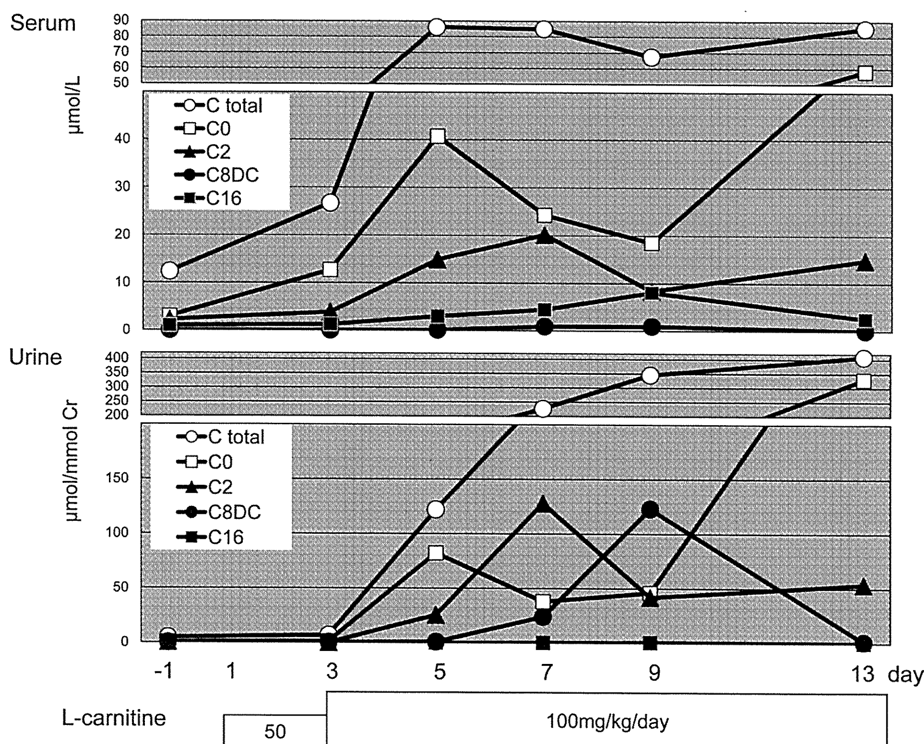

Fig. 1. Time-course of serum and urinary acylcarnitine levels measured by tandem MS. The levels of representative acylcarnitines are shown. The first day of L-carnitine supplementation is designated as day 1. Urinary carnitines were assayed using the first urine in the morning.

carnitine fractions by the enzymatic cycling method were 9.5 and 5.9 μmol/L, respectively. The initial serum acylcarnitine profile (Table 1) showed a very low free carnitine level and relatively high long-chain acylcarnitine levels. This profile was compatible with the secondary carnitine deficiency due to CPT2 or translocase deficiency.

After confirmation of the carnitine deficiency, we supplied her with L-carnitine orally from the 15th hospital day (day 1 in the Table 1 and Fig. 1) at a dose of 50 mg/kg/day for the first two days and 100 mg/kg/day from day 3. Blood and urinary samples were obtained before plus 3, 5, 7, 9 and 13 days after L-carnitine supplementation. During carnitine supplementation, the patient had continuous intravenous glucose infusion of 2.5 mg/kg/min until day 11. We analyzed the serum and urinary acylcarnitines by tandem mass analysis, as previously reported (Mueller et al. 2003; Kobayashi et al. 2007a,b). Table 1 shows details of the analyses. Fig. 1 shows the changing patterns of free carnitine (C0), acetyl-carnitine (C2), C8DC representing medium-chain dicarboxylic acylcarnitines, and C16 representing long-chain acylcarnitines in the serum and urine. Urinary excretion of C0 and acylcarnitines remained at very low levels on day 3. Sequential peaks of free carnitine (day 5), acetylcarnitine (day 7), and long-chain acylcarnitines (day 9) were found in the serum, which corresponded to peaks of free carnitine, acetylcarnitine, and dicarboxylic medium-chain acylcarnitines in the urine.

The fatty liver and hepatomegaly improved as judged by an abdominal CT scan on the 26th hospital day (day 13). Informed consent for a skin biopsy, enzyme assay, and DNA was obtained from the parents. CPT2 activity in the patient's fibroblasts was 0.18 nmol/min/mg of protein (3 controls; 0.82, 1.27, and 1.26 nmol/min/mg of protein), confirming the diagnosis of CPT2 deficiency.

Now the patient is 4 years of age. After carnitine supplementation, she did not experience hypoglycemia at all. She is being treated with 1,000 mg L-carnitine/day (current body weight 19.8 kg). Her growth and development are within normal ranges. She had some rhabdomyolysis attacks (the highest CK recorded was 16,769 IU/L) during a febrile illness even after L-carnitine supplementation.

Discussion

The diagnosis of CPT2 deficiency was first suspected by the data on urinary organic acid analysis and acylcarnitine analysis and was confirmed by enzyme assay using fibroblasts. Our patient is a compound heterozygote of a previously reported E174K mutation from the father and an unknown mutation from the mother which was not detected by exon sequencing. According to an in vitro expression analysis of mutant CPT2 cDNAs carrying E174K, the mutant E174K protein was present as much as a wild type protein and retained 10% residual CPT2 activity (Wataya et al. 1998). This "mild" mutation from the father, together with possible null mutation from the mother, may result in an infantile form of CPT2 deficiency.

Initially, she developed secondary carnitine deficiency. Chronic administration of pivalate-conjugated antibiotics is

a major cause of secondary carnitine deficiency even in healthy children (Stanley 2004). Ten days before the onset of the Reye-like syndrome, she had a cold and was given Cefteram pivoxil (CFTM-PI) for four days. The initial serum acylcarnitine profile showed no elevation of hydroxy-C5 carnitine, nor of pivaloylcarnitine. While the antibiotic might have contributed to secondary carnitine deficiency in part, the acute attack with fasting was more likely the course of the low carnitine in the patient at presentation.

The time-course changes in the serum and urinary acylcarnitine levels after L-carnitine supplementation were studied. These changing profiles suggest that accumulated and potentially toxic long-chain acylcarnitines in the mitochondria were eliminated from the body by day 13. The majority of accumulated long-chain acylcarnitines in the mitochondria may be eliminated by the following steps: 1) a large amount of accumulated long-chain acylcarnitines should be transferred from the mitochondrial matrix by carnitine acylcarnitine translocase if there is a sufficient amount of free carnitine outside of the mitochondrial matrix; 2) then peroxisomal beta-oxidation reduces the chain length of such accumulated fatty acids; 3) the resultant medium-chain fatty acids can be catalyzed in the mitochondria, or further ω-oxidized into dicarboxylic acids in the microsomes; 4) these medium-chain DC and their carnitine conjugates can be excreted into the urine efficiently. It took several days for oral L-carnitine administration to increase the serum carnitine levels, probably because the intracellular stores were depleted and it took several days for them to be replenished. Thereafter, the administration increased the excretion of abnormal acylcarnitines, some of which had probably accumulated within the tissues.

It is noteworthy that the acetylcarnitine in both the serum and the urine was a predominant acylcarnitine on day 7 (Fig. 1). Elevation of acetylcarnitine in the serum and urine indicates the presence of enough acetyl-CoA in the mitochondria and the availability of acetyl-CoA for carnitine acyltransferase reactions in the cells, and might account for the increased beta-oxidation rates upon L-carnitine therapy (Fontaine et al. 1996). In general, acetylcarnitine is a major acylcarnitine in healthy controls and is regarded as a marker of undisturbed beta-oxidation (Costa et al. 1998). Since CPT2-deficient patients have beta-oxidation restrictions of long-chain acyl-CoA, L-carnitine supplementation may increase beta-oxidation of medium-chain acyl-CoAs, which could be supplied via peroxisomal beta-oxidation of long-chain acyl-CoA.

Carnitine supplementation in the treatment of long-chain beta-oxidation defects is still controversial. In patients with a defect in the mitochondrial beta-oxidation spiral, when a preceding L-carnitine deficiency is normalized, and transport into the mitochondria of long-chain fatty acids is also normalized, acyl-CoAs accumulate instead of being oxidized by the defective reaction and, consequently, in such cases, free CoA is depleted in the mitochondria (Yoshino et al. 2003). This may be true in beta-oxidation defects such as very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency and trifunctional protein deficiency. Studies on VLCAD-deficient mice suggested carnitine supplementation results in the induction of acylcarnitine production in various tissues and significant accumulation of potentially toxic intermediate acylcarnitines in tissues (Liebig et al. 2006; Primassin et al. 2008). However, blockage of the CPT2 step causes the accumulation of long-chain acylcarnitines but does not primarily cause the accumulation of intermediate CoA esters in beta-oxidation.

