infancy within three degrees of relationship. He had normal psychomotor development and had not been vaccinated against influenza.

On arrival, he was comatose and had a temperature of 38.9 °C, heart rate of 136 beats per minute, and blood pressure of 106/62 mm Hg. Neither arrhythmia nor cardiac hypertrophy was seen in the electrocardiogram or echocardiography. Blood examination showed marked liver dysfunction and ammonemia (aspartate aminotransferase, 4282 IU/l; alanine aminotransferase, 1750 IU/l; ammonia, 156 μg/dl). Blood gas analysis showed marked acidosis (pH 6.964, pCO_2 59.6 mm Hg, HCO_3 11.2 mol/l, BE −23.7 mmol, and lactate 9.0 mmol/l). Blood glucose was 128 mg/dl under intravenous infusion. Influenza encephalopathy was diagnosed and intensive therapy, including mechanical ventilation, steroid, and heart stimulants, was started. A few hours later, he developed cardio-pulmonary arrest and died 36 h after developing pyrexia. This clinical course led us to suspect Reye's syndrome and mitochondrial disorders. The parents consented to resection of the patient's liver and skin fibroblasts. Urine organic acid analysis, blood amino acid profile, and carnitine profile did not show any findings suggestive of congenital metabolic disorders. Microscopical finding showed microvesicular fatty droplets in hepatic cytoplasm in hematoxylin-eosin and oil red O staining (Fig. 1), that was compatible with Reye's syndrome. The grade of histological hepatic changes was milder than the fulminant clinical course.

The activities of respiratory chain complexes (Co) I, II, III, and IV were assayed in the crude post-600 g supernatant of the liver and in isolated mitochondria from skin fibroblasts as described previously [2]. The activity of each complex was presented as a percent ratio relative to the mean value obtained from 12 healthy controls. The activities of Co I, II, III, and IV were also calculated as the percent relative to citrate synthetase (CS), a mitochondrial enzyme marker, or Co II activity [2]. Liver respiratory chain complex I activities were very low, but CS, Co II, III, and IV activities were normal. In contrast to the liver, the fibroblast complex I activity was normal (Table 1).

The expression of the mitochondrial respiratory chain Co I, II, III, and IV proteins in the liver and fibroblasts were examined by Western blotting using blue native polyacrylamide gel electrophoresis (BN-PAGE) according to methods described previously [3]. The results of BN-PAGE are shown in Fig. 2. The band corresponding to Co I was not visible; while, the intensities of the Co II, III, and IV bands remained normal. Several base substitutions were detected by polymerase chain reaction, but there was no pathogenic mutation in the genomic DNA extracted from the autopsied liver tissue.

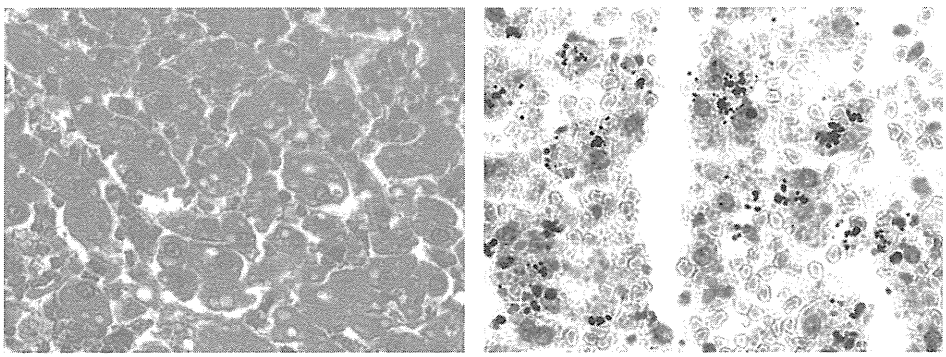
3. Discussion

Mitochondrial malfunction has been described in influenza encephalopathy. There are no reports of mitochondrial respiratory chain diseases, although disorders of fatty acid oxidation have been discussed [1]. Complex I deficiency was first recognized in 1979 by Morgan-

Table 1
Enzyme assay of respiratory chain complexes.

%	Co I	Co II	Co III	Co IV	CS
Liver					
% of normal	7.2	57.9	122.3	161.0	78.1
CS ratio	9.2	74.1	155.0	203.8	–
Co II ratio	12.3	–	212.2	272.2	–
Fibroblast					
% of normal	82.0	83.1	72.9	97.3	120.4
CS ratio	66.2	66.8	56.5	76.3	–
Co II ratio	98.2	–	83.7	112.5	–

Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthase.
Enzyme activities are expressed as a % of the mean relative activity of the normal control and relative to CS and Co II.



A. Hematoxylin-Eosin staining (×400) B. Oil Red O staining (×400)

Fig. 1. Autopsy liver samples show preserved hepatic architecture with scattered distribution of micro-vesicular fatty droplets in the hepatic cytoplasm (A). Marked congestion, focal necrosis, and mild inflammatory cellular infiltration without fibrosis were noted. Fat deposition was also suggested with oil red O staining (B). The grade of histological hepatic changes was milder than the fulminant clinical course.

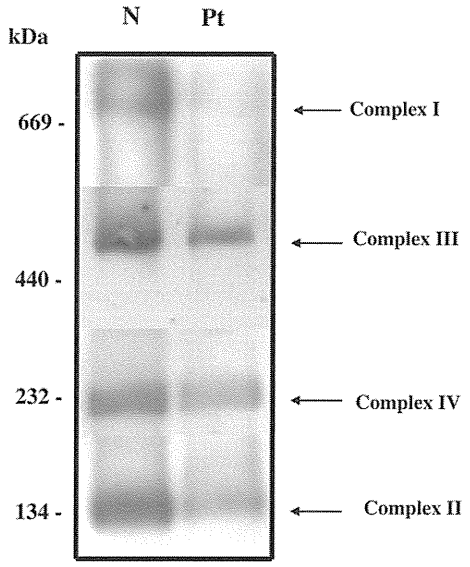


Fig. 2. Blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis of liver respiratory chain enzymes showed markedly decreased protein expression of complex I, while the protein bands of complex II, III, and IV were comparable to the control (N) samples.

Hughes; yet, studies have not progressed because of technical difficulties. More recently, complex I deficiency was regarded as the most common energy generation disorder. The manifestations range from typical mitochondrial diseases, such as Leigh syndrome, to obscure conditions such as slow regression or intractable secretory diarrhea [4].

Complex II activity has been shown to be more labile than complex I when measuring respiratory chain enzymes in patients with a wide range of metabolic disorders, liver failure, or liver disease [5]. In the present case, only complex I activity was very low; this indicates primary complex I deficiency rather than a secondary effect of influenza A infection. Complex I includes seven mitochondrial DNA-encoded subunits and at least 39 nuclear-encoded subunits. In our case, no mutation was detected in the mitochondrial DNA (mtDNA). The detection rate for mutations in mitochondrial or nuclear DNA in complex I deficiency is as small as 20% [6,7].

In the present case, complex I was deficient only in the liver, not in fibroblasts. Mitochondrial respiratory complex disorders can show clinical and biochemical tissue specificity [2,4,6,8,10]. For this reason, it is difficult to diagnose by suspension cells or serum enzyme assays. The possible mechanisms of tissue specificity are tissue-specific subunits of complex I [9], the ratio between normal and mutant mtDNA in a specific tissue [7], and tissue differences in RNA processing [10]. To our knowledge, very few cases with liver-specific complex I deficiency have been reported [2,8]. These reported cases had chronic neurological symptoms such as epilepsy, hypotonia, or developmental regression, with the exception of one case that had severe cardiomyopathy in early infancy [2]. There was one case without evidence of liver dysfunction [8]. Clinically there was no definite difference from usual Co I deficiency. One reason for the small number of cases is that the liver is not the prime diagnostic tissue. Respiratory chain complex deficiency is usually confirmed by tissue biopsy. Muscle is usually the prime diagnostic tissue, and cultured skin fibroblasts are also often analyzed [10]. False-negative diagnostic results may occur because the liver is not examined.

This case was determined to be complex I deficiency by BN-PAGE Western blotting and determination of enzyme activities. This is the first report of respiratory chain complex I deficiency in influenza encephalopathy. We suggest there may be many undiagnosed cases of this metabolic disorder. Here, we described a healthy child, who had never been suspected of having any disease, diagnosed with a metabolic disorder after acute encephalopathy with subsequent death. Future studies are needed to focus on the development of a method to detect this inborn metabolic disorder before onset.

