patients have mtDNA depletion, and that some mechanisms other than mtDNA depletion might participate in the multiple MRC deficiency observed in these patients.

In the present case, serum methylmalonic acid accumulation and low ^{14}C-propionate fixation capacity suggested disturbance of methylmalonic acid metabolism. Elevated methylmalonic acid may result from the accumulation of succinyl-coenzyme A under the assumption that accumulated succinyl-CoA inhibits the reaction catalyzed by MCM or causes an equilibrium shift, leading to the accumulation of methylmalonyl-coenzyme A, which is converted to methylmalonic acid. As usual, increased levels of C4DC are detected in patients with severe MCM deficiency during acute crises. It is suggested that the C4DC of the present patient was associated with an increased level of succinylcanritine due to accumulated succinyl-coenzyme A.

In conclusion, we identified two novel *SUCLG1* mutations in a Japanese female patient with neonatal lactic acidosis and prolonged mild methylmalonic aciduria. For patients showing these combined manifestations, MRC activities and mutations of *SUCLG1* or *SUCLA2* should be screened for.

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ORIGINAL ARTICLE

HRAS mutants identified in Costello syndrome patients can induce cellular senescence: possible implications for the pathogenesis of Costello syndrome

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Costello syndrome (CS) is a congenital disease that is characterized by a distinctive facial appearance, failure to thrive, mental retardation and cardiomyopathy. In 2005, we discovered that heterozygous germline mutations in *HRAS* caused CS. Several studies have shown that CS-associated *HRAS* mutations are clustered in codons 12 and 13, and mutations in other codons have also been identified. However, a comprehensive comparison of the substitutions identified in patients with CS has not been conducted. In the current study, we identified four mutations (p.G12S, p.G12A, p.G12C and p.G12D) in 21 patients and analyzed the associated clinical manifestations of CS in these individuals. To examine functional differences among the identified mutations, we characterized a total of nine HRAS mutants, including seven distinct substitutions in codons 12 and 13, p.K117R and p.A146T. The p.A146T mutant demonstrated the weakest Raf-binding activity, and the p.K117R and p.A146T mutants had weaker effects on downstream c-Jun N-terminal kinase signaling than did codon 12 or 13 mutants. We demonstrated that these mutant HRAS proteins induced senescence when overexpressed in human fibroblasts. Oncogene-induced senescence is a cellular reaction that controls cell proliferation in response to oncogenic mutation and it has been considered one of the tumor suppression mechanisms *in vivo*. Our findings suggest that the *HRAS* mutations identified in CS are sufficient to cause oncogene-induced senescence and that cellular senescence might therefore contribute to the pathogenesis of CS.
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Keywords: Costello syndrome; HRAS; phenotype-genotype; RAS/MAPK; senescence

INTRODUCTION

Costello syndrome (CS, OMIM 218040) is a genetic disorder that is characterized by a distinctive facial appearance, loose skin, failure to thrive, mental retardation, cardiomyopathy and a predisposition to tumor formation.[1] Patients with CS have an estimated 13% chance of developing tumors, usually rhabdomyosarcoma, neuroblastoma or bladder cancer.[2] Previously, we identified heterozygous germline *HRAS* mutations in patients with CS.[3] It has been suggested that the CS diagnosis should be applied only to patients with a mutation in *HRAS* because of the high risk of malignancies associated with *HRAS* mutations and the relative homogeneity of the CS phenotype.[4]

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A total of 14 *HRAS* missense mutations and one duplication mutation have been reported in 185 patients with CS[3,5–23] or congenital myopathy with excess of muscle spindles.[24] Most of these mutations have previously been reported as somatic and oncogenic mutations in various tumors. More than 90% of the mutations found in CS patients are clustered in codons 12 and 13 (p.G12A/S/V/C/D/E and p.G13C/D). Other mutations, including p.Q22K, p.E37dup, p.T58I, p.E63K, p.K117R, p.A146V and p.A146T, have also been identified, albeit rarely. Although the clinical manifestations of CS appear to be homogeneous, several genotype-phenotype correlations have been reported. Previous studies have also suggested that CS patients with the p.G12A mutation may have an increased risk of malignancy, compared with patients with p.G12S.[7] Patients with the p.G12C mutation had a more severe CS phenotype; these individuals developed severe hypertrophic cardiomyopathy and died in the neonatal period. Patients with p.K117R or p.A146V had a milder and more unusual CS phenotype, compared with patients with mutations in codon 12 or 13. Though detailed analyses of some mutants have been performed,[13,25–28] a comprehensive comparison of the substitutions identified in patients with CS has not been conducted.

The activated RAS/mitogen-activated protein kinase (MAPK) pathway generally stimulates cell proliferation, but it can also result in antiproliferation under certain conditions. Overexpressing HRAS p.G12V in human and murine fibroblasts caused oncogene-induced senescence (OIS),[29–31] which protects cells from proliferating in the presence of oncogene-induced damage.[32,33] OIS is a cellular reaction that controls cell proliferation in response to oncogenic mutation and is considered a tumor suppression mechanism *in vivo*.[34,35] Studies of a zebrafish model of CS, which expresses *HRAS* p.G12V, have shown that progenitor cells in the adult heart and brain undergo cellular senescence, suggesting that OIS in adult progenitor cells contributes to the development of CS. We hypothesized that OIS would be a key mechanism of the clinical manifestations in patients with CS, including short stature, osteoporosis and tumor suppressive effects. However, it has not been verified that HRAS mutants other than p.G12V cause cellular senescence.

The three aims of this study were the following: (1) to examine the detailed clinical manifestations of CS in patients with *HRAS* mutations, (2) to characterize a large panel of HRAS mutants to look for differences among various mutations located in codon 12/13 and to compare the effects of mutants in codon 12/13 with those of p.K117R/p.A146T, and (3) to clarify whether *HRAS* mutants other than p.G12V can cause OIS. To address these issues, we analyzed the *HRAS* mutations in CS patients and studied the Raf-binding activity, downstream signaling and ability to cause senescence of a large panel of HRAS mutants.

MATERIALS AND METHODS

Patients

A total of 31 patients suspected of having CS were recruited to the study. The diagnosis of CS was evaluated by clinical geneticists. All patients had sporadic cases. The study was approved by the Ethics Committee of the Tohoku University School of Medicine.

Mutation analysis

We sequenced the *HRAS* genes of all patients in the study to confirm the diagnosis of CS. After obtaining written informed consent, genomic DNA was isolated from the peripheral leukocytes of patients. Four coding exons of *HRAS* from 31 CS patients were sequenced. Each *HRAS* exon with flanking intronic sequences was amplified using primers based on sequences obtained from GenBank (GenBank accession no. NT035113). The M13 reverse or forward sequence was added to the 5′ end of the polymerase chain reaction primers for use, as a sequencing. polymerase chain reaction was performed in a 30 µl reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphate, 10% (v/v) dimethyl sulfoxide, 0.4 pmol each primer, 100 ng genomic DNA and 2.5 units of Taq DNA polymerase. The reaction consisted of 35 cycles of denaturation at 94 °C for 15 s, annealing at 57 °C for 15 s and extension at 72 °C for 30 s. The products were gel-purified and sequenced on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Plasmids

To introduce exogenous wild-type or mutated HRAS into cultured cells, we constructed plasmids encoding wild-type or mutant *HRAS* cDNAs. Human *HRAS* cDNA in pUSEamp was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The plasmid was digested with *Eco*RI and subcloned into pBluescript KSII+ (Stratagene, La Jolla, CA, USA). Substitutions generating p.G12V (c.35G>T), p.G12A (c.35G>C), p.G12S (c.34G>A), p.G12C (c.34G>C), p.G12D (c.35G>A), p.G13C (c.37G>C), p.G13D (c.38G>A), p.K117R (c.350A>G) or p.A146T (c.436G>A) were introduced using the QuikChange Site-Directed mutagenesis kit (Stratagene). All mutant and wild-type constructs were verified by sequencing. The full-length wild-type and mutant *HRAS* cDNAs were digested with *Eco*RI and subcloned into the pBabe-puro retroviral vector (GenHunter, Nashville, TN, USA) and the pCAGGS expression vector (gifted by Dr Jun-ichi Miyazaki of Osaka University). The pBabe-zeo-Ecotropic Receptor plasmid (Addgene plasmid 10687, Addgene Inc., Cambridge, MA, USA) was obtained from Addgene.

Cell culture and senescence-associated β-galactosidase staining

NIH 3T3 cells, human fibroblast BJ cells and the Phoenix Ampho and Eco packaging cell lines were purchased from the American Tissue Culture Collection (Manassas, VA, USA). NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium containing 10% calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. BJ and Phoenix cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. To characterize the phenotypes of cells overexpressing wild-type or mutated HRAS, senescence associated β-galactosidase staining was performed with the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Beverly, MA, USA) according to the manufacturer's protocol.

Ras activation assay

We performed RAS activation assays to clarify the functional differences among the HRAS mutants identified in patients with CS. The Ras activation assay kit was purchased from Millipore (Billerica, MA, USA). NIH 3T3 cells were plated in 6-well plates at 1.5×10^5 cells per well. Cells were transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA) with 1 µg wild-type or mutant *HRAS* construct. The assay was performed according to the manufacturer's protocol.

