mental disorders, and diabetes or other metabolic diseases of unknown cause, we plan to conduct research based on the assumption that such cases include those caused by abnormalities in genes identified in MRCD patients.

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Patient Report

Case of an infant with hepatic cirrhosis caused by mitochondrial respiratory chain disorder

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Abstract The patient had hepatomegaly with liver dysfunction at the age of 1 month. Magnetic resonance imaging performed at the age of 1 year showed multiple nodules of varying size in his liver. We were able to examine the mitochondrial respiratory chain function in the liver biopsy samples because all other differential diagnoses for hepatic cirrhosis had been ruled out. Complex I and IV activities were below the normal level (<30%) of the citrate synthase (CS) ratio. Liver blue native polyacrylamide gel electrophoresis showed an extremely weak complex I and IV band. Liver respiratory chain complexes I and IV were found to be deficient in this patient. The histologic findings were highly suggestive of mitochondrial respiratory chain disorder. Findings of progressive liver cirrhosis changes were observed in magnetic resonance imaging at the age of 5 years. An examination of the mitochondrial respiratory chain function should be performed along with a liver biopsy if mitochondrial respiratory chain disorder is suspected as a possible differential diagnosis of idiopathic hepatitis.

Key words chronic hepatitis, infant, liver cirrhosis, mitochondrial respiratory chain complex I and IV deficiency, mitochondrial respiratory chain disorder.

Mitochondrial respiratory chain disorder (MRCD), which is caused by the loss of one or more enzyme activities in respiratory chain complexes I–IV, has many clinical manifestations in various organs and is a known cause of mitochondrial encephalomyopathy, idiopathic hepatitis and idiopathic muscle weakness. Although MRCD is one of the differential diagnoses for hepatic disorder, it is not actively diagnosed. The early diagnosis of MRCD in the liver is important because some patients will subsequently develop liver cirrhosis or liver failure.[1,2] This report is based on a boy with chronic hepatic disorder and cirrhosis who was found to have mitochondrial respiratory chain complex I and IV deficiencies during his infant period.

Case Report

The patient was a Japanese boy born at term and weighing 3296 g; he was the second child of healthy parents with consanguinity. His elder sister (3 years old) is presently in good health. The mother's brother (31 years old) was found to have hepatic dysfunction during his infant period and his condition progressed to cirrhosis during adulthood. The proband's weight gain after birth was good. Jaundice and hepatomegaly were observed at the age of 1 month and he was admitted to our hospital. Upon admission (32 days after birth), he exhibited conjunctival icterus, his liver was palpable 5 cm below the right costal margin, he had normal muscle tone and no external malformations were noted. His laboratory data on admission showed cholestatic hepatitis. Tandem mass spectrometry, urine organic acid and bile acid analysis were normal. The following differential diagnoses were ruled out: autoimmune disease, infectious disease, disorder of organic acid metabolism and fatty acid oxidation, alpha 1-antitrypsin deficiency, tyrosinemia, galactosemia, and citrin deficiency. Furthermore, respiratory disorder, abnormal findings on skin or bone, and susceptibility to infection, which are the main symptoms of Langerhans cell histiocytosis and cystic fibrosis, are not present in this patient at the current age of 6 years. Imaging studies did not reveal any congenital portal venous or portal biliary tract malformations. The patient's transaminase (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]) levels were 78–477 IU/l (AST) and 13–181 IU/l (ALT) and fluctuated with his physical condition. The patient's γ-GTP levels decreased to a normal range before the age of 6 months. Throughout the clinical course, the patient's blood lactate and pyruvic acid levels were almost always normal. Hypoglycemia was not observed during follow-up examinations. He exhibited normal growth and development. An abdominal magnetic resonance imaging (MRI) examination performed at the age of 2 months was normal except for hepatomegaly. However, an abdominal MRI performed at 1 year and 4 months showed multiple nodules of varying size in his liver, which appeared

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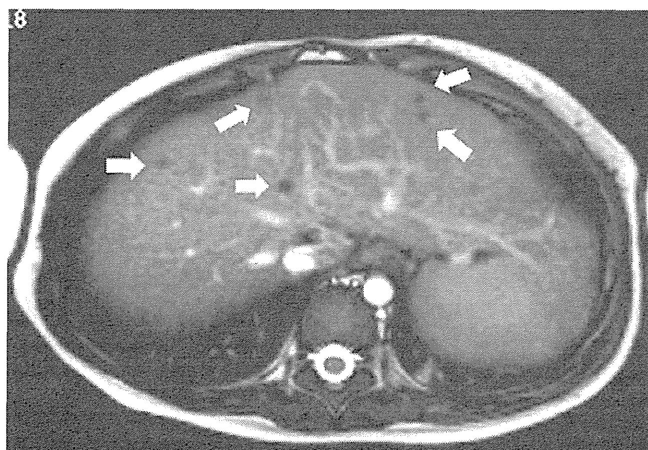

Fig. 1 Abdominal magnetic resonance image obtained at 1 year and 4 months shows multiple nodules (arrows) varying in size in the liver, which presented as a low-intensity area on T2-weighted imaging.

as low-intensity areas on T2-weighted images (see Fig. 1) and high-intensity areas on T1-weighted images without contrast enhancement. The number of nodules in his liver increased from the time of the MRI examination performed at the age of 1 year and 4 months. In addition, a transient elevation in the patient's serum ammonia levels (290 μg/dL) and impaired consciousness with the onset of fever and a poor appetite were observed at the age of 2 years. The clinical course during this episode showed positive results. Liver biopsies were performed during a laparotomy to inspect the progress of the liver cirrhosis at the age of 2 years and 1 month. He was suspected of having MRCD, which is one of the main causes of hepatic disorder, because all other differential diagnoses for hepatic cirrhosis had been ruled out. Thus, we were able to examine the mitochondrial respiratory chain function in the liver biopsy samples. Liver respiratory chain complexes I and IV were found to be deficient in this patient using both a respiratory chain enzyme assay (Table 1) and a liver blue native polyacrylamide gel electrophoresis (BN-PAGE).[3] Complex I and IV activities were below the normal level (<30%)[4] of the CS ratio. Liver BN-PAGE showed an extremely weak complex I and IV band in this patient. In addition, the rate of mtDNA and nDNA (quantitative polymerase chain reaction) was about 95.4% (normal level). Mitochondrial DNA depletion syndrome was ruled out. The macroscopic anatomy showed diffuse nodules on the surface of the liver. The microscopic findings for the liver are shown in Figure 2. Coenzyme Q, vitamin C, vitamin E, and carnitine therapy were initiated at an age of 2 years and 3

