, *Gorilla gorilla*, *Macaca mulatta*, *Pan troglodytes*, *Pongo pygmaeus*, *Tarsius syrichta* and *Tupaia belangeri*, which were identified using Gene Tree (http://www.ensembl.org). DNA samples from 70 Japanese volunteers were used for the control cohort of normal Japanese.

RESULTS

Cytogenetic analyses

In patient 1, aCGH analysis revealed an aberration in the contiguous 11 probes at 18q12.3 with the mean $\log_2$ ratio of −1.02306 (figure 2A). This indicated a 986 kb loss of genomic copy number at 18q12.3; molecular karyotyping was determined as arr chr18q12.3q12.3 (40 282 934–41 269 199)x1. The deletion exclusively contained *SETBP1* and was confirmed by FISH analysis showing only one signal from the targeted probes (supplemental online figure S1). FISH analysis using the same probes showed no abnormality in either parent, indicating a de novo deletion (data not shown).

In patient 2, aCGH showed an 850 kb deletion within the chromosomal region 18q12.3 (chr18:40 233 803–41 088 224) (figure 2B). The deletion was confirmed by FISH, and both parents were found to be normal by conventional chromosome analysis and FISH analysis with the same locus-specific probe, indicating a de novo occurrence (data not shown). The only referenced gene within the deleted region was *SETBP1*. The two neighbouring genes, *BC051727* and *AK123972*, were non-coding. *TSLC14A2* (NM_007163) encodes a renal tubular urea transporter of the solute carrier family 14, not related to the phenotype of the patient.

Expression of SETBP1

In comparison with the normal control, SETBP1 RNA expression in the skin fibroblasts derived from patient 1 was reduced to 0.53, 0.60 and 0.41 (mean 0.51), and the lower SETBP1

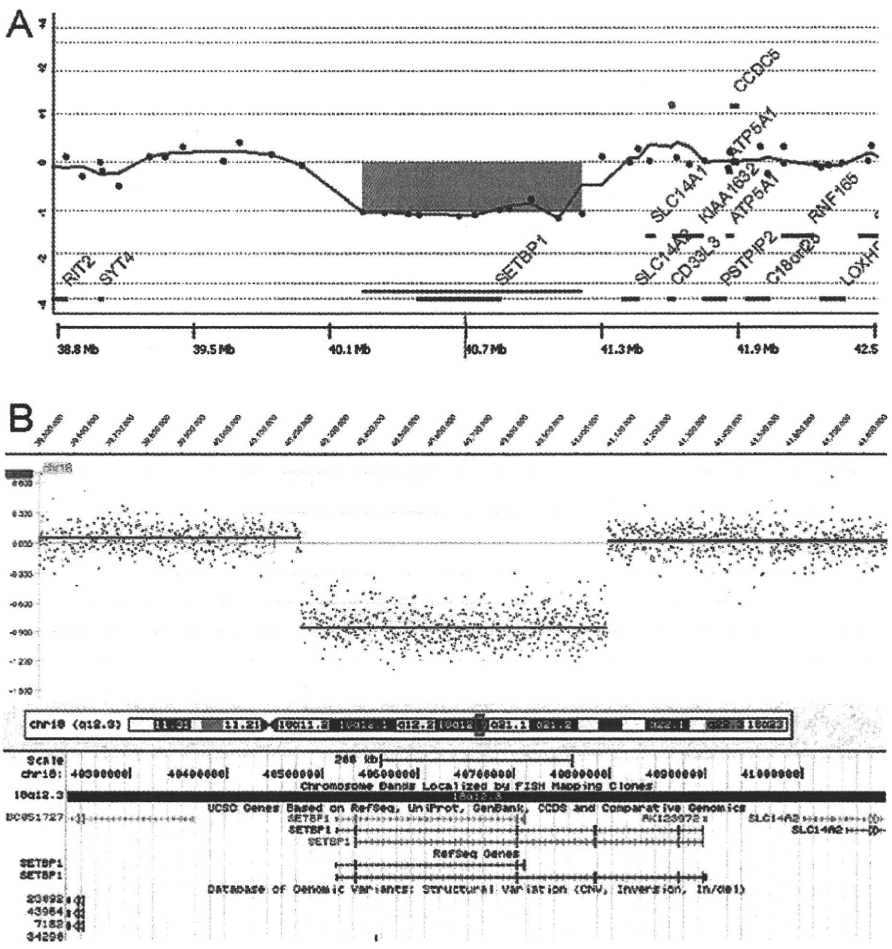

Figure 2 Microarray-based comparative genomic hybridisation identifies small deletions including *SETBP1* in patient 1 (A) and patient 2 (B). DNA copy number changes are represented by the negative $\log_2$ ratio below the baseline showing the deletions. (B) The square in the chromosome ideogram indicates the chromosomal position of the deletion; genes contained within the deletion are depicted below (http://genome.ucsc.edu).