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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.

Subjects	Disease	Age (years)	BMI	Height (cm)	Acylcarnitine (nmol/ml)									AG (fmol/ml)	DAG (fmol/ml)	A/D ratio
					C4	C6	C8	C10:1	C10	C12	C14	C16	C18			
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

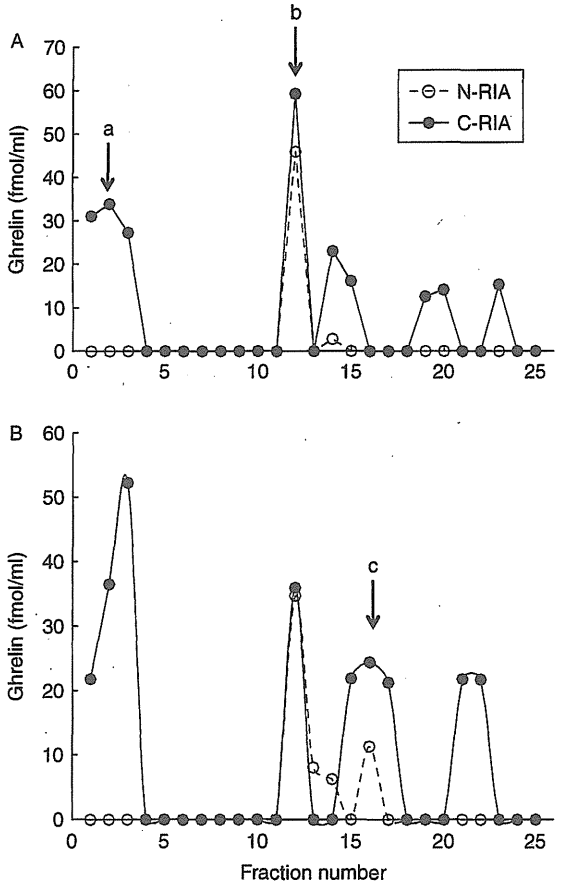

Figure 1 Representative RP-HPLC profiles of ghrelin immunoreactivity in patients with MCADD (A) and GA2 (B). Closed circles, data obtained using a RIA for a ghrelin C-terminal region (C-RIA); open circles, data obtained using a RIA for a ghrelin N-terminal region (N-RIA). Patient plasma extracts from 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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Case report

Two Japanese patients with Leigh syndrome caused by novel *SURF1* mutations

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Abstract

We report two patients with Leigh syndrome that showed a combination of facial dysmorphism and MRI imaging indicating an *SURF1* deficiency, which was confirmed by sequence analysis. Case 1 is a 3-year-old girl with failure to thrive and developmental delay. She presented with tachypnea at rest and displayed facial dysmorphism including frontal bossing, lateral displacement of inner canthi, esotropia, maxillary hypoplasia, slightly upturned nostril, and hypertrichosis dominant on the forehead and extremities. Case 2 is an 8-year-old boy with respiratory failure. He had been diagnosed as selective complex IV deficiency. Case 2 displayed facial dysmorphism and hypertrichosis. Since both patients displayed characteristic facial dysmorphism and MRI findings, we sequenced the *SURF1* gene and identified two heterozygous mutations; c.49+1 G>T and c.752_753del in Case 1, and homozygous c.743 C>A in Case 2. For patients with Leigh syndrome showing these facial dysmorphism and hypertrichosis, sequence analysis of the *SURF1* gene may be useful.

Keywords: Leigh syndrome; *SURF1* deficiency; Facial dysmorphism; Hypertrichosis

1. Introduction

Leigh syndrome (OMIM 256000) is a progressive neurodegenerative disorder with the usual onset in infancy or early childhood. It is a genetically heterogeneous disease and the most common cause is a molecular defect in mitochondrial energy production system, including the respiratory chain complexes and pyruvate dehydrogenase complex. An isolated generalized defect of complex IV, (Cytochrome C oxidase) is the most common biochemical abnormalities found in Leigh syndrome [1]. Leigh syndrome with *SURF1* mutations, which encode the putative assembly protein of complex IV, have been reported [2] with specific clinical features of facial dysmorphism [3], hypertrichosis [4], and MRI findings [5]. Here, we report two patients with these clinical features and novel *SURF1* mutations.

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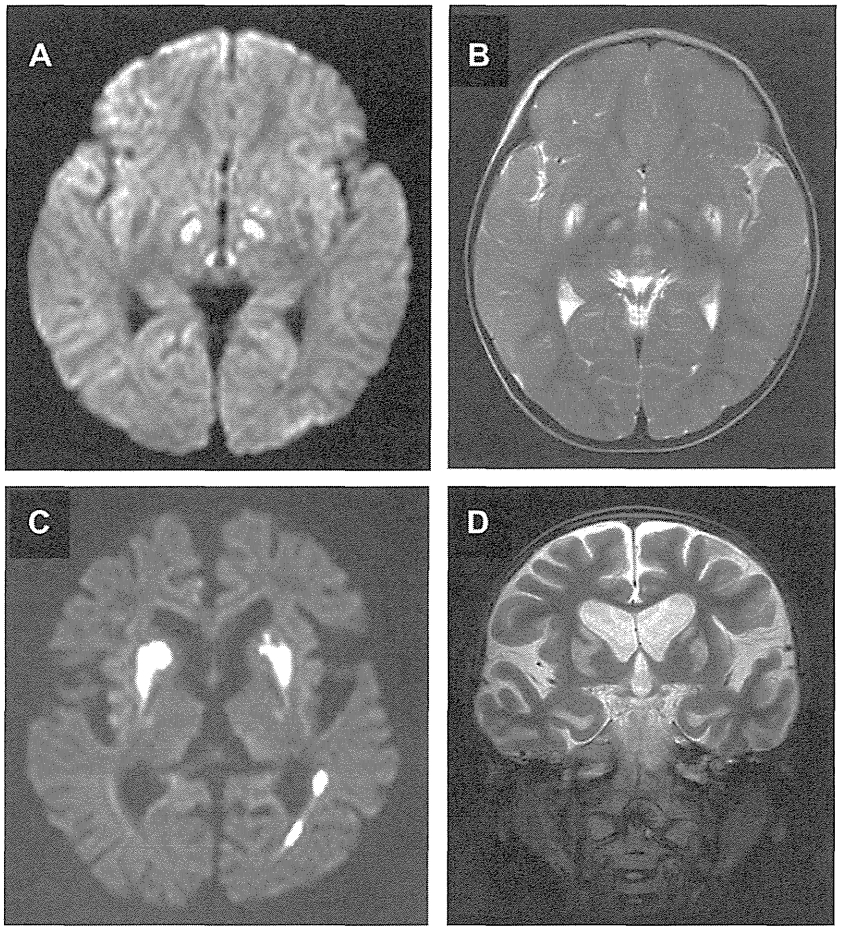

Fig. 1. Diffusion-weighted (A and C) and T2-weighted (B and D) magnetic resonance imaging of the brain in Case 1 at 2 years and 5 months of age (A and B), and in Case 2 at 7 years and 6 months of age (C and D). In Case 1, the bilateral substantia nigra (A), subthalamic nucleus (A and B), red nucleus (A), medial parts of the midbrain (A and B) and putamen (B) show the signal hyperintensity. In Case 2, bilateral striatum reveal hypeintensity (C and D). The left optic radiation is also involved in Case 2 (C) and the global cerebral hemisphere is atrophic (C and D).

2. Case reports

2.1. Case 1

Case 1 is 3-year-old female that was referred to our hospital for an evaluation of failure to thrive and developmental delay at 2 years. She was born to healthy nonconsanguineous Japanese parents. The neonatal period was unremarkable. She held her head upright at 3 months of age, and sat at the 6 months. At the 9 months, she was able to walk independently while holding on to furniture. Her development did not progress thereafter, and she has not walked alone and only speaks using jargon. She was conscious, alert and presented with tachypnea at rest. She displayed facial dysmorphism including frontal bossing, lateral displacement of inner canthi, esotropia, maxillary hypoplasia, slightly upturned nostril, and hypertrichosis dominant on the forehead and extremities. Mild opthalmoplegia and ptosis were noted. She manifested generalized mild hypotonia, truncal ataxia and normal deep tendon reflexes with negative Babinski's signs. Serum lactate was elevated at 35.7 mg/dl. MRI showed signal hyperintensity of the bilateral putamen, subthalamic nucleus, red nucleus and brain stem on T2-weighted images (T2WI) and diffusion-weighted images (DWI) (Fig. 1). The enzyme analysis of the respiratory chain complexes were not performed in this patient.

