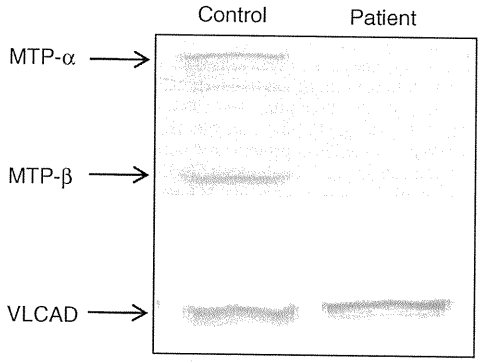

Fig. 1. Western blot analysis of cultured skin fibroblasts of mitochondrial trifunctional protein. MTPα- and MTPβ- were not detectable in the patient's fibroblasts.MTPα- and MTPβ-, α- and β-subunits of MTP, respectively; VLCAD, very long-chain acyl-CoA dehydrogenase.

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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Clinical and molecular investigation of 19 Japanese cases of glutaric acidemia type 1

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ABSTRACT

Glutaric acidemia type 1 (GA1) is a metabolic disease caused by a deficiency of glutaryl-CoA dehydrogenase (GCDH). Untreated patients mostly develop severe striatal degeneration. More than 200 mutations have been reported in the *GCDH* gene, and common R402W and IVS10-2A>C were found in Caucasian and Chinese/Taiwanese, respectively. However, in Japan, genetic mutations have only been reported in a few cases. Herein, we report the clinical and molecular basis of GA1 in 19 Japanese patients, including six previously reported patients. All cases showed high urinary glutaric acid excretion. Eleven patients were severely impaired (three patients died), three had mild impairment, and five showed normal development. Four of 5 patients that developed normally were detected in the presymptomatic stage by neonatal or sibling screening. Nineteen mutations in 26 alleles were identified, and eight of them (89 or 90delC, Y155C, IVS4+2T C, G244S, Q352X, G354A, K361E, and 1144-1145delGC) were novel. S305L (12.1%, 4/34 alleles) was found in several cases, suggesting that this mutation is a common mutation. In contrast, R402W was not identified and IVS10-2A>C was only found in one allele, suggesting that Japanese patients with GA1 show allelic heterogeneity and have a different genetic background to patients from other countries. One of a pair of sisters with the same mutations (M339V/S305L) lacking residual activity was severely retarded, whereas the older girl remains asymptomatic at 22 years of age, indicating that genotype does not necessarily predict GA1 phenotype. We consistently found that there was no association between genotype and phenotype. However, children with mild impairment were diagnosed and treated earlier than severely impaired cases {4.7 ± 2.5 months (range: 2–8 months) vs. 11.6 ± 12.7 months (range: 4–51 months)}. Our results suggest that early detection and treatment but not genotype are associated with better patient outcome, reinforcing the importance of neonatal screening.

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1. Introduction

Glutaric aciduria type 1 (GA1, OMIN 231670) is an autosomal recessive metabolic disorder caused by deficiency of glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) [1,2]. GCDH is located in the mitochondrial matrix and acts in the intermediate steps of lysine, hydroxylysine, and tryptophan metabolisms [3]. The clinical manifestations of GA1 include extrapyramidal symptoms, developmental regression, and macrocephaly, appearing most often after acute encephalopathic crises, which are accompanied by bilateral marked enlargement of the sylvian fissure and degeneration of the striatum [1], and in addition, extrastriatal abnormalities [4] and abnormal hemodynamic changes [5]. Its biochemical characteristics include the accumulation of glutaric acid (GA), and 3-hydroxyglutaric acid, which can be detected by gas chromatography (GC/MS), and glutarylcarnitine, which can be identified by electrospray ionization/tandem mass spectrometry (MS/MS) [1,2]. It has been reported that GA1 can be classified into two types based on the level of excreted GA: the high excretion form (GA > 100 mmol/mol creatine) and the low excretion form (GA < 100 mmol/mol creatine) [6].