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Case report

Molecular analysis of a presymptomatic case of carnitine palmitoyl transferase I (CPT I) deficiency detected by tandem mass spectrometry newborn screening in Japan

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Abstract

Carnitine palmitoyl transferase I (CPT I) deficiency is a rare disorder of long-chain fatty acid oxidation. It is one of the metabolic diseases detectable by tandem mass spectrometry. We report herein a presymptomatic CPT I deficiency detected in a Japanese female newborn by tandem mass spectrometry newborn screening. A mutation analysis of the *CPT1A* gene revealed two novel mutations, p.R446X and p.G719D.
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Keywords: Carnitine palmitoyl transferase I; CPT IA; Tandem mass spectrometry; Newborn screening

1. Introduction

The β-oxidation of long-chain fatty acids is an important source of energy production, especially during times of increased energy demand, such as fasting, illness, or prolonged exercise. Carnitine palmitoyl transferase I (CPT I) is the key enzyme of long-chain fatty acid oxidation. CPT I deficiency generally occurs with febrile or gastrointestinal illness, when energy demands are increased. Clinical symptoms range from recurrent hypoketotic hypoglycemia to Reye-like syndrome and sudden death [1].

More than 20 metabolic diseases, CPT I deficiency among them, can now be screened by tandem mass spectrometry on dried blood spots [2]. CPT I deficiency is characterized by decreased levels of long-chain acyl-carnitines such as palmitoylcarnitine (C16) and stearoylcarnitine (C18), and increased levels of free carnitines (C0). According to a tandem mass spectrometry pilot study in Japan, the deficiency is detected in about 1 out of every 200,000 newborns.

We herein report a patient with presymptomatic CPT I deficiency who was discovered by tandem mass spectrometry newborn screening. The results of sequencing analysis of *CPT1A* gene revealed a novel nonsense mutation (p.R446X) and a novel missense mutation (p.G719D).

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2. Case report

The patient is the first child of healthy nonconsanguineous Japanese parents with no family history of metabolic disease or neuromuscular disease. At the late-phase of pregnancy, intrauterine growth retardation was detected. The patient was born by cesarean section because of breech presentation. Her birth weight, height, and head circumference were 2230 g, 48.0 cm, and 32.0 cm, respectively.

The patient was admitted to our hospital at 1 month of age, when tandem mass spectrometry newborn screening disclosed an elevation in free carnitine (C0 140 μM: cutoff, lower than 90) and a decreased level of palmitoylcarnitine (C16 0.03 μM). Hypotonia and hepatomegaly were absent on physical exanimation. Her body weight gain was about 40 g/day, with breast milk feeding. Biochemical testing uncovered no particular abnormal findings. The carnitine profile in dried blood spots revealed an elevation of free carnitine (C0 105 μM) and decreased levels of long-chain acyl-carnitines (C16 0.09 μM, C18 0.043 μM). The ratio of free carnitine to the sum of long-chain acyl-carnitines {C0/(C16 + C18)} was 789, which suggested a diagnosis as CPT I deficiency (cut off <100). No metabolic acidosis (pH 7.357, PCO_2 42.1 mmHg, HCO_3 23.6 meq/L, BE −2), hypoglycemia (blood sugar 105 mg/dl), or renal tubular acidosis was observed. Urine organic acid analysis was normal.

Enzymatic analysis in blood revealed a low level residual CPT I activity of 11–26% of control. Sequencing analysis of 18 exons from exon 2 to exon 19 in the *CPT1A* gene was performed with the written informed consent of her parents. The results showed two novel mutations: c.1339C > T (p.R446X) in exon 11 and c.2156G > A (p.G719D) in exon 18 (Fig. 1). The p.R446X mutation was transmitted from her father; the other mutation (p.G719D) was transmitted from her mother (data not shown).

The patient is now given a low-fat diet with supplementation of medium-chain triacylglycerol (MCT) milk. On earlier occasions when she fell sick, hypoglycemia was prevented by early intervention with glucose infusion. On the latest examination at 3 years, her psychomotor development was appropriate for her age.

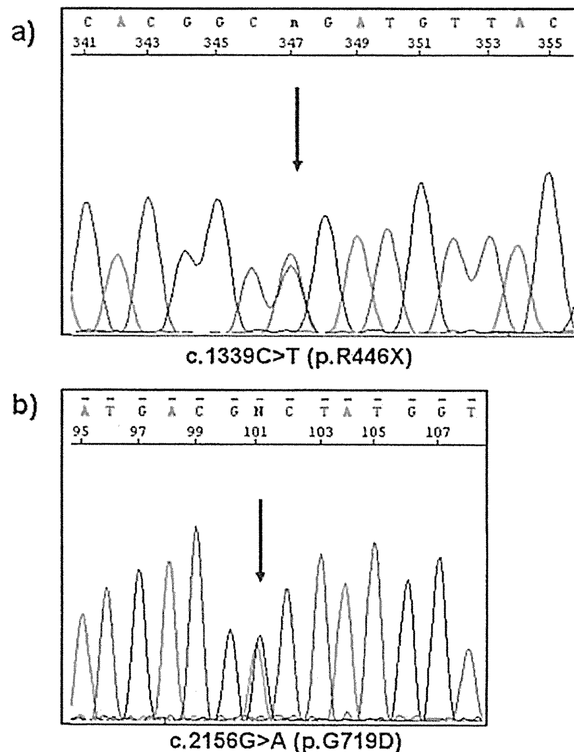

Fig. 1. (a) A C-to-T substitution at c.1139 in exon 11 was detected in a heterozygous pattern. This c.1339C > T substitution created a stop codon (p.R446X). (b) A G-to-A substitution at c.2156 in exon 18 was found in a heterozygous pattern. The c.2156G > A substitution changed the codon of glycine at 719 to aspartic acid (p.G719D).

3. Discussion

Most CPT I-deficient patients present recurrent episodes of coma and seizure due to hypoketotic hypoglycemia. With tandem mass spectrometry newborn screening, patients in a presymptomatic state can be detected. Our patient seems to have developed normally, without severe metabolic crisis, up to the present. Tandem mass spectrometry screening allows early medical intervention for patients with fatty acid oxidation defect.

Enzyme assay and/or mutational analysis are necessary to confirm the diagnosis. In most individuals with CPT I deficiency, residual enzyme activity is 1–5% of control [3]. In contrast, the residual enzyme activity of the myopathic type of the Inuit is 15–25%. Our patient had residual activity of 11–26% of control, a level as high as that of the myopathic type.

More than 20 mutations responsible for the CPT I deficiency have been identified in the *CPT1A* gene [4–6]. Most of the mutations seem to be unique or restricted to only a few pedigrees, except p.G710Q in the Hutterite population and p.P479L in the Inuit population [3,7]. Sequencing for the present patient revealed a novel nonsense mutation (p.R446X) and a novel missense mutation (p.G719D). The p.G719D mutation proved to be absent in 50 unrelated controls (data not shown). The glycine at 719 of CPT I is conserved in mouse, rat, horse, and zebra fish. These data suggest the substitution appears not to be a polymorphism, but a disease-causing mutation. A clear genotype–phenotype correlation has been reported only between the p.P479L mutation (common mutation in Inuit) and adult-onset myopathic presentation with high residual activity. The data on our present patient suggest that the mutant pG719D-CPT I protein might have relatively high residual activity, as the other mutation was a nonsense mutation. An

expression study will be necessary to confirm this hypothesis.

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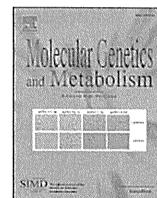

A patient with mitochondrial trifunctional protein deficiency due to the mutations in the *HADHB* gene showed recurrent myalgia since early childhood and was diagnosed in adolescence☆

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ABSTRACT

Mitochondrial trifunctional protein (MTP) is a multienzyme complex involved in the metabolism of long-chain hydroxyacyl-CoA, a product of the fatty acid β-oxidation cycle. MTP is an α4β4 hetero-octomer encoded by two different genes: *HADHA* (OMIM 600890) and *HADHB* (OMIM 143450). MTP deficiency induces three different types of presentation: (1) a lethal phenotype with neonatal onset (severe); (2) a hepatic phenotype with infant onset (intermediate); and (3) a neuromyopathic phenotype with late-adolescent onset (mild). While acylcarnitine analysis has revealed increased levels of long-chain hydroxyacylcarnitine in blood when an MTP deficiency exists, the neuromyopathic type is usually asymptomatic and does not always result in an abnormality in acylcarnitine analysis results. We report here the case of a 13-year-old girl with recurrences of intermittent myalgia since her early childhood, for whom the disorder had not been definitely diagnosed. Since she was referred to our hospital because of rhabdomyolysis, we have repeatedly performed blood acylcarnitine analysis and found slight increases in long-chain 3-OH-acylcarnitine levels, on the basis of which we made a chemical diagnosis of MTP deficiency. Immunoblot analysis of skin fibroblasts revealed loss of α- and β-subunits of MTP. In addition, analysis of the *HADHB* gene, which encodes long-chain 3-ketoacyl-CoA thiolase, one of the enzymes constituting MTP, identified compound heterozygous mutations of c.520 C>T (p.R141C) and c.1331 G>A (p.R411K).