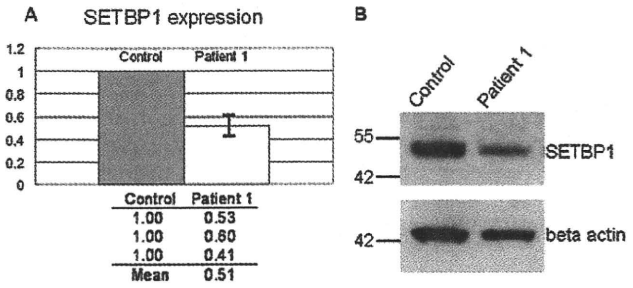

Figure 3 Expression studies. (A) SETBP1 RNA expression ratio analysed by real-time PCR. Raw data are given beneath the histogram. SETBP1 expression in the patient was about half that found in the control. (B) Western blotting of SETBP1. A total of 10 μg protein was separated in the gels. SETBP1 protein can be seen to be decreased in the patient. Beta actin was used as the internal control. Molecular mass (kDa) is indicated on the left of the gel.

protein concentration was also confirmed by western blotting (figure 3A,B).

Cohort study for *SETBP1* mutations

We identified 18 nucleotide changes including 11 non-synonymous and seven synonymous mutations, but no nonsense and no truncation mutations (table 1). The seven synonymous and four non-synonymous mutations, V231L, A390V, V1101I and P1130T, which were already listed in the single-nucleotide polymorphism database, were benign single-nucleotide polymorphisms. Four missense mutations (R627C, E958G, G1067S and W1242C; data not shown) located on the conserved sequence regions compared with the homologous genes from other species were not identified in normal control samples. However, W1242C was found in a healthy parent. Q1558L was also inherited from a healthy parent. The codon positions of E1466D and P1526Q were conserved among species and included in the important regions, SET-binging region and PPLPPPPP repeat, respectively. However, the patients' phenotypes were not similar to the presenting patient or SGS. Thus there was no definite pathogenic mutation. The sequence of the remaining *SETBP1* allele in patient 1 contained no nucleotide alterations.

DISCUSSION

In this study, we identified two patients with de novo chromosomal microdeletions in 18q12.3 that included *SETBP1* exclusively. *SETBP1* haploinsufficiency was suggested to be pathogenic. The patients exhibit moderate developmental delay and distinctive facial features, including prominent forehead, sparse eyebrows, mild ptosis with periorbital fullness. Patient 2 in particular showed a striking discrepancy between expressive speech impairment and conserved receptive speech, which has also been previously observed in patients with larger deletions in del(18)(q12.3q12.3). The complete and exclusive loss of one copy of *SETBP1* in our patient in correlation thus suggests an essential role for *SETBP1* in expressive speech development.

Schinzel *et al* reported on three patients with del(18)(q12.2q21.1) showing muscular hypotonia, seizures, behavioural disorders, and a pattern of minor dysmorphic features including prominent forehead, ptosis of the upper eyelids, full periorbital tissue, epicanthic folds and strabismus.[5] These phenotypic characteristics are similar to those in the cases presented here. Tinkle *et al* reported on a patient with del(18)(q12.2q21.1) with

Table 1 Identified nucleotide alteration in the cohort study

Nucleotide position*	Change	Amino acid change*	Location	Number of alleles that showed nucleotide changes	Conserved/ not conserved†	Function‡	Trio analyses	Results of population study	In silico database
c. 691	G>C	V231L	Exon 4	4	Not conserved				rs11082414
c. 1169	C>T	A390V	Exon 4	1	Not conserved				rs8091231
c. 1879	C>T	R627C	Exon 4	3	Conserved			None	None
c. 1911	G>A	P637P (synonymous)	Exon 4	1	-				None
c. 1932	C>T	S644S (synonymous)	Exon 4	2	-				rs3744824
c. 2607	C>T	S869S (synonymous)	Exon 4	12	-	The Ski homology region			None
c. 2873	A>G	E958G	Exon 4	1	Conserved			None	None
c. 3199	G>A	G1067S	Exon 4	1	Conserved			None	None
c. 3301	G>A	V1101I	Exon 4	90	Conserved				rs3744825
c. 3372	C>T	G1124G (synonymous)	Exon 4	1	-				None
c. 3388	C>A	P1130T	Exon 4	66	Conserved				rs1064204
c. 3726	G>C	W1242C	Exon 4	1	Conserved		Familial	None	None
c. 3825	A>G	S1275S (synonymous)	Exon 4	2	-				rs8096662
c. 4010	G>C	S1337S (synonymous)	Exon 5	1	-	SET-binding region			None
c. 4398	G>T	E1466D	Exon 6	3	Conserved	SET-binding region			None
c. 4563	C>G	P1521P (synonymous)	Exon 6	1	-	PPLPPPPP repeat			None
c. 4577	C>A	P1526Q	Exon 6	1	Conserved	PPLPPPPP repeat			None
c. 4673	A>T	Q1558L	Exon 6	1	Conserved		Familial		None