Luciferase assay

We used luciferase assays to examine the effect of the identified mutations on the RAS pathway. NIH 3T3 cells were plated in 12-well plates at 1×10^5 cells per well. After 24 h, cells were transiently transfected with 700 ng pFR-luc, 10 ng pFA2-Elk1 or 10 ng pFA2-cJun, 7 ng phRLnull-luc and 35 ng wild-type or mutant *HRAS* construct, using Lipofectamine Plus (Invitrogen). At 18 h after transfection, the cells were serum starved in Dulbecco's modified Eagle medium for 24 h. Cells were then harvested in passive lysis buffer, and luciferase activity was assayed using the Promega Dual-Luciferase assay kit (Promega, Madison, WI, USA). Renilla luciferase expressed by phRLnull-luc was used to normalize the transfection efficiency. The experiments were performed in triplicate. Statistical analysis was performed with Tukey's multiple comparison test.

Western blotting

We performed western blotting against molecular markers of premature senescence to confirm their expression in cells overexpressing HRAS. Cells were harvested at the indicated times, washed in ice-cold phosphate-buffered saline and lysed on ice in lysis buffer (10 mM Tris-HCl, pH 7.5 and 1% sodium

dodecyl sulfate). Lysates were boiled for 5 min and centrifuged at 13 000 *g* for 10 min at 4 °C. Protein concentrations were estimated using the Lowry or Bradford method (BioRad, Hercules, CA, USA), and each lysate was adjusted to equalize the protein concentrations. Equal volumes of lysates were mixed with 2×sodium dodecyl sulfate sample buffer and boiled for 5 min. Electrophoresis was performed on 5–15% sodium dodecyl sulfate–polyacrylamide gels. After separation, proteins were transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature and incubated overnight at 4 °C with one of the following primary antibodies: HRAS (sc-520, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-p44/42MAPK, p44/42MAPK (#9102 and #9101, respectively, Cell Signaling Technology, Danvers, MA, USA), p16 (sc-468, Santa Cruz Biotechnology), phospho-p53 (Ser15) (#9284, Cell Signaling Technology) or β-actin (A5316, Sigma, St. Louis, MO, USA). Detection was performed using the enhanced chemiluminescence method (Amersham, GE Healthcare UK, Amersham, UK), with the appropriate peroxidase-conjugated secondary antibody.

Retroviral gene transfer

We generated cells that stably overexpressed wild-type or mutant HRAS by retroviral gene transfer. Phoenix cells (5×10^6) were plated in a 10 cm dish, incubated for 24 h and then transfected with 18 μg of retroviral plasmid using Fugene6 (Roche Applied Science, Mannheim, Germany). After 48 h, the virus-containing medium was filtered through a 0.45-μm filter and supplemented with 4 μg/ml polybrene (Sigma) to collect the virus (first supernatant). Viruses were collected after an additional 24 h as before (second supernatant). BJ fibroblasts were plated at 6×10^5 cells per 10 cm dish and incubated overnight. For infections, the culture medium was replaced with the first viral supernatant and incubated at 37 °C for 8 h, after which the second viral supernatant was added. Infected cell populations were selected 40 h later, using 2 μg/ml puromycin or 200 μg/ml zeocin. The ecotropic retrovirus receptor was introduced into the BJ human fibroblasts by infecting cell populations with an amphotropic vector (pBabe-zeo-ecotropic receptor produced in Phoenix Ampho cells), allowing subsequent infection with ecotropic viruses.

RESULTS

Mutation analysis in patients with CS

Genomic sequencing analysis of 32 individuals with confirmed or suspected CS revealed four different missense mutations in 21 patients: a heterozygous 34G>A mutation (p.G12S) in 16 patients, a heterozygous 35G>C mutation (p.G12A) in three patients, a heterozygous 34G>T change (p.G12C) in one patient, and a 35G>A change (p.G12D) in one patient.

The clinical data for 21 CS mutation-positive patients are shown in Table 1. Curly and/or sparse hair (21/21), failure to thrive (21/21), coarse facial appearance (20/20), deep palmar/planter creases (20/21), soft, loose skin (18/21) and relative macrocephaly (17/21) were observed at high frequency in patients with CS, as previously reported.[1,3] Laryngomalacia (soft larynx), which has been reported in several patients with CS,[36–38] was observed in three patients. One patient had hypertension, which was also observed in a mouse model of CS.[39] One patient had glycogen storage disease type III, as previously reported by Kaji *et al.*,[40] accompanied by a p.G12S mutation. Bladder cancer was observed in one patient.

One patient (NS 223) with *HRAS* p.G12C had severe clinical manifestations of CS and was treated with pravastatin.[41] She was born at 23 weeks of gestation with extremely low birth weight (766 g, >90th percentile), even though her mother had received tocolytic therapy. Her Apgar scores were 3 and 7 at 1 and 5 min, respectively. She required mechanical ventilation. Extubation was attempted periodically beginning at day 70, but it was unsuccessful until she turned 2 years old, because of her laryngomalacia and increased mucus secretion. Hypertrophic cardiomyopathy was first observed on day 38. The patient was given propranolol and cibenzoline to control the gradual progression of hypertrophic cardiomyopathy. Cardiac arrest after extubation occurred on day 192 and the patient was successfully resuscitated. Papillomas developed at approximately 11 months of age. Erosion and itching of skin were not well controlled by topical steroids or antihistamines. Pravastatin (0.2 ~ 0.4 mg/kg/day) was administered in anticipation of its suppressive effect on RAS, beginning when she was 16 months old. Thereafter, the papillomas disappeared once and appeared again, but were less numerous than when they first appeared. The effects of pravastatin on hypertrophic cardiomyopathy were not obvious. The patient was discharged from the hospital at 2 years of age.

Analysis of mutant HRAS activation states and effects on the downstream pathway

We performed RAS activation assays to elucidate functional differences among the mutants identified in patients with CS. We transfected NIH 3T3 cells with wild-type *HRAS* or one of the nine *HRAS* mutants identified in patients with CS. We found an increase in guanosine triphosphate (GTP)-bound HRAS in all cells transfected with *HRAS* p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C, p.G13D, p.K117R and p.A146T. We did not detect any differences among the increases of GTP-bound HRAS in the cells transfected with *HRAS* p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C, p.G13D and p.K117R. The increase in the level of GTP-bound HRAS-p.A146T was milder than that of other mutants.

Next, we examined the effect of the identified mutations on the RAS pathway by studying the activation of ELK1 and c-Jun in transfected NIH 3T3 cells. ELK1 and c-Jun are the main nuclear targets of extracellular signal-regulated kinase and c-Jun N-terminal kinase, respectively. We transfected the pFR-luc trans-reporter vector, the pFA2-ELK1 or pFA2-cJun vector and the phRLnull-luc vector into NIH 3T3 cells and determined the relative luciferase activity (RLA) in each cell line. The basal RLA in cells transfected with active MEK1 or MEKK constructs showed a three-fold increase, compared with cells transfected with wild-type *HRAS* cDNA (Figure 1a). A significant increase in RLA was observed upon transfection with ELK1 and HRAS p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C, p.G13D, p.K117R and p.A146T (Figure 1b). The RLA of c-Jun was significantly increased in cells transfected with HRAS p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C and p.G13D (Figure 1c). In these assays with ELK1 and c-Jun, we observed no significant difference among RLAs in the cells transfected with *HRAS* p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C and p.G13D. These results suggest that HRAS-p.K117R and p.A146T had a weaker effect on the c-Jun N-terminal kinase pathway than the other mutants.

Cellular senescence in human fibroblasts transfected with HRAS mutants

The HRAS p.G12V mutant causes a senescence phenotype when transduced into human diploid fibroblasts. To examine the ability of the various mutants identified in patients with CS to cause senescence, we introduced wild-type or mutated *HRAS* cDNAs into human fibroblast BJ cells, using retroviral gene transfer. Figure 2a shows these cells six days after infection. Wild-type HRAS-induced cells exhibited a narrow and elongated morphology and they were not flat like senescent cells. They proliferated at levels similar to cells transfected with empty vector. In contrast, the p.G12V, p.G12A, p.G12S, p.G12C, p.G12D, p.G13C, p.G13D, p.K117R and p.A146T mutants produced cells with a senescence phenotype, exhibiting flat, enlarged and multivacuolated morphology and prominent nucleoli. Senescence

