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

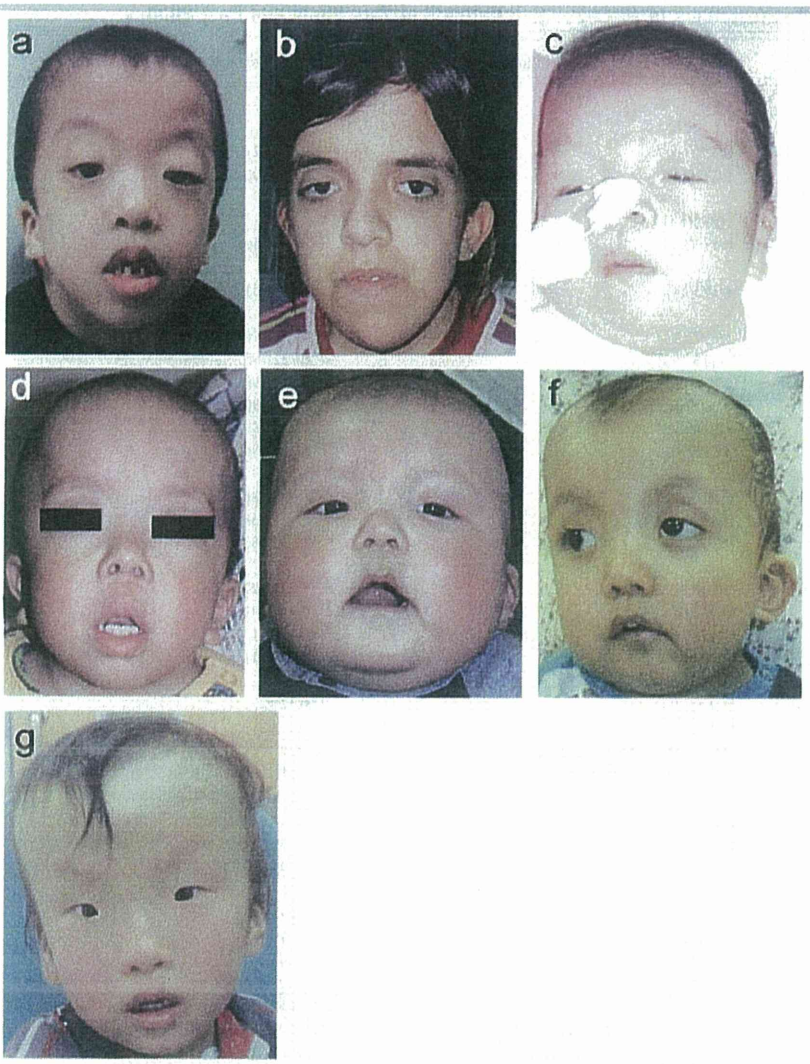

Figure 1. Facial appearance of patients with *RAF1* mutations. **a–f:** patients with p.S257L mutations. **a:** NS135; **b:** NS146; **c:** NS215; **d:** NS256; **e:** NS258 at 6 months; **f:** 2 years and 4 months; **g:** NS222 with p.S427G. [Color figure can be viewed in the online issue, which is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