*Nucleotide and amino acid positions indicate NM_015559 sequence with the first initiation codon ATG at position 1.
†Conserved or not conserved was determined by comparison with the other species.
‡Functional domains were obtained from Minakuchi *et al* (2001).[12]

long-term survival, and concluded that life expectancy is minimally reduced.[6]

Although the previously reported chromosomal anomalies were identified at chromosomal G-banded levels, in more recent reports deletions in 18q12.2q21.1 were characterised by molecular techniques, and common features in the patients' phenotypes were reported.[7 8] The critical region for the phenotype of patients was narrowed to the 18q12.3-q21.1 region by Cody *et al*[9] and Buysse *et al*[10] (figure 4), who proposed a new syndrome involving expressive speech delay. They hypothesised that genes within the region may be specific to the neural and motor planning domains necessary for speech. However, deletions described so far contain numerous genes, including *SETBP1*, not allowing a phenotype–genotype correlation for haploinsufficiency of *SETBP1* exclusively.

Our findings correlate the phenotypes of the two patients with the exclusive complete loss of one copy of *SETBP1*. There is significant phenotypic overlap with the previously reported del (18)(q12.2q21.1) syndrome, suggesting a major contribution of the deletion of *SETBP1* to these phenotypes, as it has been described in contiguous deletion syndromes. The discrepancy between expressive and receptive language abilities in our patients appears to be a unique characteristic in the *SETBP1* deletion phenotype. The complete and exclusive loss of one copy of *SETBP1* in our patients in correlation with their phenotypes suggests an essential role for *SETBP1* in expressive speech development, although the exact function of the gene remains unknown.

SETBP1 encodes SET binding protein 1 expressed in numerous tissues including fetal brain. Its fusion with nucleoporin 98 kDa (*NUP98*) by chromosomal translocation has been shown in acute T-cell lymphoblastic leukaemia,[11] and the SET binding protein has been proposed to play a key role in the mechanism of SET-related leukaemogenesis and tumorigenesis by regulatory function in the nucleus.[12] Hoischen *et al* recently identified mutations in *SETBP1* to be causative of SGS, which is