2.2. Case 2

Case 2 is 8-year-old male on ventilation that was transferred to our hospital for tracheostomy. He was born at term to healthy, nonconsanguineous parents. He had been able to get cruising by 12 months. At 19 months, he presented with neurodevelopmental regression and ataxia. Laboratory investigation revealed elevated cerebrospinal fluid lactate and pyruvate. Brain MRI showed signal hyperintensity of the bilateral basal ganglia, midbrain and medulla oblongata on T2WI. Fibroblast analysis confirmed a decreased amount and activity of complex IV in the respiratory chain complexes

(Fig. 2). He displayed facial dysmorphism including synophrys and micrognathia, hypertrichosis, thoracic deformity and generalized hypotonia and elevated deep tendon reflexes with positive Babinski's signs. MRI showed that the bilateral cerebral hemisphere were globally atrophic and signal hyperintensity of the bilateral optic radiation, putamen, basal ganglia including subthalamic nucleus, and brain stem on T2WI. The left optic radiation, bilateral putamen and globus pallidus also showed high signal intensity on DWI (Fig. 1).

3. Genomic DNA sequencing, RT-PCR and sequencing

Genomic DNA was prepared from white blood cells using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). PCR of all exons and exon–intron boundaries of the *SURF1* gene was performed with specific primers using Ex Taq PCR version 1.0 kit (Takara, Shiga, Japan) according to the manufactures instruction (Suppl. Table 1). Total RNA was extracted from leukocytes using Trizol reagent and amplified with the SMART™ mRNA amplification method (Clontech, Mountain View, CA). The amplified mRNA was subjected to reverse transcription with Prime Script reverse transcriptase (Takara, Shiga, Japan) using Oligo (dT) primers. RT-PCR was performed using primers at exons 1 and 9 of the *SURF1* gene, according to the manufacture's instruction (Suppl. Table 1). Patients and families participating in the gene analysis gave written informed consent to the gene analysis, which was approved by the ethical committee of Kanagawa Children's Medical Center.

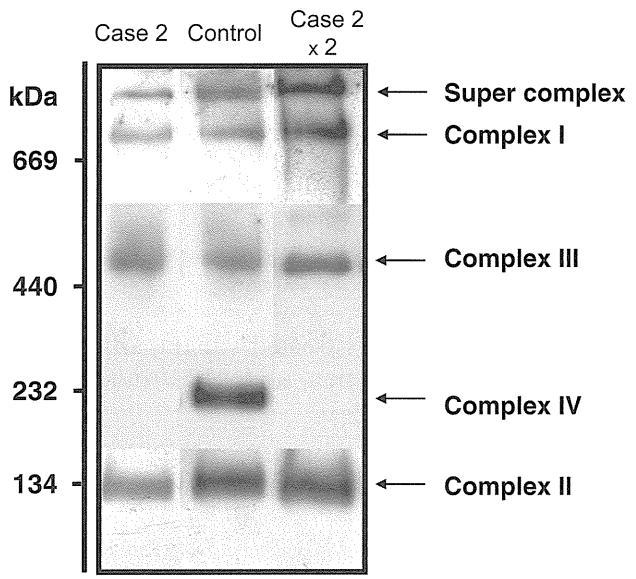

Fig. 2. Analysis of respiratory chain complex amount by blue native polyacrylamide gel electrophoresis in Case 2. Mitochondria isolated from Case 2 and normal control fibroblasts were solubilized in dodecyl maltoside and subjected to BN-PAGE and Western blotting [9]. In x 2 lane, the amount of protein loaded was twice. The amount of fully assembled complex IV was shown to be dramatically decreased in Case 2. The amount of complexes I, II, and III were all comparable to those in the normal control. In vitro enzyme assay [10] also revealed deficiencies of complex IV: the activities of complex I, II, III and IV relative to that of citrate synthase were 137%, 238%, 124% and 12%, respectively.

4. Results

4.1. Case 1

We identified two novel heterozygous mutations: a maternal c.49+1 G>T splice site mutation in intron 1 and a paternal c.752_753del in exon 8. This deletion resulted in a frame shift at amino acid 251(Gln251) causing a stop codon in exon 8 (Fig. 3). The c.49+1 G>T splice site mutation changes the highly conserved G nucleotide at position +1 of the donor splice site (5′ss) in intron 1. We attempted to characterize the splicing outcome of this sequence variation by RT-PCR analysis from patient's blood. Sequence analysis of the RT-PCR reaction detected only the allele with the c.752_753delAG mutation, which implies the presence of a nonsense mediated decay or instability of mRNA from the allele with the c.49+1 G>T splice site mutation.

4.2. Case 2

Sequence analysis of the *SURF1* gene revealed a novel homozygous c.743 C>A, p.Ala248Asp in exon 7. Both parents of this patient were heterozygous for this mutation (Fig. 3). This mutation changes highly conserved Alanine to Aspartate. This mutation was not found in 100 control alleles.

5. Discussion

Molecular elucidation of Leigh syndrome is challenging since many enzymes are involved, such as mitochondrial respiratory chain complexes I, II, III, IV, and V, and components of the pyruvate dehydrogenase complex. Mutation analysis in DNA is more complicated, even after focusing on respiratory complex IV. Mitochondrial-encoded *MTCO3* and nuclear-encoded *COX10*, *COX15*, *SCO2*, and *SURF1*, have been reported as the cause of Leigh syndrome [6,7]. Our two cases presented with mental retardation, failure to thrive, respiratory dysfunction, facial dysmorphism and hypertrichosis. Facial dysmorphism including micrognathia and hypertrichosis especially in the extremities have been reported to be distinctive and characteristic feature of *SURF1* gene mutation [3,4]. Our two cases underscore the importance of *SURF1* analysis in Leigh syndrome with facial dysmorphism and hypertrichosis. However, not all patients with this gene mutation carry these symptoms. Although facial dysmorphism has been also

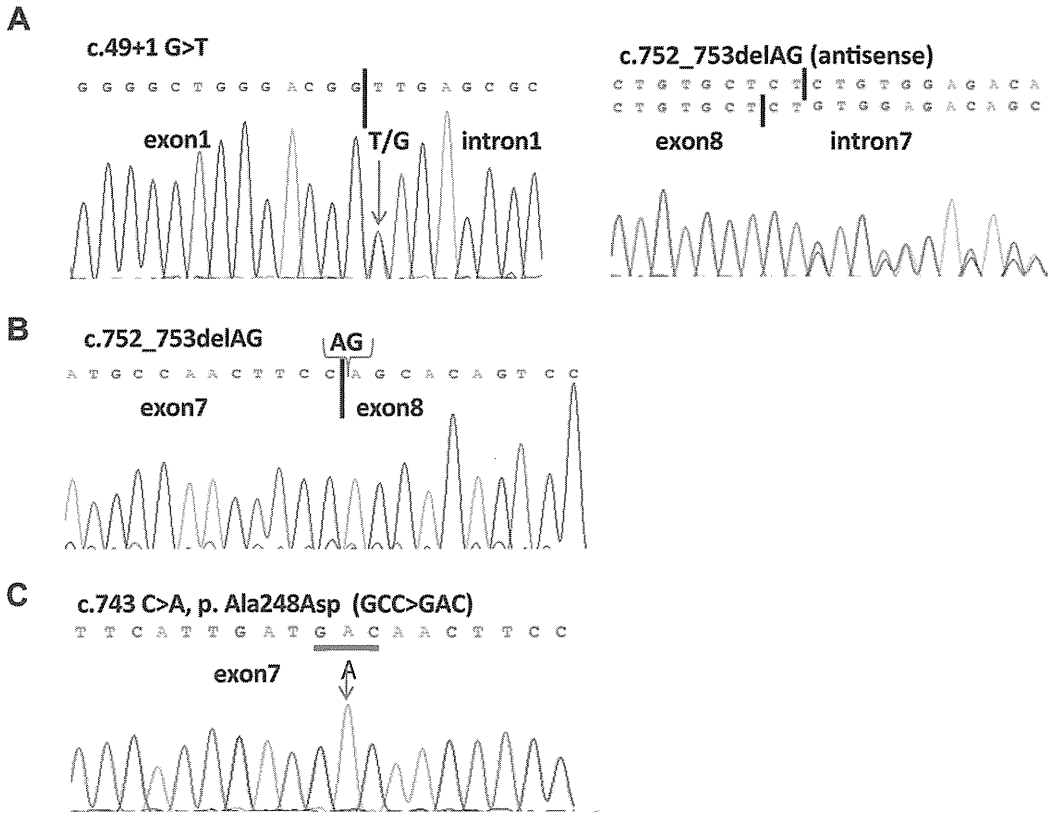

Fig. 3. Analysis of the *SURF1* gene. A chromatogram of the two novel heterozygous mutations; c.49+1 G>T and c.752_753del in Case 1 (A) and homozygous c.743 C>A in Case 2 (C). Panel B shows the chromatogram of cDNA from Case 1. The mutations are shown on the sense strand except for the right panel of A (antisense).

reported in Leigh syndrome with pyruvate dehydrogenase complex, hypertrichosis has not been described [8].