Table 1 Respiratory chain enzyme assay in the liver of the patient

	Complex I	Complex II	Complex III	Complex IV	CS
% of normal	14	37	62	15	54
CS ratio (%)	26	67	111	27	
Complex II ratio (%)	38		165	40	

months. The patient continues to exhibit normal physical and mental development after diagnosis. His weight was 21.9 kg (+0.3SD score) and height was 117.5 cm (+0.6SD score) at the age of 6 years. However, the patient's transaminase levels were 56–311 IU/L (AST) and 31–174 IU/L (ALT), and findings of the follow-up MRI at the age of 5 years suggested progressive changes in liver cirrhosis. MRI demonstrated right lobe atrophy, enlargement of the left lobe, and an irregular edge border of the liver (Fig. 3a,b). MRI revealed a well-circumscribed mass 16 × 11-mm (see arrow) in liver segment VI (Fig. 3c). In addition to this mass, MRI demonstrated nodules 4–8 mm in size in the liver parenchyma, which were visualized as slightly hyperintense lesions on the T2-weighted images and as hypointensities on the T1-weighted images (Fig. 3c).

Discussion

We reported a boy with chronic hepatic disorder and cirrhosis who was found to have mitochondrial respiratory chain complex I and IV deficiency during his infant period. In our experience, deficiencies of complexes I and IV account for about 15% of all diagnosed cases of MRCD in Japanese subjects. Nine clinical case reports of complex I and IV deficiencies, including adult subjects, were reported between 1988 and 2010. No specific manifestations of complex I and IV deficiency were observed in the past reports. The patient's blood lactate/pyruvate rate was almost normal in the clinical course. However, a normal lactate level does not exclude respiratory chain defects in MRCD, including mitochondrial hepatopathy.[5,6] The molecular and genetic causes of complex I and IV deficiency are not clear.

The histological findings of liver biopsy specimens from patients with primary mitochondrial hepatopathies reveal individual, non-specific histologic and ultrastructural findings, with predominant microvesicular steatosis and canalicular cholestasis.[7] Periportal and centrilobular fibrosis are characteristic features, and the dropout of broad bands of hepatocytes leads to micronodular cirrhosis.[7] Thus, the histologic findings of this case were highly suggestive of MRCD. In addition, if electron micrographs revealed morphological abnormality of mitochondria in liver biopsies, they would have been useful for confirming diagnosis of MRCD.

With respect to MRI findings, nodules which were found at the age of 1 year were not detected at the age of 5 years. The nodules in Figure 1 might be regenerative nodules (RN) associated with hepatic cirrhosis, because RN typically appear as hypointense lesions on T2-weighted images[8] and the imaging findings at the age of 5 years were typical of hepatic cirrhosis. Furthermore, focal nodular hyperplasia, which is one of the important differential diagnoses of hepatic nodules in infants, was excluded on the basis of the high signal intensity in the non-enhanced T2-weighted images.[9] However, these nodules were so small that they were difficult to evaluate by MRI or histopathology.

Findings of progressive liver cirrhosis changes were observed in a liver MRI at the age of 5 years (Fig. 3a–c). The 16 × 11-mm mass in Figure 3c (see arrow) was visualized as a hyperintensity on opposed-phase T1-weighted gradient-echo images and as a slightly low-intensity area on the T2-weighted images. Focal

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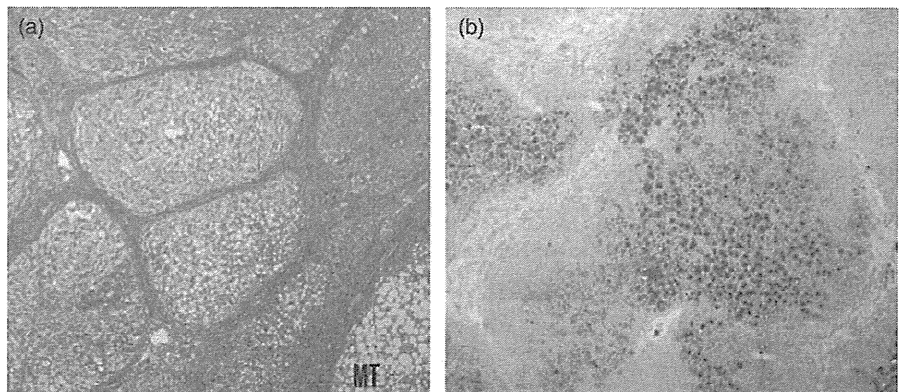

Fig. 2 (a) Masson trichrome stain: Microscopic findings in the liver show the division of a hepatic lobule into nodules by bridging fibrosis in the liver tissue. (b) Sudan III stain: Liver tissues show heterogeneous hepatic steatosis in each septum. Portal fibrosis was observed in liver tissues without inflammation (not shown).

nodular hyperplasia was excluded because the mass did not show the high-intensity on the non-enhanced T2-weighted images. The size remained unchanged as compared to the previous year. Thus, the mass was suspected to be a regenerative nodule or adenomatous hyperplasia, associated with hepatic cirrhosis. In addition to this mass, MRI in Figure 3c demonstrated nodules 4–8 mm in size in the liver parenchyma. Although these nodules were found to contain lipids inside, as they were visualized as low-intensity areas on opposed-phase T1-weighted gradient-echo images and as high-intensity areas on in-phase images, they were too small to evaluate in detail.

The early and accurate diagnosis of MRCD is important because appropriate therapy and guidance can be provided to the patient and his/her family before the condition worsens. MRCD is difficult to diagnose because the clinical manifestations do not depend on the type of complex deficiency. Some previous patients have died of hepatic failure during the neonatal period or infancy, while other patients never develop hepatic disease despite long-term follow-up observation.[1] However, it is conceivable that a regular screening for secondary liver cancer is necessary for the patient with progressive cirrhosis, along with MRCD during his infant period.[10]

With respect to diagnosis, regular ultrasound or CT examinations are needed for infants with idiopathic chronic hepatitis because multiple nodules in the liver gradually appeared in the patient. Furthermore, we conclude that an examination of the mitochondrial respiratory chain function should be performed along with a liver biopsy if MRCD is suspected as a possible differential diagnosis of idiopathic hepatitis under the signs of liver cirrhosis, as in this case.

