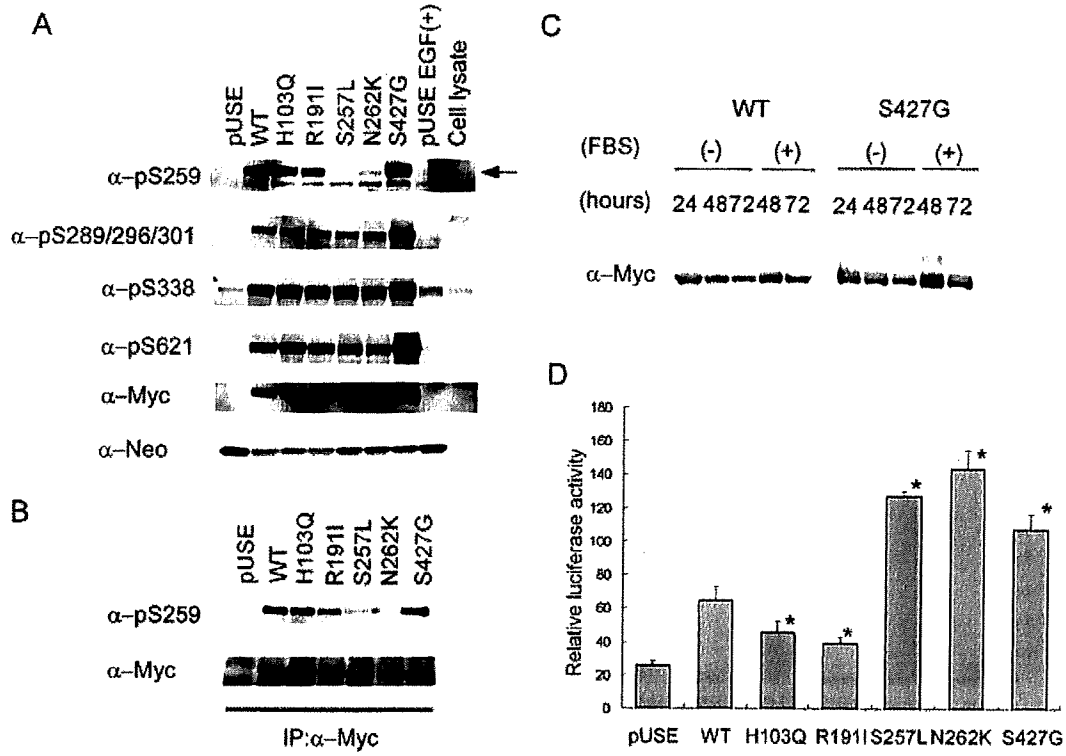

Figure 2. Analysis of phosphorylation status, degradation, and effect on downstream signaling in RAF1 mutants identified in this study. **A:** Phosphorylation status of wild-type (WT) RAF1 and mutants. Expression levels of RAF1 proteins and their phosphorylation levels were detected with different antibodies 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

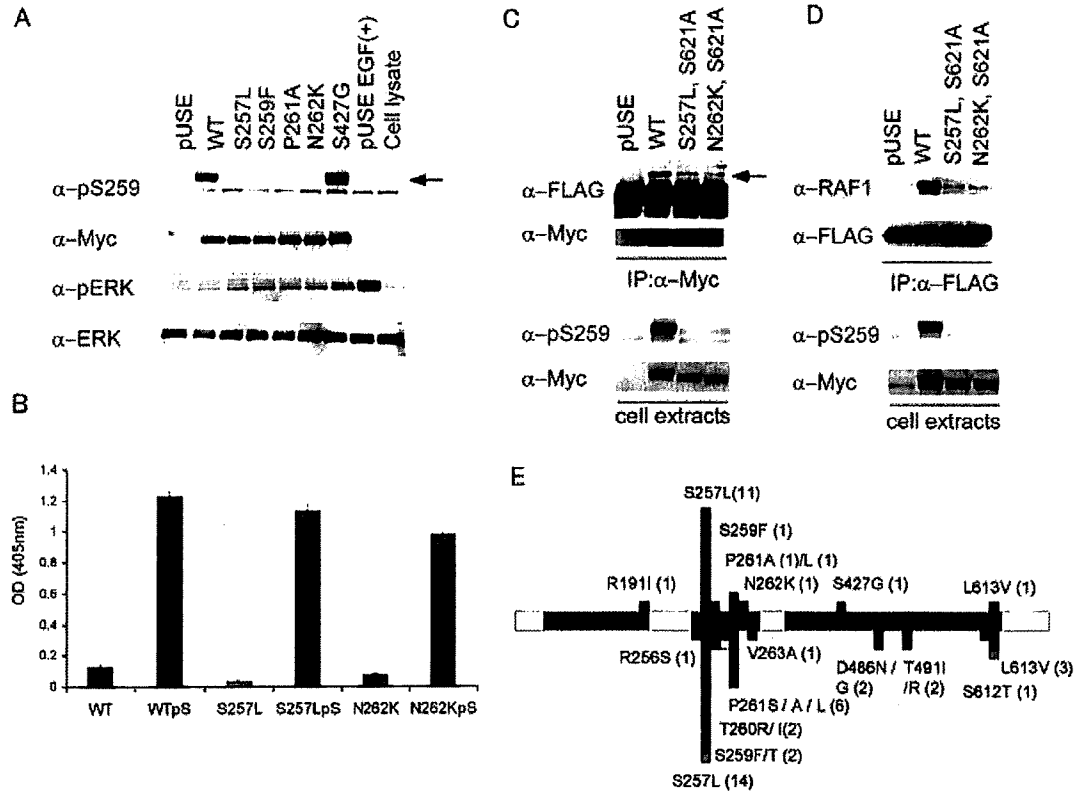

Figure 3. Phosphorylation of S259, binding to 14-3-3 and ERK activation of mutants located in the CR2 domain. **A:** Phosphorylation status of WT and mutants located in the CR2 domain. Phosphorylation of S259 was not observed in cells expressing p.S257L, p.S259F, p.P261A, and p.N262K. In order to examine 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