To date, more than 100 patients of Leigh disease with *SURF1* mutations have been reported [6,7]. To our knowledge, this is the first report of a mutation in intron 1, suggesting the need to scan whole exons and exon/intron boundaries.

Common MRI findings of Leigh syndrome are symmetric lesions in the brainstem, basal ganglia, thalamus and spinal cord, Leigh syndrome with *SURF1* mutation have been reported to involved the subthalamic nuclei, medulla, inferior cerebellar peduncles, and substantia nigra [5]. In addition, Case 2 showed signal hyperintensities in bilateral optic radiation on T2WI and DWI, which has not been reported previously in Leigh syndrome with *SURF1* mutations. Since Case 2 had never shown severe hypoxemia, this finding may be significant in patients with *SURF1* mutation or may appear in a progressed stage of disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.braindev.2012.02.007.

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Metabolic autopsy with postmortem cultured fibroblasts in sudden unexpected death in infancy: Diagnosis of mitochondrial respiratory chain disorders

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ABSTRACT

Mitochondrial respiratory chain disorders are the most common disorders among inherited metabolic disorders. However, there are few published reports regarding the relationship between mitochondrial respiratory chain disorders and sudden unexpected death in infancy. In the present study, we performed metabolic autopsy in 13 Japanese cases of sudden unexpected death in infancy. We performed fat staining of liver and postmortem acylcarnitine analysis. In addition, we analyzed mitochondrial respiratory chain enzyme activity in frozen organs as well as in postmortem cultured fibroblasts. In heart, 11 cases of complex I activity met the major criteria and one case of complex I activity met the minor criteria. In liver, three cases of complex I activity met the major criteria and four cases of complex I activity met the minor criteria. However, these specimens are susceptible to postmortem changes and, therefore, correct enzyme analysis is hard to be performed. In cultured fibroblasts, only one case of complex I activity met the major criteria and one case of complex I activity met the minor criteria. Cultured fibroblasts are not affected by postmortem changes and, therefore, reflect premortem information more accurately. These cases might not have been identified without postmortem cultured fibroblasts. In conclusion, we detected one probable case and one possible case of mitochondrial respiratory chain disorders among 13 Japanese cases of sudden unexpected death in infancy. Mitochondrial respiratory chain disorders are one of the important inherited metabolic disorders causing sudden unexpected death in infancy. We advocate metabolic autopsy with postmortem cultured fibroblasts in sudden unexpected death in infancy cases.

1. Introduction

Sudden unexpected death in infancy (SUDI) is defined as sudden unexpected death occurring before 12 months of age. If SUDI remains unexplained after thorough investigations, it is classified as sudden infant death syndrome (SIDS). The more common causes of SUDI are infection, cardiovascular anomaly, child abuse, and metabolic disorders. However, the many potential inherited metabolic disorders are more difficult to diagnose at autopsy as compared to cardiovascular defects and serious infection. Inherited metabolic disorders may, therefore, be underdiagnosed as a cause of SUDI or misdiagnosed as SIDS. Fatty acid oxidation disorders (FAODs) are one type of the inherited metabolic disorders and may cause as much as 5% of SUDI cases after thorough investigations including metabolic autopsy [1–5]. In a review of SUDI cases with respect to potential FAODs, we found a case of carnitine palmitoyltransferase II deficiency [6]. In that study, we performed fat staining of liver, postmortem acylcarnitine analysis, and genetic analysis, advocating the importance of metabolic autopsy in SUDI cases.

Mitochondrial respiratory chain (MRC) disorders were first identified in 1962 [7]. MRC disorders have a frequency of about at least 1:5000 newborns and are the most common disorders among inherited metabolic disorders [8]. However, there are few published reports regarding the relationship between MRC disorders and SUDI. Studies of MRC disorders have not progressed because of technical difficulties or variability in clinical manifestations [9]. In sudden death cases especially, clinical features are unclear and postmortem changes complicate molecular analysis.

In the present study, we performed metabolic autopsy in 13 Japanese cases of SUDI in order to determine whether MRC disorders could be detected or not. We performed fat staining of liver and postmortem

Abbreviations: CS, citrate synthetase; FAODs, fatty acid oxidation disorders; MRC, mitochondrial respiratory chain; OXPHOS, oxidative phosphorylation; SIDS, sudden infant death syndrome; SUDI, sudden unexpected death in infancy.

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acylcarnitine analysis according to the previous methods. In addition, we analyzed MRC enzyme activity in frozen organs as well as in postmortem cultured fibroblasts. With such metabolic autopsy, we were able to detect one probable case and one possible case of MRC disorders. These cases might not have been identified without metabolic autopsy. MRC disorders are important diseases causing SUDI and metabolic autopsy might be helpful for forensic scientists and pediatricians to diagnose MRC disorders that might not otherwise be identified.

2. Materials and methods

2.1. Subjects

Between October 2009 and September 2011, forensic autopsy was performed on 588 cases at our institute, 22 of whom were under 12 months of age. Following macroscopic examination, nine cases could be diagnosed but 13 cases (Table 1) did not have any characteristic appearance and remained undiagnosed. In this study, we reviewed these 13 undiagnosed cases (8 males, 5 females) with age ranging from 1 to 10 months.

2.2. Autopsy

Autopsies were performed within 24 h following death. Blood was obtained from the femoral vein. Heart and liver specimens were immediately cut and frozen at −80 °C. Dermis, which was cut and sterilized, was cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum, 1% penicillin streptomycin glutamine, and 2.5% amphotericin B (Life Technologies, Indianapolis, IN). Once cultures were established, fibroblasts were frozen at −80 °C.

2.3. Sudan III staining

Liver samples preserved in 4% phosphate-buffered formaldehyde solution were frozen, cut into 10-μm sections, and stained by the Sudan III method for fat staining.

2.4. Postmortem blood acylcarnitine analysis by tandem mass spectrometry

Whole blood samples obtained at autopsy were blotted onto one spot on Guthrie cards. They were subjected to acylcarnitine analysis by tandem mass spectrometry and compared with the previously determined normal range [6].

Table 1
SUDI cases.

Case no.	Age/sex	Height/weight (cm/kg)	Circumstances	Fever	Remarks
1	4 mo/M	68/7.5	Sleeping	−	
2	10 mo/F	70/8.8	Sleeping	−	Sister: undiagnosed encephalitis
3	10 mo/F	71/7.7	Sleeping	+	Cesarean section
4	9 mo/M	67/7.5	Sleeping	−	
5	4 mo/M	60/5.7	Sleeping	−	Hydrocephalia
6	6 mo/M	68/8.0	Sleeping	−	
7	1 mo/F	51/3.6	Sleeping	−	Twins, preterm birth
8	10 mo/M	72/9.9	Sleeping	−	Developmental disease (right side of the body paralysis)
9	6 mo/F	64/8.9	Sleeping	−	Bronchitis
10	4 mo/M	65/7.4	Sleeping	−	Cesarean section
11	1 mo/M	58/4.8	Sleeping	−	
12	5 mo/M	59/4.2	Sleeping	−	Preterm birth
13	2 mo/F	53/3.9	Sleeping	−	Low-birth-weight infant

Abbreviations: F, female; M, male; mo, month; SUDI, sudden unexpected death in infancy.