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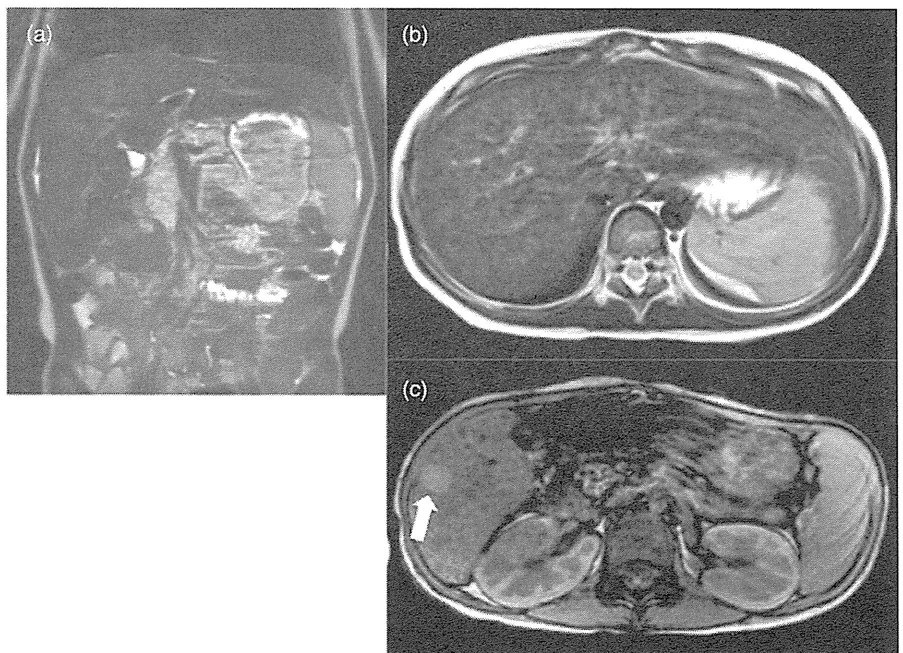

Fig. 3 (a) T2-weighted magnetic resonance image (MRI) demonstrates right lobe atrophy, enlargement of the left lobe, and an irregular edge border of the liver at the age of 5 years. (b) T2-weighted MRI shows marked hyperintensity in the periportal region. The hepatic parenchyma appears heterogeneously enhanced in the delayed phase. (c) T1-weighted MRI revealing a well-circumscribed 16 × 11-mm mass (see arrow) in liver segment VI. This mass is visualized as a hyperintensity on opposed-phase T1-weighted gradient-echo images and as a slightly lower-intensity area on the T2-weighted images. In addition to this mass, MRI demonstrates nodules 4–8 mm in size in the liver parenchyma, which are visualized as slightly hyperintense lesions on the T2-weighted images and as hypointensities on the T1-weighted images.

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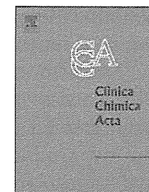

Oxysterol changes along with cholesterol and vitamin D changes in adult phenylketonuric patients diagnosed by newborn mass-screening

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ABSTRACT

Background: Phenylketonuria (PKU) possibly leads to hypocholesterolemia and lowered vitamin D (VD) status. Metabolism of oxysterols linking with those of cholesterol and VD has never been examined in PKU.
Methods: Blood oxysterols along with blood phenylalanine, lipids and VD were examined for 33 PKU adults aged 21–38 years and 20 age-matched healthy controls.
Results: Total- and low-density cholesterols, and 25-hydroxy VD_3 were decreased significantly in the PKU group (cholesterols, 10% decrease; 25-hydroxy VD_3 35% decrease vs. the control group). 24S-hydroxycholesterol (24S-OHC) eliminating brain cholesterol, and 27-OHC and 7α-hydroxycholesterol (7α-OHC) representing peripheral and hepatic cholesterol elimination, respectively, were significantly decreased in PKU group: 24S-OHC, 25% decrease, $p<.01$; 27-OHC and 7α-OHC, 35–40% decrease, $p<.001$. 7β-Hydroxycholesterol (7β-OHC) reflecting oxidative stress was increased significantly in PKU group ($p<.05$). 7α-OHC and 27-OHC levels in PKU group always showed similar values, regardless of other parameters while the 24S-OHC and 7β-OHC levels decreased and increased, respectively, showing significant correlations with phenylalanine level ($p<.005$). 27-OHC level showed a significant positive correlation with the 25-hydroxy VD_3 level in this group ($p<.001$).
Conclusion: Blood oxysterol changes predominate over blood cholesterol changes and influence on VD status in adult PKU patients.

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1. Introduction

Phenylketonuria (PKU: OMIM, 261600) is an autosomal recessive disorder caused by deficiency of hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1). Diverse unfavorable effects of hyperphenylalaninemia on the central nervous system in children have been well described [1–5]. Unless phenylalanine intake is restricted, they develop convulsions, developmental delay, and mental retardation. Additionally, evidence that high plasma phenylalanine concentrations possibly cause behavioral

Abbreviations: PKU, phenylketonuria; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; VD, vitamin D; PTH, parathyroid hormone; 7α-OHC, 7α-hydroxycholesterol; 7β-OHC, 7β-hydroxycholesterol; 24S-OHC, 24S-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol.

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abnormalities, cognitive impairment, and emotional disturbance in adults has been reported increasingly [6,7].

Aside from the clinical presentations, various metabolic changes in PKU, particularly changes related to neurological manifestations, have been studied [1–5]. Among them, the most frequently explored are the low productions of neurotransmitters such as serotonin, dopamine and catecholamine, together with disturbance in the transport systems [1–5]. In addition, several studies have demonstrated the possibility that enhanced oxidative stress is, in part, associated with neurological manifestations in PKU [8–12]. Probably, multiple metabolic factors are associated with the clinical presentations of the disease. Nevertheless, information related to the biochemistry in PKU remains insufficient.

Oxysterols are metabolites originated from cholesterol, and some oxysterols have been implicated in the pathophysiology of neurological disorders [13–18]. Oxysterols are also metabolically connected to vitamin D (VD) (Fig. 1). In PKU, cholesterol and VD productions have