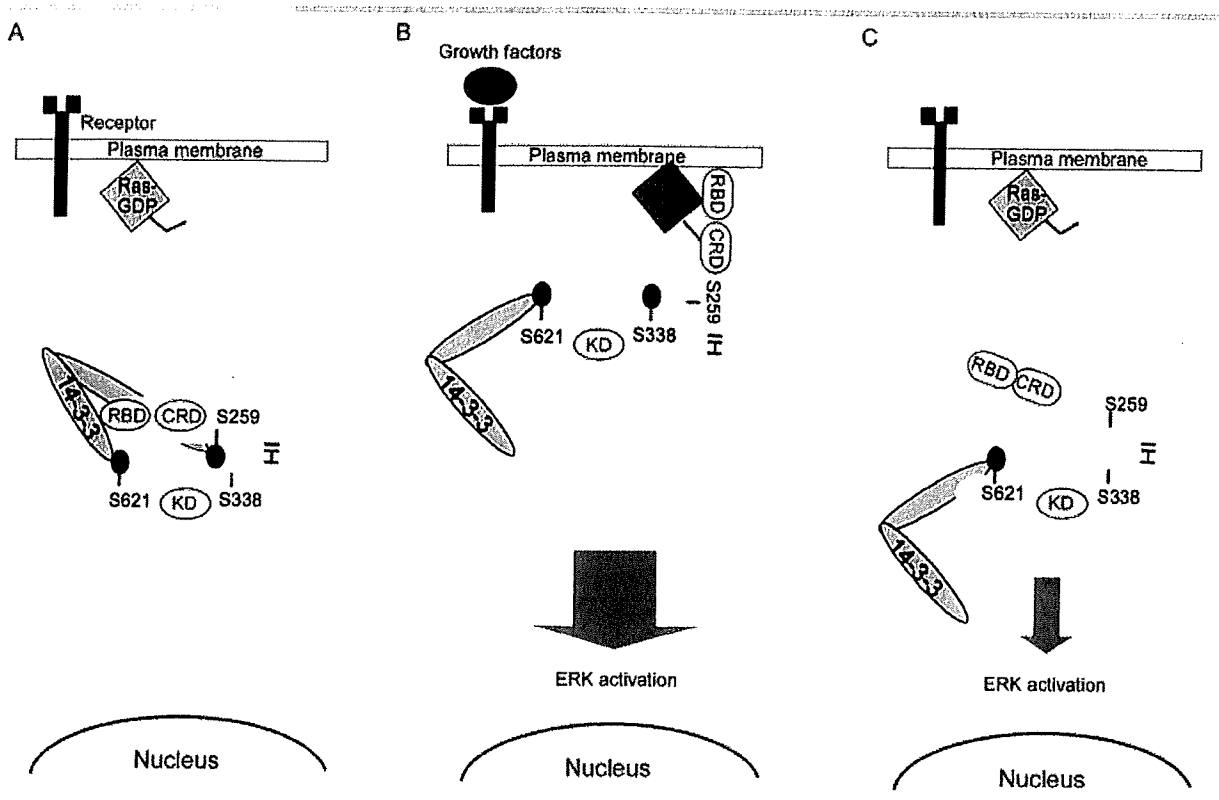

Figure 4. Schematic model of WT and mutant activation. **A:** In an inactive state, RAF1 is phosphorylated on S259 and S621 and is bound to 14-3-3. **B:** In growth-factor stimulation, the GTP-bound RAS binds to the CR1 domain of RAF1, which displaces 14-3-3. S259 is dephosphorylated by protein phosphatase 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.

Acknowledgments

The authors wish to thank the patients and their families who participated in this study. We are grateful to physicians who referred the patients and to Kumi Kato and Miyuki Tsuda for technical assistance. This work was supported by Grants-in-Aids from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Japan Society for the Promotion of Science, and The Ministry of Health Labour and Welfare to Y.M. and Y.A. and by an outstanding Senior Graduate Student award from Tohoku University Graduate School of Medicine to T.K.

References

Allanson JE, Hall JG, Hughes HE, Preus M, Witt RD. 1985. Noonan syndrome: the changing phenotype. Am J Med Genet 21:507–514.

Aoki Y, Niihori T, Kawame H, Kurosawa K, Ohashi H, Tanaka Y, Filocamo M, Kato K, Suzuki Y, Kure S, Matsubara Y. 2005. Germline mutations in HRAS proto-oncogene cause Costello syndrome. Nat Genet 37:1038–1040.

Aoki Y, Niihori T, Narumi Y, Kure S, Matsubara Y. 2008. The RAS/MAPK syndromes: novel roles of the RAS pathway in human genetic disorders. Hum Mutat 29:992–1006.

Bentires-Alj M, Kontaridis MI, Neel BG. 2006. Stops along the RAS pathway in human genetic disease. Nat Med 12:283–285.

Brems H, Chmara M, Sahbatou M, Denayer E, Taniguchi K, Kato R, Somers R, Messiaen L, De Schepper S, Fryns JP, Cools J, Marynen P, Thomas G, Yoshimura A, Legius E. 2007. Germline loss-of-function mutations in SPRED1 cause a neurofibromatosis 1-like phenotype. Nat Genet 39:1120–1126.

Carta C, Pantaleoni F, Bocchinfuso G, Stella L, Vasta I, Sarkozy A, Digilio C, Palleschi A, Pizzuti A, Grammatico P, Zampino G, Dallapiccola B, Gelb BD, Tartaglia M. 2006. Germline missense mutations affecting KRAS isoform B are associated with a severe Noonan syndrome phenotype. Am J Hum Genet 79:129–135.

Dhillon AS, von Kriegsheim A, Grindlay J, Kolch W. 2007. Phosphatase and feedback regulation of Raf-1 signaling. Cell Cycle 6:3–7.