MTP deficiency is considered an extremely rare disorder, as only five cases (lethal phenotype, two patients; hepatic phenotype, two patients; and neuromyopathic phenotype, one patient) have thus far been reported in Japan. However, it is likely that the neuromyopathic phenotype of MTP deficiency has not yet been diagnosed among patients with recurrences of intermittent myalgia and rhabdomyolysis, as in our patient reported here.

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1. Introduction

Mitochondrial trifunctional protein (MTP) is a multienzyme complex involved in the metabolism of long-chain hydroxyacyl-CoA, a product of the fatty acid β-oxidation cycle [1]. When an abnormality exists in this complex, the fatty acid β-oxidation cycle fails to supply an adequate amount of energy, resulting in three different types of presentation: a lethal phenotype with neonatal onset, in which hepatic and/or cardiac muscular disturbance occurs early in infancy, causing sudden death; a hepatic phenotype with infant onset, in which non- or low-ketotic hypoglycemia occurs; and a neuromyopathic phenotype with late-adolescent onset, in which muscular symptoms such as intermittent myalgia or rhabdomyolysis occur [2,3]. The diagnosis is based on increased levels of long-chain 3-OH-acylcarnitine, as demonstrated by blood acylcarnitine analysis using ESI-MS/MS. However, the neuromyopathic phenotype is usually asymptomatic and frequently shows no abnormal test results; therefore, its definite diagnosis may require an extended length of time.

A 13-year-old girl with repeated myalgia since early childhood, in whom MTP deficiency had not been diagnosed, was referred to our hospital because of rhabdomyolysis. We repeatedly performed blood acylcarnitine analysis, found slight increases in long-chain 3-OH-acylcarnitine levels, and finally diagnosed MTP deficiency by Western blot and genetic analysis.

☆ Databases: HADHA OMIM:600890, GDB:434026, GenBank:NM_000182HADHB OMIM:143450, GDB:344953, GenBank:NM_000183

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2. Materials and methods

2.1. Case

The 13-year-old girl was the first child of non-consanguineous parents, and was born at 42 weeks of gestation after an uneventful pregnancy. Her birth weight was 3032 g. One month after birth, an apparent life-threatening event (ALTE) developed; however, she showed normal growth and development. From the age of 3 years, she complained of pain in her leg muscles after walking over a long distance. From the age of 9 years, the frequency of intermittent acute muscle pain increased and even mild exercise occasionally caused severe discomfort in her leg muscles. The symptom was often triggered by infection and menstruation. She experienced bouts of muscle pain after hard exercise such as running on a school field day or hiking on a school excursion. After hard exercise, she felt difficulty in moving because of unusual severe pain in her generalized muscles. Serum acylcarnitine analysis performed at 10 years of age revealed slightly increased levels of long-chain acylcarnitine, on the basis of which very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency was suspected. However, her VLCAD activity was normal and the disorder had not been definitely diagnosed.

At the age of 13 years, she was admitted to Kobe University Hospital with severe myalgia over the whole body. On admission, hematological and biochemical investigations revealed markedly elevated serum creatine kinase (22,885 IU/L), aldolase (62.6 IU/L), and myoglobin (2960 ng/mL). Plasma free and total carnitine and total keton bodies concentration were normal (Table 1). Echocardiography and electocardiography did not reveal any evidence of a cardiomyopathy.

Table 1
Laboratory findings upon hospitalization.

<Blood biochemistry>			Reference range
AST	529	IU/L	13–31
ALT	205	IU/L	8–34
LDH	909	IU/L	115–217
CK	22,885	IU/L	46–168
CK-MB	434	IU/L	0–25
Aldolase	62.6	IU/L	2.2–5.5
Myoglobin	2960	ng/mL	0–60
BUN	11	mg/dL	9–22
Cre	0.34	mg/dL	0.5–1.3
T.chol	167	mg/dL	146–219
TG	58	mg/dL	28–149
Glu	180	mg/dL	61–92
Lactic acid	19.1	mg/dL	3–17
Pyruvic acid	1.47	mg/dL	0.3–0.94
Total ketone bodies	39	μmol/L	26–122
Acetoacetate	24	μmol/L	13–69
β-hydroxy butylate	15	μmol/L	0–76
Total carnitine	57	μmol/L	45–91
Free carnitine	45.7	μmol/L	36–74
Acylcarnitine	11.3	μmol/L	6–23
<Acylcarnitine analysis>			Reference range
(Blood spot)			
C14:1	0.21	μM	<0.4
C14-OH	0.12	μM	<0.12
C16:1	0.15	μM	<0.785
C16:0-OH	0.18	μM	<0.12
C18-OH	0.066	μM	<0.1
C18:1-OH	0.15	μM	<0.07
(Serum)			
C14:1	0.4	μM	<0.1
C14-OH	0.2	μM	<0.1
C16:1	0.2	μM	<0.1
C16:0-OH	0.16	μM	<0.8
C18-OH	0.077	μM	<0.05
C18:1-OH	0.13	μM	<0.7

2.2. Methods

2.2.1. Urine organic acid and acylcarnitine analysis

Urine organic acid and blood acylcarnitine analysis from dried blood spots and serum were performed by GC/MS and ESI-MS/MS, respectively, as described in detail previously [4].

2.2.2. Cell culture

Fibroblasts from the patient were cultured in Eagle's minimum essential medium containing 10% fetal calf serum and antibodies (100 μg/mL each of penicillin and streptomycin; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan).

2.2.3. Western blot analysis

Western blot analysis was performed following 12.5% SDS/PAGE [5] using rabbit polyclonal antibodies raised against purified MTP protein as the primary antibody. Bound antibodies were visualized using the ImmunoPure NBT/BCIP Substrate Kit (Promega, Madison WI, USA). Protein concentrations were determined using the Bio-Rad protein assay protocol (Bio-Rad Laboratories, Hercules, CA).

2.2.4. Mutation analysis

Genomic DNA was extracted from the patient's fibroblasts using a QIAamp DNA Micro Kit (Qiagen GmbH Hilden, Germany). We designed 20 sets of primers for amplification of *HADHA* (one for each exon, including 5′ and 3′ splice sites), and 16 sets for amplification of *HADHB*. Each exon was amplified by polymerase chain reaction (PCR) and directly sequenced as described previously [6].

3. Results

3.1. Urine organic acid and acylcarnitine analysis

Urine organic acid analysis performed at the same time revealed slight ketosis (as indicated by slightly increased excretion of 3-OH-butyrate), low-ketotic dicarboxylic aciduria (as indicated by increased excretion of adipate and suberate), and 3-OH dicarboxylic aciduria (as indicated by increased excretion of 3-OH-sebacate and 3-OH-dodecanedioate).

Acylcarnitine analysis of blood spots at the time of hospitalization revealed increases in the levels of long-chain 3-OH-acylcarnitines (C14-OH, C16-OH, C18-1-OH). The changes indicated an MTP deficiency. On the other hand, elevation of both long-chain acylcarnitines (C14-1, C16-1) and long-chain 3-OH-acylcarnitines (C14-OH, C18-OH) in serum suggested a VLCAD deficiency (Table 1). These findings strongly suggested long-chain fatty acid metabolism disorder, VLCAD or MTP deficiency, however, it was difficult to distinguish between the two. So acylcarnitine analysis was performed another four times, with inconsistent results: slightly increased levels of long-chain 3-OH-acylcarnitine (C16-OH, C18-1-OH), a feature of MTP deficiency, were noted on two occasions, while no abnormality was noted on the other two occasions. On the basis of these results and her clinical manifestations, we made a chemical diagnosis of a mild form of MTP deficiency, in which neither symptoms nor abnormal laboratory findings are noted during attack-free intervals.

3.2. Western blot analysis

Western blot analysis using samples extracted from the patient's skin fibroblasts detected neither α- nor β-subunits of MTP (Fig. 1), whereas both subunits were detected in control fibroblasts. These findings showed that the patient had an MTP deficiency.

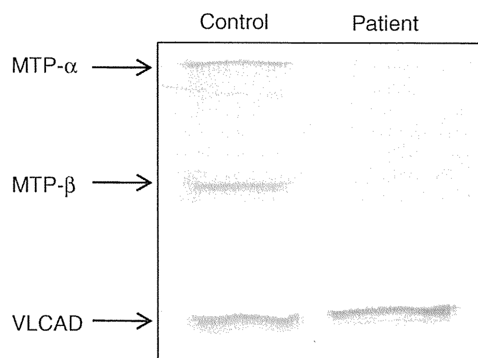

Fig. 1. Western blot analysis of cultured skin fibroblasts of mitochondrial trifunctional protein. MTPα- and MTPβ- were not detectable in the patient's fibroblasts.MTPα- and MTPβ-, α- and β-subunits of MTP, respectively; VLCAD, very long-chain acyl-CoA dehydrogenase.