Table 1 Clinical findings and *HRAS* mutations in our CS patients

Patients	*NS71*	*NS123*	*NS125*	*NS132*	*NS137*	*NS139*	*NS156*	*NS157*	*NS167*	*NS181*	*NS198*	*NS217*
Gender	F	F	F	F	M	F	M	F	M	M	M	M
Age	9 months	11 years	17years	3 years	10 years	7 months	2 years 3 months	17 years	3 months	3 years	1 year 2 months	4 years 6 months
Paternal age at birth (years)	39	29	42	37	30	35	34	34	37	33	31	40
Maternal age at birth (years)	28	26	27	31	28	35	36	36	34	33	31	37
Growth and development												
Postnatal failure to thrive	+	+	+	+	+	+	+	+	+	+	+	+
Mental retardation	+	+	+	+	+	+	+	+	+	+	+	+
Craniofacial characteristics												
Relative macrocephaly	+	+	+	+	+	+	+	+	+	+	+	+
Coarse facial appearance	+	+	+	+	+	+	+	+	+	+	+	+
Muscloskeltal characteristics												
Short neck	+	+	+	+	+	+	+	+	–	+	–	+
Hyperextensive fingers	+	+	+	+	–	+	+	+	+	–	–	–
Tight Achilles tendon	–	+	+	+	+	–	+	–	–	–	–	+
Abnormal foot position	+	+	+	+	NA	+	NA	–	–	+	–	+
Skin characteristics												
Curly, sparse hair	+	+	+	+	+	+	+	+	+	+	Curly	+
Soft, loose skin	+	+	+	+	+	+	+	+	–	+	+	+
Deep palmer/ planter creases	+	+	+	+	+	+	+	+	+	+	+	+
Cardiac defect												
Hypertrophic cardiomyopathy	+	–	+	–	+	+	NA	+	–	+	–	–
Others	PS	–	–	–	–	–	PAC	Anomalous septum in the right atrium	VSD, arrhythmia	Atrial tachycardia	–	ASD, PSVT, PVC, CAR
Neoplasia												
Papillomata	–	–	+	–	–	–	NA	+	+	–	+	–
Other tumors		Bladder cancer							Heart neoplasia			
Others			GH deficiency		GSDIII	Chiari I, syringomyelia	Pyrolic stenosis	Congenial stridor, GH deficiency	Hypoplastic nails	Hypertention		Hydronephrosis, GER, laryngomalacia
HRAS mutation												
Nucleotide substitution	c.34G>A	c.35G>C	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A	c.34G>A
Amino acid substitution	p.G12S	p.G12A	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S	p.G12S

Table 1 Continued

Patients	*NS223*	*NS231*	*NS239*	*NS248*	*NS254*	*NS263*	*NS299*	*NS318*	*NS324*	*Total*
Gender	F	F	M	M	F	M	F	F	F	
Age	6 months	5 months	18 years	5 years	2 months	1 month	3 years	1 month	1 year 6 months	
Paternal age at birth (years)	34	27	27	NA	37	35	34y	33	33	
Maternal age at birth (years)	36	27	26	30	34	36	35y	32	33	
Growth and development										
Postnatal failure to thrive	+	+	+	+	+	+	+	+	+	21/21
Mental retardation	+	+	+	+	NA	+	+	+	+	20/20
Craniofacial characteristics										
Relative macrocephaly	–	+	+	–	+	+	–	–	+	17/21
Coarse facial appearance	+	+	+	+	+	+	+	+	+	21/21
Muscloskeltal characteristics										
Short neck	–	+	NA	NA	+	+	+	–	–	14/19
Hyperextensive fingers	–	+	–	+	+	–	–	+	+	13/21
Tight Achilles tendon	+	NA	–	+	–	–	–	+	+	10/20
Abnormal foot position	–	–	NA	NA	NA	–	–	+	+	9/16
Skin characteristics										
Curly, sparse hair	+	Curly	Curly	+	+	+	Curly	+	Curly	21/21
Soft, loose skin	–	+	+	+	+	+	–	+	+	18/21
Deep palmer/planter creases	+	–	+	+	+	+	+	+	+	20/21
Cardiac defect										
Hypertrophic cardiomyopathy	+	–	+	+	+	+	+	+	+	14/20
Other	PAC	PVC	–	–	–	–	–	PAC	PAC	
Neoplasia										
Papillomata	+	–	+	–	–	–	–	–	–	6/20
Other tumors										
Others	Prabastatin administration	Laryngomalasia, hydrocephallus	GH deficiency, Arnold Chiari, scoliosis	Empty sella, GH deficiency, hypothyroidism, hypogonadism, syringomyelia		Hyperinsulinemia		Laryngomalasia seizure	Laryngomalasia	
HRAS mutation										
Nucleotide substitution	c.34G>T	c.35G>A	c.34G>A	c.34G>A	c.34G>A	c.35G>C	c.34G>A	c.35G>C	c.34G>A	
Amino acid substitution	p.G12C	p.G12D	p.G12S	p.G12S	p.G12S	p.G12A	p.G12S	p.G12A	p.G12S	

Abbreviations: –, absent; +, present; ASD, atrial septal defect; F, female; GER, gastroesophageal relfux; GH, growth hormone; GSDIII, glycogen storage disease III; M, male; NA, not available; PAC, premature atrial contraction; PS, pulmonic stenosis; PSVT, paroxysmal supraventricular tachycardia; PVC, premature ventricular contraction; VSD, ventricular septal defect.

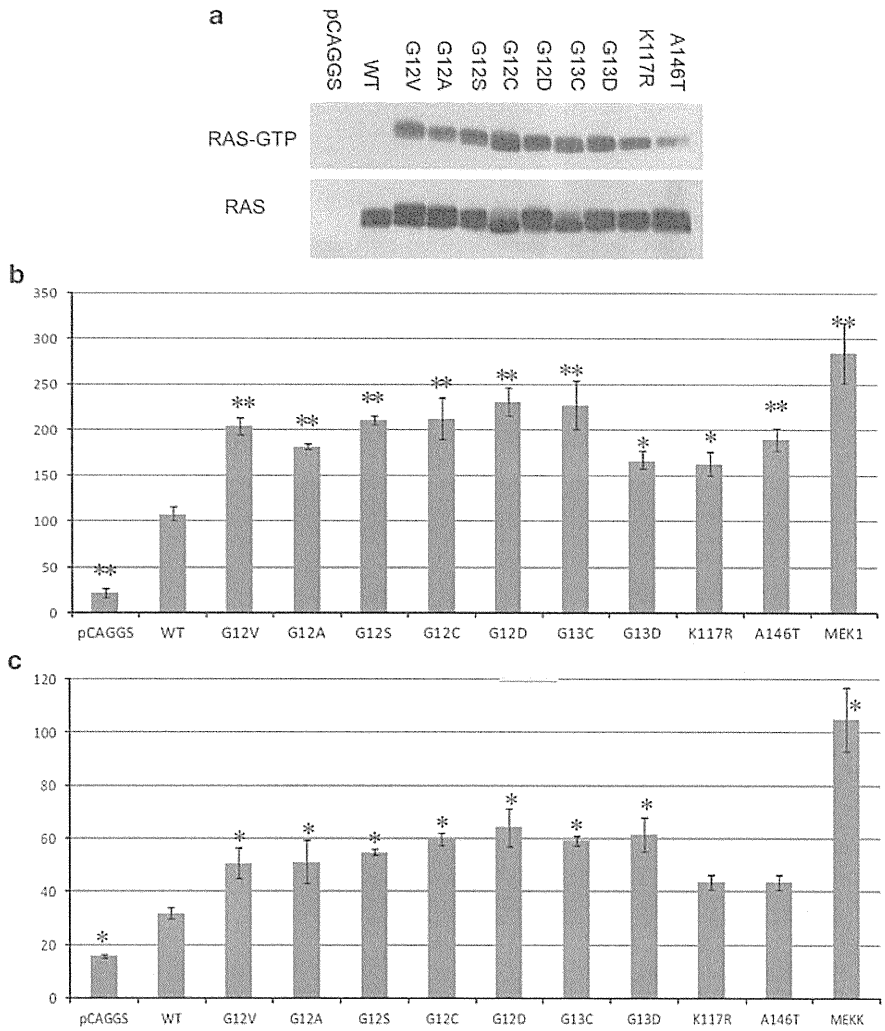

Figure 1 Functional characterization of HRAS mutants. (**a**) Ras-guanosine triphosphate (GTP) in NIH 3T3 cells transfected with wild-type or mutant HRAS constructs. HRAS protein levels were similar in NIH3T3 cells expressing each protein and were subsequently used as a loading control. (**b**, **c**) Stimulation of ELK (**b**) and c-Jun (**c**) transcription by *HRAS* mutants. The ELK-and c-Jun-GAL4 vectors and GAL4-luciferase trans-reporter vector were transiently co-transfected with various *HRAS* constructs into unstimulated NIH 3T3 cells. Relative luciferase activity (RLA) was normalized to the activity of a co-transfected control vector (phRLnull-luc) expressing *Renilla reniformis* luciferase. The results are expressed as the means and s.d. from triplicate samples. MEK1 and MEKK were used as positive controls. WT, wild type. $^{*}P<0.05$; $^{**}P<0.01$ compared with WT.

associated β-galactosidase staining confirmed that these cells showed cellular senescence.

Two downstream signaling pathways, p53 and Rb-p16, are activated during cellular senescence. To examine oncogene induced cellular senescence at the molecular level, we assessed senescence markers, including phosphorylated extracellular signal-regulated kinase, phosphorylated p53 and p16, in cells expressing HRAS mutant proteins (Figure 2b). As expected, phosphorylated p53 (Ser15) and p16 levels, as well as phospho-extracellular signal-regulated kinase levels, were significantly increased in the cells transfected with HRAS mutants relative to cells transfected with mock vector or wild-type HRAS. These results demonstrate that not only p.G12V, but also the other eight CS-related HRAS mutants, can cause OIS.

DISCUSSION

In this study, we identified four *HRAS* mutations in 21 patients with CS and evaluated their detailed clinical manifestations of the disease in these patients. Biochemical analyses, including a GTP binding assay and luciferase assays to detect ELK and c-Jun trans-activation, showed that there were no significant differences among the analyzed mutations in codon 12/13. The p.A146T mutant demonstrated the weakest Raf binding activity, and the p.K117R and p.A146T mutants had weaker effects on downstream c-Jun N-terminal kinase signaling than mutants in codon 12 or 13. Our results indicated that all HRAS mutants detected in CS patients were able to cause OIS.