Digilio MC, Conti E, Sarkozy A, Mingarelli R, Dottorini T, Marino B, Pizzuti A, Dallapiccola B. 2002. Grouping of multiple-lentigines/LEOPARD and Noonan syndromes on the PTPN11 gene. Am J Hum Genet 71:389–394.

Ferrero GB, Baldassarre G, Delmonaco AG, Biamino E, Banaudi E, Carta C, Rossi C, Silengo MC. 2008. Clinical and molecular characterization of 40 patients with Noonan syndrome. Eur J Med Genet 51:566–572.

Hennekam RC. 2003. Costello syndrome: an overview. Am J Med Genet C Semin Med Genet 117:42–48.

Jongmans M, Sistermans EA, Rikken A, Nillesen WM, Tamminga R, Patton M, Maier EM, Tartaglia M, Noordam K, van der Burgt I. 2005. Genotypic and phenotypic characterization of Noonan syndrome: new data and review of the literature. Am J Med Genet A 134A:165–170.

Ko JM, Kim JM, Kim GH, Yoo HW. 2008. PTPN11, SOS1, KRAS, and RAF1 gene analysis, and genotype–phenotype correlation in Korean patients with Noonan syndrome. J Hum Genet 53:999–1006.

Light Y, Paterson H, Marais R. 2002. 14-3-3 antagonizes Ras-mediated Raf-1 recruitment to the plasma membrane to maintain signaling fidelity. Mol Cell Biol 22:4984–4996.

Lo FS, Lin JL, Kuo MT, Chiu PC, Shu SG, Chao MC, Lee YJ, Lin SP. 2008. Noonan syndrome caused by germline KRAS mutation in Taiwan: report of two patients and a review of the literature. Eur J Pediatr 168:919–923.

Mendez HM, Opitz JM. 1985. Noonan syndrome: a review. Am J Med Genet 21:493–506.

Mercer KE, Pritchard CA. 2003. Raf proteins and cancer: B-Raf is identified as a mutational target. Biochim Biophys Acta 1653:25–40.

Musante L, Kehl HG, Majewski F, Meinecke P, Schweiger S, Gillessen-Kaesbach G, Wieczorek D, Hinkel GK, Tinschert S, Hoeltzenbein M, Ropers HH, Kalscheuer VM. 2003. Spectrum of mutations in PTPN11 and genotype–phenotype correlation in 96 patients with Noonan syndrome and five patients with cardio-facio-cutaneous syndrome. Eur J Hum Genet 11:201–206.

Narumi Y, Aoki Y, Niihori T, Sakurai M, Cave H, Verloes A, Nishio K, Ohashi H, Kurosawa K, Okamoto N, Kawame H, Mizuno S, Kondoh T, Addor MC, Coeslier-Dieux A, Vincent-Delorme C, Tabayashi K, Aoki M, Kobayashi T, Guliyeva A, Kure S, Matsubara Y. 2008. Clinical manifestations in patients with SOS1 mutations range from Noonan syndrome to CFC syndrome. J Hum Genet 53:834–841.

Niihori T, Aoki Y, Narumi Y, Neri G, Cave H, Verloes A, Okamoto N, Hennekam RC, Gillessen-Kaesbach G, Wieczorek D, Kavamura MI, Kurosawa K, Ohashi H, Wilson L, Heron D, Bonneau D, Corona G, Kaname T, Naritomi K, Baumann C, Matsumoto N, Kato K, Kure S, Matsubara Y. 2006. Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. Nat Genet 38:294–296.

Noble C, Mercer K, Hussain J, Carragher L, Giblett S, Hayward R, Patterson C, Marais R, Pritchard CA. 2008. CRAF autophosphorylation of serine 621 is required to prevent its proteasome-mediated degradation. Mol Cell 31:862–872.

Pandit B, Sarkozy A, Pennacchio LA, Carta C, Oishi K, Martinelli S, Pogna EA, et al. 2007. Gain-of-function RAF1 mutations cause Noonan and LEOPARD syndromes with hypertrophic cardiomyopathy. Nat Genet 39:1007–1012.

Razzaque MA, Nishizawa T, Komoike Y, Yagi H, Furutani M, Amo R, Kamisago M, Momma K, Katayama H, Nakagawa M, Fujiwara Y, Matsushima M, Mizuno K, Tokuyama M, Hirota H, Muneuchi J, Higashinakagawa T, Matsuoka R. 2007. Germline gain-of-function mutations in RAF1 cause Noonan syndrome. Nat Genet 39:1013–1017.

Reynolds JF, Neri G, Herrmann JP, Blumberg B, Coldwell JG, Miles PV, Opitz JM. 1986. New multiple congenital anomalies/mental retardation syndrome with cardio-facio-cutaneous involvement—the CFC syndrome. Am J Med Genet 25:413–427.

Roberts AE, Araki T, Swanson KD, Montgomery KT, Schiripo TA, Joshi VA, Li L, Yassin Y, Tamburino AM, Neel BG, Kucherlapati RS. 2007. Germline gain-of-function mutations in SOS1 cause Noonan syndrome. Nat Genet 39: 70–74.

Rodriguez-Viciana P, Tetsu O, Tidyman WE, Estep AL, Conger BA, Cruz MS, McCormick F, Rauen KA. 2006. Germline mutations in genes within the MAPK pathway cause cardio-facio-cutaneous syndrome. Science 311:1287–1290.

Schubbert S, Zenker M, Rowe SL, Boll S, Klein C, Bollag G, van der Burgt I, Musante L, Kalscheuer V, Wehner LE, Nguyen H, West B, Zhang KY, Sistermans E, Rauch A, Niemeyer CM, Shannon K, Kratz CP. 2006. Germline KRAS mutations cause Noonan syndrome. Nat Genet 38:331–336.

Tartaglia M, Kalidas K, Shaw A, Song X, Musat DL, van der Burgt I, Brunner HG, Bertola DR, Crosby A, Ion A, Kucherlapati RS, Jeffery S, Patton MA, Gelb BD. 2002. PTPN11 mutations in Noonan syndrome: molecular spectrum, genotype-phenotype correlation, and phenotypic heterogeneity. Am J Hum Genet 70: 1555–1563.