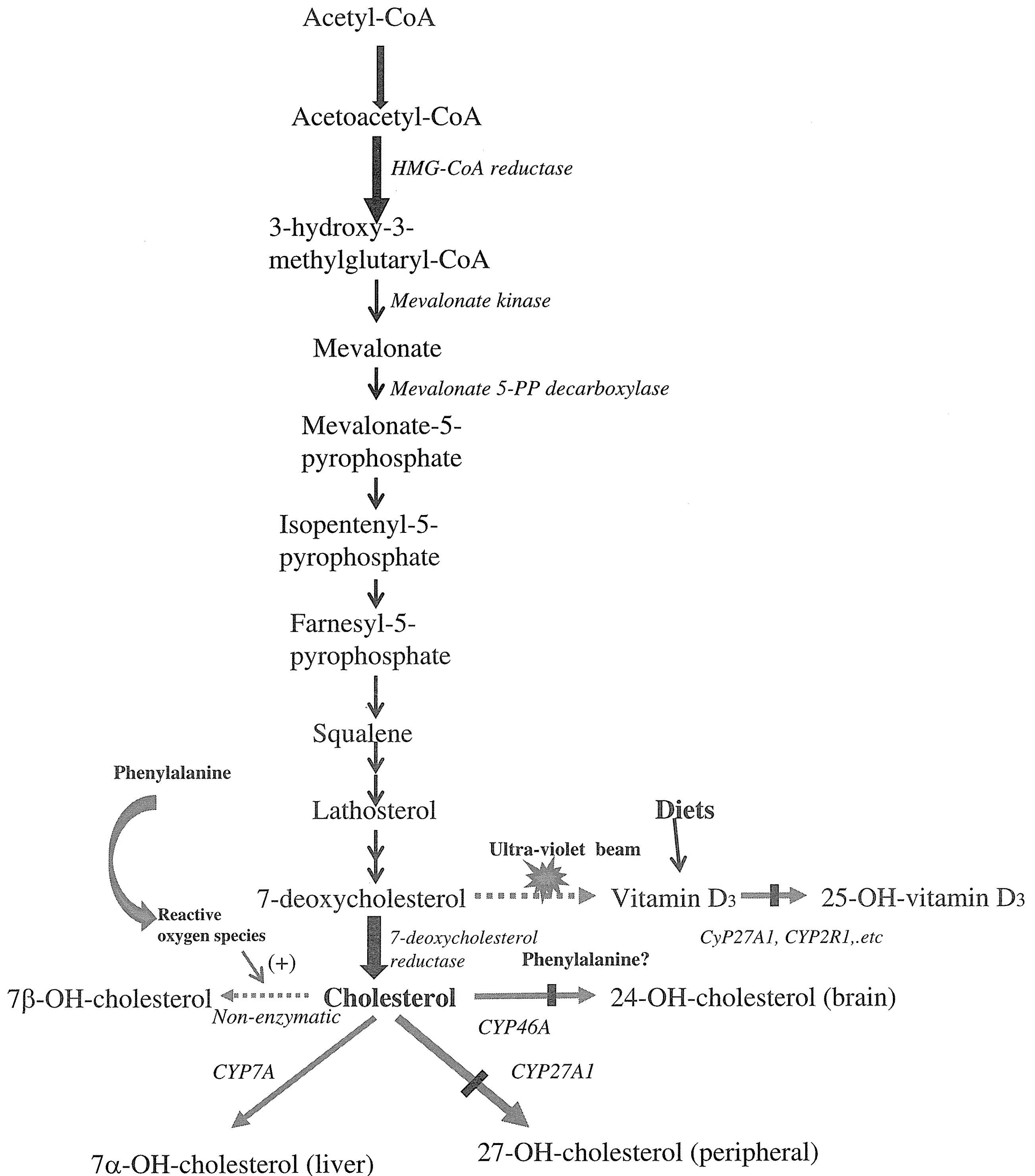

Fig. 1. Metabolic map illustrating cholesterol, oxysterol and vitamin D productions.

been suggested to be suppressed, although the mechanisms remain unclear [1,19–26]. These backgrounds of PKU encouraged us to investigate oxysterols in PKU.

In this report, we describe blood oxysterols associated to phenylalanine, lipids, and VD in adult PKU patients.

2. Subjects and methods

2.1. Subjects and sample collection

We enrolled 33 PKU patients (18 female, 15 male) with ages ranging between 21 and 38 years (mean ± SD, 28.1 ± 5.1 years, Table 1). Phenylalanine hydroxylase (PAH) deficiency was diagnosed by the analysis of dihydropteridine reductase activity in erythrocytes, biopterin loading test, and/or pteridine analysis in urine (1). Patients were all found to have hyperphenylalaninemia by mass screening at around 5 days of age. After the diagnoses, they exclusively received phenylalanine-restricted diets. After the age of 20 years, restrictions of phenylalanine differed greatly among patients. Some patients continued to receive strictly restricted diets. Others received mildly restricted or less restricted diets. Results showed that plasma phenylalanine levels differed greatly among individuals, but intra-individual changes were small. As control subjects, we enrolled 20 healthy volunteers (10 female, 10 male) with ages of 21–38 years old (mean ± SD, 28.9 ± 4.5 years).

Table 1
Characteristics of 33 phenylketonuric patients and 20 healthy age-matched controls.

	Healthy controls	PKU patients
Age (y)	28.9 ± 4.5	28.9 ± 5.3
Number (M/F)	22 (10/10)	33 (15/18)
BMI (kg/m^2)	23.7 ± 2.2	23.1 ± 1.9
Dietary intake	2234 ± 261	1890 ± 265[c]
Energy (kcal/day)		
Protein (g/day)	80 ± 15	71 ± 17[c]
Natural protein (g/day)	66 ± 14	50 ± 13[d]
Fat (g/day)	57 ± 13	55 ± 21
Calcium (mg/day)	1170 ± 387	1094 ± 339
Vitamin D	120 ± 23	107 ± 21
Phenylalanine (μmol/l)	30 ± 15	1019 ± 380[d]
TP (g/dl)	7.5 ± 0.3	7.2 ± 0.3
Albumin (g/dl)	4.6 ± 0.2	4.3 ± 0.2
Urate (mg/dl)	4.7 ± 0.5	4.5 ± 0.5
Creatinine (mg/dl)	0.4 ± 0.1	0.4 ± 0.1
ALT (IU/l)	11 ± 3	9 ± 2
AST (IU/l)	18 ± 4	18 ± 3
TC (mg/dl)	173 ± 26	155 ± 24[b]
TG (mg/dl)	82 ± 23	81 ± 20
LDL-C (mg/dl)	99 ± 18	87 ± 20[b]
HDL-C (mg/dl)	57 ± 7	54 ± 11
Oxysterol		
7α-(OH) cholesterol (ng/ml)	47.6 ± 21.9	30.9 ± 17.1[b]
7β-(OH) cholesterol (ng/ml)	10.3 ± 3.0	13.5 ± 8.7[b]
24S-(OH) cholesterol (ng/ml)	49.3 ± 9.9	37.6 ± 6.8[b]
27-(OH) cholesterol (ng/ml)	94.8 ± 26.4	55.4 ± 14.1[c]
Markers for cholesterol synthesis and absorption		
Lathosterol (μg//ml)	1.37 ± 0.52	1.05 ± 0.33[b]
Campesterol(μg//ml)	2.33 ± 1.29	2.57 ± 1.11
Sitosterol (μg//ml)	2.31 ± 0.80	2.20 ± 0.73
7-deoxycholesterol (μg//ml)	0.23 ± 0.08	0.16 ± 0.05[b]
Bone markers [a]		
25-(OH) vitamin D_3 (ng/ml)	28.9 ± 2.3	19.9 ± 2.1[d]
1.25-$(OH)_2$ vitamin D_3 (pg/ml)	40.7 ± 2.7	55.5 ± 3.7[d]
Intact PTH (pg/dl)	32.5 ± 3.5	37.1 ± 2.4[b]

Samples were collected approximately during the same time period.
Data are presented as mean ± SD.
BMI, body mass index; TP, total protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; PTH, parathyroid hormone.
[a] The sunlight exposure was similar between the 2 groups.
[b] $p<.05$.
[c] $p<.01$.
[d] $p<.001$ vs. controls.