2.5. Enzyme analysis

The activity of mitochondrial respiratory chain complexes I, II, III, and IV was assayed in the crude post-600-g supernatant of heart and liver, and in isolated mitochondria from skin fibroblasts as described previously [10]. The activity of each complex was presented as a percent ratio relative to the mean value [9]. The activity of complexes I, II, III, and IV was also calculated as the percent relative to citrate synthetase (CS), a mitochondrial enzyme marker or complex II activity [10].

2.6. Ethics

This study was approved by the Ethics Committee of the Osaka University Graduate School of Medicine.

3. Results

3.1. Microscopic examination

One of the common features in diagnosing MRC disorders is hepatic steatosis. We therefore performed Sudan III staining to examine whether vacuoles caused by fatty degeneration were present in hepatocytes. Diffuse microvesicular steatosis was detected in case 5 (Fig. 1A). No Sudan III-positive vacuole was detected in case 13 (Fig. 1B) and the other cases, for example, case 2 (Fig. 1C).

3.2. Postmortem blood acylcarnitine analysis

We performed acylcarnitine analysis by tandem mass spectrometry using whole blood samples. In all samples, data were within the normal range. These data suggested that no case was affected by FAODs (data not shown).

3.3. Enzyme analysis of MRC complexes in heart, liver, and cultured fibroblasts

The enzyme activity of each complex was compared with the CS ratio and complex II ratio. Lower than 20% activity of any complex in a tissue or lower than 30% activity of any complex in a cell line meets the major criteria. Lower than 30% activity of any complex in a tissue or lower than 40% activity of any complex in a cell line meets the minor criteria according to Bernier et al. [11].

In heart, 11 cases of complex I activity met the major criteria of MRC disorders and one case of complex I activity met the minor criteria (Fig. 2A). In liver, three cases of complex I activity met the major criteria of MRC disorders and four cases of complex I activity met the minor criteria (Fig. 2B). In cultured fibroblasts, one case (case 5) of complex I activity met the major criteria of MRC disorders and one case (case 13) of complex I activity met the minor criteria (Fig. 2C, Table 2). The activity of complexes II, III, and IV was maintained in almost all cases.

3.4. Diagnosis

A definite diagnosis is defined as the identification of either two major criteria or one major plus two minor criteria. A probable diagnosis is defined as either one major plus one minor criterion or at least three minor criteria. A possible diagnosis is defined as either a single major criterion or two minor criteria, one of which must be clinical [11].

All the cases had a clinical symptom of sudden death, meeting one minor criterion. In the enzyme activity, eleven cases (cases 2, 4–13) met the major criteria and we could make a probable diagnosis in these 11 cases. The other two cases (cases 1 and 3) met the minor criteria and we could make a possible diagnosis.

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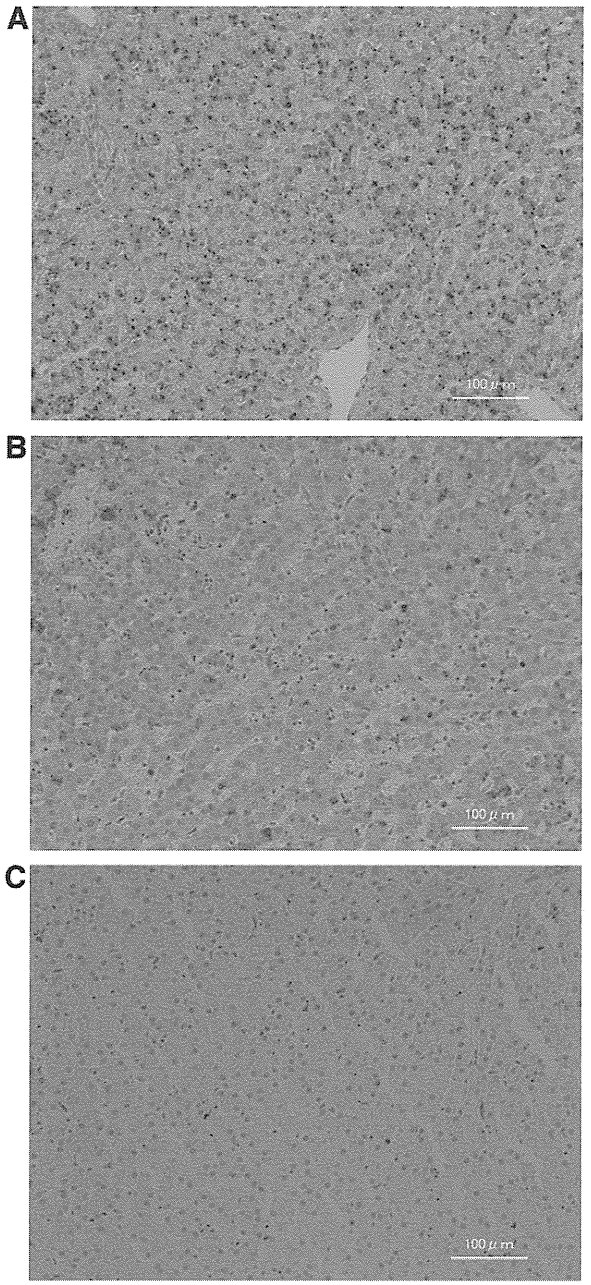

Fig. 1. Microscopic examination of liver (Sudan III staining): (A) case 5, (B) case 13, and (C) case 2. Diffuse microvesicular steatosis was detected in case 5 (A). No Sudan III-positive vacuole was detected in case 13 (B) and the other cases, for example, case 2 (C).

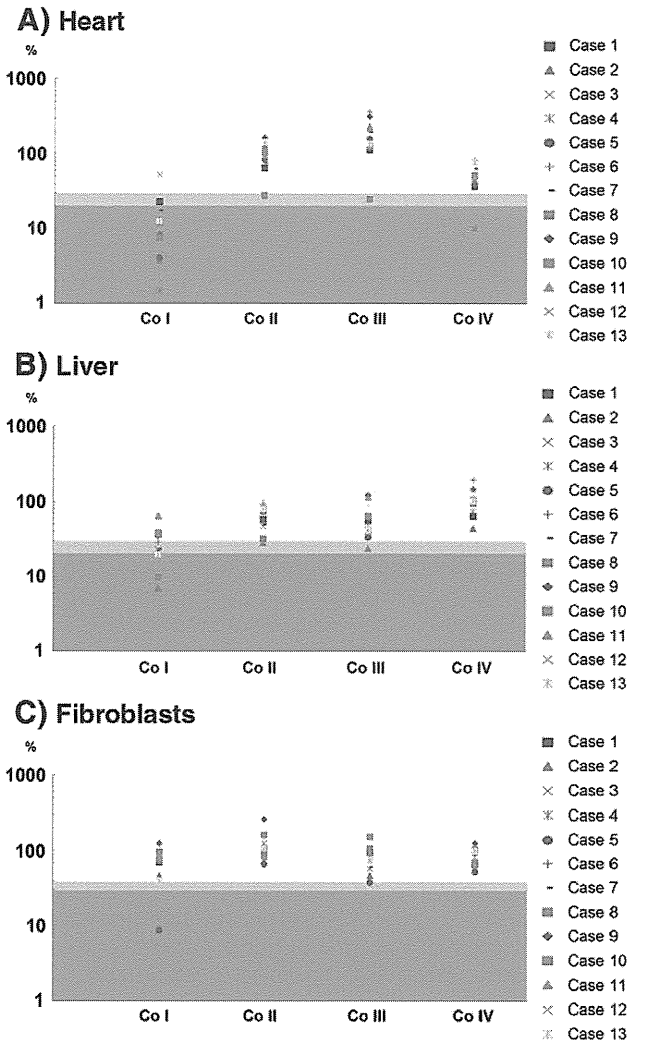

Fig. 2. Enzyme activity of MRC complexes in heart (A), liver (B), and cultured fibroblasts (C). In heart, 11 cases of complex I activity were under 20% of the CS ratio, meeting the major criteria and one case of complex I activity was under 30% of the CS ratio, meeting the minor criteria (A). In liver, three cases of complex I activity were under 20% of the CS ratio, meeting the major criteria and four cases of complex I activity were under 30% of the CS ratio, meeting the minor criteria (B). In cultured fibroblasts, one case (case 5) of complex I activity was under 30% of the CS ratio, meeting the major criteria and one case (case 13) of complex I activity was under 40% of the CS ratio, meeting the minor criteria (C). The activity of complexes II, III, and IV was maintained in almost all cases. The enzyme activity of each complex was compared with the CS ratio. Lower than 20% activity in a tissue or lower than 30% activity in a cell line (dark blue) meets the major criteria. Lower than 30% activity in a tissue or lower than 40% activity in a cell line (light blue) meets the minor criteria. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Mitochondria are essential organelles that exist in all nucleated mammalian cells. They provide the energy required for normal cell function through oxidative phosphorylation (OXPHOS). OXPHOS includes MRC complexes (complexes I, II, III, and IV) and ATP synthase (complex V) [12], which use reduced coenzymes from the tricarboxylic acid cycle and molecular oxygen, generating cellular energy in the form of ATP [13].