Our study is the first to demonstrate that HRAS mutants other than p.G12V can induce senescence when they are overexpressed in human fibroblasts. The symptoms of CS seem to be caused by either hyperproliferation or hypoproliferation, coupled with growth factor resistance, which may be ascribable to DNA damage response or OIS. Postnatal cerebellar tonsillar herniation, Chiari 1 malformation,[42] deep palmar and plantar creases and papillomata may all be caused by hyperproliferation. In contrast, the poor weight gain, short stature and endocrine dysfunction observed in CS patients[43–45] might be caused by hypoproliferation. Adult brain and heart progenitor cells in a zebrafish CS model with a homozygous HRAS p.G12V mutation

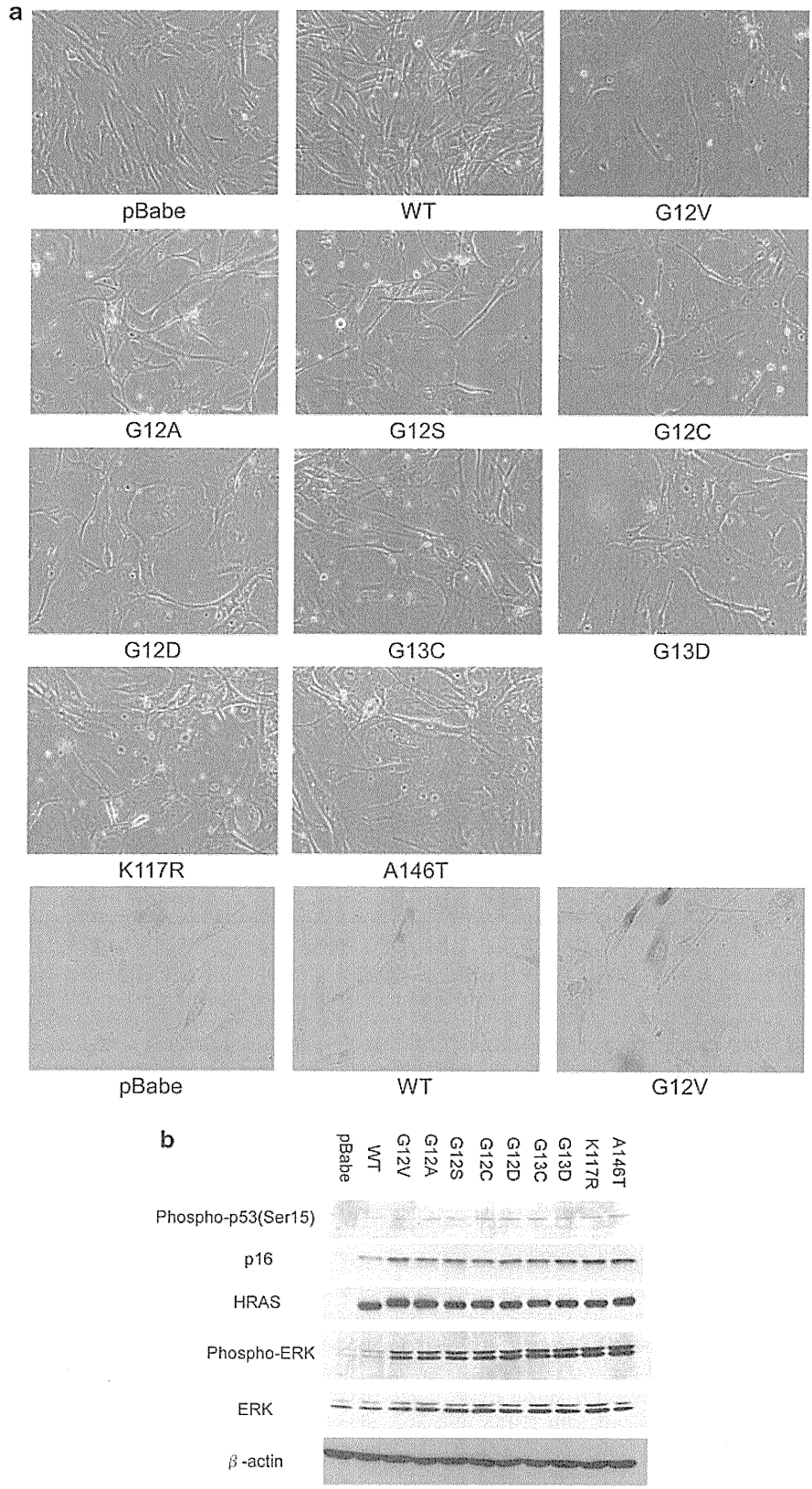

Figure 2 Effect of Costello syndrome (CS)-associated HRAS mutants on primary fibroblasts. (**a**) BJ cells transduced with retroviruses expressing wild-type or mutant HRAS. Images in the lowest tier show senescence-associated β-galactosidase staining. (**b**) Immunoblots of cellular lysates from BJ cells transduced with empty vector (pBabe) or with wild-type or mutant HRAS retroviruses.

exhibited cellular senescence, suggesting that the age-related worsening of the Costello phenotype[46] might occur, because the replicative capability of adult progenitor cells is exhausted. Osteoporosis has frequently been found in adult patients with CS,[47] suggesting that cellular senescence affects osteogenesis. However, further studies will be needed to determine whether OIS indeed contributes to the pathogenesis in patients with CS.

It has been suggested that clinical symptoms vary among patients with mutations in codon 12 or 13. In previous studies, a total of 19 CS patients have been reported to die from severe cardiomyopathy, cardiac arrhysmia, rhabdomyosarcoma, respiratory failure, multi-organ failure or sepsis. The number of fatal cases was 5/138 patients with p.G12S, 4/6 with p.G12C, 3/17 with p.G12A, 3/4 with p.G12D, 2/2 with p.G12V, 1/1 with p.G12E and 1/1 with p.E63K.[3,5–23] The mortality of patients with p.G12C or p.G12D was significantly higher than that of the patients with the more common p.G12S (P=0.026 by Fisher's exact test). Previous studies have shown that the p.G12V substitution has the highest transformative potential (p.G12V>p.G12A, p.G12S, p.G12C, p.G12D>p.G13D) and is the most frequently found mutation in human tumors.[48,49] However, our Ras activity assays and luciferase assays did not show any differences among *HRAS* codon 12/13 mutants. This may be due to the extremely high expression level of HRAS protein in our transient transfection study, which could make it difficult to detect subtle differences between mutants. Further studies will be necessary to clarify whether the high mortality in patients with p.G12C or p.G12D is due to functional differences in these mutants or due to bias because of our small sample size of patients.

Mutations at codons 117 and 146 are rare in CS and somatic cancers. Meanwhile, mutations at codons G12, G13 and Q61 have been shown to impair intrinsic and GTPase activating protein-mediated GTP hydrolysis, leading to elevated levels of cellular RAS-GTP. It has been reported that the nucleotide exchange rate of both p.K117R and p.A146V HRAS is increased, relative to wild type.[13,27,28] However, the transformational potential of p.A146V HRAS is partially activated,[27] whereas that of p.K117R-HRAS is not; its transformational activity is instead similar to that of GTPase impaired mutants.[28] Our results and those of other reports suggest that p.K117R and p.A146T have milder effects on downstream effectors than do mutations in codon 12/13.

The clinical manifestations of CS in patients with p.K117R or p.A146V mutations suggest that these alleles have distinct effects, compared with mutations in codon 12/13. Of two CS patients with a p.K117R mutation, one patient had an atypical phenotype such as microretrognatism and slightly less-pronounced plantar and palmar creases.[7] The other patient had mild craniofacial manifestations of CS.[13] One patient with the p.A146V mutation showed a mildly coarse face and did not have deep palmar creases.[6] These atypical phenotypes might be attributed to the mild effects of p.K117R or p.A146V compared with codon 12/13 mutants.

Inhibitors of the RAS/MAPK pathway could provide benefits for patients with RAS/MAPK syndromes. Statins are 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors that result in decreased isoprenylation of RAS[50] and are now widely used for the treatment of hyperlipidemia. Statins have been used to modify the clinical manifestation of neurofibromatosis type I, which is caused by a genetic defect in a negative regulator of the RAS/MAPK pathway. Studies using mouse models of NF1 (Nf1 mice) have shown that treatment with a statin reverses the cognitive deficits of these mice.[51] A randomized control trial for neurofibromatosis type I treatment with simvastatin had a negative outcome.[52] Furthermore, statins have displayed antitumor activity in experimental tumor models, though clinical antitumor effects of statins have not been established.[53] Well-designed clinical studies will be needed to determine the effects of statins or other RAS inhibitors on manifestations of CS.

In conclusion, we identified *HRAS* mutations in 21 patients and examined the clinical manifestations of mutation-positive patients. Functional analysis revealed that CS-causing mutant HRAS proteins caused OIS in human fibroblasts. These findings may help enable more accurate prognoses for patients with *HRAS* mutations and contribute to our understanding of the mechanism underlying CS pathogenesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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CLINICAL STUDY

Analysis of plasma ghrelin in patients with medium-chain acyl-CoA dehydrogenase deficiency and glutaric aciduria type II

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Abstract

Objective: Ghrelin requires a fatty acid modification for binding to the GH secretagogue receptor. Acylation of the Ser3 residue of ghrelin is essential for its biological activities. We hypothesized that acyl-CoA is the fatty acid substrate for ghrelin acylation. Because serum octanoyl-CoA levels are altered by fatty acid oxidation disorders, we examined circulating ghrelin levels in affected patients.