Tartaglia M, Mehler EL, Goldberg R, Zampino G, Brunner HG, Kremer H, van der Burgt I, Crosby AH, Ion A, Jeffery S, Kalidas K, Patton MA, Kucherlapati RS, Gelb BD. 2001. Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. Nat Genet 29:465–468.

Tartaglia M, Pennacchio LA, Zhao C, Yadav KK, Fodale V, Sarkozy A, Pandit B, Oishi K, Martinelli S, Schackwitz W, Ustaszewska A, Martin J, Bristow J, Carta C, Lepri F, Neri C, Vasta I, Gibson K, Curry CJ, Siguero JP, Digilio MC, Zampino G, Dallapiccola B, Bar-Sagi D, Gelb BD. 2007. Gain-of-function SOS1 mutations cause a distinctive form of Noonan syndrome. Nat Genet 39:75–79.

van der Burgt I. 2007. Noonan syndrome. Orphanet J Rare Dis 2:4.

Zebisch A, Haller M, Hiden K, Goebel T, Hoefler G, Troppmair J, Sill H. 2009. Loss of RAF kinase inhibitor protein is a somatic event in the pathogenesis of therapy-related acute myeloid leukemias with C-RAF germline mutations. Leukemia 23:1049–1053.

Zebisch A, Staber PB, Delavar A, Bodner C, Hiden K, Fischereder K, Janakiraman M, Linkesch W, Auner HW, Emberger W, Windpassinger C, Schimek MG, Hoefler G, Troppmair J, Sill H. 2006. Two transforming C-RAF germ-line mutations identified in patients with therapy-related acute myeloid leukemia. Cancer Res 66:3401–3408.

Zenker M, Buheitel G, Rauch R, Koenig R, Bosse K, Kress W, Tietze HU, Doerr HG, Hofbeck M, Singer H, Reis A, Rauch A. 2004. Genotype–phenotype correlations in Noonan syndrome. J Pediatr 144:368–374.

Zenker M, Horn D, Wieczorek D, Allanson J, Pauli S, van der Burgt I, Doerr HG, Gaspar H, Hofbeck M, Gillessen-Kaesbach G, Koch A, Meinecke P, Mundlos S, Nowka A, Rauch A, Reif S, von Schnakenburg C, Seidel H, Wehner LE, Zweier C, Bauhuber S, Matejas V, Kratz CP, Thomas C, Kutsche K. 2007a. SOS1 is the second most common Noonan gene but plays no major role in cardio-facio-cutaneous syndrome. J Med Genet 44:651–656.

Zenker M, Lehmann K, Schulz AL, Barth H, Hansmann D, Koenig R, Korinthenberg R, Kreiss-Nachtsheim M, Meinecke P, Morlot S, Mundlos S, Quante AS, Raskin S, Schnabel D, Wehner LE, Kratz CP, Horn D, Kutsche K. 2007b. Expansion of the genotypic and phenotypic spectrum in patients with KRAS germline mutations. J Med Genet 44:131–135.

Brief Communication

Journal of Child Neurology
Volume 24 Number 7
July 2009 868-873

10.1177/0883073808330186
http://jcn.sagepub.com

Smith-Magenis Syndrome With West Syndrome in a 5-Year-Old Girl: A Long-Term Follow-Up Study

Naomi Hino-Fukuyo, MD, Kazuhiro Haginoya, MD, Mitsugu Uematsu, MD, Tojo Nakayama, MD, Atsuo Kikuchi, MD, Shigeo Kure, MD, Fumiaki Kamada, MD, Yu Abe, MD, Natsuko Arai, MD, Noriko Togashi, MD, Akira Onuma, MD, and Shigeru Tsuchiya, MD

Smith-Magenis syndrome is characterized by multiple congenital anomalies and mental retardation caused by the heterozygous deletion of chromosomal region 17p11.2. We present a long-term follow-up study of a girl with Smith-Magenis syndrome and West syndrome. West syndrome became apparent at 7 months of age. Since then, mental retardation, particularly in terms of language development, became increasingly more obvious. The patient's spasms and hypsarrhythmia disappeared after a course of adrenocorticotropic hormone therapy, but focal seizures reappeared at the age of 3 years and 3 months. Her craniofacial dysmorphia and mental retardation became increasingly evident compared to her condition at the onset of West syndrome. Chromosome analysis detected the characteristic 17p deletion, which was then confirmed via fluorescent in situ hybridization analysis. This is the second report of a patient with Smith-Magenis syndrome and West syndrome; taken together, these results suggest that Smith-Magenis syndrome may be a further cause of West syndrome.

Keywords: Smith-Magenis syndrome; West syndrome; epilepsy; chromosomal abnormality

Smith-Magenis syndrome is characterized by multiple congenital anomalies and mental retardation presenting with a distinct phenotype.[1] This syndrome is associated with either an interstitial deletion of chromosomal region 17p11.2, which contains the retinoic acid-induced 1 gene, or a mutation of retinoic acid-induced 1 gene.[2-4] The Smith-Magenis syndrome phenotype includes distinctive craniofacial and skeletal features, infantile hypotonia, significant expressive language delay, cognitive and mental retardation, stereotypies, neurobehavioral problems, and sleep disorder because of abnormal melatonin secretion.[5-7] First described by Smith et al[2] in 1982, this syndrome occurs in all ethnic groups with an overall estimated frequency of 1/25 000.[8]

Epileptic seizures occur in 11% to 30% of individuals with Smith-Magenis syndrome.[1,8-11] Electroencephalographic abnormalities have been documented in approximately 25% of patients, even in the absence of a clinical history of seizures in 1 series.[1,11]

West syndrome is an age-dependent epileptic syndrome that occurs in infancy. West syndrome is characterized by clustering epileptic spasms, electroencephalographic hypsarrhythmia, and arrested psychomotor development. A previous report described a 9-month-old girl with Smith-Magenis syndrome associated with West syndrome,[12] but the neurological and epileptological descriptions of the patient were sparse. Here, we report a new patient with Smith-Magenis syndrome afflicted by West syndrome, for whom electroencephalographic and neurological follow-up were performed.