3.3. Mutation analysis

Analysis of the genes encoding MTP component enzymes identified compound heterozygous mutations of c.520 C>T/c.1331 G>A (p.R141C/p.R411K) in the *HADHB* gene encoding long-chain 3-ketoacyl-CoA thiolase (LCT), one of the enzymes constituting MTP. No mutation was found in the *HADHA* gene.

4. Discussion

Fatty acid metabolism plays an important role in energy supply in the body. Its pathways comprise intake of fatty acids from cell membranes, the fatty acid β-oxidation cycle, an electron transport chain, and production of ketone bodies. More than 20 enzymes and transporters are known to be involved in the metabolism.

MTP resides in the mitochondrial inner membrane, and is a multienzyme complex involved in the metabolism of long-chain hydroxyacyl-CoA. In MTP deficiency, β-oxidation of long-chain fatty acids is impaired; the deficiency is asymptomatic when the supply and demand of energy is in balance; however, if the energy supply is inadequate when the demand for energy is increased by infection, disease, exercise, or elongated intervals between meals, the body cannot handle the energy shortage. Symptoms occur in organs requiring large amounts of energy produced by the metabolism of fatty acids, such as the brain, cardiac muscle, liver, and skeletal muscle. The patient reported here began to repeatedly complain of leg pain after exercise at around the age of 3 years, and as she grew up, her muscle symptoms became more prominent. These findings led us to conclude that her clinical manifestations were of the muscular type of MTP deficiency. In addition, an ALTE occurred at the age of one month. It was assumed that this episode might also have been related to MTP deficiency.

MTP deficiency is a very rare disorder, and has thus far been reported in only about 50 patients (of whom 20 were classified as having the neuromyopathic phenotype) in Europe and North America, and in five patients (of whom only one had the neuromyopathic phenotype) in Japan. In many patients with the muscular type, the time from onset to definite diagnosis was long. Spiekerkoetter et al. investigated the clinical manifestations of 11 patients with muscular-type MTP deficiency and reported an average period from initial onset of the disorder to diagnosis of 5 years and 10 months [7]. In the one patient with muscular-type MTP deficiency reported in Japan, rhabdomyolysis began to repeatedly occur at the age of 15 years, and a definite diagnosis was made at the age of 23 years [8]. More recently, it has become possible to perform less expensive, more convenient, and highly accurate blood acylcarnitine analysis using tandem mass spectrometry, which has resulted in diagnosis of a fatty acid metabolism disorder in an increasing number of patients. MTP deficiency is characterized by increased 3-OH-acylcarnitine (C16:0, C16:1, C18:0, C18:1) in serum (or plasma) or blood spots, demonstrated by acylcarnitine analysis, whereas the analysis does not detect any abnormality when no episodic symptoms occur [9]. In our patient, we repeatedly performed blood acylcarnitine analysis and found no consistent results. In the first analysis, the profile from the blood spots indicated an MTP deficiency, while that from the serum indicated a VLCAD deficiency. It was difficult to distinguish between VLCAD and MTP deficiency on the basis of results from the first analysis. In the following analysis, a slight increase in the levels of long-chain 3-OH-acylcarnitines, a feature of MTP deficiency, was noted on some occasions, but no abnormality was detected on other occasions. Comprehensive evaluation of these acylcarnitine analysis results and her clinical manifestations were highly suggestive of MTP deficiency. Furthermore, diagnosis was confirmed by Western blot analysis and genetic analysis. When patients with recurrent myalgia and rhabdomyolysis are examined on the assumption that fatty acid disorder including MTP deficiency may be diagnosed, it is important to repeatedly perform acylcarnitine analysis using samples obtained while symptoms occur.

MTP is an octamer composed of four α-subunits that function as long-chain hydroxyacyl-CoA dehydrogenases (LCHADs) and long-chain enoyl-CoA hydratases (LCEHs), and four β-subunits that function as LCTs [10]. LCHAD and LCEH subunits are encoded by the *HADHA* gene, and LCT subunits are encoded by the *HADHB* gene. These two genes are adjacently located in the human chromosome region 2q23 [11], and consist of 20 and 16 exons, respectively [12,13]. The c.1331 G>A (p.R411K) mutation detected by the *HADHB* gene analysis in our patient is the same as that found by Orii et al. in two Japanese family lines with MTP deficiency, which they reported was mild in both patients [14]. On the other hand, the c.520 C>T (p.R141C) mutation has never been reported, and is therefore novel. Because the latter mutation was not detected in 50 normal controls and Arg141 is conserved in different species, we concluded that the c.520 C>T (p.R141C) mutation was a causative mutation.

Some investigators have compared the neuromyopathic phenotype of MTP deficiency with the lethal phenotype and found that the neuromyopathic phenotype is associated with better preserved enzyme activities and is more closely related to protein expression and clinical manifestations [15,16]. In our patient, the clinical manifestations fell into the category of the neuromyopathic phenotype, but Western blot analysis detected neither α- nor β-subunits of MTP. It has been reported that MTP exerts enzymatic activities in a stable manner only when the α- and β-subunits making up the octameric MTP are all normal, and that mutant proteins yield dominant negative effects to inhibit the activities of normal proteins [17]. However, the reason for this apparent discrepancy between the phenotype and the enzyme activities remains unclear.

5. Conclusion

In patients with recurrent muscular symptoms such as myalgia and rhabdomyolysis, a fatty acid metabolism disorder such as MTP deficiency should be a suspected etiology, even when tests performed during attack-free intervals frequently detect no abnormalities. For the purpose of diagnosing MTP deficiency in these patients, it is important to suspect the disorder on the basis of their past histories and to repeatedly perform acylcarnitine analysis when attacks occur. The biochemical findings in VLCAD and MTP deficiency can overlap, which occurred in our patient and suggest that suspected deficiency of either should lead to Western blot and genetic analysis to rule out both when appropriate. MTP deficiency is a rare disorder, and its rareness may be explained by the presence of patients with recurrent muscular symptoms in whom the disorder has not yet been diagnosed.

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原　著

カルニチンパルミトイルトランスフェラーゼ2欠損症のろ紙血血清のアシルカルニチンプロファイルの経時的変化

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要　旨

我々は，カルニチンパルミトイルトランスフェラーゼ(CPT)2欠損症の血液ろ紙，血清のアシルカルニチンプロファイルの出生後からの経時的変化を検討した．症例は日齢0の男児．切迫早産のため在胎37週0日，帝王切開にて出生した．姉がCPT2欠損症のため本症例もブドウ糖輸液を行い注意深い観察を行った．血液ろ紙，血清のアシルカルニチンプロファイルを経時的に分析し，以下の所見と姉がCPT2欠損症と酵素診断されていることから本症例は無症状であったがCPT2欠損症と化学診断した．血液ろ紙におけるC16-アシルカルニチン(C16)，C18：1アシルカルニチン（C18：1），C18-アシルカルニチン（C18）は日齢3にピークとなり，カットオフ値を超えていたがその後カットオフ値以下となった．(C16＋C18：1)/C2は生後14日までカットオフ値を超えており，スクリーニング指標として有用と考えられた．血清でもC16, C18：1, C18は日齢3にピークとなり，その後徐々に低下したが，日齢14まで常にカットオフ値を超えており，ろ紙血よりも血清におけるアシルカルニチン分析の方が確実に異常を指摘できた．ろ紙血による現行の採血時期における脂肪酸代謝異常症のスクリーニングでは，我々の症例のようにすでにC16, C18, C18：1がカットオフ値を下回り偽陰性となる可能性がある．このようなCPT2欠損症例を見逃さないためにはスクリーニング時期をより早期に設定する必要性が示唆された．

キーワード：CPT2欠損症，脂肪酸β酸化障害，アシルカルニチン，
タンデムマススペクトロメトリー，新生児マススクリーニング

はじめに

カルニチンパルミトイルトランスフェラーゼ(CPT)2欠損症は，常染色体劣性遺伝形式を示し，ミトコンドリア脂肪酸β酸化障害をきたす疾患の1つである．ミトコンドリアにおける脂肪酸β酸化系は肝臓ではブドウ糖からのエネルギー供給が低下したときなどに作動してアセチル-CoAやケトン体など代替エネルギーを産生する．また脂肪酸β酸化系は心臓や骨格筋においては安静時のエネルギー産生において重要である．長鎖脂肪酸が細胞質からミトコンドリア内に輸送される際にカルニチンシャトルが必要である．長鎖脂肪酸が活性化されたアシル-CoAはミトコンドリア外膜に存在するCPT1により，アシルカルニチンとなる．アシルカルニチンはカルニチンアシルカルニチントランスロカーゼによりミトコンドリア内膜を通過し，CPT2により再びアシル-CoAへ変換される．CPT2に異常があるとミトコンドリア内でアシルカルニチンからアシル-CoAへの変換が障害され，β酸化を受けることができず，アシルカルニチンが蓄積する．このように脂肪酸代謝が十分に行われず，エネルギー産生が低下することで発症する．

CPT2欠損症は本邦におけるタンデム型質量分析計(以下タンデムマス)によるマススクリーニング・パイロット研究などの報告によれば，比較的頻度の高い脂肪酸酸化異常症である[1)]．