Materials and methods: Blood levels of acyl (A) and des-acyl (D) forms of ghrelin and acylcarnitine of patients with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency and glutaric aciduria type II (GA2) were measured.

Results: Plasma acyl ghrelin levels and A/D ratios increased in patients with MCAD deficiency or GA2 when compared with normal subjects. Reverse-phase HPLC confirmed that *n*-octanoylated ghrelin levels were elevated in these patients.

Conclusion: Changing serum medium-chain acylcarnitine levels may affect circulating acyl ghrelin levels, suggesting that acyl-CoA is the substrate for ghrelin acylation.

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Introduction

Ghrelin, an endogenous ligand for the GH secretagogue receptor, is an acylated peptide produced by gastrointestinal endocrine cells (1). Ghrelin is the only peptide known to require a fatty acid modification. Octanoylation of the Ser3 residue is essential for ghrelin-mediated stimulation of GH secretion and regulation of energy homeostasis via increased food intake and adiposity (2, 3). Other than octanoylation (C8:0), the hormone is subject to other types of acyl modification, decanoylation (C10:0), and possibly decenoylation (C10:1) (4, 5). Recently, ghrelin *O*-acyltransferase (GOAT), which octanoylates ghrelin, was identified (6, 7). The fatty acid substrate that contributes to ghrelin acylation, however, has not been clarified, although the presumed donor is acyl-CoA.

Mitochondrial fatty acid oxidation (FAO) disorders result from genetic defects in transport proteins or enzymes involved in fatty acid β-oxidation (8, 9). The clinical phenotypes have recently been associated with a growing number of disorders, such as Reye syndrome, sudden infant death syndrome, cyclic vomiting syndrome, fulminant liver disease, and maternal complications during pregnancy (10). Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, the most common inherited defect in FAO, causes elevated serum octanoylcarnitine levels (11), reflecting elevated octanoyl-CoA levels. Glutaric aciduria type II (GA2), which is caused by defects in electron transfer flavoprotein (ETF), ETF-ubiquinone oxidoreductase, or other unknown abnormalities in flavin metabolism or transport, is characterized by elevated serum acylcarnitine levels, including octanoylcarnitine (8, 9). In carnitine palmitoyltransferase II (CPT II) deficiency and very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, serum octanoyl-CoA levels do not increase, but at times actually decrease (8, 9).

We hypothesized that octanoyl-CoA is the fatty acid substrate for ghrelin acylation. To examine this hypothesis, we measured circulating ghrelin levels in patients with MCAD deficiency (MCADD) and GA2.

Materials and methods

Subjects

Five female patients with FAO deficiency (two with MCADD one with GA2, one with CPT II deficiency (12),

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and one with VLCAD deficiency) were recruited for this study. The study protocol was approved by the ethics committee on human research at the Kyoto University Graduate School of Medicine. Written informed consent was obtained prior to enrollment.

Measurement of plasma ghrelin concentrations

Because FAO patients tend to develop hypoglycemia by fasting, it was difficult to do overnight fasting. Therefore, blood samples for ghrelin analyses were drawn from a forearm vein in the morning after fasting as long as possible. Plasma samples were prepared as described previously (13). Blood samples were immediately transferred to chilled polypropylene tubes containing Na_2EDTA (1 mg/ml) and aprotinin (Ohkura Pharmaceutical, Kyoto, Japan: 1000 kallikrein inactivator units/ml = 23.6 nmol/ml (23.6 pM)) and centrifuged at 4 °C. One-tenth volume of 1 M HCl was immediately added to the separated plasma. The acylated and desacylated forms of ghrelin were measured using a fluorescence enzyme immunoassay (FEIA; Tosoh Corp. Tokyo, Japan). The minimal detection limits for acyl and des-acyl ghrelin in this assay system were 2.5 and 10 fmol/ml respectively. The interassay coefficients of variation were 2.9 and 3.1% for acyl and des-acyl ghrelins respectively.

Reverse-phase HPLC

Reverse-phase HPLC (RP-HPLC) was performed as described previously (4, 5, 14). Briefly, plasma diluted 50% with 0.9% saline was applied to a Sep-Pak C18 cartridge pre-equilibrated with 0.9% saline. The cartridge was washed with saline and 10% acetonitrile (CH_3CN) solution containing 0.1% trifluoroacetic acid (TFA). Adsorbed peptides were eluted with 60% CH_3CN solution containing 0.1% TFA. The eluate was evaporated and separated by RP-HPLC. All HPLC fractions were quantified using RIAs for ghrelin (4, 14, 15, 16). RIAs for a ghrelin C-terminal region (C-RIA) and a ghrelin N-terminal region (N-RIA) measure des-acyl ghrelin and octanoyl-ghrelin respectively (15). A RIA for N-terminal ghrelin showed ~20–25% cross-reactivity values for the *n*-decanoylated and *n*-decenoylated forms (16). Authentic human ghrelin-(1–28) was chromatographed with the same HPLC system.

Tandem mass spectrometry

Acylcarnitines in sera and dried blood spots were measured according to previously reported methods (17, 18), without derivatization. Briefly, 3 μl serum and 110 μl methanol solutions (99%) with deuterium-labeled acylcarnitines as internal standards were mixed and centrifuged, and 5 μl of the supernatant was introduced into liquid chromatography flow of methanol/acetonitrile/water (4:4:2) with 0.05% formic acid using a SIL-20AC autoinjector (Shimadzu, Kyoto, Japan). Flow injection and electrospray ionization tandem mass spectrometric (MS/MS) analyses were performed using an API 4000 LC/MS/MS system (AB Sciex, Tokyo, Japan). Positive ion MS/MS analysis was performed in precursor ion scan mode with an m/z value of 85 for the product ion. Data were recorded for 0.7 min after every sample injection and the recorded intensities of the designated ions were averaged using Chemoview Software (Foster City, CA, USA). All samples were measured serially within 1 day.

Results

We measured plasma ghrelin concentrations in patients with MCADD and GA2 (Table 1) and also in patients with CPT II and VLCAD deficiency. Elevated C8-acylcarnitine serum levels were observed in MCADD and GA II, whereas they were unchanged or lower in CPT II or VLCAD deficiency (Table 1). Levels of acyl ghrelin but not des-acyl ghrelin appeared to be elevated in patients with MCADD or GA2 in comparison with those in patients with CPT II or VLCAD deficiency, or those in female normal subjects from a previous study.

We then performed RP-HPLC analysis of ghrelin using plasma from patient 1 with MCADD. It demonstrated an eluted peak that corresponded to *n*-octanoylated human ghrelin-(1–28) in an N-RIA and a C-RIA, indicating that the detected acyl ghrelin was octanoylated (Fig. 1A). When plasma from patient 3 with GA2 was examined using the same method, the N-RIA revealed that the major peak corresponded to *n*-octanoylated human ghrelin-(1–28) (Fig. 1B). In addition, a small peak, which corresponded to decanoylated ghrelin, was observed in fraction 16 (arrow c), reflecting that serum C10-acylcarnitine levels were also elevated in patient 3 (Table 1).

Discussion

Ghrelin is the sole peptide hormone known to have a fatty acid modification. When we started this study in 2007, the catalytic enzyme and fatty acid substrate that mediate ghrelin acylation had not been identified. During this study, the GOAT enzyme was shown to be essential for ghrelin acylation (6, 7). Octanoic acid and octanoyl-CoA were candidates for the fatty acid substrate. We hypothesized that octanoyl-CoA was the substrate, because acylation of ghrelin should be an intracellular process. In fact, Ohgusu *et al.* (19) showed that acyl-CoA can be the substrate for ghrelin acyl-modification using the *in vitro* assay system. We tested this hypothesis in patients with MCADD and GA2,

Table 1 Clinical features, serum acylcarnitine levels, and plasma ghrelin concentrations in female patients with FAO disorders.

					Acylcarnitine (nmol/ml)										
Subjects	Disease	Age (years)	BMI	Height (cm)	C4	C6	C8	C10:1	C10	C12	C14	C16	C18	AG (fmol/ml)	DAG (fmol/ml)	A/D ratio
Patients (n=5)																
1	MCAD	6	15.1	119.5	0.30	0.55	4.61	0.95	0.29	0.04	0.01	0.05	0.01	45.09	57.23	0.79
2	MCAD	11	16.0	125.3	0.07	0.36	2.26	0.40	0.20	0.02	0.02	0.07	0.01	30.11	40.83	0.74
3	GA2	6	15.8	116.1	0.39	0.31	1.24	0.32	1.86	0.35	0.12	0.16	0.05	56.55	50.80	1.11
4	CPT II def.	10	17.8	141.5	0.10	0.06	0.21	0.20	0.40	0.15	0.03	0.08	0.02	19.76	34.49	0.57
5	VLCAD def.	5	14.8	109.5	0.07	0.09	0.07	0.08	0.28	0.42	2.17	2.00	0.87	27.02	113.07	0.24
Normal subjects (n=20; mean±s.d.)[a]		32.6±10.3	20.3±1.9											19.66±11.26	47.71±43.71	0.48±0.17
Reference range (n=34; mean±s.d.)					0.25±0.09	0.04±0.02	0.07±0.06	0.08±0.05	0.13±0.12	0.06±0.05	0.03±0.02	0.09±0.04	0.04±0.02			

C8, octanoyl acylcarnitine; C10, decanoyl acylcarnitine; C10:1, decenoyl acylcarnitine; AG, acyl ghrelin; DAG, des-acyl ghrelin; def., deficiency.
[a]See reference 13. All samples were reassayed using the FEIA.

which are characterized by higher intracellular octanoyl-CoA levels. Indeed, plasma A/D ratios tended to be elevated in these FAO deficiencies. A relationship between age and ghrelin levels may exist (20, 21). Concerning children, Ikezaki reported that the circulating ghrelin levels tended to correlate negatively with age in children and adolescents, but the correlation was not significant (22). Thus, the relationship has not been confirmed yet. Although we did not compare them directly with those in age- and body mass index (BMI)-matched normal children, they appeared to be higher than those in children with CPT II and VLCAD deficiencies with similar BMIs. BMIs of these patients were comparable to those of normal Japanese female children (23). These findings support the hypothesis that octanoyl-CoA is a primary substrate for ghrelin, although medium-chain triglyceride dietary lipids are a direct source for ghrelin acylation (7, 16, 24). Moreover, GOAT is a membrane-bound molecule in the endoplasmic reticulum (ER). Although how octanoyl-CoA gets into the ER lumen is unclear, Yang *et al.* (6) speculated that GOAT might mediate the transfer of octanoyl-CoA from the cytosol to the ER lumen. Although serum acylcarnitine levels tended to correlate with acyl ghrelin levels, further studies using more patients with FAO disorders are needed to confirm this relationship.