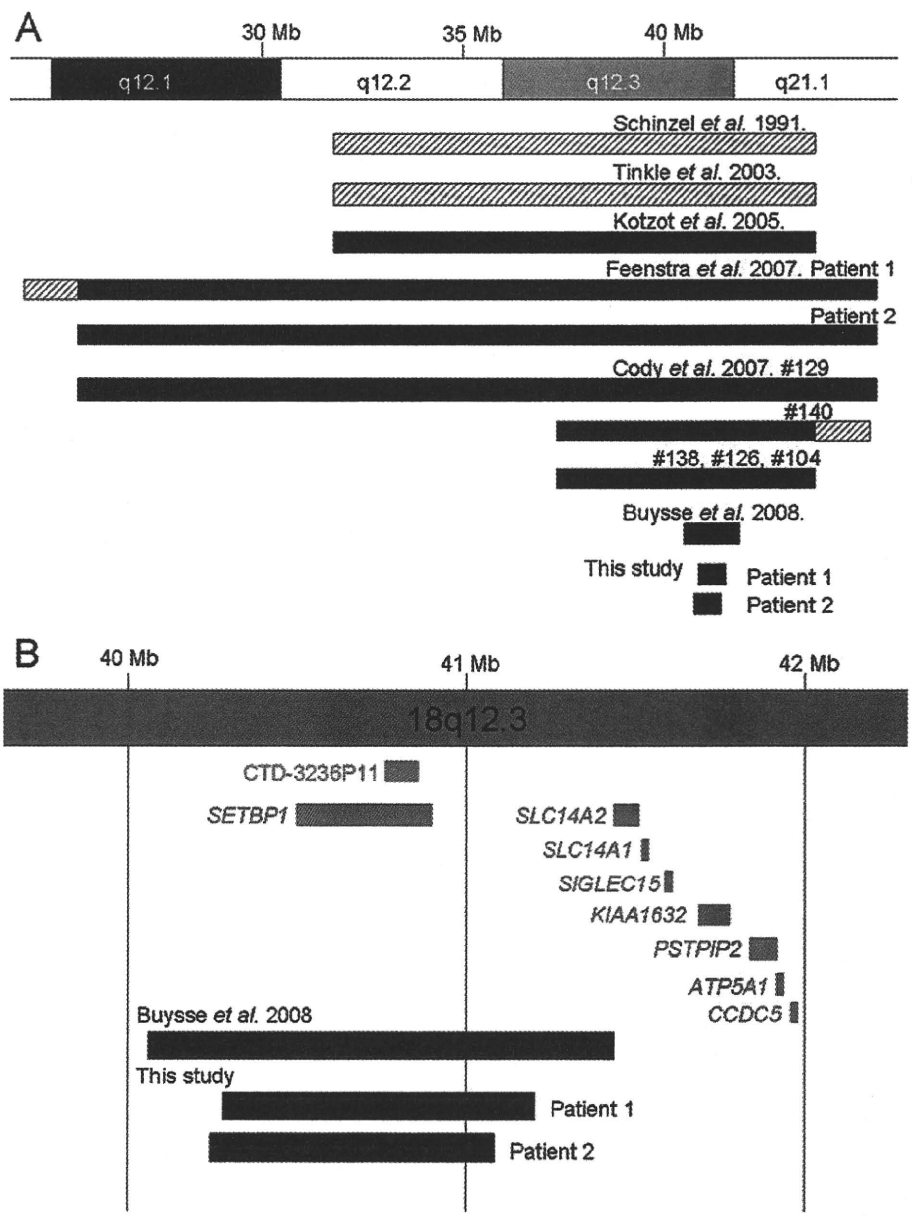

Figure 4 Comparison of the deletion regions. (A) Schematic representation of the previously reported deletions on a physical map of chromosome 18. (B) The deletion region of the patient is expanded. Bars filled with black and diagonal lines indicate definite and ambiguous deletion regions, respectively. Green and red bars indicate the position of the BAC clone used for fluorescence in situ hybridisation and the known genes, respectively.

characterised by severe mental retardation, distinctive facial features, and multiple congenital malformations.[1] Prognosis is poor, and most affected individuals die in the first decade of life. All reported mutations of *SETBP1* in patients with SGS were missense mutations in the important SET-binding region, and a gain-of-function or dominant-negative effect was suspected.[12]

As the phenotype of our two patients and the previously reported patients with del(18)(q12.2q21.1) including *SETBP1* does not resemble SGS and clinical features are generally milder, we conclude that haploinsufficiency of *SETBP1* does not cause SGS. We analysed the expression of SETBP1 by real-time PCR and western blotting, and found that SETBP1 was reduced in patient-derived skin fibroblasts, confirming that the effects of SETBP1 are allele dose-dependent. The deletion mainly affects speech, but the syndromic phenotype includes global development delay and recognisable facial dysmorphism underlying ubiquitous expression of SETBP1. As the phenotypic appearance of *SETBP1* haploinsufficiency is completely different from that of SGS, our findings support the proposed gain-of-function or dominant negative effect of the identified mutations in this gene.

There are various examples of phenotypic variability due to the different nature of mutations in the same gene. Mutations in fibroblast growth factor 3 (*FGFR3*) cause disproportionate growth in achondroplasia by gain-of-function, whereas terminal deletions of 4p including *FGFR3* cause Wolff–Hirschhorn syndrome, which does not show disproportionate growth at all, but small stature.[13 14] On the other hand, gain-of-function mutations of T-box 1 (*TBX1*) can result in the same phenotypic spectrum as haploinsufficiency caused by loss-of-function mutations or deletions in 22q11 including *TBX1*.[15]

In our study, we delimit a phenotype for haploinsufficiency of *SETBP1* distinct from the phenotype of SGS described in patients with mutations in the same gene suggesting a gain-of-function or a dominant negative effect of the mutations described. The *SETBP1* deletion phenotype seems to overlap extensively with the previously described del(18)(q12.2q21.1) syndrome, which has been characterised by moderate developmental delay, distinctive facial appearance, expressive language delay, and behavioural problems. Haploinsufficiency of *SETBP1* may thus primarily contribute to the phenotype of this contiguous gene syndrome. We did not identify pathogenic mutations on sequencing *SETBP1* in a cohort of 142 patients with developmental delay. Additional studies of the exact cellular function of *SETBP1* are needed to understand the pathogenic origin of the variable and distinct phenotypes.

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Competing interests None.

Patient consent Obtained.

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ORIGINAL INVESTIGATION

Association study of *KIBRA* gene with memory performance in a Japanese population

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Abstract

Objectives. Papassotiropoulos et al. (Science 314: p 475) discovered that a single nucleotide polymorphism (SNP) of the *KIBRA* gene (rs17070145) was associated with delayed recall performance in Caucasians. KIBRA is highly expressed in the brain and kidneys, and is reported to be involved in synaptic plasticity. Therefore, we first tried to replicate the association between the SNP and memory performance in a Japanese subjects. *Methods.* We examined the association between the SNP and memory performance measured by the Wechsler Memory Scale-Revised (WMS-R) in 187 healthy Japanese people. *Results.* The T allele carriers had significantly better verbal memory, attention/concentration and delayed recall performance than the C/C carriers (corrected $P=0.044$, 0.047 and 0.0084, respectively). Furthermore, the C/T carriers and the T/T carriers had better delayed recall performance than the C/C carriers (post hoc $P=0.0017$ and 0.0096). *Conclusions.* This data suggest that the C/C genotype might have an impact on memory performance in Asian populations as well as in Caucasian populations. Further investigation to clarify the association of the *KIBRA* gene with memory in other ethnic groups is warranted.