臨床型は大きく出生前発症型，乳幼児発症型，軽症型（骨格筋型）の3つに分類される．出生前発症型は腎異形性，大脳奇形，顔貌異常など認め，致死的である．乳児発症型は低ケトン性低血糖の発作として発症し，乳幼児突然死やReye様症候群と関連がある．軽症型（骨格筋型）は成人期に偶発性横紋筋融解症で発症する[2)]．

我々は以前1歳3か月にReye様症候群で発症したCPT2欠損症症例を経験した[3)]．今回その次子で，出生

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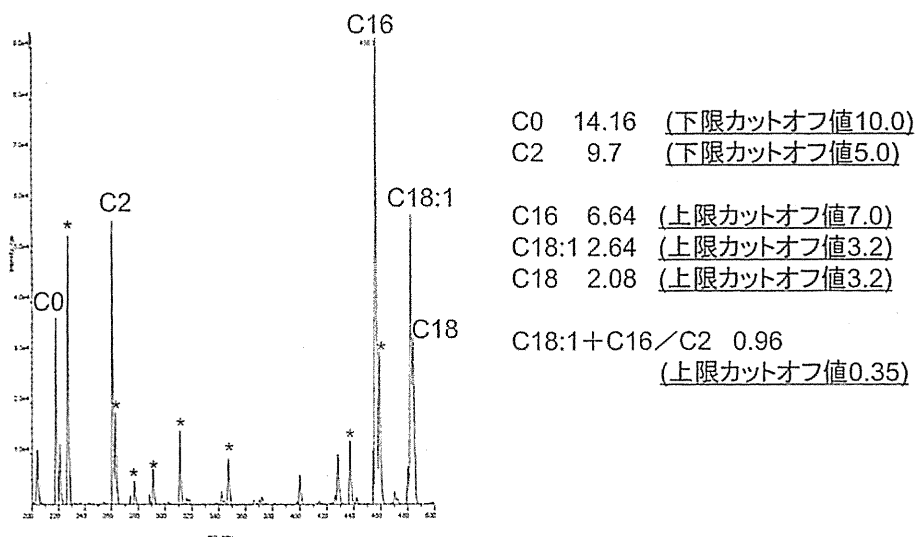

図1　生後12時間後のろ紙血アシルカルニチンプロファイル
横軸はm/z値，縦軸は相対量を示した．図右に各アシルカルニチンの定量値（μmol/l）と括弧内に上限または下限カットオフ値を示した．＊は内部標準物質．C0，C2に比較してC16，C18：1，C18などの長鎖アシルカルニチンが高く，(C18：1＋C16)/C2も上昇しており，CPT2欠損症に特徴的なパターンである．

直後から経過観察し，生後12時間後から経時的に血清，ろ紙血のアシルカルニチン推移を観察し，CPT2欠損症と化学診断できた症例を経験した．タンデムマスによる新生児スクリーニングの実施時期を考える上で貴重な経験であると考え報告する．

症　例

在胎週数37週0日，出生体重2,600g，男児．

家族歴：姉がCPT2欠損症で当科にて加療中．姉は1歳3か月にReye様症候群にて発症した．発作時の有機酸分析にて低ケトン性ジカルボン酸尿，アシルカルニチン分析にてCPT2欠損症が疑われた．線維芽細胞を用いたCPT2活性がコントロールの16%と低下しておりCPT2欠損症と診断した．ゲノムレベルでの遺伝子解析では父由来の*CPT2*遺伝子にE174K変異が同定されたが，母由来の変異は同定されなかった[3]．

母親の妊娠経過：次子妊娠にあたり，遺伝相談を実施した．姉で母由来の変異は同定されておらず，出生前に遺伝子解析を行っても保因者か患者かの区別がつけられないこと，新生児期に十分なグルコースの補給で新生児期発症を予防できる可能性が高いことを説明し，両親の希望で出生前検査は行わずに妊娠は継続された．妊娠36週6日，切迫早産にて入院．翌日緊急帝王切開となった．

出生後の経過：アプガースコア1分9点，5分10点で仮死なく出生．体温36.8℃，呼吸数54回/分，心拍数134回/分，血圧59/30mmHgで活気は良好であった．大泉門は平坦，肺野は清，心音は整，腹部は平坦で軟，筋トーヌス低下や外表奇形を認めなかった．血液生化学検査では，アンモニア値は出生後157μg/dlとやや高値であったが，生後3日には100μg/dl以下となり一過性であった．血糖値は46mg/dlと著明な低血糖（40mg/dl以下）は認めず，その後も低血糖は認めなかった．その他，胸部レントゲンではCTR47%で心拡大はなく，心臓，腎，頭部超音波検査では異常を認めなかった．

出生後10%グルコースにてグルコース注入速度(GIR)4.8mg/kg/minの糖補充を開始した．両親の承諾のもと出生後早期に遺伝子解析を実施したところ患児もE174K変異をヘテロでもつことが判明し，注意深い観察をおこなった．日齢1に10ml×8回/日から経管栄養を開始し，以後1回哺乳量を10mlずつ増量し，輸液は漸減していった．日齢5に経静脈栄養を中止し自律哺乳とした．

方　法

日齢1, 2, 3, 4, 5, 6, 7, 8, 14にろ紙血，血清を採取し，タンデムマスによるアシルカルニチン分析を島根大学において，既報の方法にて行った[4]．

結　果

生後12時間（日齢1）での血液ろ紙のアシルカルニチンの結果は，C16は6.64μmol/L，C18：1は2.64 μmol/L，C18は2.08μmol/Lと長鎖アシルカルニチンが高値であったがカットオフ値以下であった．しかし，(C18：1＋C16)/C2は0.96とカットオフ値を超えて高

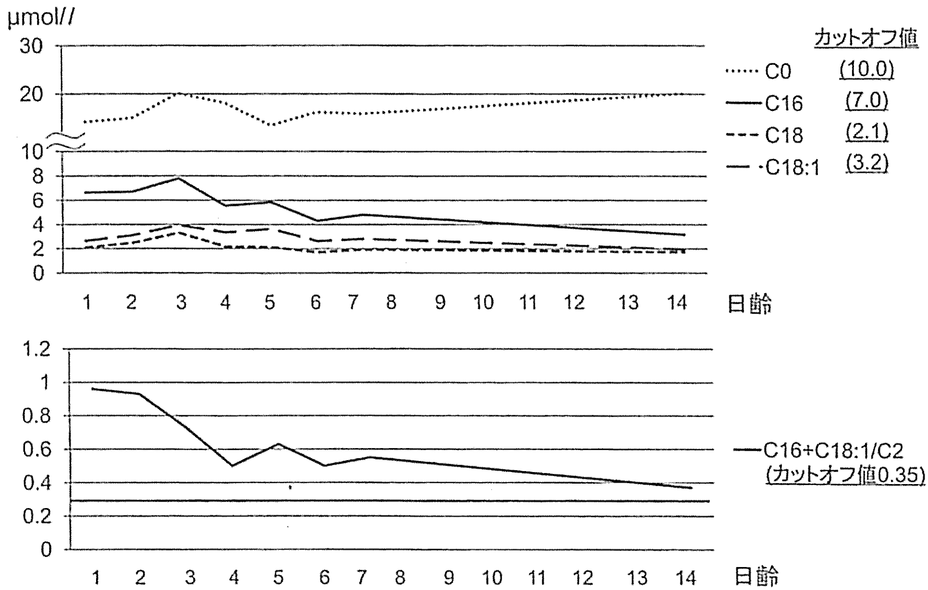

図2　血液ろ紙アシルカルニチンプロファイルの推移
上段の図では横軸は日齢，縦軸は各アシルカルニチン量．括弧内に上限または下限カットオフ値を示した．C16，C18，C18：1はいずれも日齢3にピークとなり，その後漸減した．遊離カルニチンは明らかな低値を認めない．下段の図は（C16＋C18：1）/C2を縦軸に示した．図中にカットオフ値を直線で示した．出生直後が最も高く，日齢14まで上限値を超えている．

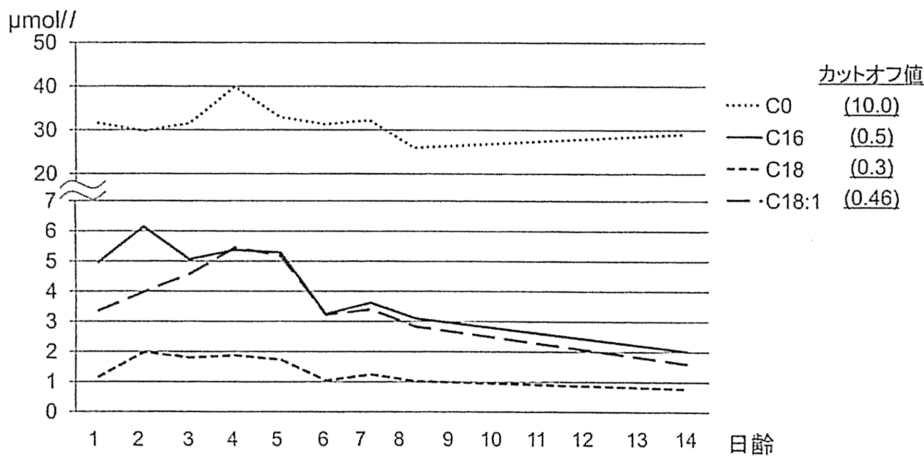

図3　血清アシルカルニチンプロファイルの推移
横軸は日齢，縦軸は各アシルカルニチン量．括弧内に上限または下限カットオフ値を示した．日齢1より明らかな長鎖アシルカルニチンの増加を認める．C16，C18，C18：1は日齢4にピークとなりその後低下した．遊離カルニチンは明らかな低値を認めない．