In addition to *n*-octanoylated ghrelin, other molecular forms of the ghrelin peptide exist, including des-acyl ghrelin lacking an acyl modification and such minor acylated ghrelin species as *n*-decanoylated ghrelin (Ser3 is modified by *n*-decanoic acid) (4, 5). Serum from a patient with GA2 showed the presence of acylated ghrelin that was not octanoylated and was possibly decanoylated (16). In a patient with GA2, intracellular levels of a variety of acyl-CoAs, including octanoyl- and decanoyl-CoAs, were increased, whereas MCADD was associated with specific elevation of octanoyl-CoA levels. In fact, the patient with GA2 had elevated octanoylcarnitine and decanoylcarnitine levels: 1.24 and 1.86 nmol/ml respectively. Nonetheless, the HPLC peak representing *n*-decanoylated ghrelin was much smaller than that representing *n*-octanoylated ghrelin. Although this is possibly because GOAT acylates ghrelin more efficiently with octanoyl-CoA than decanoyl-CoA, it is more likely because the cross-reactivity between *n*-octanoylated and *n*-decanoylated ghrelins is 20–25% in the N-RIA. In fact, the HPLC peaks of fraction 15–17 in the C-RIA, which detects similarly both *n*-octanoylated and *n*-decanoylated ghrelins, were large, strongly suggesting that a substantial amount of *n*-decanoylated ghrelin comparable to the elevated decanoylcarnitine level was present. Our observation that acyl ghrelin levels were not elevated in VLCAD and CPT II deficiencies, in which medium-chain acyl-CoAs levels are not higher, supported the idea that GOAT specifically acts on medium-chain acyl-CoAs. Although C16 and C18 levels were not increased in the patient with CPT II deficiency (Table 1), they may be normalized during

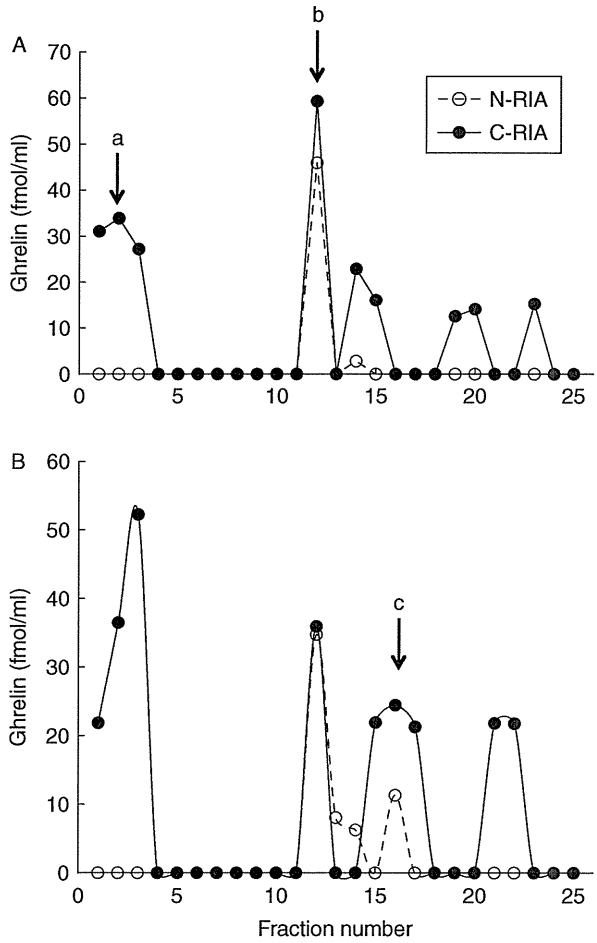

Figure 1 Representative RP-HPLC profiles of ghrelin immunoreactivity in patients with MCADD (A) and GA2 (B). Closed circles, data obtained using a RIA for a ghrelin C-terminal region (C-RIA); open circles, data obtained using a RIA for a ghrelin N-terminal region (N-RIA). Patient plasma extracts from a Sep-Pak C18 cartridge were fractionated using a Symmetry300 C18 column (5 mm packing, 3.9×150 mm, Waters). A linear gradient of 10–60% CH_3CN containing 0.1% TFA was passed over the column for 40 min at 1.0 ml/min. The fraction volume was 1.0 ml. Arrows indicate the elution positions of des-acyl human ghrelin-(1–28) (a), *n*-octanoylated human ghrelin-(1–28) (b), and *n*-decanoylated ghrelin (c).

a stable period in a mild form of CPT II deficiency (25). In fact, this patient did not manifest any marked signs or symptoms at the measurement.

Ghrelin modification with the fatty acid is essential for its biological action. Octanoylation of ghrelin may also be linked to energy homeostasis and fat metabolism. For instance, when serum *n*-octanoic acid levels increase following fat degradation, ghrelin octanoylation is enhanced, resulting in stimulation of fat synthesis. Thus, ghrelin may play an important role in energy homeostasis through its own fatty acid metabolism. Related to this concept, Kirchner *et al.* (24) speculated that signaling via GOAT and ghrelin might act as a fat sensor for exogenous nutrients and support fat storage as nutrients are ingested.

FAO deficiency contributes to such clinical problems as sudden infant death syndrome, cyclic vomiting syndrome, fulminant liver disease, and maternal complications (8, 9). Early diagnosis and appropriate management are required to reduce mortality and morbidity associated with this class of disorders. Recently, newborn screening has been expanded in this area. Measuring plasma ghrelin levels may support a diagnosis of MCADD or GA2, for example. Moreover, our results have pathophysiological implications for these disorders. Plasma ghrelin levels are changed by energy demands and food intake (e.g. glucose and fat), and ghrelin affects appetite and adiposity (2, 3). Alterations of plasma ghrelin levels in FAO disorders may reflect and/or influence the patient's metabolic status. In addition, higher acyl ghrelin levels may affect the GH/insulin-like growth factor 1 (IGF1) system. There are reports that higher AG levels would increase GH and IGF1 levels (26, 27, 28, 29) and thereby linear growth could be affected. Although none of our patients manifested markedly abnormal growth velocity, we did not measure their serum GH/IGF1 levels. Thus, further studies are warranted to detail a variety of metabolic parameters in this setting.

There are several limitations in this study. At first, the number of FAO patients tested is small. Unfortunately, the incidence of FAO patients in the Japanese population is much smaller than that in Caucasians. Although we asked pediatricians on a nationwide scale, we could successfully collect only five female patients. No adult case has yet been reported in Japan. Secondly, as mentioned above, the normal female subjects were not matched in age or BMI, although patients with MCADD and GA2 exhibited higher plasma A/D ratios than those in child CPT II and VLCAD deficiencies with similar BMIs. To supplement the correlation study, we performed RP-HPLC analysis to prove the increased octanoylation of ghrelin in MCADD and GA2 directly. Further, the presence of *n*-decanoylated ghrelin is also demonstrated in GA2. Thirdly, the disturbance in the hepatic carbohydrate regulation and the altered peripheral glucose uptake may occur in FAO patients. Hence, abnormal carbohydrate regulation could influence acyl ghrelin levels. Since none of our patients manifested abnormal fasting glucose and HbA1c levels, we speculated that no significant effects occurred.