2.2. Study design

For PKU patients and healthy controls, we tested serum levels of oxysterols together with serum levels of phenylalanine and lipids such as total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol. Serum VD_3 and intact parathyroid hormone (PTH) levels were also examined [23].

Regarding oxysterols, we examined 7-alpha-hydroxycholesterol (7α-OHC) and 27-hydroxycholesterol (27-OHC) as major precursors of bile acids reflecting the whole body cholesterol elimination, and 24S-hydroxycholesterol (24S-OHC) representing brain cholesterol elimination (Fig. 1) [13–16]. Further, we examined 7β-hydroxycholesterol (7β-OHC) reflecting the status of oxidative stress [19]. Among these oxysterols, 24S-OHC is the only one produced in the brain [13–18]. Blood was drawn from a peripheral vein in the morning after overnight fasting. The protocol was approved by the relevant institutional review boards. Parents of all patients provided written informed consent before the start of the study.

2.3. Determination of serum lipid, VD_3 and intact PTH

Serum levels of TC and TG were determined by enzymatic methods using commercial kits (Kyowa Medex Co. Ltd., Tokyo, Japan). Then HDL-C was measured using 13% polyethylene glycol (PEG 300; Wako Pure Chemical Industries Ltd., Osaka, Japan). LDL-C was measured using an enzyme immunoassay with a commercial kit (LDL-C Daiichi; Daiichi Pure Chemicals Co. Ltd.).

Serum 1.25-hydroxy VD_3 and 25-hydroxy VD_3 levels were determined, respectively, using RIA kits from Immunodiagnostic Systems Holdings plc (Boldon, UK) and Diasorin, Inc. (Stillwater, MN). Serum intact PTH level was determined using a radioimmunoassay (RIA) kit from the Nichols Institute (Quest Diagnostics, Geneva, Switzerland).

2.4. Assays for determination of oxysterols blood levels

Oxysterol concentrations in plasma samples were determined using the GC–MS method described previously with some modification [27].

Briefly, 300 μl of plasma in a glass tubes containing 150 ng of 27-hydroxycholesterol-26, 26, 26, 27, 27-D_5 (27d_5-OHC) as an internal standard was saponificated with 2 ml of 10 mol/l potassium hydroxide and 5 ml of methanol at room temperature for 1 h. After adjusting pH to 7.0 with 50% H_3PO_4, 5 ml of H_2O was added to the mixture; then oxysterols were extracted twice with 10 ml of *n*-hexane. The *n*-hexane layer was loaded immediately on the Bond Elut SI cartridge (Agilent Technologies Inc., California), which was pre-conditioned using *n*-hexane. The large excess amount of cholesterol was removed by washing with 2 ml of *n*-hexane and 10% of ethyl acetate with *n*-hexane.

Oxysterols retained onto the cartridge were eluted with 2 ml of ethyl acetate. After dryness of the eluate, the collected oxysterols were derivatized with trimethylsilyl (TMS) reagent. The derivatized oxysterols were re-suspended in 50 μl of *n*-hexane for GC–MS analysis. GC–MS analysis was conducted (GCMS-QP2010 plus; Shimadzu Corp., Kyoto) using a capillary column (30 m × 0.25 mm, 0.25 μm thickness, DB-5 ms; GL Sciences Inc., Tokyo) connected to AOC20i automatic sample injector and AOC20S autosampler. The oven temperature program was set as follows: 180 °C for 1 min, 20 °C/min to 250 °C and then 5 °C/min to 300 °C where the temperature was kept for 15 min. Helium was used as a carrier gas, and the flow rate was set at 0.96 ml/min. Injection was performed in the splitless mode and the injector temperature was kept at 230 °C. The MS ion source temperature and interface temperature were 250 °C. Samples were injected 5 μl for analysis of oxysterols.

The mass spectrometer was operated in the selected ion monitoring mode. A few ions were detected simultaneously. The ions used for analysis (m/z) and typical retention times (min) for the compounds