Key words: *KIBRA, single nucleotide polymorphism(SNP), memory, delayed recall, WMS-R*

Introduction

The mechanism of human memory is important to know fundamental brain functions. Twin and family studies have demonstrated that most cognitive traits are moderately to highly heritable, although the particular genes that underlie the heritability have only recently been investigated (Greenwood 2003). Papassotiropoulos et al. (2006) initially presented evidence that the SNP of the *KIBRA* gene (rs17070145) might be associated with human memory performance in healthy Caucasian subjects. Rs17070145 is a common T→C substitution within the ninth intron of the *KIBRA* gene (GenBank accession number NM_015238 [GenBank]). They showed that the T allele carriers of the *KIBRA* gene had better delayed recall performance than the C/C carriers. They also showed that high levels of truncated KIBRA transcripts are expressed in memory-related brain regions in humans, additionally high levels of KIBRA are expressed in hippocampus of mice. Furthermore, hippocampal activation in the T allele carriers was higher than that in the C/C carriers during memory

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retrieval by functional magnetic resonance imaging (MRI). Thus, the *KIBRA* gene has lately attracted considerable attention on the mechanism of memory. There were six replication studies that supported a significant effect of the *KIBRA* SNP on cognition in healthy subjects (Almeida et al. 2008; Nacmia et al. 2008; Bates et al. 2009; Schaper et al. 2008; Preuschhof et al. 2009; Zhang et al. 2009) and two studies failed to confirm the association between the *KIBRA* SNP and memory performance (Need et al. 2008; Need et al. 2009).

KIBRA is a cytoplasmic protein, highly expressed in the brain and kidneys and represents a new member of the family of signal transducers. It contains two amino-terminal WW domains, a C2 like domain and a carboxyl-terminal glutamic acid-rich stretch (Kremerskothen et al. 2003). It interacts with dynein light chain 1 to activate the oestrogen receptor (Rayala et al. 2006) and discoidin domain receptor 1 to modulate collagen-induced signalling (Hilton et al. 2008). Recently, protein kinase Mζ, a brain-specific variant of PKCζ, was reported to be combined with KIBRA and supposed to modulate molecular pathways of memory formation (Yoshihama et al. 2009).

Although these results strongly suggest that the *KIBRA* SNP should contribute interindividual differences of human memory function, at least two issues remain to be clarified. First, the previous studies demonstrated different memory performance between T allele carriers and C/C carriers (Papassotiropoulos et al. 2006; Almeida et al. 2008; Nacmias et al. 2008; Need et al. 2008; Schaper et al. 2008; Bates et al. 2009; Zhang et al. 2009). The difference among three genotypes of the *KIBRA* SNP and their association with memory performance is unclear. Second, the genotype distribution of the *KIBRA* SNP is largely different among ethnics according to the National Center for Biotechnology Information database of genetic variation (dsSNP) (T allele frequencies in Caucasian: 26%, in Japanese: 81%, in Chinese: 78%, in African: 66%, respectively). It would be possible that genotype effects of the *KIBRA* SNP on memory performance in Asian populations may differ from those in Caucasian populations. To clarify these two issues, we examined a possible association between the *KIBRA* SNP and memory performance in 187 Japanese healthy volunteers.

Materials and methods

Subjects

One hundred and eighty seven healthy subjects participated in this study (92 males and 95 females, the age range; 20–65 years, mean age ± standard deviation (S.D.); 35.9 ± 11.5 years, mean Intelligent Quotient (IQ) ± S.D.; 108.8 ± 11.9). We obtained all the data including IQ and memory performance from the database and research bioresource of healthy controls with genomic DNA in Human Brain Phenotype Consortium (http://www.med.osaka-u.ac.jp/pub/psy/www/jp/labo/sp/consortioum.htm). All the subjects were biologically unrelated Japanese and met the following criteria; (1) were recruited by local advertisements for Human Brain Phenotype Consortium at a single-institution, Osaka University, (2) had no first- or second-degree relatives with psychiatric disease, (3) were excluded if they had neurological or medical conditions that could potentially affect the central nervous system, such as substance abuse or dependence, atypical headache, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, cancer in an active stage, cerebrovascular disease, epilepsy or seizures, (4) were excluded if they had any psychiatric diseases and/or received psychiatric medication, (5) were excluded if the IQ was under 70. They were screened for psychiatric disease with the non-patient edition of the modified structured clinical interview for the Diagnostic and Statistical Manual-Fourth Edition Axis I disorders (SCID-I/NP) (First et al. 1997). Tables I and II (and Supplementary Table I available at: http://informahealthcare.com/doi/abs/10.3109/15622971003797258) show characteristics of subjects according to genotypes. After a description of the study, written informed consent was obtained from every subject. This study was carried out in accordance with the World Medical Association's Declaration of Helsinki and was approved by the ethics committee at Osaka University.