In summary, we have demonstrated increased levels of acyl ghrelin in patients with MCADD or GA2, which are also characterized by increased intracellular octanoyl-CoA levels. These findings provide mechanistic insights into the biosynthesis of ghrelin. Furthermore, analyzing plasma ghrelin levels may help elucidate pathophysiological processes in FAO deficiencies and aid in the diagnosis of these disorders. Detailed studies using more patients are certainly needed.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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原　著

劇症肝不全として発症したミトコンドリアDNA枯渇症候群の新生児例

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要　旨

小児劇症肝不全の約半数は乳児期に発症し，死亡率は70～80％にも及ぶが，発症原因が明らかでないことも多い．新生児期に劇症肝不全で発症し，ミトコンドリアDNA枯渇症候群が原因と考えられた女児例を経験したので報告する．日齢13より嘔吐を認め，日齢15に哺乳力低下と傾眠傾向があり入院した．血清トランスアミナーゼ値の上昇，高アンモニア血症，低血糖および高ビリルビン血症を認めた．著明な凝固機能異常と傾眠傾向（小児肝性昏睡分類II度）から劇症肝不全と診断し，人工肝補助療法を開始したが，小児肝性昏睡分類IV度へと進行した．肝不全に対して肝移植を検討したが，平坦化した脳波所見から重篤な脳障害が残る可能性が高いと考えられたこと，また両親が肝移植を希望しなかったことから，内科的治療を継続した．日齢47，出血性ショックのために死亡した．剖検では肝重量は16gと著明に萎縮し，病理組織所見では，肝細胞が脱落していた．肝臓と心筋のミトコンドリア呼吸鎖酵素活性を測定したところ，呼吸鎖複合体I活性が低下しており，劇症肝不全の原因としてミトコンドリア呼吸鎖異常症が考えられた．肝臓のミトコンドリアDNAと核DNA量比が31.7％と低下していたためミトコンドリアDNA枯渇症候群と診断した．新生児における原因不明の肝不全では，ミトコンドリア呼吸鎖異常症も念頭に置き，呼吸鎖酵素活性を測定することが診断に重要であると考えられた．

キーワード：劇症肝不全，ミトコンドリア呼吸鎖異常症，ミトコンドリアDNA枯渇症候群，新生児

はじめに

小児劇症肝不全は死亡率が高く，特に1歳以下では予後不良とされている．原因は代謝性25％，ウイルス性22％，薬剤性8％，自己免疫性2％，不明43％と小児では原因不明が最も多くなっている[1]．また，ミトコンドリア呼吸鎖異常症はミトコンドリア呼吸鎖の機能障害によって引き起こされる疾患であり，5,000～7,000人に1人の頻度とされている．多臓器にわたり様々な症状を呈するが，主に骨格筋，心筋，中枢神経をはじめ，肝臓，腎臓，膵臓など広範囲な腹腔内の臓器も障害される．その中で肝不全を呈するミトコンドリア呼吸鎖異常症としては，ミトコンドリアDNA（mtDNA）枯渇症候群（MDS），Pearson症候群，Leigh脳症などが知られている．MDSには大きく分けて筋型，脳筋型，肝脳型の3種の発症形式があり，中でも肝脳型では出生直後から6か月の間に症状が出現することが多い[2]．近年，罹患臓器を用いた呼吸鎖の酵素活性やBlue Native Polyacrylamide Gel Electrophoresis（BN-PAGE）でのイムノブロット法などの酵素解析により，診断が困難とされていたミトコンドリア呼吸鎖異常症の迅速な診断が可能となった．今回われわれは，MDSが原因と考えられた新生児劇症肝不全例を経験したので報告する．

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症　例

症例：日齢15，女児

主訴：哺乳力低下，傾眠傾向

妊娠分娩歴：妊婦健診では異常を認めなかった．在胎38週3日，羊水過少を認めたため分娩誘発目的にK病院に入院し，翌日に経腟分娩となった．胎盤には梗塞等の異常は認めなかった．出生体重は2,254g，Apgar Scoreは1分値9点/5分値10点であった．妊婦検診での母体HBs抗原は陰性であった．栄養は人工乳であった．

家族歴：母親は2経妊0経産．これまでの流産の原因は不明である．近親者に代謝性疾患なし．血族結婚なし．

表1　入院時検査所見

〈血算〉		〈生化学〉		〈免疫血清〉	
WBC	24,300 /μl	T-bil	37.7 mg/dl	IgG	824 mg/dl
RBC	299×10⁴ /μl	D-bil	13.4 mg/dl	IgA	4 mg/dl
Hb	10.4 g/dl	AST	232 IU/*l*	IgM	44 mg/dl
Hct	31.6 %	ALT	141 IU/*l*	〈ウイルス検査〉	
Plt	24.6×10⁴ /μl	LDH	561 IU/*l*	HBs Ag	(−)
〈血液ガス（動脈血）〉		γGTP	155 IU/*l*	HCV Ab	(−)
pH	7.354	TG	23 mg/dl	風疹 IgM	(−)
pCO_2	34.4 mmHg	TBA	54.6 μM/*l*	EB-VCA IgM	(−)
pO_2	48.1 mmHg	ChE	483 IU/*l*	Real time PCR（血清）	
HCO_3^-	18.7 mmol/*l*	CRP	0.0 mg/dl	HSV-1	(−)
BE	−6.0 mmol/*l*	NH_3	482 μg/dl	HSV-2	(−)
〈凝固線溶系〉		乳酸	76.2 mg/dl	CMV	(−)
PT	＜5 %	ピルビン酸	2.63 mg/dl	VZV	(−)
APTT	＞120 sec	フェリチン	13,664 ng/ml	HHV-6	(−)
HPT	＜10 %			HHV-7	(−)

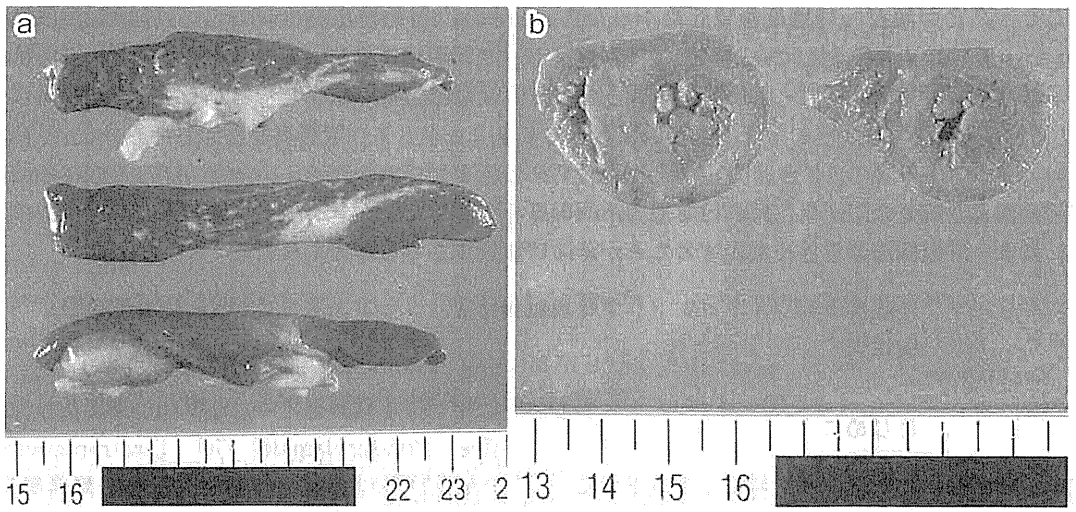


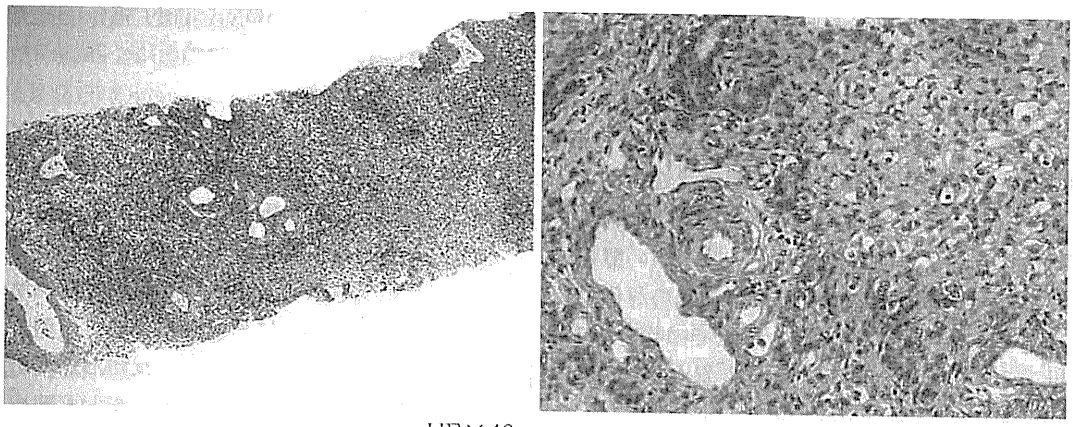
図1　剖検所見
a．肝臓：肝臓は16.9gと著明な萎縮があり，脂肪沈着を認めた．
b．心筋：心筋の肥厚を認めた．

現病歴：日齢13．嘔吐と皮膚黄染を主訴に近医を受診した．ミノルタ経皮黄疸計では12.5mg/dlであった．翌日には嘔吐が頻回になり，日齢15に哺乳力低下と傾眠傾向を認めたため近医を再診した．血液検査では血清トランスアミナーゼ値の上昇（AST 283IU/*l*，ALT 183IU/*l*），高アンモニア血症（1,371μg/dl），低血糖（3mg/dl），高ビリルビン血症（T-Bil 42.3mg/dl）を認めたため，精査加療目的に当院へ救急搬送となった．

入院時現症：体重2,431g，心拍数180/分，呼吸数38/分，血圧61/43mmHg，体温36.7℃．全身状態は活気がなく，四肢の硬直を認めた．皮膚は著明に黄染し，腹部は膨隆していたが，肝臓は触知できなかった．顔貌異常，外表奇形は認めなかった．

入院時検査所見（表1）：高ビリルビン血症，血清トランスアミナーゼの上昇，凝固機能異常（PT：5%未満）を認めた．血中乳酸およびピルビン酸値の著明な上昇と，血液ガス分析では軽度の代謝性アシドーシスを認めた．