Case

A 5-year-old girl had been born to nonconsanguineous healthy parents after 37 weeks of gestation. No family

Received November 12, 2008. Accepted for publication November 29, 2008.

From the Department of Pediatrics, Tohoku University School of Medicine, Sendai, Japan (NH-F, KH, MU, TN, AK, YA, NA, ST); Department of Pediatric Neurology, Takuto Rehabilitation Center for Children, Sendai, Japan (KH, AO); Department of Medical Genetics, Tohoku University School of Medicine, Sendai, Japan (SK, FK); and Eko-Ryoikuen, Hospital Home for Children and Persons with Severe Motor and Intellectual Disabilities, Sendai, Japan (NT).

The authors have no conflicts of interest to disclose with regard to this article.

Address correspondence to: Naomi Hino-Fukuyo, MD, Department of Pediatrics, Tohoku University School of Medicine, Seiryo-machi 1-1, Aoba-ku, Sendai 980-8574, Japan; e-mail: naomi-h@zc4.so-net.ne.jp.

Hino-Fukuyo N, Haginoya K, Uematsu M, et al. Smith-Magenis syndrome with West syndrome in a 5-year-old girl: a long-term follow-up study. *J Child Neurol.* 2009;24:868-873.

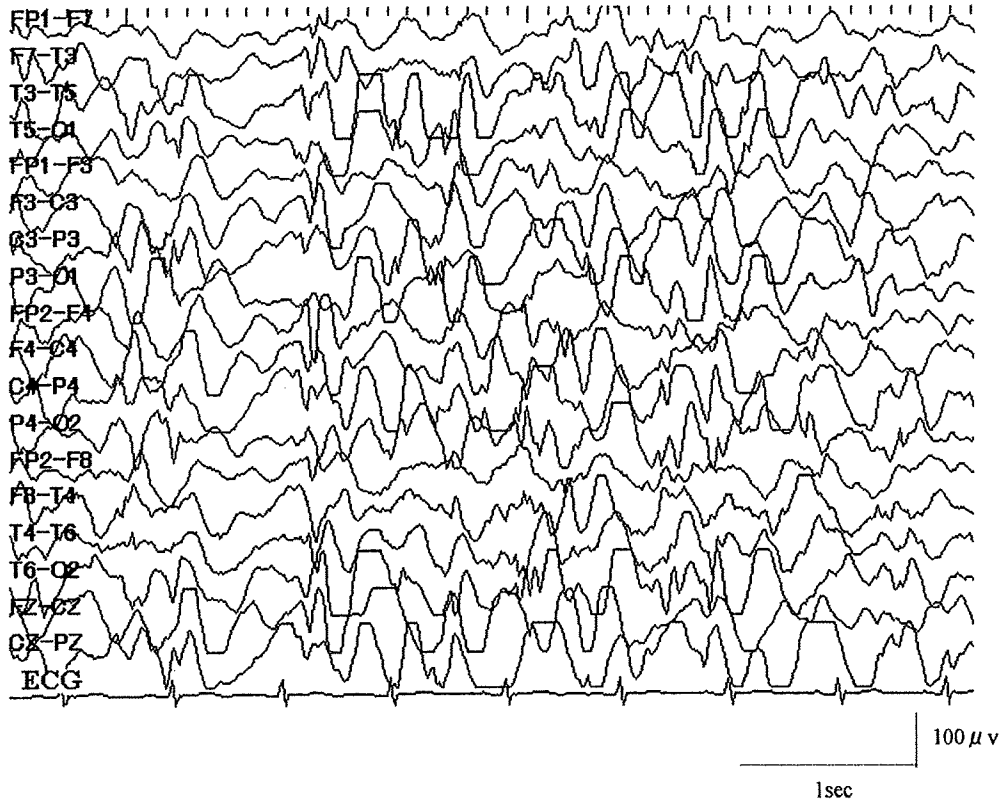

Figure 1. Interictal electroencephalography showing hypsarrhythmia at 7 months of age.

history of neurological conditions was apparent and the patient has no siblings. The neonatal period was unremarkable. The patient had a birth weight of 2106 g (–2.2 SD), a body length of 42 cm (–3.5 SD), and a head circumference of 29.5 cm (–2.7 SD). Psychomotor development was normal until the first seizure episode occurring at 7 months of age. Because the patient exhibited brief spasms in clusters, and interictal electroencephalography showed hypsarrhythmia (Figure 1), she was diagnosed with West syndrome and admitted to Tohoku University Hospital. A mild developmental delay was apparent on admission (eg, she was unable to sit up at the time of examination). Neurological examinations were unremarkable except for mild hypotonia. Laboratory examinations, including a full blood count, routine blood chemistry, urinalysis, amino acids, blood levels of lactate and pyruvate, and urine organic acids were within normal ranges. Brainstem auditory evoked potentials and visual evoked potentials were normal. Routine cerebrospinal fluid examination was negative. G-banding cytogenetic analysis showed a normal 46, XX karyotype. Magnetic resonance imaging revealed a small T2 high-intensity lesion of unknown etiology at the posterior lateral aspect of the left lateral ventricle (Figure 2A). Retinal observation was normal. Spasms were refractory to pyridoxine and valproic acid. After 4 days of adrenocorticotropic hormone (ACTH) therapy, spasms disappeared; electroencephalography showed no hypsarrhythmia but focal spikes were apparent in the left frontal to central areas, which resolved after replacing valproic acid with nitrazepam.