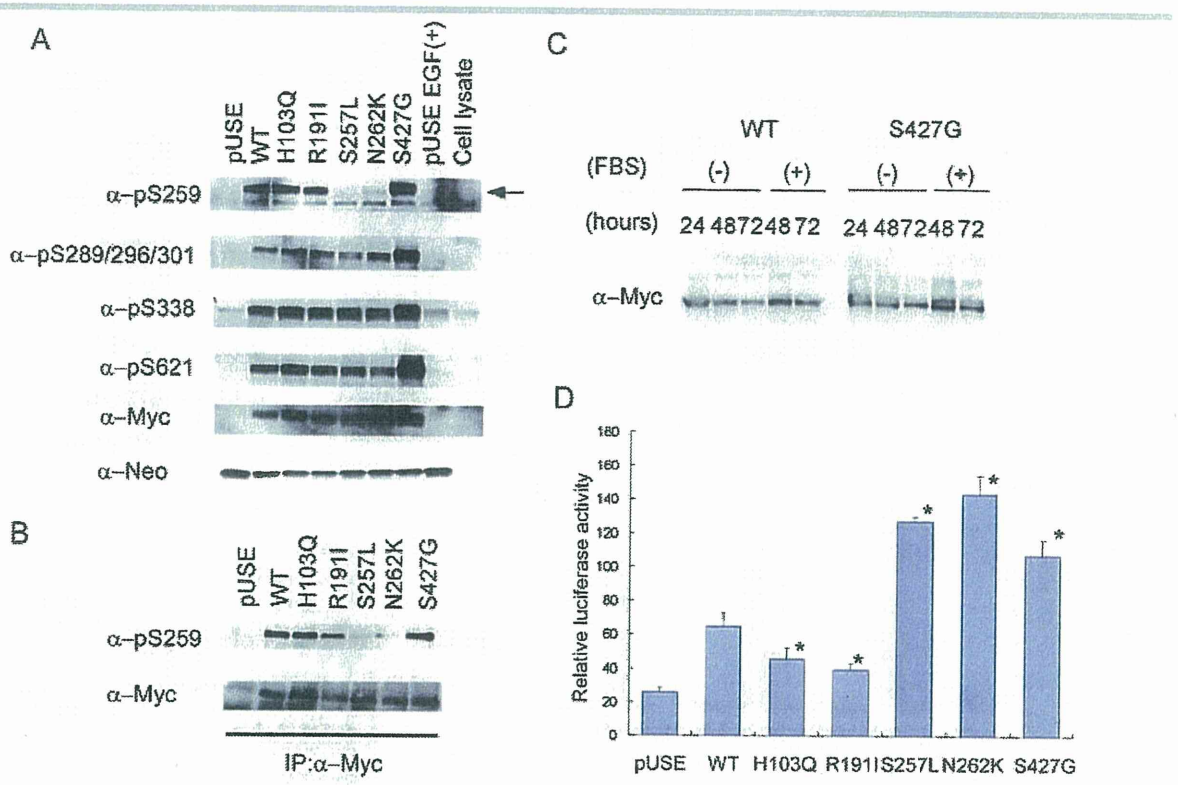

Figure 2. Analysis of phosphorylation status, degradation, and effect on downstream signaling in RAF1 mutants identified in this study. **A:** Phosphorylation status of wild-type (WT) RAF1 and mutants. Expression levels of RAF1 proteins and their phosphorylation levels were detected with different antibodies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