入院後経過：血液検査所見から急性肝炎重症型と診断した．さらに傾眠傾向（小児肝性昏睡分類にてII度）を認めたため，劇症肝不全と判断した．高ビリルビン血症に対する治療も兼ねて直ちに交換輸血を開始し，その後人工肝補助療法へと移行した．人工肝補助療法として，持続的血液濾過透析を継続し，血漿交換療法を連日行った．同時に，プロスタグランディンE2の投与を開始し，劇症肝不全の原因としてウイルス性



HE×40　　HE×400

図2　肝臓組織所見　日齢47（入院第32病日）
ほとんどの肝細胞は脱落し，組織球に置き換わっていた．

表2　ミトコンドリア呼吸鎖複合体の酵素活性

Liver	CoI	CoII	CoIII	CoIV	CS
% of normal	7.3	17.1	42.4	56.7	175.2
CS ratio (%)	4.1	9.7	23.8	31.7	
CoII ratio (%)	42.5		256.5	327.5	

Heart	CoI	CoII	CoIII	CoIV	CS
% of normal	13.7	48.1	49.1	39.6	79.8
CS ratio (%)	16.3	60.6	56.7	47.9	
CoII ratio (%)	24.1		85.0	71.6	

CoI：complexI　CoII：complexII　CoIII：complexIII
CoIV：complexIV　CS：citrate synthase
光化学的に呼吸鎖複合体Ⅰ～Ⅳの酵素活性とクエン酸合成酵素（CS）を測定し，CSとの相対活性を正常対照と比較した．肝臓と心臓において複合体Ⅰの著明な低下を認め，CS比で20%以下となっていたため呼吸鎖複合体Ⅰ欠損症と診断した．

または新生児ヘモクロマトーシスも否定ができないため，アシクロビルおよびデスフェラールの投与も開始した．凝固能異常による出血傾向に対し，ビタミンKとメシル酸ナファモスタットの投与を開始した．治療開始8時間経過した時点での脳波所見は低振幅徐波を示しており，また肝性昏睡はⅣ度に進行した．治療によりアンモニアおよび血清ビリルビンは速やかに低下したものの，凝固機能の改善は認めなかった．日齢21，入院第6病日に頭部MRIを施行したところ，T1強調像において対称性に淡蒼球から中心溝付近に異常高信号を認め，高アンモニア血症に伴う脳障害が示唆された．また腹部MRIでは肝萎縮を認めたが，ヘモクロマトーシスに特徴的なT2強調像での著明な低信号は認めなかった．両親に対して，平坦化した脳波所見から重篤な脳障害が残る可能性が高いと考えられること，また本邦における小児劇症肝不全の転帰[1]に関して説明を行った上で，治療戦略の1つとして肝移植の可能性を提示したが，移植の希望はなかった．内科的治療を継続したが，著明な凝固機能異常が遷延し，肺出血と消化管出血による出血性ショックのため日齢47に死亡した．入院後に行った検査（表1）では，ウイルス学的検査は陰性，尿中ウイルス培養陰性，その他にも遊離脂肪酸，有機酸/脂肪酸代謝異常症スクリーニング検査はそれぞれ正常であった．アミノ酸分析では，肝不全のために汎アミノ酸高値を認めた．新生児ウイルス関連血球貪食症候群（VAHS）も否定できなかったため，日齢40に骨髄生検を施行したところ，一部に空胞を伴うマクロファージの赤血球貪食像を認めたが，VAHSにみられる広範囲な血球貪食像は認めなかった．

剖検所見（図1）：肝臓は16.9gと著明に萎縮し，肝全体に脂肪滴を認めた．また，心臓では左心室壁の肥厚を認めた．両親の同意が得られず，脳組織の評価は施行しなかった．

肝臓組織所見（図2）：ほとんどの肝細胞は脱落し，線維化を伴っていた．鉄染色は陰性であった．

ミトコンドリア呼吸鎖酵素活性（表2）：肝臓および心筋のミトコンドリア呼吸鎖複合体活性の測定を行った．光化学的に呼吸鎖複合体I～IVの酵素活性とクエン酸合成酵素（CS）の活性を測定し，CS及び複合体IIとの相対活性を正常対照と比較した．呼吸鎖の酵素活性は，Kirdyら[3]の方法を用いて，酵素活性の評価はBernierら[4]の基準を用いて行った．肝臓では複合体Iの著明な低下があり，複合体IIの低下も認めた．心筋では明らかな複合体I活性の選択的低下を認めた．

ミトコンドリアDNAの比較定量，及び遺伝子検査：肝臓及び心筋のDNAを精製したのち，real time PCRを用いて，ミトコンドリアDNA（mtDNA）量と核DNA（nDNA）量の比較定量（quantitative PCR）を，Heら[5]の方法に基づき施行した．その結果，mtDNAの枯渇が判明した（mtDNA/nDNA　肝：31.7%，心筋：26.1%，35%未満を有意な低下とした[6]）．そこで肝脳型MDSの代表的な責任遺伝子である*DGUOK*，*MPV17*，*POLG*について検査した．mtDNA複製に関与するDNA polymerase γ（POLG（1434-40G＞A/））の遺伝子変異を片方のアリルに認めたが，同変異部位はイントロン領域でもあることから，本疾患の原因としての関連は低いと考えられた．現在のところ，肝臓と心筋に異常をきたすMDSとして報告があるのはPOLGだけである[7]．

上記の結果から遺伝子異常は明らかにできなかったものの，MDSによる呼吸鎖複合体I欠損症に伴った新生児劇症肝不全症と診断した．MDSでは一次的には複合体IIの低下は生じないが，自験例では肝不全に伴い肝臓の複合体IIの活性が二次的に低下した可能性が高いと考えられた．

考　察

自験例では確定診断のため肝生検を考慮したが，生存中は凝固機能異常とともに著明な肝萎縮のために肝臓の針生検が施行できず診断に苦慮した．入院当初，代謝性アシドーシス，高乳酸血症，高アンモニア血症を認めていたため，肝不全症状を呈するミトコンドリア呼吸鎖異常症を疑い，剖検による肝臓組織を利用して呼吸鎖活性の検索を行ったところミトコンドリア呼吸鎖複合体Iの欠損があり診断に至った．また複合体IIの低下もみられたが，MDSではmtDNAの支配領域に関係のない複合体IIまで2次的に低下してくることも多い[8]．特に肝障害が進行するとミトコンドリア障害に加え，フリーラジカル等の産生も起こり複合体IIを中心とした2次的低下はよくみられる現象で[9]，一次的なものと区別しにくい場合もあり，臨床像，生化学検査，病理組織，mtDNA定量，遺伝子検査など多角的な検討が必要となる．

複合体Iは45個のサブユニットからなり，電子伝達系酵素の中で最も大きく，mtDNAとnDNAの両支配を受けている．このうち7個のサブユニットはmtDNAにコードされている．また，複合体I欠損症がnDNAの遺伝子異常の場合には，生後早期に呼吸不全や代謝性アシドーシス，フロッピーインファントで見つかり，比較的早期に死亡する乳児重症型が多いとされる[10]．mtDNAが極端に減少したMDSでは，さまざまな臓器障害を呈するが，肝障害なども報告されている[11]．軽度の肝腫大や脂肪化を伴う無症候性の症例から数週間で進行して肝不全に至る症例まである．臨床症状としては，筋緊張低下や嘔吐，成長障害，アシドーシス，低血糖を呈する．肝脳型MDSでは出生直後から6か月の間に症状が出現するとされ，84%が5か月以下の発症であったと報告されている[2]．呼吸鎖異常症の約半数にmtDNA欠乏が存在するとされ，さらにその中の64%が新生児期に肝障害が存在していたとしている[6]．呼吸鎖複合体活性の低下を認めた場合，MDSを疑う根拠になるため[12]，自験例でもmtDNAを測定したところ，MDSの診断に至った．

mtDNAとnDNAのPCR比較定量は，組織内での比を正常コントロール群と比較するものであり，フレッシュな組織が採取されているのであれば通常は剖検後でも正確な数値が出る[13]．しかし自験例のように広範な細胞脱落の場合にどのくらい誤差が出るかは不定であり，MDSか否かは最終的には遺伝子検索によると思われる．自験例では心筋も併せてmtDNA量が低下しているためMDSの可能性が極めて高いと考える．

高乳酸血症はミトコンドリア疾患に特徴的とされている．自験例では高乳酸血症を伴う代謝性アシドーシスが続いていたため，ミトコンドリア呼吸鎖の酵素活性を測定する契機となった．しかし，呼吸鎖複合体I欠損症の20%では血中乳酸値が正常であり，また髄液中の乳酸値も約23%が正常であると報告されている[10]．従って，低血糖やケトアシドーシスなど検査所見などからミトコンドリア呼吸鎖異常症を疑うような症例では，乳酸値が正常でも確定診断のために肝生検を施行し，呼吸鎖活性を検索することが推奨されている[14]．

また，ミトコンドリア呼吸鎖異常症では胎児期から何らかの異常を認め，流産や低出生体重児，胎児水腫を伴う症例が報告されている[15]．自験例も低出生体重児のため，胎児期から子宮内発育遅延（IUGR）があったと考えられる．肝臓をはじめとした臓器障害は胎児期から存在していたものと推測され，出生後，肝臓の