The infantile or early neonatal period demands high energy. Patients with MRC disorders are unable to produce adequate energy, which may thus compromise them in the first days of life or during infancy. MRC disorders affect most organ systems and present variable clinical manifestations from prenatal complications through acute neonatal decompensation and death to adult-onset disorders. Therefore, it is not surprising that MRC disorders are also one of the causes of SUDI. However, there are few reports on a relationship between MRC disorders and SUDI [12,14].

We have previously reviewed SUDI cases with respect to FAODs and found a case of carnitine palmitoyltransferase II deficiency [6]. In that study, we advocated the importance of metabolic autopsy [15], including fat staining of liver, postmortem acylcarnitine analysis, and genetic analysis. Using this protocol, most FAODs, some amino acid oxidation disorders, and some organic acid oxidation disorders could be diagnosed.

However, MRC disorders are difficult to diagnose. First, they present variable clinical manifestations and non-specific features such as failure to thrive or hepatic, cardiac, renal, gastrointestinal, endocrine, hematological, or other symptoms [10,16]. Second, although blood

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Table 2
Enzyme assay of mitochondrial respiratory chain complexes in cultured fibroblasts.

	Enzyme activity (%)[a]			
	Co I	Co II	Co III	Co IV
Case 5				
CS ratio	9	66	38	53
Co II ratio	13	–	71	58
Case 13				
CS ratio	39	106	76	98
Co II ratio	37	–	73	92

Abbreviations: Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthetase.
[a] Relative to mean CS and Co II of the normal controls.

lactate levels and muscle morphology can be used as a screening test, some confirmed patients were normal [10]. Third, genomic mutational analysis is difficult because MRC complexes are composed of 13 subunits encoded by mitochondrial DNA and over 70 subunits encoded by nuclear genes. In addition, nuclear genes are related to many assembly factors, membrane dynamics, nucleotide transport synthesis, and mitochondrial DNA replication and expression. Therefore, enzyme analysis still remains the most significant diagnostic tool. A definite diagnosis thus requires enzyme analysis [8].

In the present study, we performed enzyme analysis in frozen heart, frozen liver, and cultured fibroblasts. Eleven cases were supposed to be a probable diagnosis and two cases were supposed to be a possible diagnosis. However, it seemed unlikely that such a high proportion would have real MRC disorders. Did we have to take the effect of postmortem changes into consideration?

For forensic autopsy, organ specimens are often preserved in formaldehyde solution and sometimes frozen. These specimens are susceptible to postmortem changes and, therefore, correct enzyme analysis is hard to be performed. Based on the previous report that artifactual loss of complex II activity in autopsy samples preceded that of complex I and the data that complex II activity in the present study was maintained, this low complex I activity might be decreased before death. However, postmortem changes cannot be completely ruled out and this low complex I activity may not therefore be consistent with premortem activity.

We therefore analyzed activity in cultured fibroblasts. Cultured fibroblasts are not affected by postmortem changes and, therefore, reflect premortem information more accurately. In cultured fibroblasts, one case (case 5) of complex I activity met the major criteria and one case (case 13) of complex I activity met the minor criteria. In case 5, complex I activity was distinctively decreased. Sudan III staining of the case revealed hepatic steatosis, consistent with Reye-like syndrome. Reye-like syndrome is one of the characteristic features of MRC disorders [9]. We could therefore make a probable diagnosis (case 5) and a possible diagnosis (case 13) from metabolic autopsy with postmortem cultured fibroblasts.

Case 5 had hydrocephalia and case 13 was a low-birth-weight infant. However, neither was severe. Macroscopic examination did not reveal any abnormal appearance and microscopic examination showed no pathological findings except for steatosis. These cases might not have been identified without postmortem cultured fibroblasts. As with such cases, some MRC disorders reveal no clinical manifestation and no pathological characteristic. We believe it is important to perform metabolic autopsy with postmortem cultured fibroblasts when encountering SUDI cases.

We emphasized the advantage of metabolic autopsy with cultured fibroblasts. First, despite lacking obvious preceding symptoms, MRC disorders could be diagnosed. Second, cultured cells are the only method to retrieve premortem information from the deceased. Third, even frozen samples are affected by postmortem changes and may lead to a false positive diagnosis. However, we have to discuss the disadvantage. MRC disorders showed tissue specificity and the activity of cultured fibroblasts represent normal in some cases. Some of the low complex I activity in heart or liver could represent premortem MRC disorders despite normal activity in cultured fibroblasts. Thus, other molecular investigations may well be added to enzyme analysis. Recently, systematic gene analysis using next-generation sequencing has been reported for the diagnosis of patients with MRC disorders [17]. Further investigations are thus needed.

In conclusion, we detected one probable case and one possible case of MRC disorders among 13 Japanese cases of SUDI. MRC disorders are one of the important inherited metabolic disorders causing SUDI. We advocate metabolic autopsy with postmortem cultured fibroblasts in SUDI cases.

Acknowledgments

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Review

Molecular pathology of MELAS and L-arginine effects☆,☆☆

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ABSTRACT

Background: The pathogenic mechanism of stroke-like episodes seen in mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) has not been clarified yet. About 80% of MELAS patients have an A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene, which is the base change at position 14 in the consensus structure of $tRNA^{Leu(UUR)}$ gene.
Scope of review: This review aims to give an overview on the actual knowledge about the pathogenic mechanism of mitochondrial cytopathy at the molecular levels, the possible pathogenic mechanism of mitochondrial angiopathy to cause stroke-like episodes at the clinical and pathophysiological levels, and the proposed site of action of L-arginine therapy on MELAS.
Major conclusions: Molecular pathogenesis is mainly demonstrated using ρ^0 cybrid system. The mutation creates the protein synthesis defects caused by 1) decreased life span of steady state amount of $tRNA^{Leu(UUR)}$ molecules; 2) decreased ratio of aminoacyl-$tRNA^{Leu(UUR)}$ versus uncharged $tRNA^{Leu(UUR)}$ molecules; 3) the accumulation of aminoacylation with leucine without any misacylation; 4) accumulation of processing intermediates such as RNA 19, 5) wobble modification defects. All of these loss of function abnormalities are created by the threshold effects of cell or organ to the mitochondrial energy requirement when they establish the phenotype. Mitochondrial angiopathy demonstrated by muscle or brain pathology, as SSV (SDH strongly stained vessels), and by vascular physiology using FMD (flow mediated dilation). MELAS patients show decreased capacity of NO dependent vasodilation because of the low plasma levels of L-arginine and/or of respiratory chain dysfunction. Although the underlying mechanisms are not completely understood in stroke-like episodes in MELAS, L-arginine therapy improved endothelial dysfunction.
General significance: Though the molecular pathogenesis of an A3243G or T3271C mutation of mitochondrial $tRNA^{Leu(UUR)}$ gene has been clarified as a mitochondrial cytopathy, the underlying mechanisms of stroke-like episodes in MELAS are not completely understood. At this point, L-arginine therapy showed promise in treating of the stroke-like episodes in MELAS. This article is part of a Special Issue entitled Biochemistry of Mitochondria.

1. Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (OMIM 540000), characterized by an early onset of stroke-like episodes, was first described by Pavlakis and colleagues in 1984 [1]. At least 39 distinct mitochondrial DNA mutations have been associated with MELAS [2], about 80% of MELAS patients have an A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene (OMIM 590050) [3–5]. Although more than 25 years have passed since MELAS was first defined clinically and pathologically, the pathogenesis of the stroke-like episodes is still uncertain. Mitochondrial angiopathy with degenerative changes in small arteries and arterioles, which has been reported in many MELAS patients [6,7], is suggested by the observation of strong succinate dehydrogenase activity in the wall of blood vessels (SSVs) [8]. In spite of the fact that many therapeutic trials have been conducted to cure mitochondrial disorders, no trial has been successful, though several clinical trials are still on-going. Based on the hypothesis that stroke-like episodes in MELAS are caused by segmental impairment of vasodilatation in intracerebral arteries, we use L-arginine in MELAS patients during the acute phase to cure the symptoms or to

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decrease the frequency and/or the severity of the stroke-like episodes [9,10,11]. This review aims to give an overview on the actual knowledge about the pathogenic mechanism of mitochondrial cytopathy at the molecular levels, the possible pathogenic mechanism of mitochondrial angiopathy to cause stroke-like episodes in the clinical and pathophysiological levels, and the proposed site of action of L-arginine therapy on MELAS.

2. Molecular pathophysiology of mitochondrial cytopathy in MELAS

2.1. Characteristics of $tRNA^{Leu(UUR)}$ gene and structure stabilization of mutant

A point mutation in the structural gene for a tRNA may be expected to result in a deficiency in translation. However, inhibition of translation due to a mutated tRNA gene may occur at several levels. The base change at position 14 in the consensus structure of $tRNA^{Leu(UUR)}$ is an invariant A in bacterial and cytosolic eukaryotic tRNAs and is typically involved in the tertiary folding of classical tRNAs (Fig. 1A) [12]. Because of above reason, the A3243G mutation is primarily thought to disrupt the tertiary interaction between the highly conserved np A14 (>90% for adenine) and U8, a binding that stabilizes the L-shaped tertiary fold [13,14], which results in partially folded tRNA transcripts into the L-shaped structure with an acceptor branch but with a floppy anticodon branch [15]. The mutant tRNA is able to adapt to the synthetase, but results in incorrect tRNA processing and enzyme maturation and accordingly defects in a variety of biochemical pathways. The mutation may directly affect the mitochondrial tRNA function in translation, such as structure stabilization, methylation, amino-acylation, and codon recognition, or alternatively, may affect recognition of the tRNA by an enzyme not directly involved in translation, such as the enzymes which process the large polycistronic transcripts of the mtDNA.

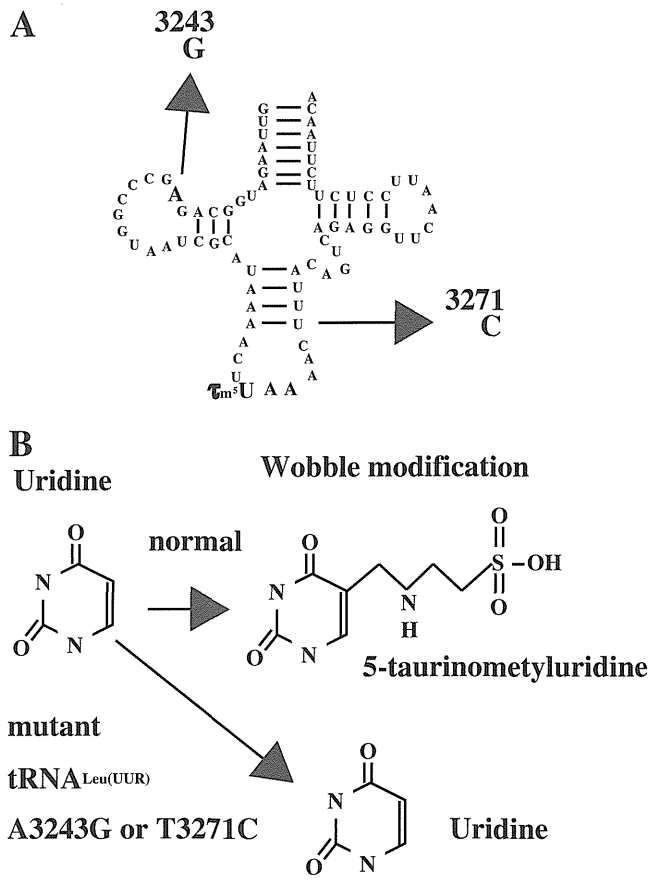

Fig. 1. $tRNA^{Leu(UUR)}$ structure and wobble modification. $tRNA^{Leu(UUR)}$ structure. An A to G change at position 14 in the consensus structure of $tRNA^{Leu(UUR)}$, which is thought to disrupt the tertiary folding of classical tRNAs [12], results in partially folded tRNA transcripts into the L-shaped structure with an acceptor branch but with a floppy anticodon branch [14,15]. B. Wobble modification The wild-type $tRNA^{Leu(UUR)}$ contains an unknown modified uridine at the wobble position and that this modification occurs at the uracil base [35], however its modification is absent in the $tRNA^{Leu(UUR)}$ with a mutation at either np A3243G or T3271C. The wobble modified uridine in the wild-type $tRNA^{Leu(UUR)}$ is 5-taurinomethyluridine (sm5U). The U on the bold indicates the unmodified uridine present in the mutant tRNAs.

2.2. ρ^0 cybrid system in MELAS

King et al. developed the technologies whereby the mitochondria from cells derived from patients are transferred to a cell line lacking mtDNA (so called ρ^0 cybrid system), which allowed to conduct the study of the genotype-phenotype relationships in mitochondrial function [16,17]. In this manner, it is possible to create trans-mitochondrial cell lines containing different proportions of mutated mtDNA from 0% to 100%, and to study the effects of a given mutant load on the activity of respiratory chain complexes, mitochondrial respiration and cell growth, as well as mitochondrial tRNA stability, methylation, aminoacylation, codon recognition and threshold effects. First application of this technique to an A3243G mutation related to molecular basis of MELAS, has been reported by Chomyn et al. [18], and King et al. [19] independently. Mutant transformants showed protein synthesis defects clearly, and demonstrated that there was the direct evidence between single nucleotide change at 14th position of an A to G transition in the mitochondrial $tRNA^{Leu(UUR)}$ gene and mitochondrial dysfunction. However, the reduction in labeling of the various mitochondrial translation products in mutant was not correlated with their UUR-encoded leucine content. King also reported the similar effects in transformants having a T3271C mutation [19]. This ρ^0 cybrid system becomes the orthodox and powerful tool when one evaluates the pathogenicity of any nucleotide changes in the mitochondrial DNA.

2.3. Transcription termination of mitochondrial RNAs in MELAS

The mammalian mitochondrial tRNAs are transcribed as part of larger polycistronic RNAs, in which the tRNA sequences are contiguous or nearly contiguous to the rRNA sequences and the protein-coding sequences (Fig. 2). The ribosomal gene region appears to be transcribed 50–100 times more frequently than the other H-strand genes [20]. In these polycistronic molecules, the tRNA structures are believed to act as recognition signals for the processing enzymes which make precise endonucleolytic cleavages at the 5' and 3' ends of the tRNA sequences in the primary transcripts, yielding the mature rRNAs, mRNAs, and tRNAs [21]. The ribosomal DNA transcription unit, one of three polycistronic transcription units of human mtDNA, terminates at the 3'-end of the 16S rRNA gene just before the $tRNA^{Leu(UUR)}$ gene. This transcript, corresponding to the ribosomal genes, is processed to yield the mature rRNAs and, due to its very high rate of synthesis, is responsible for the bulk of the rRNA formation [22]. Transcription termination is mediated by a protein factor (mTERF: mitochondrial termination factor) which specifically binds within the $tRNA^{Leu(UUR)}$ gene, and which promotes termination of transcription (Fig. 3A) [22,23]. Since this mutation is located exactly in the middle of termination protein binding domain, the A3243G mutation in the $tRNA^{Leu(UUR)}$ gene has been shown in vitro to impair the binding of this protein factor and to affect the efficiency of transcription termination at the end of the 16S rRNA gene [23]. However, in vivo analysis using ρ^0 cybrid system provided no evidence to support above data. There were no alterations of size of the $tRNA^{Leu(UUR)}$ or of the immediately downstream-encoded ND1 mRNA or of the 16S rRNA, as detectable by changes in their electrophoretic mobility [18]. The steady-state amounts of mitochondrial rRNAs, mRNAs, and $tRNA^{Leu(UUR)}$ are not significantly affected by the MELAS mutation in ρ^0 cybrid system. The discrepancy of the data described above may be explained by the possibility that the