The patient was monitored as a patient with cryptogenic West syndrome receiving nitrazepam. Her development delay, particularly in language development, became increasingly obvious despite complete seizure control. In the following months, the patient acquired some motor skills, such as the ability to sit on her own at 1 year and the ability to walk on her own at 2 years and 3 months of age. However, she had uttered no meaningful word at 3 years of age.

At 3 years and 3 months of age, the patient had a relapse of bilateral or hemifacial clonic seizures that appeared around her mouth without disturbance of consciousness and occasionally developed into secondarily generalized seizures. Seizures usually occurred on awakening 1 to 3 times per month. Interictal electroencephalography showed independent spikes in the right mid-temporal area and left central to parietal area. At this time, a second chromosomal analysis was performed because of obvious mental retardation and peculiar facial features. Chromosomal analysis

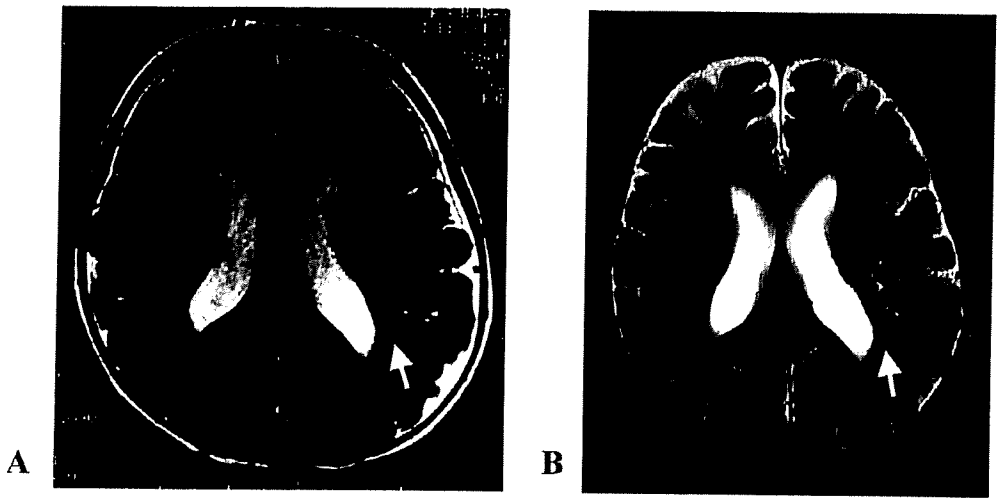

Figure 2. Brain magnetic resonance imaging at 7 months of age and 5 years of age. T2-weighted brain magnetic resonance imaging at 7 months (A) revealed a small lesion of unknown etiology (arrow) at the posterior lateral aspect of the left lateral ventricle, which remained unchanged at 5 years of age (B). An enlargement of the lateral ventricle on both sides was also apparent.

revealed the characteristic 17p deletion commonly seen in Smith-Magenis syndrome, which was later confirmed by fluorescent in situ hybridization. The probe we used contains serine hydroxymethyltransferase gene, topoisomerase III gene, flightless II gene, and lethal giant larvae homolog 1 gene.

The patient's seizures became more frequent at 4 years and 3 months of age. Seizures began with facial twitching, followed by generalized tonic seizure with severe cyanosis. Interictal electroencephalography showed spikes and sharp waves in the right frontal area. The seizures were finally controlled by nitrazepam, zonisamide, phenobarbital, and clobazam. After 10 months, the patient developed head-drop seizure in clusters and head dropping followed by bilateral tonic seizure. Interictal electroencephalography showed multifocal spikes in the bilateral frontal area, anterior-temporal area, right posterior-temporal area, and mid-frontal area. Ictal electroencephalography corresponding to single head-drop seizures showed diffuse slow waves, followed by low-amplitude diffuse fast waves with focal spikes in the left mid-temporal area (Figure 3A), whereas ictal electroencephalography corresponding to head dropping followed by bilateral tonic seizures showed diffuse suppression with fast waves built up to spike bursts without any lateralization (Figure 3B). Simultaneous surface electromyography showed cessation of neck muscle activity during head dropping, which was considered to be an atonic seizure of focal origin, although her ictal electroencephalography showed no localized findings. Various antiepileptic drugs including phenytoin, topiramate, and ethosuximide were ineffective in controlling her seizures.

At present, the patient is of relatively small stature with a height of 102 cm (−1.5 SD), a weight of 15 kg (−1.2 SD), and a head circumference of 47.8 cm (−1.5SD). A complete evaluation of the heart was normal. She exhibits brachycephaly, midfacial hypoplasia, a prominent forehead, upslanted palpebral fissures, narrow-spaced eyes, a broad nose with a depressed root, short and stubby hands, and clinodactyly of the fifth fingers (Figure 4). The small lesion of unknown etiology at the posterior lateral aspect of the left lateral ventricle of T2-weighted brain magnetic resonance imaging at 5 years of age (Figure 2B) remained unchanged. An enlargement of the lateral ventricle on both sides was also apparent. She showed good motor milestones in comparison to language development. At 5 years and 2 months age, the patient's developmental quotients for language and gross motor development were 11 and 65, respectively, based on the Enjoji scale of infant development. Behavioral disturbances, including temper tantrums, attention deficit, self-injury, and hyperactivity, have also gradually become evident. She also exhibits a sleep disorder characterized by early morning waking and fragmented and shortened total sleep cycle.