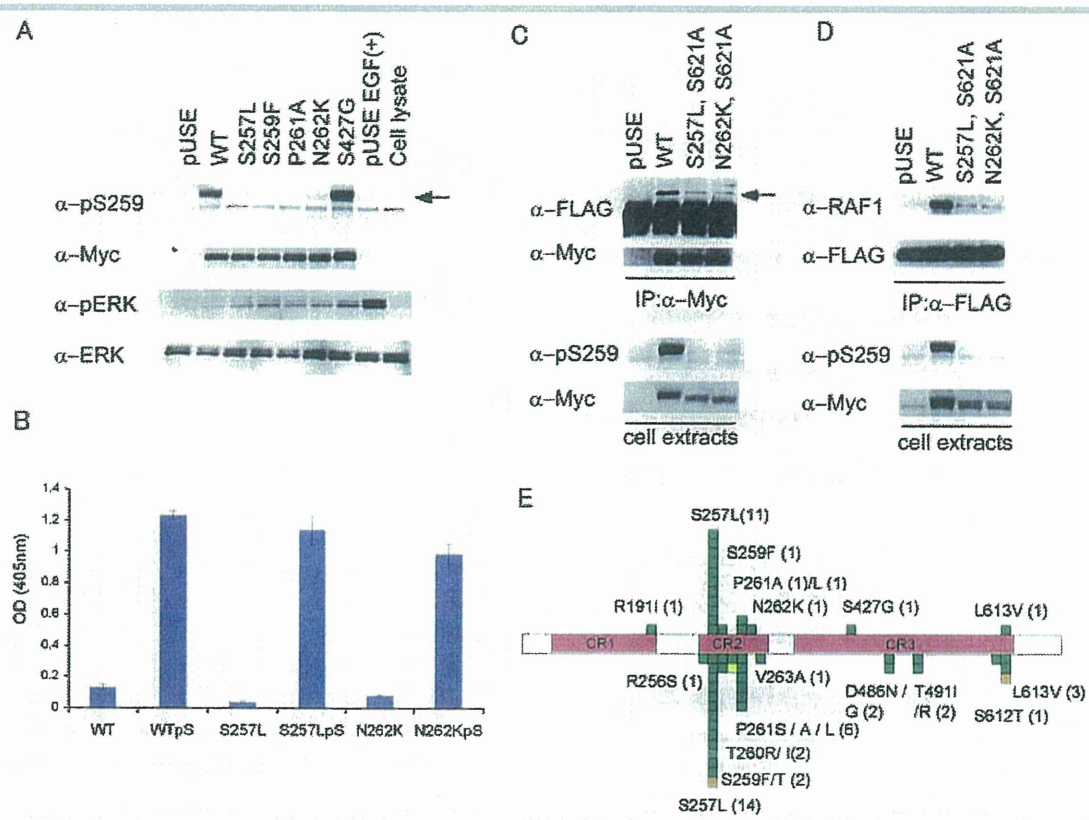

Figure 3. Phosphorylation of S259, binding to 14-3-3 and ERK activation of mutants located in the CR2 domain. **A:** Phosphorylation status of WT and mutants located in the CR2 domain. Phosphorylation of S259 was not observed in cells expressing p.S257L, p.S259F, p.P261A, and p.N262K. In order to examine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