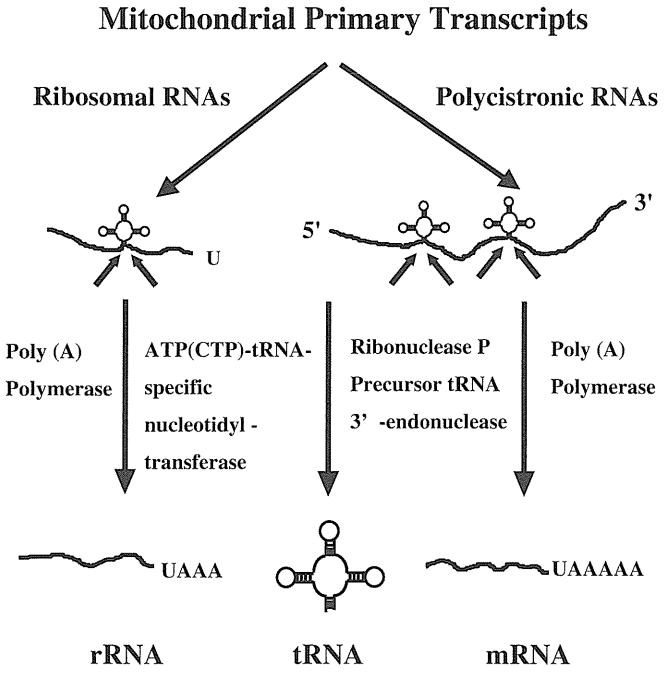

Fig. 2. Mammalian mitochondrial transcription system. The human mitochondrial RNAs are transcribed as a larger polycistronic RNAs, in which the tRNA sequences are contiguous or nearly contiguous to the rRNA sequences and the protein-coding sequences.

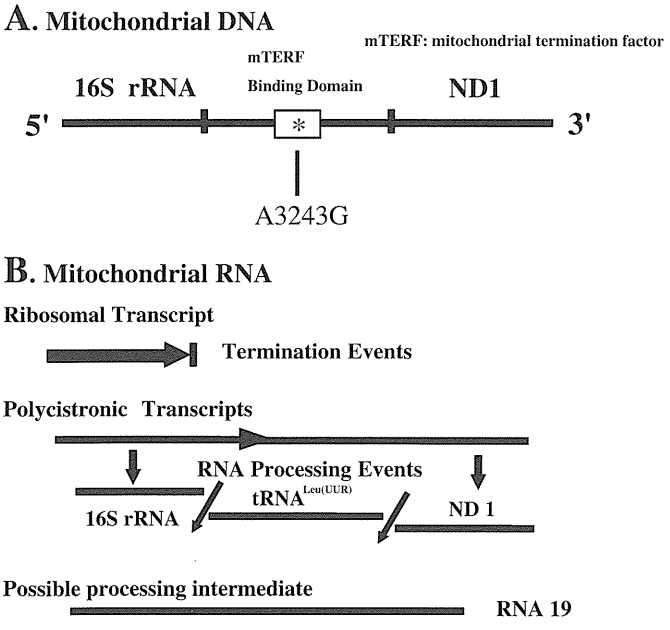

Fig. 3. Post-transcriptional modification. A. Transcription termination. The ribosomal gene region appears to be transcribed 50–100 times more frequently than the other H-strand genes [20]. Transcription termination is mediated by a protein factor (mTERF: mitochondrial termination factor) which specifically binds within the $tRNA^{Leu(UUR)}$ gene, and which promotes termination of ribosomal transcription. B. Post-transcriptional modification and RNA 19 The increase of RNA 19, corresponding to the 16S rRNA + $tRNA^{Leu(UUR)}$ + ND 1 genes, found in mutant $tRNA^{Leu(UUR)}$ cybrids clearly demonstrate that RNA processing is not occurring in mutant cybrids as efficiently as in wild-type cybrids [19]. RNA 19 is also accumulated in muscle specimens from 8 MELAS patients [26]. The proportion of mutated RNA in RNA 19 fraction is always higher than those in the percentage of mutation in mitochondrial DNA, suggesting that the A3243G mutation exhibited dominant negative effects on the mitochondrial RNA processing events, resulting in the accumulation of RNA 19 transcripts in these patients [28–30].

reduction in affinity of mTERF for the mutated target sequence is compensated by hyper-expression of the protein. Anyway, using genetic, biochemical, and morphological techniques, it was found that the mutant, but not wild-type cybrids, displayed quantitative deficiencies in cell growth, protein synthesis, and respiratory chain activity [19].

2.4. Processing of polycistronic transcripts in MELAS

It was found that there was an accumulation of a previously unidentified RNA transcript in mutant cybrids (A3243G or T3271C), designated as RNA 19, corresponds to the 16S rRNA + $tRNA^{Leu(UUR)}$ + ND1 genes, which are contiguous in the mtDNA (Fig. 3B) [19]. The ratios of mtDNA-encoded rRNAs to mRNAs were not found to be altered in these in vitro experiments. In order to analyze whether the MELAS mutation is associated with errors in transcription termination and processing of the polycistronic transcripts in the region of the mutation, it was performed fine mapping of the mature transcripts derived from the 16S rRNA, $tRNA^{Leu(UUR)}$, and ND 1 genes in both wild-type and mutant cybrids. It was also analyzed the steady-state levels of $tRNA^{Leu(UUR)}$ by high-resolution RNA transfer hybridizations. It was found that mutation has no effect in vivo on the accuracy of transcription termination at the end of the ribosomal RNA genes, on the precise endonucleolytic cleavage of the polycistronic RNA at $tRNA^{Leu(UUR)}$, or on the post transcriptional addition of -CCA at the 3′ end of $tRNA^{Leu(UUR)}$ [24]. On the other hand, the experiments using plasmids carrying $tRNA^{Leu(UUR)}$ inserts (wild type, as well as A3243G) which designated to evaluate the endonucleolytic 3′-end processing and CCA addition at the tRNA 3′ terminus, showed that A3243G mutation reduced 2.2 fold of the efficiency of 3′-end cleavage, and almost has no abnormal effects on CCA addition [25].

2.5. Accumulation of RNA 19 in MELAS cybrids and organs from patients

The increased amounts of the transcript corresponding to the 16S rRNA + $tRNA^{Leu(UUR)}$ + ND 1 genes, designated as RNA 19, found in mutant $tRNA^{Leu(UUR)}$ cybrids clearly demonstrate that RNA processing is not occurring in mutant cybrids (A3243G or T3271C) as efficiently as in wild-type cybrids [19]. It was demonstrated that RNA 19 is accumulated in muscle specimens from 8 MELAS patients who have a heterogeneous percentage of mutation (58% to 99%) in the A3243G of $tRNA^{Leu(UUR)}$ gene [26]. An increase in the levels of RNA 19 was observed in nearly all tissues examined from these patients, which do not provide evidence for tissue-specific differences in mitochondrial RNA processing. The elevation of steady-state levels of RNA 19 have also reported in skeletal muscle and fibroblasts of a patient with mitochondrial myopathy and a complex I deficiency who harbored an A to G transition in $tRNA^{Leu(UUR)}$ gene at position 3302 [27]. Thus, altered RNA processing may be associated with other point mutations in $tRNA^{Leu(UUR)}$ gene associated with MELAS. It also analyzed a mutated proportion of RNA 19 in an RNA fraction obtained from sampled skeletal muscles from 6 unrelated patients with MELAS. The proportion of mutated RNA in RNA 19 fraction exceeded 95% in all patients, although the percentage of mutation in mitochondrial DNA ranged from 54 to 92, suggesting that the A3243G mutation exhibited dominant negative effects on the mitochondrial RNA processing events, resulting in the accumulation of RNA 19 transcripts in these patients [28–30]. The protein synthesis defect has been proposed to be due to stalling of translation by pseudoribosomes that have incorporated RNA 19, an incompletely processed transcript reported to accumulate in A3243G, T3271C and A3302G mutant cells, in place of 16 S rRNA, or possibly to defective posttranscriptional modification of the $tRNA^{Leu(UUR)}$ (Fig. 4) [31]. Though the reason why RNA 19 was elevated in patients who have the point mutation of $tRNA^{Leu(UUR)}$ gene is unknown, we believe that elevated levels of RNA 19 may play an important role in the pathogenesis of this disorder.