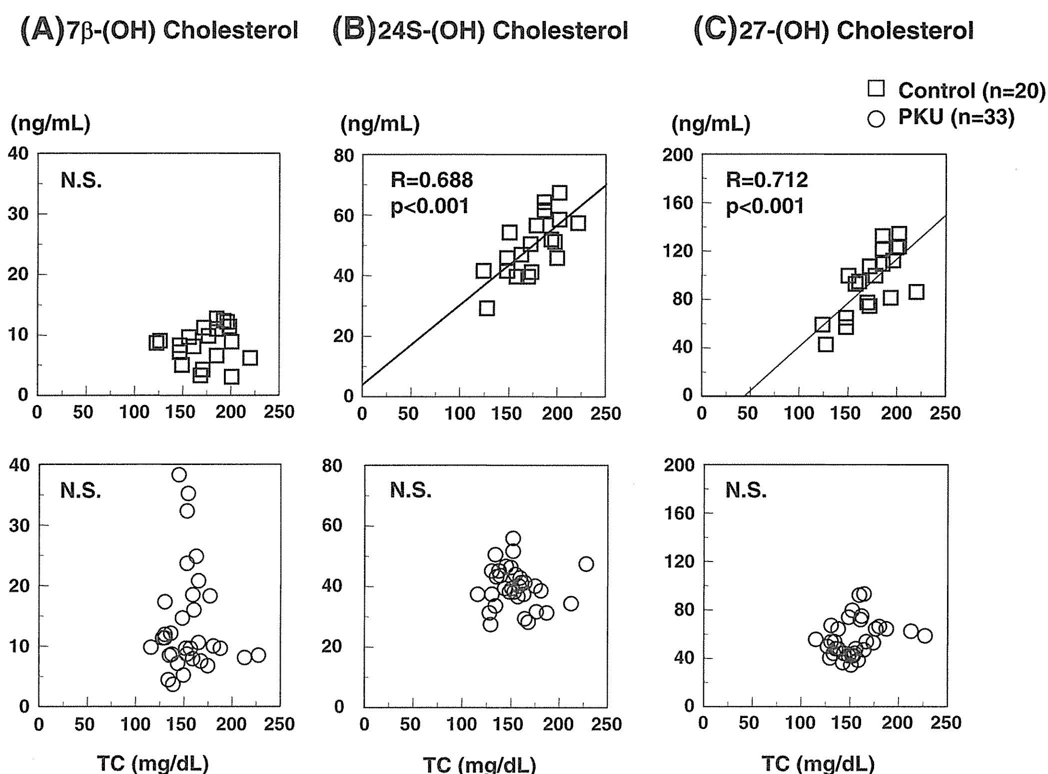

Fig. 2. Scatter graphs of oxysterol levels against total cholesterol level in control and PKU groups. TC, total cholesterol.

were the following: 7α-OHC, 456, 15.5; 7β-OHC, 456, 17.4; 24S-OHC, 413, 20.1; 27-OHC, 131, 20.6. Using this system, intra-assay and inter-assay CVs were within 10 and 15%, respectively.

2.5. Statistic analyses

Differences between values of patients and those of controls were estimated using Student's *t*-test. The relation between each pair of parameters was estimated using Pearson's correlation test. All results for which $p \leq .05$ were inferred as statistically significant.

3. Results

3.1. Daily nutrition and liver function in PKU patients compared with controls

Average daily energy and protein intakes in PKU patients were lower than those in controls, but the average fat and calcium and vitamin D intakes were not different. Between these two groups, no significant difference in age, body mass index, and liver function test was found (Table 1).

3.2. Lipid levels in PKU patients compared with controls

Total- and LDL-cholesterol levels in PKU patients were significantly lower than those in the controls ($p = 0.03$). The mean values were nine-tenths of the control values. Triglycerides and HDL-cholesterol levels were not significantly different (Table 1).

3.3. V D and intact PTH levels in PKU patients compared with controls

25-Hydroxy VD_3 level was significantly decreased in the PKU group ($p = 0.0008$) and the mean value was about two-thirds of that in the control value. On the contrary, 1.25-hydroxy VD_3 and intact parathyroid hormone levels were 1.4- and 1.15-folds increased, respectively, and showed significant differences with respective control levels; 1.25-hydroxy VD_3, $p = 0.0007$; intact PTH, $p = 0.04$ (Table 1).

3.4. Oxysterol levels in PKU patients compared with controls

7α-OHC, 24S-OHC, and 27-OHC levels in PKU group were significantly lower than those in the control group (Table 1). The mean values were about two-thirds, four- fifths and three-fifths for the respective control values. In contrast, the 7β-OHC level in PKU group was significantly higher than that in the control group (Table 1).

3.5. Correlations between oxysterols and lipids in controls and PKU patients

In the control group, 27-OHC and 24-OHC levels but not other oxysterols showed significant positive correlations with the total cholesterol level (Fig. 2; upper panels). In PKU group, no such significant correlation was found (Fig. 2: lower panels).

3.6. Correlations between oxysterols and phenylalanine in PKU patients

7β-OHC and 24S-OHC levels but not 27-OHC and 7α-OHC levels showed significant correlations with phenylalanine levels (7β-OHC, $p = 0.004$: 24S-OHC, $p = 0.0008$) (Fig. 3A and B). Among the PKU patients with phenylalanine levels exceeding 650–700 μmol/l, the 7β-OHC and 24S-OHC levels were out of the normal ranges at high rates. However, their 27-OHC levels were under a normal range at high rates, irrespective of the phenylalanine level (Fig. 3C).

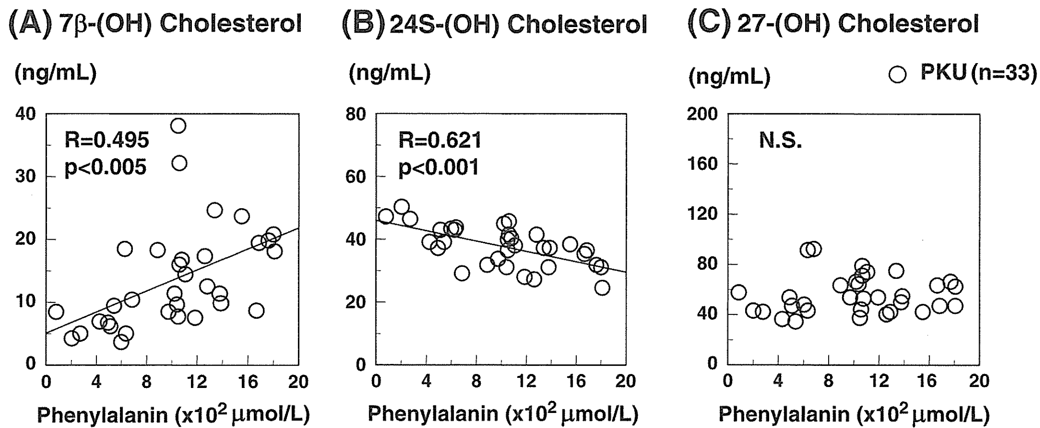

Fig. 3. Scatter graphs of oxysterol levels against phenylalanine level in the PKU group.

3.7. Correlations between oxysterols and VD in PKU patients

27-OHC level showed a significant positive correlation with 25-hydroxy VD_3 level but not with 1.25-hydroxy VD_3 and intact PTH levels (Fig. 4). Other oxysterols never showed significant correlations with bone parameters.

4. Discussion

This study demonstrated changes of blood oxysterols that predominated over those of total- and LDL-cholesterols in adult PKU. Blood levels of 24S-OHC, 27-OHC, and 7α-OHC were considerably decreased in adult PKU patients. On the contrary, 7β-OHC was considerably increased.

The control group exhibited significant correlations between 27-OHC, total- and LDL-cholesterol levels. However, the PKU group lacked such cholesterol–oxysterol correlation. In the PKU group, 24S-OHC and 7β-OHC levels were correlated significantly with the phenylalanine level, suggesting that plasma phenylalanine, to some degrees, influences on 24S-OHC and 7β-OHC metabolisms, although not directly. In this affected group, 27-OHC showed a significant positive correlation with 25-hydroxy VD_3 as a representative marker for VD status.