Genetic analysis

Venous blood was collected from the subjects and genomic DNA was extracted from whole blood according to standard procedures. A SNP (*KIBRA*: rs17070145) was genotyped by using the TaqMan 5'-exonuclease allelic discrimination assay described in the previous study (Hashimoto et al. 2006, 2007).

Cognitive tests

A full version of the Wechsler Memory Scale-Revised (WMS-R) (Wechsler 1987), which is generally used to measure memory functions, was administered to subjects. The four indices of the WMS-R (verbal memory, visual memory, attention/concentration and delayed recall) were used for the analysis. Delayed recall is an ability to recall information after 30 minutes of intervening activity. IQ data was col-

Table I. Demographic characteristics and memory performance of the Japanese healthy subjects between T allele carriers and C/C allele carriers.

	C/C	C/T, T/T	*P* value
Number of subjects	7	180	
Age[a]	34.7 (9.7)	36.0 (11.6)	$t(185) = -0.30$, $P = 0.77$
Gender (M/F)[b]	6/1	86/94	$X^2(1, N = 187) = 3.88$, $P = 0.049^*$
Education years[a]	15.7 (0.8)	15.5 (2.4)	$t(185) = 0.27$, $P = 0.789$
IQ[a]	103.0 (14.4)	109.1 (11.8)	$t(185) = -1.33$, $P = 0.186$
WMS-R indices[c]			
Verbal memory	97.4 (15.3)	111.4 (12.9)	$F_{1, 184} = 6.59$, $P = 0.011^*$
Visual memory	103.7 (12.3)	109.5 (9.7)	$F_{1, 184} = 1.47$, $P = 0.23$
Attention/concentration	97.6 (11.8)	108.6 (14.0)	$F_{1, 184} = 6.47$, $P = 0.012^*$
Delayed recall	96.6 (16.7)	112.3 (11.8)	$F_{1, 184} = 9.74$, $P = 0.0021^{**}$

M, male; F, female. WMS-R, Wechsler Memory Scale-Revised. Data are means ± SD.
$^*P < 0.05$. $^{**}P < 0.01$.
[a]Differences in clinical characteristics between genotypes were analyzed by using t-test for age, education years and IQ.
[b]Differences in clinical characteristics between genotypes were analyzed by using the chi-square test for gender.
[c]Differences in indices of WMS-R among genotypes were analyzed by using ANCOVA.

lected by using the full-scale version of the Wechsler Adult intelligence Scale (WAIS)-III ($n = 140$), the shortened version of the WAIS-Revised (WAIS-R) ($n = 44$) or the full-scale version of the WAIS-R ($n = 3$), as described previously (Ohi et al. 2009).

Statistical analysis

Statistical analysis was carried out using SPSS for Windows version 16.0 (SPSS Japan Inc., Tokyo, Japan). The presence of Hardy–Weinberg equibrium was examined by using χ^2-test for goodness of fit. Group comparisons of demographic data were performed by using χ^2-test for a categorical variable (gender), *t*-test or analysis of variance (ANOVA) for continuous variables, as appropriate. As there was a significant difference in gender between the T allele carriers and the C/C carriers, we conducted analysis of covariance (ANCOVA) for WMS-R indices with gender as a covariate (Table I). Group comparisons of WMS-R indices among three genotype groups were analyzed by ANOVA (Table II) and those between the C allele carriers and the T/T carriers were analyzed by *t*-test (Supplementary Table I available at: http://informahealthcare.com/doi/abs/10.3109/15622971003797258). Bonferroni correction was applied for multiple testing to assess the effects of the *KIBRA* genotype on four indices of the WMS-R. Post hoc comparisons were performed after ANOVA by using Bonferroni correction. We used G*Power 3.1 for power calculation (Faul et al. 2009). All *P* values reported are two tailed. Statistical significance was defined at $P < 0.05$.

Table II. Demographic characteristics and memory performance of the Japanese healthy subjects among C/C carriers, C/T carriers and T/T carriers.

	C/C	C/T	T/T	*P* value
Number of subjects	7	56	124	
Age[a]	34.7 (9.7)	36.9 (12.5)	35.6 (11.1)	$F_{2, 184} = 0.270$, $P = 0.76$
Gender (M/F)[b]	6/1	25/31	61/63	$X^2(2, N = 187) = 4.20$, $P = 0.12$
Education years[a]	15.7 (0.8)	15.4 (2.5)	15.5 (2.4)	$F_{2, 184} = 0.12$, $P = 0.89$
IQ[a]	103.0 (14.4)	107.8 (11.6)	109.6 (11.8)	$F_{2, 184} = 1.34$, $P = 0.26$
WMS-R indices[c]				
Verbal memory	97.4 (15.3)	111.6 (12.3)	111.3 (13.2)	$F_{2, 184} = 3.89$, $P = 0.022^*$
Visual memory	103.7 (12.3)	110.4 (7.3)	109.1 (10.6)	$F_{2, 184} = 1.53$, $P = 0.22$
Attention/concentration	97.6 (11.8)	108.0 (13.3)	108.8 (14.4)	$F_{2, 184} = 2.14$, $P = 0.12$
Delayed recall	96.6 (16.7)	110.9 (9.5)	112.9 (12.6)	$F_{2, 184} = 6.36$, $P = 0.0021^{**}$