値[1]であった（図1）．

血液ろ紙アシルカルニチンプロファイルの推移を示す（図2）．C16, C18, C18：1はいずれも日齢3にピークとなり，その後漸減した．遊離カルニチンは日齢3に20.1μmol/Lまで上昇した後，若干の低下傾向を示したが，カットオフ値以下にはならなかった．CPT2欠損症のスクリーニング指標である（C16＋C18：1）/C2の値の変化は，特に出生直後が最も高い結果となっており，以後徐々に低下しているが，日齢14まで上限値を超えていた．

次に血清カルニチンのプロファイルを示す（図3）．日齢1の結果にて，C16は4.97μmol/L，C18：1は3.36μmol/L，C18は1.16μmol/Lと長鎖アシルカルニチンの増加が認められ，姉がCPT2欠損症と酵素診断されていることを考えてCPT2欠損症と化学診断した．血清C16, C18, C18：1は日齢4にピークとなりその後低下した．血清中遊離カルニチンは日齢4の39.87μmol/Lをピークに低下傾向となった．

考　察

CPT2欠損症では，血清中アシルカルニチン分画においてC2の低下，C16，C18，C18：1などの長鎖アシルカルニチンの上昇や(C16+C18：1)/C2の上昇をスクリーニング指標にして精査，診断に結びつける．

本症例における血液ろ紙ではC16，C18，C18：1の長鎖アシルカルニチンは日齢3にピークとなったが，上限カットオフ値をやや超える程度であった．しかし，血清中のC16，C18，C18：1は少なくとも日齢14まではカットオフ値を超えており，血清でのアシルカルニチン分析のほうが血液ろ紙に比較してより確実に異常を指摘できることが分かった．(C16+C18：1)/C2の値に関しては，血液ろ紙においては日齢14までカットオフ値を超えていた．血液ろ紙でスクリーニングを行う場合を行う場合，(C16+C18：1)/C2をより重視すべきであると考えられた．

遊離カルニチンは新生児期早期には低下を認めなかった．しかし，遊離カルニチンの低値を伴う二次性カルニチン欠乏が乳幼児発症型CPT2欠損症の患児でみられる[2][5]ため今後注意が必要である．患児の姉も発症時に遊離カルニチンの著明な低値を認めていた．

カルニチン欠乏がみられた場合はL-カルニチンの補充が重要である．また，カルニチン欠乏が明らかになる前に予防的な投与を考慮してもよいと思われる．

CPT2欠損症を含む先天代謝異常症のタンデムマススクリーニングは欧米をはじめとして各国で新生児マススクリーニングに導入されており，脂肪酸酸化異常症の早期発見に寄与している．血液ろ紙による新生児マススクリーニングの施行時期は，アメリカでは日齢1～2[6]に行われ，本邦におけるパイロットテストには日齢4～6のろ紙血が利用されている．本症例の結果では，血液ろ紙では長鎖アシルカルニチンのピークが日齢1～3にあり，その後減少していた．このことから日齢4～6に採取したろ紙血によるタンデムマススクリーニングでは，すでに長鎖アシルカルニチンは低下し始めており偽陰性となる可能性がある．そのため，本邦におけるスクリーニング採血時期を海外と同様にさらに早い時期に行う必要性があるのではないかと考えられる．一方で血清アシルカルニチンではいずれの時期でもカットオフ値を超えていた．血液ろ紙分析でカットオフ値を超えていたC14：1アシルカルニチンが経過観察中にカットオフ値を下回った極長鎖アシル-CoA脱水素酵素欠損症症例が報告されており[7]，スクリーニングの再検査や経過追跡には血清アシルカルニチン分析を行うことがよいと考えられる．

また，本症例においては出生時にCPT2欠損症を疑わせるような症状は認めなかったが，生後12時間後の検体からすでにCPT2欠損症を示唆するアシルカルニチンプロファイルであった．特に本患者で同定されている*CPT2*遺伝子のE174K変異は，日本人成人型で同定された変異で，10%程度の残存活性を持っている変異である[8]．母由来の変異は同定されていないが，少なくともCPT2の残存活性をもつために姉は新生児型でなく，乳幼児期発症型になったと考えられる．このような残存活性を持つ症例において，さらに持続的に糖補充をしていたにもかかわらず生後12時間からすでに血液ろ紙，血清のいずれにおいても異常が指摘された．

新生児早期からタンデムマス解析で異常を指摘できることから，新生児タンデムマススクリーニングの普及により，このような症例の発症前診断が可能となり，早期の治療的介入，指導により，発症の回避が可能になると思われた．

結　語

CPT2欠損症の血液ろ紙，血清のアシルカルニチンプロファイルの経時的変化を観察した．生後12時間後の検体からすでにCPT2欠損症を示唆するアシルカルニチンプロファイルであった．ろ紙による現行の採血時期におけるスクリーニングでは，すでにカットオフ値を下回っている可能性があり，スクリーニング時期をより早期に設定する必要性があるのではないかと考えられた．

日本小児科学会の定める利益相反に関する開示事項はありません．

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Carnitine Palmitoyltransferase-2 (CPT2) Deficiency：Time-dependent Changes of Acylcarnitine Profiles in Dried Blood Spots and Serum after Birth

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We analyzed time-dependent changes of acylcarnitine profiles in dried blood spots and serum samples after birth in a CPT2-deficient patient. The boy was born at 37 weeks gestation via Caesarean section. Since his sister had CPT2 deficiency, he was carefully followed with intravenous glucose infusion from birth to day 6. Although he had no clinical symptoms, he was also diagnosed as CPT deficiency based on the family history and acylcarnitine analyses. In the acylcarnitine analyses using dried blood spots, peak levels of C16, C18, and C18：1 acylcarnitines, which are the usual screening markers for CPT2 deficiency, were above their upper cutoff values on day 3. However, their levels decreased and were under the cutoff values thereafter. The ratio C16+C18：1/C2 was above the upper cutoff values until day 14, indicating that the ratio is a useful screening marker for CPT2 deficiency. In contrast, for acylcarnitine analyses using serum, although the peak levels of C16, C18, and C18：1 acylcarnitines were also detected on day 3, their levels declined gradually but still were above their upper cutoff values until day 14. These facts indicate that acylcarnitine analyses using serum detected this abnormality more effectively than using dried blood spots. Therefore, screening for fatty acid oxidation using dried blood spots on day 5 may result in a false-negative result since the values of C16, C18, and C18：1 acylcarnitines were under their cutoff values in our CPT2 deficient patient. Screening earlier than on day 5 may be considered to detect CPT2-deficient patients like this case.

シンポジウム：タンデムマス導入による新生児マススクリーニングの新時代

タンデムマス・スクリーニングに向けた簡易な酵素活性測定法の開発

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要　約　タンデムマス新生児スクリーニングの実施地域が拡大し、発見される先天代謝異常症罹患児も増加しているが、これを真に日本の子供たちにとって有益なものとするためには、迅速で正確な確定診断法を整備・維持することが必要である。そこで我々は、国内での試験研究の初期から、高速液体クロマトグラフィ（HPLC）を用いた簡易な酵素診断法の開発と応用に取り組んできた。これはHPLCシステムがひとつあれば、どの施設でも導入できるものであり、本スクリーニング検査の長期的な継続を支えるために、国内の複数の施設で実施可能な体制の構築を提案したい。

緒　言

1997年の国内タンデムマス・スクリーニング試験研究開始[1)]から12年余りが経過し、各種対象疾患全体では約1万人に1人の罹患者が発見されている[2-4)]。疾患毎に考えれば、多くても数万人に1人程度の稀少疾患20種類以上について確定診断法が求められる。主な検査法は尿中有機酸分析・酵素活性測定・遺伝子解析だが、いずれも健康保険適応外の研究的検査にとどまっている。今後、罹患者の発見・診断が「日常業務」に近づいていかざるを得ない中で、必要な確定診断体制を長期的に維持する方策が問われている。