Aside from distinct hyperphenylalaninemia, cholesterol synthesis might be suppressed in PKU [1,20–22]. Reduced activities of two key enzymes for cholesterol synthesis, 3-hydroxy-3methylglutaryl-CoA reductase and mevalonate-5-pyrophosphate decarboxylase, have been suggested in PKU (Fig. 1) [20–22]. In the PKU group, total- and LDL-cholesterol levels were about 10% decreased as compared to the respective control levels, consistent with such precedent reports. Plasma levels of 7-dehydrocholesterol (7-DC) and lathosterol as cholesterol precursors were about 20% decreased in PKU group while cholesterol absorption markers such as campesterol and sitosterol remained unchanged (Table 1). Oxysterols are oriented from cholesterol, and, therefore, it would be likely that productions of oxysterols such as 7α-OHC, 27-OHC and 24S-OHC might be influenced by cholesterol synthesis.

24S-OHC is the brain specific cholesterol elimination product made by the neuronal specific cholesterol 24-hydroxylase (CYP46A) [13,28]. Reduced plasma concentration was found as a consequence of neurodegeneration [13,15,28]. However, it is possible that plasma concentration might rise concomitantly with increased brain cholesterol turnover. It has been shown that plasma cholesterol never enter into brain, passing through blood–brain barrier, and that suppression of cholesterol synthesis possibly lead to the decrease of plasma 24S-OHC [13,17]. So, the decrease of 24S-OHC in PKU group might be, in part, attributable to the reduced cholesterol synthesis in the brain and/or disturbed conversion from cholesterol to 24S-OHC.

Recent studies have shown the effects of 24S-OHC to prevent amyloid plaque formation as seen in Alzheimer disease and to promote growth of brain cells [29–32]. Probably, 24-OHC has several crucial biological functions in the neurological system. Therefore, we will pursuit the neurological consequence of long-standing decrease of 24-OHC in adult PKU.

27-OHC is a product of CYP27A, which expression is abundant in peripheral tissues but rather poor in the liver, [13,33–35]. This oxysterol is the first step of cholesterol elimination via acidic bile acids pathway

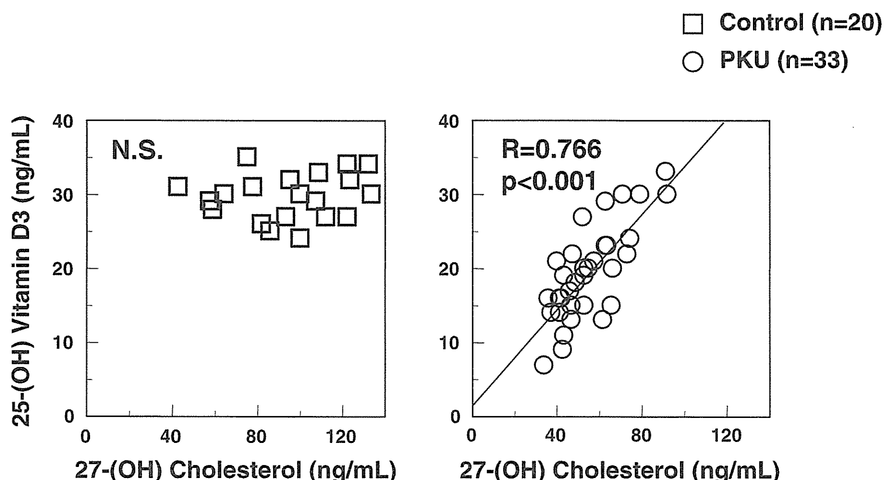

Fig. 4. Scatter graph of 25-hydroxy vitamin D_3 level against 27-hydroxycholesterol level in the PKU group.

[13,29–35]. Unlike cholesterol, 27-OHC passes through blood–brain barrier easily and enters into the brain. A positive correlation between cholesterol and 27-OHC level in the circulation, together with considerable amount of 27-OHC uptake by the human brain from the circulation, has been shown [36,37].

The decrease of 27-OHC was prominent and disproportionate to the decrease of total- and LDL-cholesterol in PKU patients. The 27-OHC level was always two-thirds of control level against the lipid and phenylalanine levels. The data obtained from the control group, together with earlier reports, support that blood 27-OHC level shows a positive correlation with blood total-and/or LDL-cholesterol levels [13,36,37]. Contradictorily, it has been shown that the effect of statins inhibiting 3-hydroxy-3methylglutaryl-CoA reductase on blood 27-OHC is often obscure even though the decrease of cholesterol level is clear [18]. On the other hand, Björkhem et al. reported that blood 27-OHC level greatly increased in Smith–Lemli–Opits syndrome (OMIM, 270400) manifesting poor cholesterol synthesis together with accumulation of 7-deoxycholesterol (7-DC) due to 7-DC reductase deficiency (EC 1. 3. 1. 21) (Fig. 1) [17].

At this time, we do not have a convincing explanation for a distinct decrease of blood 27-OHC independent of blood total-and/or LDL-cholesterol levels. Our recent study showed that vitamin D_3, which is originated from 7-DC, was also decreased in disproportion to the total- and LDL-cholesterol levels in PKU [23]. This study showed that 27-OHC level is strongly correlated with 25-hydroxy VD_3 level in PKU patients. Considering that both 27-OHC and 25-hydroxy VD_3 productions are greatly regulated by CYP27A activity, it is likely that CYP27A activity is suppressed in them (Fig. 1) [38].

The decrease of 7α-OHC, a product of 7α-hydroxylase (CYP7A) in the liver, also predominated over the decreases of total- and LDL-cholesterol, exhibiting no correlation with phenylalanine level. 7α-Hydroxylase (CYP7A) plays a central role in the bile acid synthesis and cholesterol elimination in the liver [33,35]. We inferred that the considerable decrease of 7α-OHC might reflect liver cholesterol pool.

7β-OHC production is promoted by oxidative stress. Therefore, this oxysterol is regarded as a parameter for oxidative stress [13,9]. 7β-OHC was increased in PKU patients. Particularly patients having a phenylalanine level of more than 650–700 μmol/l often exhibited high levels (Fig. 2). Our recent study provides evidence that oxidative stress is enhanced in PKU according to the phenylalanine level [10].

Thus, this study suggested changes of oxysterol together with those of cholesterol metabolism and VD status. However, to gain a better understanding of cholesterol and oxysterol metabolism in PKU, more comprehensive studies covering key enzymes should be necessary.

In summary, this study showed variable changes in oxysterols in adult PKU patients. To identify the unfavorable effects of altered oxysterol metabolism on the nervous system and the bone will be our future project.