M, male; F, female. WMS-R, Wechsler memory scale-revised. Data are means ± SD.
$^*P < 0.05$. $^{**}P < 0.01$.
[a]Differences in clinical characteristics among genotypes were analyzed by using ANOVA for age, education years, IQ and indices.
[b]Differences in clinical characteristics among genotypes were analyzed by using the chi-square test for gender.
[c]Differences in indices of WMS-R among genotypes were analyzed by using ANOVA.

Results

We examined the associations between the *KIBRA* SNP (rs17070145) and four indices of the WMS-R in 187 healthy Japanese subjects. The sample was composed of subjects carrying the C/C genotype (3.7%), the C/T genotype (39.9%) and the T/T genotype (66.3%). The genotype distributions of the SNP were in Hardy–Weinberg equilibrium ($\chi^2=0.046$, $P=0.83$).

According to the initial study (Papassotiropoulos et al. 2006), we categorized the subjects into two groups (the T allele carriers vs. the C/C carriers) (Table I). There was no significant difference in age, education years or IQ between the groups except for gender ratio ($\chi^2=3.88$, $P=0.049$). The T allele carriers had significant better memory performance in verbal memory ($F_{1,184}=6.59$, $P=0.011$), attention/concentration ($F_{1,184}=6.47$, $P=0.012$) and delayed recall ($F_{1,184}=9.74$, $P=0.0021$) than the C/C carriers, while there was no significant difference in visual memory ($F_{1,184}=1.47$, $P=0.23$) between genotype groups. The association of the SNP with verbal memory, attention/concentration and delayed recall survived after correction for multiple testing (corrected $P=0.044$, 0.047 and 0.0084, respectively). These results support that the T allele carriers could have better verbal memory, attention/concentration and delayed recall performance than the homozygous C subjects.

We next categorized the subjects into three genotype groups (the C/C genotype group vs. the C/T genotype group vs. the T/T genotype group) (Table II). There was no significant difference in age, education years, IQ or gender ratio between the groups. Significant genotype effects were found in verbal memory ($F_{2,184}=3.89$, $P=0.022$) and delayed recall ($F_{2,184}=6.36$, $P=0.0021$), while no significant difference was found in visual memory ($F_{2,184}=1.53$, $P=0.22$) and attention/concentration ($F_{2,184}=2.14$, $P=0.12$). The association of delayed recall survived after correction for multiple testing (corrected $P=0.0086$). However, the association of verbal memory was no longer positive after correction for multiple testing (corrected $P=0.088$). Post hoc analysis revealed that the T/T carriers and the C/T carriers had better delayed recall performance than the C/C carriers, respectively (post hoc $P=0.0017$, 0.0096) (Figure 1). Thus, the homozygous C allele might have recessive effects on delayed recall performance in our sample.

We finally categorized the sample into two groups (the C allele carriers vs. the T/T carriers) (Supplementary Table I available at: http://informahealthcare.com/doi/abs/10.3109/15622971003797258). There was no significant difference in age, education years, IQ or gender ratio between groups. No significant difference was observed between groups in any memory indices. These results suggest that there is no association of the *KIBRA* SNP with memory performance between the C allele carriers and the T/T carriers.

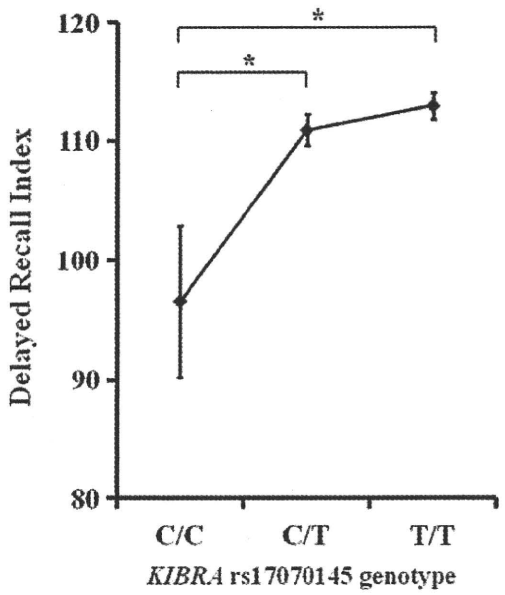

Figure 1. Delayed recall performance according to the KIBRA rs17070145 polymorphism. Mean scores of delayed recall with the C/C ($n=7$), the C/T ($n=56$) and the T/T ($n=124$) genotypes are shown (Bars represent SE). Group comparisons of delayed recall index among three genotype groups were analyzed by using ANOVA. Post hoc comparisons were performed by using Bonferroni correction. Significant differences were compared with the C/C genotype. $^*P < 0.01$.