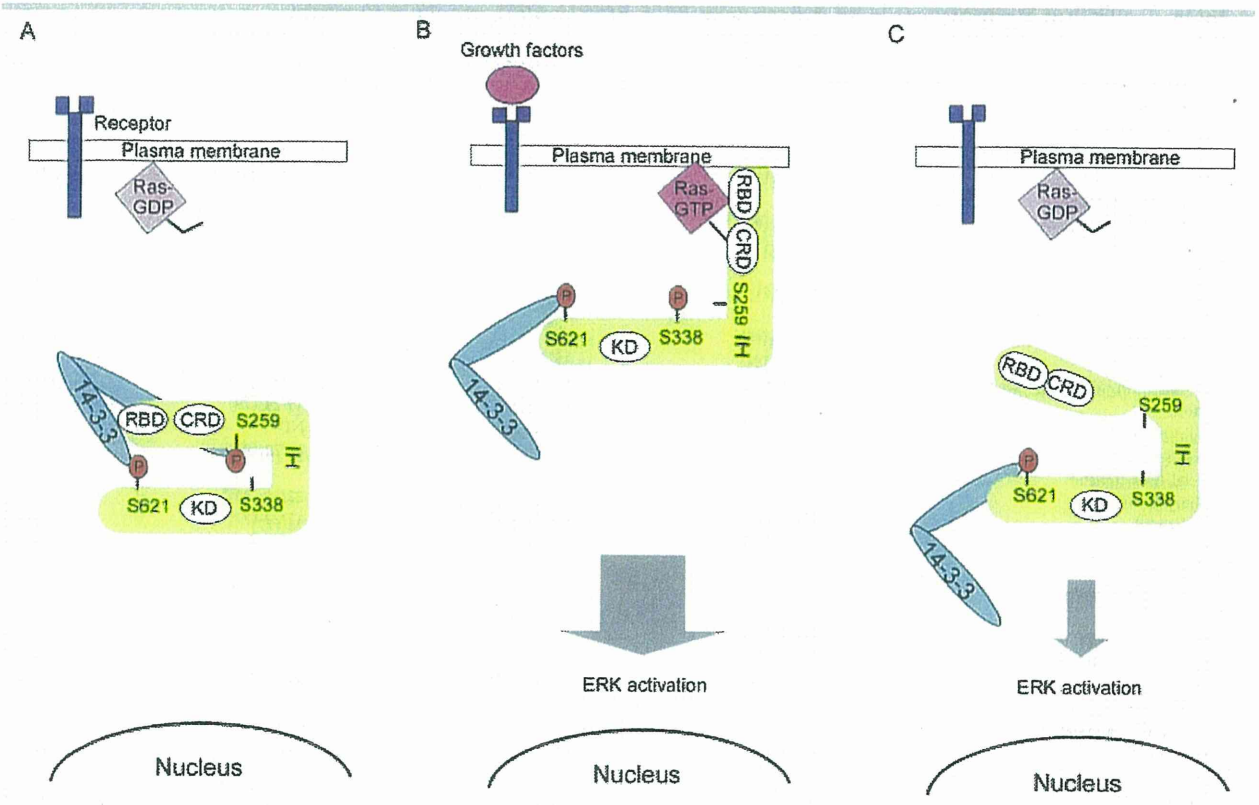

Figure 4. Schematic model of WT and mutant activation. **A:** In an inactive state, RAF1 is phosphorylated on S259 and S621 and is bound to 14-3-3. **B:** In growth-factor stimulation, the GTP-bound RAS binds to the CR1 domain of RAF1, which displaces 14-3-3. S259 is dephosphorylated by protein phosphatase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

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.

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＜診断へのアプローチ―疾患別診断＞

アミノ酸・有機酸代謝異常症を見逃していませんか？

呉　繁夫*

はじめに

先天代謝異常症を見逃さないためには，「疑う」ことが第一歩になる。先天代謝異常症の疑いをもち，検査を実施しようとした場合，検査法の概略とその適応を理解しておく必要がある。先天代謝異常症の検査として，代謝産物の濃度を測定する「化学診断」，責任酵素の活性を測定する「酵素診断」，遺伝子変異の存在を証明する「遺伝子診断」の3種類がある。アミノ酸代謝異常症や有機酸代謝異常症の診断において最も効率が良く，広く行われている診断法は，化学診断である。化学診断は，分析の手間や経費が少ない，重症度の推定，治療効果の判定が可能，などの特徴があり，先天代謝異常症の診断にはきわめて有効な診断法である。ここでは，アミノ酸代謝異常症や有機酸代謝異常症の化学診断を主に解説する。しかしながら，疾患によっては化学診断よりも遺伝子診断や酵素診断のほうが診断効率の良い疾患も存在することも念頭におき診断を進める必要がある。

I　アミノ酸と有機酸

アミノ酸からアミノ基が外れた（脱アミノされた）化合物を有機酸とよぶ（図1）。有機酸はカルボキシル基をもつので，カルボン酸ともよぶ。アミノ酸代謝異常症は，アミノ酸を直接の基質とする，ないしはそのすぐ下流の酵素の遺伝的欠損により特定のアミノ酸濃度の上昇をひき起こす。一方，有機酸代謝異常はさらに下流の酵素の欠損により生じるため，アミノ酸濃度の変化を伴うものと伴わないものが存在する。有機酸の種類は，アミノ酸とは比べものにならないほど多様であるため，個々の化合物の分離・同定がアミノ酸に比べて難しくなる。またアミノ酸には，特異的な発色剤であるニンヒドリンが存在し，検出の面でも有機酸に比べて容易であった。このため，1970年にはすでに多くのアミノ酸代謝異常症が発見されていたのに対し，多くの有機酸代謝異常症の発見は，ガスクロマトグラフの開発・普及を待たなければならなかった。最近では，タンデム・マススペクトロメトリー（MS/MS）を用いた新生児スクリーニングも実施されるようになった。ここでは，アミノ酸と有機酸の代表的な分析法である，アミノ酸分析機，ガスクロマトグラフ・質量分析機（GC/MS），MS/MSによる分析を紹介する。