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Case report

Liver-specific mitochondrial respiratory chain complex I deficiency in fatal influenza encephalopathy

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Abstract

We report on a 4-year-old boy who died from influenza encephalopathy. The clinical course and microscopic findings of the autopsied liver were compatible with Reye's syndrome. We examined the mitochondrial respiratory chain function by blue native polyacrylamide gel electrophoresis (BN-PAGE), western blotting, and respiratory chain enzyme activity assays. The activity of liver respiratory chain complex (CO) I was markedly decreased (7.2% of the respective control activity); whereas, the other respiratory chain complex activities were substantially normal (CO II, 57.9%; CO III, 122.3%; CO IV, 161.0%). The activities of CO I–IV in fibroblasts were normal (CO I, 82.0%; CO II, 83.1%; CO III, 72.9%; CO IV, 97.3%). The patient was diagnosed with liver-specific complex I deficiency. This inborn disorder may have contributed to the fatal outcome. We propose that relying only on fibroblast respiratory chain complex activities may lead to the misdiagnosis of liver-specific complex I deficiency.
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Keywords: Influenza encephalopathy; Reye's syndrome; Mitochondria; Complex I deficiency; Liver-specific

1. Introduction

Influenza encephalopathy is a critical complication of influenza infection. Although the pathological mechanism is poorly understood, mitochondrial malfunction is suggested to play a role in the pathogenesis [1]. We describe a boy with liver-specific mitochondrial respiratory chain complex I deficiency who developed fatal encephalopathy associated with influenza A infection. The possible contribution of the mitochondrial respiratory chain disorder to the clinical course is discussed.

2. Case report

A 4-year-old Japanese boy developed pyrexia. He was treated with acetaminophen once and visited the family doctor. Influenza A infection was diagnosed by nasal antigen test in a clinic and he was treated with oseltamivir. He was admitted to a nearby hospital due to a generalized seizure in the evening; then, he was transferred to our institute because of highly elevated serum transaminase. He was the first child born to healthy parents with no consanguinity. No other child had died in early

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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.

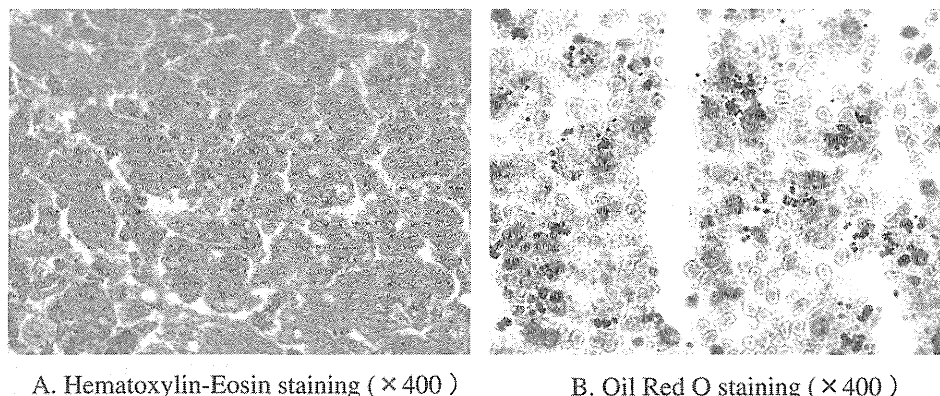
3. Discussion

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.

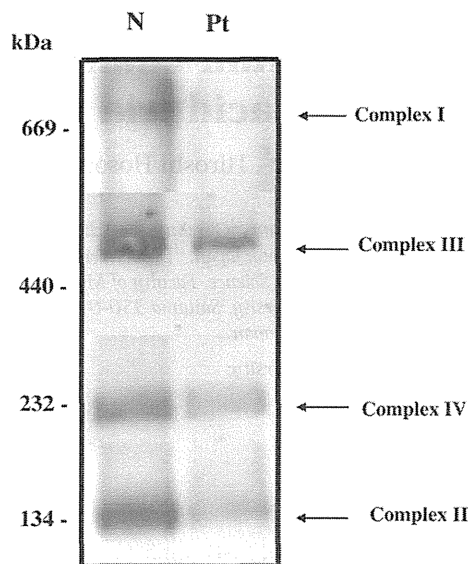


Fig. 2. Blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis of liver respiratory chain enzymes showed markedly decreased protein expression of complex I, while the protein bands of complex II, III, and IV were comparable to the control (N) samples.

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) 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

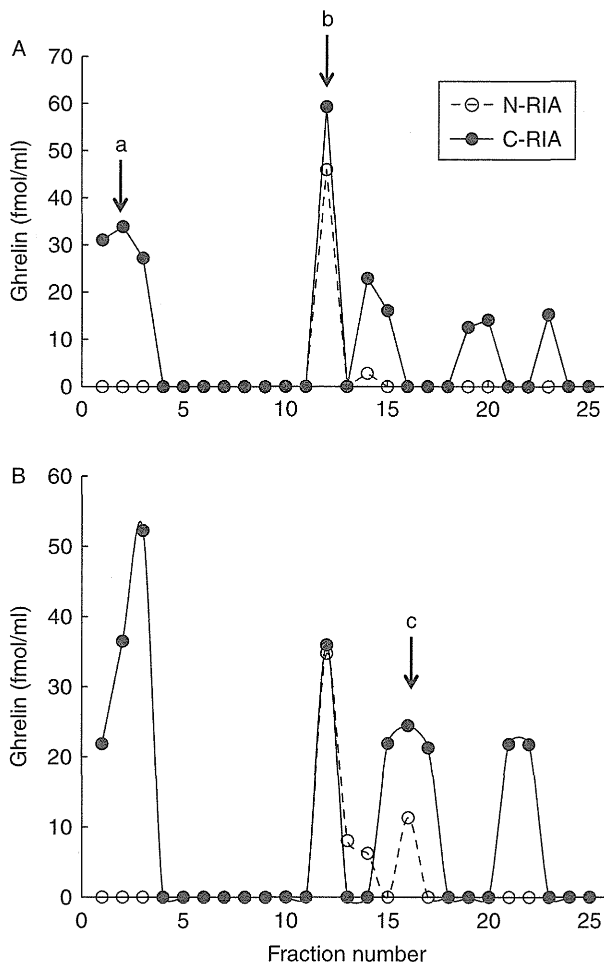

Figure 1 Representative RP-HPLC profiles of ghrelin immunoreactivity in patients with MCADD (A) and GA2 (B). Closed circles, data obtained using a RIA for a ghrelin C-terminal region (C-RIA); open circles, data obtained using a RIA for a ghrelin N-terminal region (N-RIA). Patient plasma extracts from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