広島大学小児科では、高速液体クロマトグラフィ（HPLC）を用いた酵素活性測定法を用意して発見症例に応用してきた。1台のHPLCシステムでメープルシロップ尿症（MSUD）・メチルマロン酸血症（MMA）・プロピオン酸血症（PA）・イソ吉草酸血症（IVA）・グルタル酸尿症I型（GA1）・HMG-CoAリアーゼ欠損症（HMGLD）・MCAD欠損症（MCADD）・VLCAD欠損症（VLCADD）が診断できる[5)]。本稿では、今後われわれの酵素診断法が各地のタンデムマス・スクリーニング関連施設で導入されることへの期待を込めて、実際的な側面を含めて紹介したい。

酵素活性測定法

各酵素の反応条件を表1に示す。粗酵素源には末梢血リンパ球を使用している。静脈採血でヘパリン血を採取し、遠隔地からの依頼の場合は室温輸送で翌日にリンパ球分離を行う。リンパ球ペレット作成後、そのまま酵素活性測定に進む場合は、マイクロチューブを氷冷しながら超音波破砕する。測定を翌日以降にする場合は、水分を十分に除去の上－80℃で凍結保存し、freeze & thawにて粗酵素液を調製する。MSUDの場合は、超音波破砕では恐らく分枝鎖α-ケト酸脱水素酵素複合体が解離するために酵素反応が進まず、freeze & thawにて調製する必要がある。（※粗酵素溶液調製の詳細については拙論[6,7)]を参照されたい。）

診断に当たっては、被験者1名と正常対照者1名について、反応サンプル2本の平均値から無反応対照サンプル1本の値を差し引いて酵素活性値としている。サンプル1本の酵素量をリンパ球1×10^6個（MSUDとGA1では2×10^6個）分とし、タンパク濃度測定を省略してリンパ球当たりの活

表1　酵素活性測定法の諸条件一覧

酵素名*	基質	補助因子	緩衝液	粗酵素細胞数	反応停止	HPLC 移動相	反応産物
Mut	Methylmalonyl-CoA 3mM	Adenosyl-cobalamin 0.5mM	Tris sulfate 100mM (pH 7.5)	1×10^6 (#1)	0.3N $HClO_4$	NaH_2PO_4 (pH 2.5) 100mM Methanol 12%	Succinyl-CoA
PCC	Propionyl-CoA 0.5mM	$NaHCO_3$ 30mM ATP 3mM $MgCl_2$ 5mM	K_2HPO_4 80mM (pH 7.0)	1×10^6	0.3N $HClO_4$	NaH_2PO_4 (pH 2.5) 100mM Methanol 17%	Methylmalonyl-CoA
HMGL	3-Hydroxy-3-methyl-glutaryl-CoA 2mM	DTT 5mM $MgCl_2$ 20mM	Tris-HCl 100mM (pH 8.2)	1×10^6	0.3N $HClO_4$	NaH_2PO_4 (pH 2.5) 100mM Methanol 13%	Acetyl-CoA
BCKADC	2-Ketoisocaproic acid 7.5mM Coenzyme A Li_3 salt 7.5mM	TPP 1mM NAD^+ 4mM $MgCl_2$ 35mM	Tris-HCl 50mM (pH 7.5) EDTA 0.2mM	2×10^6 (#2)	0.3N $HClO_4$	NaH_2PO_4 (pH 4.0) 100mM Acetonitrile 15%	Isovaleryl-CoA
IVDH	Isovaleryl-CoA 1mM	PMS 2mM FAD 0.1mM	K_2HPO_4 80mM (pH 7.0)	1×10^6	0.3N $HClO_4$	NaH_2PO_4 (pH 4.0) 100mM Acetonitrile 13%	3-Methylcrotonyl-CoA
GDH	Glutaryl-CoA 3mM	PMS 2mM FAD 0.1mM	K_2HPO_4 80mM (pH 7.0)	2×10^6	0.3N $HClO_4$	NaH_2PO_4 (pH 2.5) 100mM Methanol 15.5%	3-Hydroxybutyryl-CoA
MCAD	n-Octanoyl-CoA 2mM	$FcPF_6$ 1mM	K_2HPO_4 80mM (pH 7.0)	1×10^6	Acetonitrile	NaH_2PO_4 (pH 4.0) 100mM Acetonitrile 28%	2-Octenoyl-CoA
VLCAD	Palmitoyl-CoA 2mM	PMS 2mM FAD 0.1mM	K_2HPO_4 80mM (pH 7.0)	1×10^6 (#3)	Acetonitrile	NaH_2PO_4 (pH 4.0) 100mM Acetonitrile 49%	2-Hexadecenoyl-CoA
調製液の容量**	50μl			50μl	100μl	移動相流速 1.5ml/min	紫外吸光度計で定量(260 nm)

＊ Mut; methylmalonyl-CoA mutase, PCC; propionyl-CoA carboxylase, HGML; 3-hydroxy-3-methylglutaryl-CoA lyase, BCKADC; branched-chain α-ketoacid dehydrogenase complex, IVDH; isovaleryl-CoA dehydrogenase, GDH; glutaryl-CoA dehydrogenase, MCAD; medium-chain acyl-CoA dehydrogenase, VLCAD; very-long-chain acyl-CoA dehydrogenase.
＊＊ BCKADC活性測定系では、試薬類の混合溶液と粗酵素溶液の容量は、それぞれ60μl, 40μlとしている。
#1 リンパ球を破砕する際の溶媒として0.25mM NaCN水溶液を使用。
#2 リンパ球を破砕する際の溶媒として50mM Tris-HCl (pH 7.5) + 0.2mM EDTA混合液を使用。
#3 リンパ球を破砕する際の溶媒として 0.4% タウロデオキシコール酸水溶液(界面活性剤)を使用。

性値(産物 pmol/min/細胞 10^6 個)を算出する。従って、ヘパリン血検体から 3×10^6 個 (MSUD, GA1 では 6×10^6 個) 以上のリンパ球を回収する必要がある。

新生児スクリーニングへ応用するには、採血量が現実的な範囲に収まらなければならない。この点について厳密な検討は行っていないが、当科へ精査来診した被験児からは通常 3ml 程度を採取している。依頼を受ける場合も同程度の採血量を要請しており、実際に届く検体量は 2-5ml といったところである。このように採取された 43 検体から回収されたリンパ球数 (平均± SD) は 13.1±5.1 × 10^6 個で、ごく一部のケースを除いて必要数以上が得られていた (図 1)。

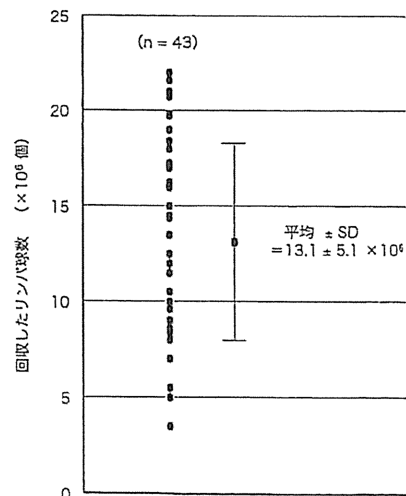

図1　タンデムマス・スクリーニング陽性となった新生児の血液検体から回収したリンパ球数

採血量の正確な記録はないが、新生児 43 例から 1 人当たり 2-5ml 程度のヘパリン加静脈血を採取して回収したリンパ球数の分布を示す。我々の酵素活性測定法では、被検者 1 名の診断に 3×10^6 個 (一部の酵素では 6×10^6 個) のリンパ球を粗酵素源として使用しており、3mL 程度の採血量があれば、ほぼ必要量が得られると思われる。

所要時間

リンパ球を分離してペレットを作成するのに約2時間を要している。そのまま酵素反応を進める場合、リンパ球の超音波破砕と反応溶液の調製に所要30分程度として、酵素によって10-60分反応させ、停止後に遠心10分間で除タンパクするので、測定サンプルができ上がるまでに1-2時間かかる。1サンプル当たりのHPLC分析時間は酵素により10-40分で、被験者と対照で計6サンプルを測定するには、1時間強で完了するもの（MSUD, MCADD）から4-5時間を要するもの（MMA）まで幅広くなっている。

以上を合計すると、被験児1名を酵素診断する所要時間は5-10時間程度となる。まとまった時間の確保が難しい場合は、リンパ球ペレットを凍結保存して酵素反応を後日に行っている。さらに測定サンプルも凍結保存ができるので、一部のサンプルのHPLC分析を翌日以降に分割するなどして、短い空き時間を有効利用するようにしている。

必要経費

酵素によって様々なビタミン類が必要になるが、金額的には基質となるアシルCoAがほとんど

表2　各種の補酵素A誘導体の試薬価格と1症例の酵素診断に要する経費

疾患	試薬	価格(円)（※Sigma 社製品）	(内容量)	1回調製量 (100μl)	酵素診断に要する経費(円/1症例)
MSUD	Coenzyme A Li3 salt	67,100	(1g)	75mM	395
PA	Propionyl-CoA	67,800	(25mg)	5mM	1,117
IVA	Isovaleryl-CoA	29,400	(10mg)	10mM	2,503
MCADD	n-Octanoyl-CoA	60,100	(25mg)	20mM	4,297
HMGLD	HMG-CoA	73,700	(25mg)	20mM	5,375
VLCADD	Palmitoyl-CoA	74,100	(25mg)	20mM	5,963
GA1	Glutaryl-CoA	27,800	(10mg)	30mM	7,352
MMA	Methylmalonyl-CoA	137,400	(25mg)	30mM	14,296