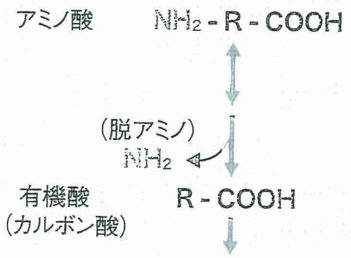

図1　アミノ酸と有機酸

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II アミノ酸と有機酸の定量

1．アミノ酸分析機

古くは，体液中のアミノ酸濃度の測定には，ペーパークロマトグラフなどでアミノ酸を分離し，ニンヒドリン反応で検出・定量が行われていた。1934年に発見されたフェニルケトン尿症は，尿を用いたこの分析手法が基本となっていた。その後，アミノ酸を効率よく分離する液体クロマトグラフ（LC）が開発され，分離された各分画のニンヒドリン反応を自動で行うアミノ酸分析機が開発された。これが現在のアミノ酸分析機の基本になっている（図2）。血清中のアミノ酸は比較的高濃度に存在するため，ニンヒドリン反応による定量で十分測定可能であるが，濃度の低い髄液中アミノ酸濃度の測定の目的などでは，その検出感度に問題があった。アミノ酸をあらかじめ蛍光標識し，その後分離・定量を行う方法により微量アミノ酸を分析する方法も開発されている。

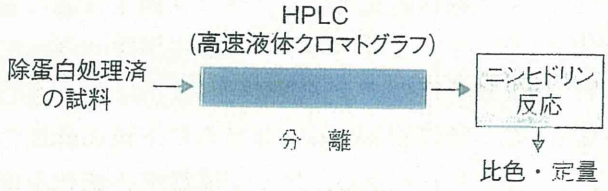


図2　アミノ酸分析計

2．ガスクロマトグラフ・質量分析機（GC/MS）

ガスクロマトグラフは，気化させた試料を細長いカラムを通すことにより分離させる技術で，カラム壁（シリカ）と相互作用しやすい有機酸はカラムから出てくる時間が遅くなる性質を利用して分離している。ガスクロマトグラフの分離精度はきわめて高いが，分離された有機酸の同定・定量に難があった。この問題を解決したのが，質量分析装置（MS）との合体であり，通常GC/MSとよばれる（図3）。有機酸内の分子を安定同位体で置換した内部標識物質を使用することで，正確な同定・定量が可能になった。

有機酸分析は主に尿を検体として用いて行う。有機酸分画をあらかじめ抽出し，GC/MSで分析

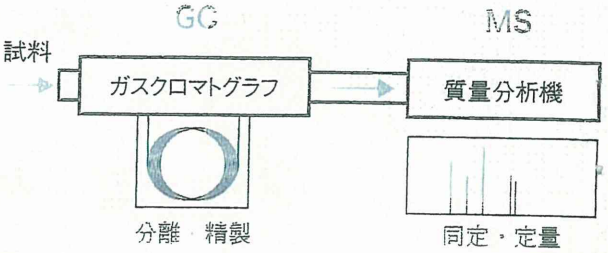


図3　GC/MS

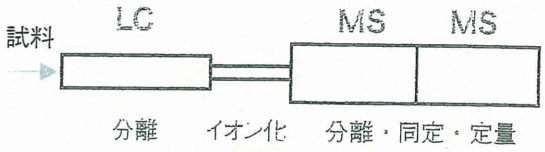


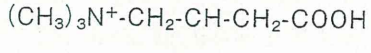
図4　MS/MS（LC/MS/MS）

する方法と抽出を行わずに分析する方法とがある。抽出過程で回収率の悪い有機酸が診断に重要である疾患の場合，後者が有用となる。抽出を行わない場合，尿中に大量に存在する尿素の影響をなくすため，尿をウレアーゼにより処理後，GC/MSにて分析を行う。抽出を行わないと検体中にアミノ酸，糖，核酸など分析対象の種類が非常に多様になるため，より高度の分離能・同定能をもつGC/MSが必要になる半面，多くの物質濃度の情報を得ることが可能になる。

3．タンデム・マススペクトメトリー（MS/MS）

MS/MS分析では，検体をLCで分離後，高電圧下で荷電液滴を作成し，溶媒を蒸発させた後に，質量分析機に導入している（図4）。したがって，LC/MS/MSとよぶこともある。新生児スクリーニングで利用さている MS/MS の主な分析対象はアミノ酸とアシルカルニチンである。アシルカルニ