Discussion

Here, we report a case of Smith-Magenis syndrome associated with West syndrome, for which we have performed a long-term electroencephalographic follow-up. The diagnosis of Smith-Magenis syndrome is based on the clinical recognition of a unique phenotype involving physical, developmental, and behavioral aspects. Infants are described as "cherubic" with a Down syndrome–like appearance. However, many of the features of Smith-Magenis syndrome may be relatively subtle in infancy and

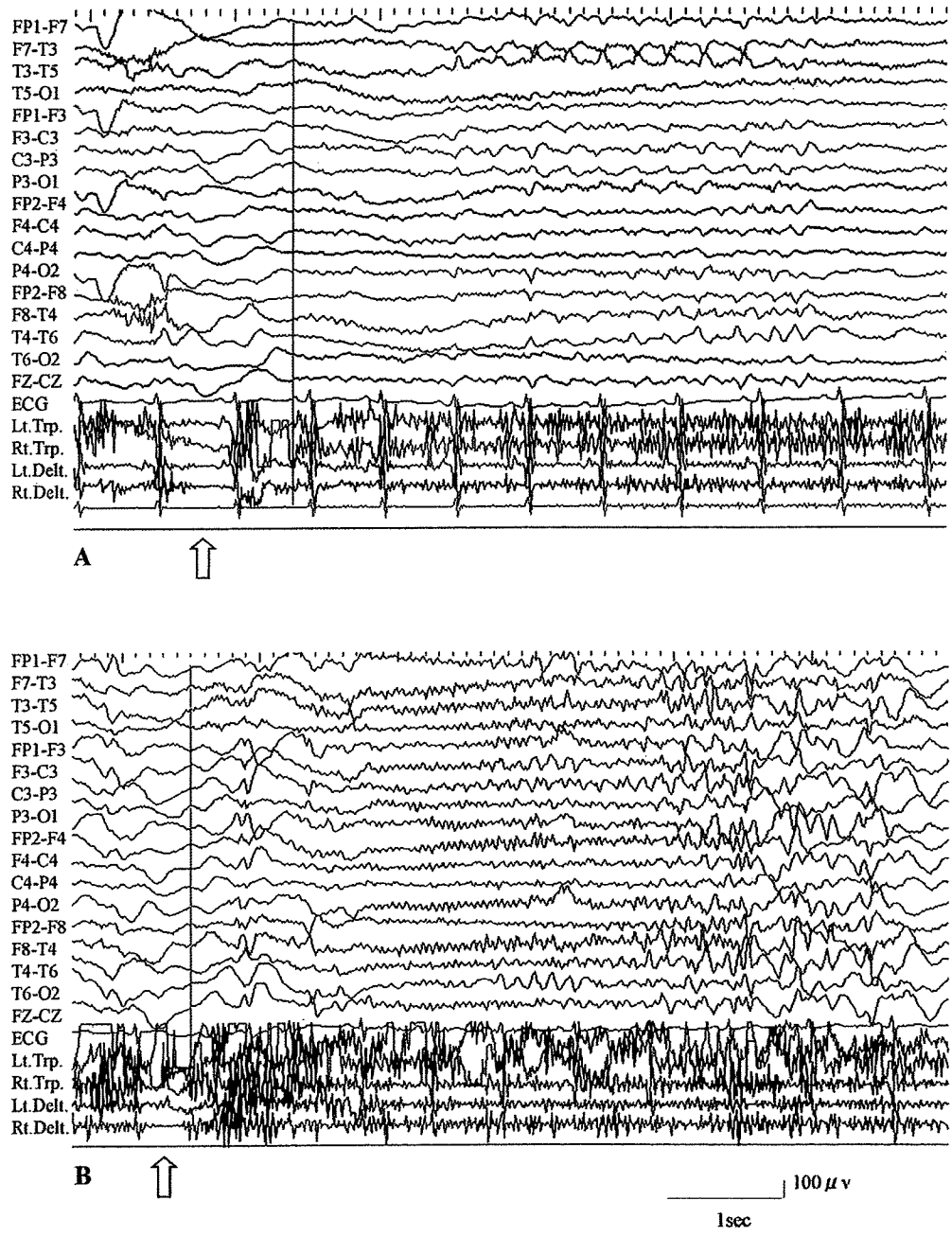

Figure 3. Ictal electroencephalography and surface electromyography of a single head-drop seizure (arrow) (A) and head dropping followed by bilateral tonic seizure (arrow) (B). Delt, deltoid muscle; Lt, left; Rt, right; trap, trapezius muscle.

early childhood and become more recognizable with advancing age. Most children are diagnosed in mid-childhood when the features of the disorder are most recognizable and striking.

The diagnosis of Smith-Magenis syndrome is based on clinical findings and can be confirmed via routine G-banded cytogenetic analysis, provided the resolution is adequate (550 band or higher), or fluorescent in situ hybridization analysis. These analyses are performed to detect the characteristic 3.5-Mb interstitial deletion of chromosomal region 17p11.2, which contains the crucial retinoic acid–induced 1 gene. All 17p11.2 deletions