MSUD；メープルシロップ尿症，PA；プロピオン酸血症，IVA；イソ吉草酸血症，MCADD；中鎖アシルCoA脱水素酵素欠損症，HMGLD；HMG-CoAリアーゼ欠損症，VLCADD；極長鎖アシルCoA脱水素酵素欠損症，GA1；グルタル酸尿症I型，MMA；メチルマロン酸血症

(1) 分析時間が最も長いメチルマロン酸血症の場合の移動相（Nacarai 製）

NaH_2PO_4	¥1,700／500g	0.1M NaH_2PO_4	880mL
蒸留水	¥2,350／3L	＋メタノール	120mL
メタノール	¥3,400／3L	＝¥861／L	

流速 1.5mL/分×40分/サンプル×6サンプル＝360mL → 1例診断当たり¥310

(2) 有機溶媒使用量が最も多い VLCAD 欠損症の場合の移動相（Nacarai 製）

NaH_2PO_4	¥1,700／500g	0.1M NaH_2PO_4	510mL
蒸留水	¥2,350／3L	＋アセトニトリル	490mL
メタノール	¥6,400／3L	＝¥1,465／L	

流速 1.5mL/分×25分/サンプル×6サンプル＝150mL → 1例診断当たり¥329

図2　酵素反応のHPLC分析で消費する移動相溶液の経費

Nacarai社製品のカタログ価格に基づいた概算例を示す。メチルマロン酸血症とVLCAD欠損症以外の対象疾患の場合は、分析時間・有機溶媒使用量とも、両疾患の場合の中間となるため、必要経費はより低額となる。実際の移動相使用量は、サンプル分析前後の送液分や再測定の必要などから、計算例の2-3倍程度となることが多い。

を占める。Sigma社2009年版カタログ上の価格と、被験児1名の診断のために調製する使用量から求めた必要額を表2に示す。また、HPLC分析用の移動相については、分析時間が最も長いMMAと、有機溶媒濃度が最も高いVLCADDの場合での必要額を図2に示す。以上より、最も高額となるMMAの場合は、分析前後の移動相消費も見込むと以下のようになる。

Methylmalonyl-CoA（Sigma; ¥137,400/25mg）2.6mg　=約14,000円

Coenzyme B12（Sigma; ¥10,200/100mg）0.5mg　=約50円

移動相（蒸留水880mL＋メタノール120mL＋NaH_2PO_4 10g）500mL　=約450円

結局、被験児1名の酵素診断に必要な消耗品の経費のほとんどは、基質となるアシルCoAが占めており、従って概算額はほぼ表2の通りとなる。

他のランニングコストとしては、HPLCカラムの費用がかかる。各酵素に共通して使用する逆相カラムは、移動相の有機溶媒（メタノールまたはアセトニトリル）に応じて2本を交換して使用しており、測定頻度にもよるが、年1回程度更新すると2本で約10万円が必要になる。

有用性と課題

1．除外診断における有用性

スクリーニングで陽性となった新生児の両親・家族には、罹患・非罹患を問わず、できるだけ早期に診断を明確にすることが求められる。その場合、真の罹患児については、異常代謝産物の分析や遺伝子解析などの方法で、陽性所見に基づいて診断することができ、それは必ずしも難しいことではない。一方、異常代謝産物の蓄積が軽度であったり安定状態では消失するような場合や、遺伝子変異が同定困難あるいは新規の1塩基置換である場合などは、これらの方法では確定診断も除外診断も不明確になる。酵素活性測定は、被験児の状態や変異の種類に関係なく罹患・非罹患を区別することが可能で、特に酵素反応産物が十分に生成するという「陽性所見」で偽陽性例を積極的に除外診断できる点が特に優れている。

実際の応用結果として、広島県のタンデムマス新生児スクリーニング陽性例の診断結果を表3に示す。陽性32例中18例を罹患者と診断し、そのうち11例で酵素活性低下を確認した（※SCADDの2例は変異酵素発現系での活性測定結果であり[8]、CPT1D例の酵素活性測定は千葉県こども病院・高柳正樹先生による）。一方、除外診断された14例では6例が正常レベルの酵素活性値に依拠していた。また、グルタル酸尿症II型（GA2）疑いで非罹患とした2例中1例と、シトルリン低値でオルニチントランスカルバミラーゼ欠損症（OTCD）罹患者とは判断されなかった1例は、異常代謝産物の陰性所見だけが根拠となっており、完全に除外診断できたとは言い難い。GA2疑いで非罹患とした別の1例は、リンパ球β酸化能測定が実用化されていたため[9]、その正常所見によって除外診断することが可能であった。

広島県および国内各地のタンデムマス新生児スクリーニング陽性例（※およびMSUD疑い例）の酵素診断結果をまとめたものが表4である。52例中35例を酵素欠損症罹患者と判定したが、裏返せば除外診断を要するケースが約1/3もあったことになる。このように、タンデムマス新生児スクリーニングでは非罹患者の除外診断も大きな課題であり、その解決には酵素診断が最適と言える。

2．残存活性の定量的評価

酵素診断では、残存活性の高さによって重症度を推定できることが期待されるところである。当科の酵素診断法のうち、この面で最も有用性が高いと考えられるのはMCADDについてのものである。図3に示すように、急性発症後に診断された罹患児群の残存活性は、1例を除いて正常対照平均値の5%未満に分布しているのに対し、発症前に罹患者と診断された群の残存活性はより高い値まで広がりを示しており、新生児スクリーニングでは多様な重症度の症例が発見されうることが示唆された。

一方、有症状例の診断依頼数が最も多いMMAの場合は、遅発性で最重症型ではないと考えられる症例でも、我々の方法では酵素活性値が測定感

表3　広島県のタンデムマス新生児スクリーニング陽性例の診断結果

	疾患	陽性	罹患	確定診断所見		除外診断所見		除外例に関する備考
有機酸代謝異常症	PA	7	4	尿中有機酸異常	4	酵素活性正常	3	
				酵素活性低下	4			
				遺伝子変異同定	3			
	MMA	2	1	尿中有機酸異常	1	酵素活性正常	1	
				酵素活性低下	1			
	HCSD	1	1	尿中有機酸異常	1			
				遺伝子変異同定	1			
	MCG	2	0			尿中有機酸正常	2	母体酵素欠損に起因する一過性の異常と判定。
	小計	12	6					
脂肪酸代謝異常症	MCADD	5	3	尿中有機酸異常	2	酵素活性正常	2	2例中1例は低出生体重児でMCTオイルを投与されていた。
				酵素活性低下	3			
				遺伝子変異同定	2			
	GA2	4	2	尿中有機酸異常	2	尿中有機酸正常	2	1例は完全な除外診断には至っていない。
						β酸化能正常	1	
	CTD	3	0			カルニチン補充→中止後再低下なし	3	2例はNICU管理中の栄養不良による低カルニチン血症と判断。1例はリスクなし。
	SCADD	2	2	尿中有機酸異常	2			
				酵素活性低下	2			
				遺伝子変異同定	2			
	CPT1D	1	1	酵素活性低下	1			
				遺伝子変異同定	1			
	小計	15	8					
尿素回路異常症	OTCD	3	2	血中アミノ酸異常	2	血中アミノ酸正常	1	完全な除外診断には至っていない。
				尿中有機酸異常	2	尿中有機酸正常	1	
				遺伝子変異同定	1			
	ASA	1	1	血中アミノ酸異常	1			
				尿中有機酸異常	1			
	CD	1	1	遺伝子変異同定	1			
	小計	5	4					
合計		32	18	1999年4月—2010年1月 受検総数221,506人(受検率 75.4%)				患者発見率　1/12,305人

PA；プロピオン酸血症，MMA；メチルマロン酸血症，HCSD；ホロカルボキシラーゼ合成酵素欠損症，MCG；3-メチルクロトニルグリシン尿症，MCADD；中鎖アシルCoA脱水素酵素欠損症，GA2；グルタル酸尿症Ⅱ型，CTD；カルニチントランスポーター異常症，SCADD；短鎖アシルCoA脱水素酵素欠損症，CPT1D；カルニチンパルミトイルトランスフェラーゼ-Ⅰ欠損症，OTCD；オルニチントランスカルバミラーゼ欠損症，ASA；アルギニノコハク酸尿症，CD；シトリン欠損症

度以下となるケースが多く、重症度の推定は困難な結果となっている。MMAの場合、放射性同位体標識基質を使用する従来法に比べて、HPLC法では感度が低いものと思われ、基質や酵素量（細胞数）を増やすことで改善できるかも知れないが、費用や採血量の問題があり実現できていない。