1）遊離カルニチン



2）アシルカルニチン

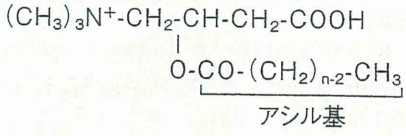


図5　遊離カルニチンとアシルカルニチン

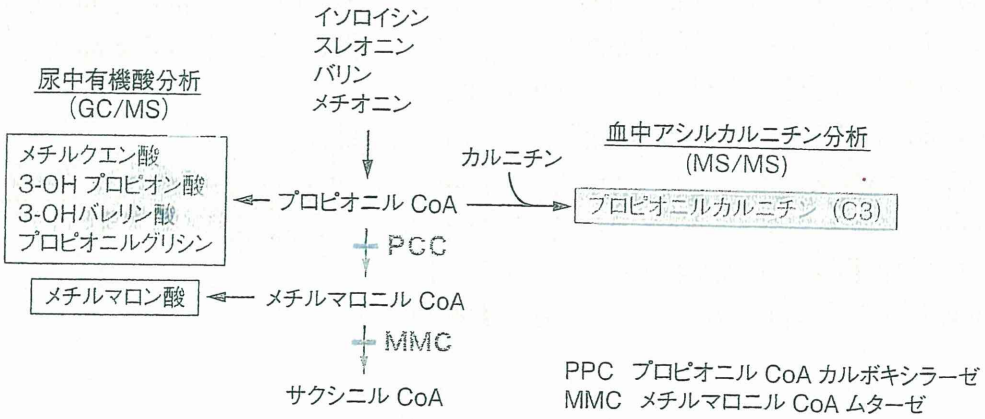


図 6 尿中有機酸分析と血中アシルカルニチン分析

チンはアシル CoA とカルニチンの結合したもので（図 5），これを分析することで，脂肪酸代謝や有機酸代謝で生じた異常代謝産物のスクリーニングが可能になる。アシルカルニチン分析の場合，アシル基の鎖長や水酸基の有無などの情報しか得られないので，複数の疾患をまとめて診断することになる。

III 血中アミノ酸分析

各自治体で行われている新生児マススクリーニングでは，フェニルアラニン，メチオニン，ロイシン，などの血中濃度を従来のガスリー法，高速液体クロマトグラフなどで測定されている。高フェニルアラニン血症の場合，フェニルケトン尿症などのフェニルアラニン水酸化酵素欠損症のほか，テトラヒドロビオプテリン代謝異常症が含まれる。高メチオニン血症の場合，ホモシスチン尿症のほか，肝炎，胆道閉鎖症，門脈短絡などの疾患との鑑別が必要になる。問題となるのが，シトリン欠損症などのアミノ酸キャリア欠損による疾患である。シトリン欠損症の場合，約 40%の症例は新生児スクリーニングで，メチオニン，フェニルアラニン，ガラクトースなどの高値を指摘される。多くは，フェニルアラニンとガラクトースなど複数の項目で異常が見出される。残りの症例は，新生児肝炎や胆道閉鎖症の疑いで発見される。異常は一過性のことが多く，検査時期を逸するとアミノ酸やガラクトースの異常は検出できなくなる。シトリン欠損症には，アミノ酸分析などの化学診断のみで診断を確定することは困難で，*SLC25A13* 遺伝子の変異検索により診断を確定する必要がある。

IV 尿有機酸分析

代表的な有機酸代謝異常症であるメチルマロン酸血症やプロピオン酸血症に罹患している患児が MS/MS による新生児スクリーニングを受けた場合を考えてみる。両疾患の代謝マップを図 6 に示す。プロピオン血症で蓄積するプロピオニル CoA やメチルマロン酸血症で蓄積するメチルマロニル CoA はいずれもカルニチンと結合すると，C3（プロピオニルカルニチン）となる。したがって，スクリーニングの結果報告では，両疾患とも

Key Points

1. アミノ酸・有機酸代謝異常症が疑われる場合，まず血清アミノ酸分析と尿有機酸分析を行う。とくに症状があるときの検体採取は重要である。
2. タンデムマス試験による新生児スクリーニングでは，頻度の高いメチルマロン酸血症とプロピオン酸血症は「C3（プロピオニルカルニチン）の高値」として検出される。
3. 新生児スクリーニングでフェニルアラニンやメチオニンなど複数のアミノ酸濃度の異常が検出される場合は，シトリン欠損症を考慮する。