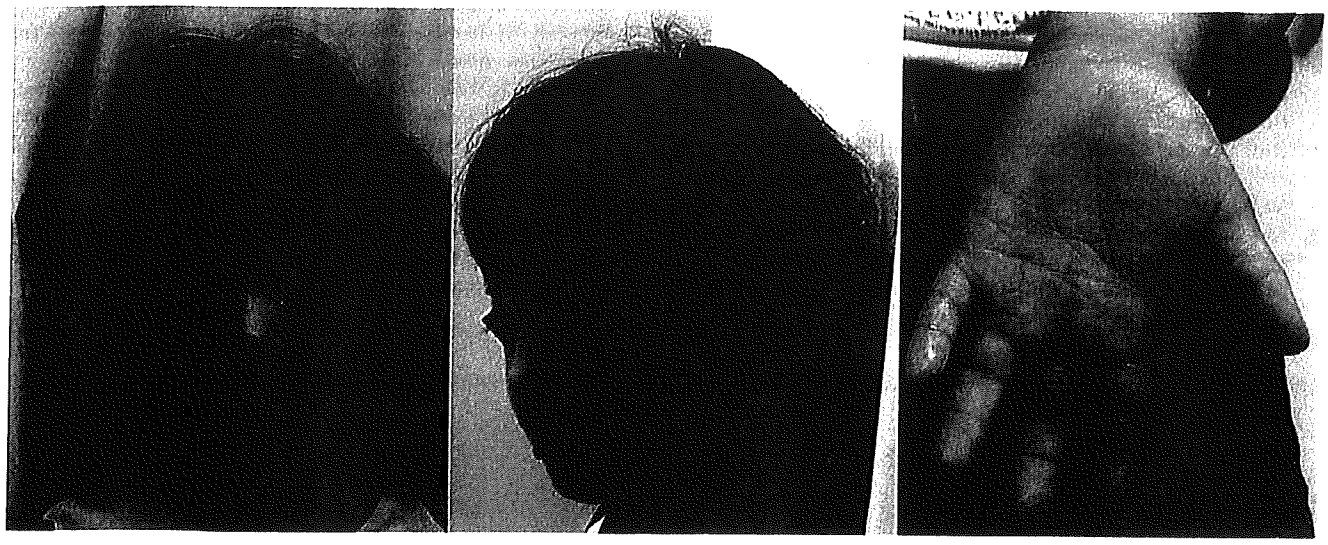

Figure 4. Facial features and fingers of the patient at 4 years of age. Brachycephaly, midfacial hypoplasia, a prominent forehead, upslanted palpebral fissures, narrow-spaced eyes, a broad nose with a depressed root, short and stubby hands, and clinodactyly of the fifth fingers are apparent.

associated with Smith-Magenis syndrome include a deletion of retinoic acid–induced 1.[13] Alternatively, some individuals develop Smith-Magenis syndrome as a result of mutation, rather than deletion, of the retinoic acid-induced 1 gene, which can be detected via direct sequencing. Other affected individuals have atypical (smaller or larger) deletions involving 17p11.2.[14] Studies indicate that approximately 90% of cases have a fluorescent in situ hybridization-detectable deletion, with approximately 70% of cases exhibiting the characteristic 3.5-Mb deletion,[13,14] the remaining 30% shows atypical deletions. In the present case, chromosomal analysis performed early in childhood was normal, which we assume to have been the result of laboratory error, and a second chromosomal analysis revealed the 17p deletion, which was then confirmed via fluorescent in situ hybridization analysis.

Among 60 previously reported cases of Smith-Magenis syndrome, 18% (11/60) exhibited seizures, and abnormal epileptiform electroencephalography activity recorded via prolonged video-electroencephalography was identified in 30% of patients.[10] Parents reported initial seizure phenomena suggestive of either absence or partial seizures (7/11), generalized convulsions indicative of generalized tonic-clonic seizures (4/11), and drop attacks (2/11), which are considered to be atonic seizure.[10] Generalized epileptiform patterns were the most common (73%).[10] Therefore, cortical hyperexcitability and epilepsy may represent an important component of the Smith-Magenis clinical phenotype.[10] According to a previous report, Smith-Magenis syndrome is not characterized by any single seizure type or electroencephalographic pattern, although complex partial seizures are most common.[11] No ictal events, however, have been analyzed thus far via video-electroencephalography. The present patient most likely progressed from West syndrome to focal epilepsy with multifocal spike discharges.

Pediatric neurologists should be aware of this underdiagnosed syndrome as a further cause of West syndrome, although the pathophysiological contributions of Smith-Magenis–related gene deletions in West syndrome remain to be elucidated.

References

1. Greenberg F, Lewis RA, Potocki L, et al. Multi-disciplinary clinical study of Smith-Magenis syndrome (deletion 17p11.2). *Am J Med Genet*. 1996;62:247-254.
2. Smith AC, McGavran L, Robinson J, et al. Interstitial deletion of (17) (p11.2p11.2) in nine patients. *Am J Med Genet*. 1986;24: 393-414.
3. Seranski P, Hoff C, Radelof U, et al. RAI1 is a novel polyglutamine encoding gene that is deleted in Smith-Magenis syndrome patients. *Gene*. 2001;270:69-76.
4. Slager RE, Newton TL, Vlangos CN, Finucane B, Elsea SE. Mutation in RAI1 associated with Smith-Magenis syndrome. *Nat Genet*. 2003;33:466-468.
5. Potocki L, Glaze D, Tan DX, et al. Circadian rhythm abnormalities of melatonin in Smith-Magenis syndrome. *J Med Genet*. 2000;37:428-433.
6. Leersnyder H, Blois MC, Claustrat B, et al. Inversion of the circadian rhythm of melatonin in the Smith-Magenis syndrome. *J Pediatr*. 2001;139:111-116.
7. Potocki L, Shaw CJ, Staniewicz P, Lupski JR. Variability in clinical phenotype despite common chromosomal deletion in

Smith-Magenis syndrome [del(17)(p11.2p11.2)]. *Genet Med.* 2003;5:430-434.
8. Greemberg F, Guzzetta V, Montes de Oca-Luna R, et al. Molecular analysis of the Smith-Magenis syndrome: a possible contiguous gene syndrome associated with del (17)(p11.2). *Am J Hum Genet.* 1991;49:1207-1218.
9. Gropman AL, Elsea S, Duncan WC, Smith AC. New developments in Smith-Magenis syndrome (del 17p11.2). *Curr Opin Neurol.* 2007;20:125-134.
10. Goldma AM, Potocki L, Walz K, et al. Epilepsy and chromosomal rearrangements in Smith-Magenis syndrome (del(17)(p11.2p11.2). *J Child Neurol.* 2006;21:93-98.
11. Gropman AL, Smith AC, Greenberg F. Neurologic aspects of the Smith-Magenis syndrome. *Ann Neurol.* 1998;44:561.
12. Roccella M, Parisi L. The Smith-Magenis syndrome: a new case with infant spasms. *Minerva Pediatr.* 1999;51:65-71.
13. Vlangos CN, Wilson M, Blancato J, Smith AC, Elsea SH. Diagnostic FISH probes for del (17)(p11.2p11.2) associated with Smith-Magenis syndrome should contain the RAI1 gene. *Am J Med Genet.* 2005;132:278-282.
14. Vlangos CN, Yim DK, Elsea SH. Refinement of the Smith-Magenis syndrome cirtical region to approximately 950 kb and assessment of 17p11.2 deletions. Are all deletions created equally? *Mol Genet Metab.* 2003;79:134-141.