Discussion

In this study, we first examined the genotype effect of the *KIBRA* SNP among three genotype groups in healthy Japanese subjects. The C allele might have recessive effect on delayed recall performance. Moreover, it revealed that the *KIBRA* SNP had an impact on memory performance in Asian populations as well as in Caucasian populations, though the allelic distribution of the *KIBRA* rs17070145 is different between ethnic groups (T allele frequencies in Caucasian: 26%, in Japanese: 81%, in Chinese: 78%, in African: 66%, respectively).

Since the initial association study, several studies have investigated the relationship between the *KIBRA* SNP and memory performance using various methods. In the initial study, the Rey Auditory Verbal Learning Test(AVLT — a standardized test of learning a list of 30 unrelated nouns (Rosenberg et al. 1984)), Buschke's Selective Remaining Test, and

10-min delayed recall of semantically unrelated picture stimuli were used in three healthy Caucasian cohorts. They found that the T allele (the T/T combined with the C/T) carriers had better delayed recall performance than the C/C carriers (Papassotiropoulos et al. 2006). In terms of replicating the original finding, Schaper et al. examined the association between the *KIBRA* SNP and memory performance by AVLT in a small sample (Schaper et al. 2008). They found that the T allele (the T/T combined with the C/T) carriers had significantly better total recall and delayed recognition performance than the homozygous C/C carriers. Almedia et al. found that the T allele carriers had significant better performance in immediate recall, delayed recall and recognition performance than the C/C carriers measured by the cognitive battery of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Almeida et al. 2008). Bates et al. (2009) found that the *KIBRA* SNP was related to delayed recall performance by using AVLT in subjects with asymptomatic atherosclerosis for randomized controlled trial of aspirin. Preuschhof et al. (2010) also replicated the beneficial effect of the *KIBRA* T allele on episodic memory performance and found that the *KIBRA* and the *CLSTN2* interactively modulate episodic memory performance in healthy subjects. In this way, these previous studies supported that the T allele has a more beneficial effect on episodic memory than the C allele. On the other hand, Zhang et al. (2009) suggested that the T allele carriers had less cognitive flexibility than the C/C carriers in European Americans but not in African Americans, and that current smoking status moderates this influence of the *KIBRA* SNP on cognitive performance. The other two studies did not support the association between the SNP and memory performance (Need et al. 2008, 2009). These inconsistent findings among the studies may have been led to by the possibility of publication bias, age of the subjects, differences between the memory tests which were used in the studies.

In our study, the *KIBRA* SNP had an effect on attention/concentration ($P=0.047$), similar to the previous study which suggested the association between the *KIBRA* SNP and immediate memory performance (Almeida et al. 2008); however, the *KIBRA* SNP was not associated with attention in the original study (Papassotiropoulos et al. 2006). These inconsistent findings between the studies may be led by the possibility of differences between the memory tests and ethnic groups. When we controlled the attention/concentration for verbal memory and delayed recall, genotype effects were found in delayed recall ($P=0.029$), while no significant difference was found in verbal memory ($P=0.074$).

Since the *KIBRA* gene is associated with memory in normal subjects, there are two studies to examine the association of the *KIBRA* SNP with risk for developing Alzheimer's disease (AD). One study reported that the T allele carriers of the *KIBRA* SNP had increased risk of late-onset AD (Rodriguez-Rodriguez et al. 2009); however, another study indicated that the C/C carriers had increased risk for late-onset AD (Corneveaux et al. 2010). No association between the *KIBRA* SNP and mild cognitive impairment (MCI) was found in an aged cohort without dementia (Almeida et al. 2008). Subjective memory complaints with the T allele carriers performed more poorly than those with the C/C carriers on long-term memory tests (Nacmias et al. 2008), which was opposite genotype effect found in healthy subjects including our data (Papassotiropoulos et al. 2006; Almeida et al. 2008; Schaper et al. 2008; Bates et al. 2009; Preuschhof et al. 2009). These inconsistencies among genetic association studies in patients and inconsistencies between normal control studies and studies in memory impairments could be due to the differential role of the *KIBRA* gene in a certain pathological state.

The limitation of our study is that we had a small sample size of the C/C genotype group compared with the other two genotype groups. A larger population would avoid this problem. The effect sizes f in our study were medium; 0.19 in verbal memory and 0.23 in delayed recall. It is similar to the previous studies (the effect size $d=0.2$–0.7; small to large) (Papassotiropoulos et al. 2006; Schaper et al. 2008). When we performed power calculations, the post hoc powers in our study were 0.73 in verbal memory and 0.88 in delayed recall.

Further independent study with larger sample size is warranted in the other Asian population. More investigations to clarify the association of the *KIBRA* gene with memory performance in other ethnic groups would be required. To clarify the function of the *KIBRA* gene may lead to further understandings about the brain function and the pathology of neuropsychiatric disorders with memory disturbances.

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