に C3 高値として報告される。精査・鑑別のためには尿を用いた GC/MS 分析が必要になる。プロピオン酸血症の場合，プロピオニル CoA の代謝産物である，メチルクエン酸や 3-OH-プロピオン酸が尿中に検出される。一方，メチルマロン酸血症の場合，メチルマロニル CoA の代謝産物であるメチルマロン酸が尿中排泄される。このように，MS/MS によるアシルカルニチン分析は，有機酸血症の確定診断には向かないが，スクリーニング検査として有用性が高い。

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9. 代　謝

アミノ酸代謝異常症（フェニルケトン尿症，楓糖尿症など）

inborn error of amino acid metabolism

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新生児マス・スクリーニング（NBS）で発見されるアミノ酸代謝異常症であるフェニルケトン尿症，楓糖尿症，ホモシスチン尿症の3疾患について，診断のポイント，基本病態，食事療法や薬物療法について述べる．

診断のポイントと基本病態

1．フェニルケトン尿症（phenylketonuria：PKU）　新生児マス・スクリーニング（newborn screening：NBS）で，血中フェニルアラニン（Phe）値が2～4 mg/dL以上を異常として見出される．便宜上，血中Phe濃度が16.5 mg/dL以上をPKU，それ未満を高フェニルアラニン血症と分類している．Pheをチロシンに転換するPhe水酸化酵素の遺伝的欠損により発症する，常染色体劣性遺伝病である．無治療の場合，精神発達遅滞・色素異常などの症状を呈する．

2．楓糖尿症（メープルシロップ尿症）　NBSで，血中ロイシン濃度の高値として発見される．分岐鎖アミノ酸（branched-chain amino acid：BACC）であるロイシン，イソロイシン，バリンの異化経路である，BACC脱水素酵素複合体を構成する酵素の遺伝的欠損により発症する常染色体劣性遺伝病である．無治療の場合，嘔吐，意識障害，呼吸障害などの急性発作を呈し，精神発達遅滞を高率に伴う．

3．ホモシスチン尿症　NBSで，血中メチオニン（Met）濃度の高値として発見される．ほとんどが，シスタチオニンβ合成酵素（cystathionine beta-synthase：CBS）の遺伝的欠損により発症する常染色体劣性遺伝病である．出生時にはほとんどが無症状で，無治療の場合，1歳過ぎから精神発達遅滞，3歳頃から骨格異常による高身長，四肢指伸長，水晶体脱臼，血栓症・塞栓症による脳梗塞や心筋梗塞が高率に発症する．

治療の実際

前述3疾患の食事療法の基本は，低蛋白食に各治療乳を組み合せ，指標となるアミノ酸の血中濃度を目標範囲に維持することにある．

1．フェニルケトン尿症　Pheを含まないPKU治療乳を与え，許容範囲のPheを食品中の蛋白から摂取することで，血中Phe濃度を目標範囲に維持することが治療の基本となる[1]．初期治療は入院で行う．母乳ないしは一般粉乳と治療乳（商品名：雪印新フェニルアラニン除去ミルク®，雪印新ペプチドロフェ®など）とを組み合せ，週2～3回血中アミノ酸濃度を測定し，血中Phe濃度を2～4 mg/dLになるよう両者の摂取量を決定する．初期治療ミルクの投与量が決まったら，外来フォローとする．乳児期は月2回，それ以後は最低月1回，血中アミノ酸分析や生化学検査を行う．随時，成長や知能の評価を行う．Phe濃度の年齢別目標範囲は，乳児～幼児期前半は2～4 mg/dL，乳児期後半～小学校低学年は3～6 mg/dL，小学校高学年は3～8 mg/dL，中学生は3～10 mg/dL，それ以降は3～15 mg/dLとする．1日の摂取熱量は，同年齢の小児に合せる．蛋白摂取が低下すると体蛋白質の異化が進み，かえってPhe濃度が上昇するため，1日の総蛋白摂取量は，乳児期で2 g/kg，幼児期で1.5～1.8 g/kg，学童期以降は1.0～1.2 g/kg以上となるようにする．食事療法は，生涯継続する必要がある．成人後も，食事療法を中止すると統合失調症やうつ病に似た精神障害を高率に発症することが知られている．

2．楓糖尿症　NBSで血中ロイシン濃度の高値が指摘されたらただちに入院させ，自然蛋白質摂取を中止し，治療乳（商品名：雪印新ロイシン・イソロイシン・バリン除去ミルク®）とカロリー補給を行う．これにより体蛋白質の異化を抑え，同化へと導き，血中BACC濃度の低下を図るのが治療原則である[2]．BACCのうち，中間代謝産物の毒性が強いロイシンの血中濃度を指標とし，目標範囲に維持する．急性期の治療は，BACC除去ミルクと十分なカロリーを経管的に注入する．嘔吐・下痢などの消化管症状が強く経管栄養が実施できないときには高カロリー輸

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液を実施し，脂肪製剤やブドウ糖液を投与する．以上の治療を開始しても神経症状の改善がみられないときには，血液透析を行う．急性期の治療中は血中アミノ酸分析を随時行い，ロイシン濃度をモニターするとともに，イソロイシンやバリンの欠乏の有無にも注意する．イソロイシンやバリンの欠乏が生じると蛋白同化速度が鈍り，ロイシン濃度の低下速度も鈍る．神経症状が消失し，血中ロイシン濃度が 10 mg/dL 以下に低下した後は，慢性期の治療を開始する．離乳前は母乳やミルクを BACC 供給源とし，離乳後は低蛋白食を BACC 供給源とし治療乳を併用することで，血中ロイシン濃度を 2～5 mg/dL に維持する．蛋白制限に加え，同年齢の小児と同等のカロリー補給が必要である．

3．ホモシスチン尿症　治療の基本は，低 Met，高シスチン食事を与え，血中 Met 濃度を 1 mg/dL 以下に保つことにある[3]．許容量の Met を含む低蛋白食と治療乳（商品名：雪印新低メチオニンミルク®，明治メチオニン除去フォーミュラ®）を用いる．これらの治療乳はいずれも，シスチンが強化されている．大部分は，ビタミン B_6に反応しないタイプであるが，反応する症例も存在するため，生後 6 カ月時に反応性を確認する．治療乳を一般粉乳に変え，ピリドキシンの大量投与（250 mg/日）を 10 日間行い，前後の血中 Met 濃度，ホモシステイン濃度の低下の有無を調べる．反応性がある場合にはビタミン B_6投与量を漸減し，最小有効投与量を決める．ビタミン B_6反応性の症例は，わが国ではまれである．ビタミン B_6大量投与で呼吸不全や肝障害を呈する症例があるので，ビタミン B_6負荷試験は入院のうえ十分な観察下で行う．学童期に入ると，食事療法のみでは血中 Met を 1 mg/dL 以下に維持できない症例が多くなる．その際は，ベタイン（200～250 mg/kg/日 分 3）を併用する．ベタイン併用時には，Met でなく血中ホモシステイン濃度が指標となる．血中ホモシステイン濃度を 20～50 μM 以下に保ち，血中 Met は 15 mg/dL を超えないようにベタイン投与量を調整する．

私の治療方針

PKU を含む Phe 水酸化酵素欠損症のなかで，同酵素の補酵素であるテトラヒドロビオプテリン（BH_4）に反応する症例が存在することを，1999 年に筆者らが報告した[4]．その後，BH_4治療の有効性は広く認められ，保険適用となった．現在，わが国で約 30 名がこの治療を受け，Phe 制限食を緩和ないし中止することに成功している．本症の診断や治療の詳細は日本先天代謝異常学会の設置した「BH_4委員会」に照会のこと（連絡先：熊本大学小児科内 BH_4委員会事務局，E-mail：JSIMD@kumamoto-u.ac.jp）．

最新ガイドライン/エビデンス

特殊ミルク共同安全開発委員会が編集した「改訂 2008 食事療法ハンドブック－アミノ酸代謝異常症・有機酸代謝異常症のために」（非売品）が，恩賜財団母子愛育会より入手可能である．

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