Since GA1 was first described in 1975 [3], more than 200 different mutations have been reported [7–9], and its frequency was estimated to be approximately 1 in 100,000 newborns [2]. Although almost all mutations are private, several common mutations have been identified, including A421V in the Amish Community [10], IVS 1+5G T in Canadian Oji-Cree Indians [11], and E365K in Irish travelers [8]. R402W is the most frequent mutation in the European population [6,8], and IVS10-2A C is relatively common in China [12] and Taiwan [13]. In Japan, the frequency of GA1 has been estimated to be approximately 1 in 210, 000 newborns, based on a newborn screening pilot study [14,15]. However, mutations have only been characterized in a few cases [16] since the first description of a Japanese case in 1987 [17]. Herein, we investigated the clinical and molecular aspects of 19 Japanese patients with GA1.

2. Subjects and methods

2.1. Subjects

We studied 19 Japanese patients who were diagnosed with GA1 based on their urinary organic acid profiles and/or blood acylcarnitine

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analysis. The diagnoses were confirmed by analyzing the *GCDH* gene and/or GCDH activity.

The mutations of 6 cases (cases 2–5, 12, and 19) were reported previously (cases 4, 12, and 19: [16], cases 2, 3, and 5: Japanese domestic journal). In this study, we analyzed the mutations in 13 cases (cases 1, 6–11, and 13–18). Among the 13 patients, 4 cases (case 6, 7, 10, and 11) were previously described in case reports [18,19]. No family demonstrated consanguineous marriage.

2.2. DNA sequencing

Genomic DNA was isolated from skin fibroblasts using a Qiamp DNA Microkit (QIAGEN GmbH, Hilden, Germany) and from peripheral blood lymphocytes using the DNA Quick II kit (Dainippon Pharmaceuticals, Osaka, Japan). Each exon of *GCDH* including the intron/exon boundaries was PCR-amplified for 30 cycles using the conditions shown in Supplemental Table 1. The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany) and sequenced using the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA) or the CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA, USA). The structure of the human *GCDH* gene was obtained from the GenBank database (ENSG00000105607). Informed consent to perform DNA analysis was obtained from the parents of the patients. Our study protocol was approved by the Ethics Committee of the Shimane University Faculty of Medicine.

3. Results

3.1. Clinical characteristics

The clinical features of 19 Japanese GA1 patients (10 boys and 9 girls) are summarized in Table 1. Cases 4 and 19 and cases 15 and 18 were siblings. Fifteen of the 19 cases were symptomatic patients. Three (cases 1–3) of 19 cases were detected in a newborn screening pilot study, and one (case 4) was an asymptomatic sibling case that was detected at 2 years of age. To evaluate their outcomes, we classified them into three groups based on disability score [20] that included motor disability, cognitive function, and speech: a) the severe handicap group (disability score 7–9), b) the mild impairment group (disability score 4–6), and c) the normal developmental group (disability score 3) (Supplemental Table 2).

Eleven of the 19 cases were classified into severe handicap group (three of them died), 3 cases belonged to mild impairment group, and 5 cases showed normal development (Fig. 1). The mean age at onset of the symptomatic cases was 5.7 m (range: 4–8 m) in the severe handicap group, 2.3 m (range: 2–3 m) in the mild impairment group, and 6 m in case 4 of the normal development group who suffered from macrocephaly. The mean age at diagnosis was 11.6 m (range: 4–51 m) in the severe handicap group, 4.7 m (range: 2–8 m) in the mild impairment group, and 27 m (range: 24–30 m) in the normal development group, except for the 3 cases diagnosed by newborn screening. Macrocephaly was observed in 31.6% of patients (6/19). All 19 cases showed high urinary glutaric acid excretion. Cranial CT and/or MRI demonstrated frontotemporal atrophy and striatum signal abnormalities in all cases involving mild impairment or severe handicap. In contrast, three of five cases in the normal development group demonstrated mild changes by neuroimaging.

3.2. Clinical manifestations of patients

No cases had a past history except for cases 1, 6, 7, and 9. None of the cases showed abnormal development before the onset of GA1. Immediately after the diagnosis of GA1, all cases were treated with dietary restriction, L-carnitine administration, and prompt intravenous fluid infusions for catabolic states such as recurrent vomiting and diarrhea. In addition, a GABA analogue and vitamin B2 were given to the 14 and 8 cases, respectively.

3.2.1. Normal development group

Cases 1–3 were detected prior to displaying any specific symptoms by a newborn screening program using MS/MS. Case 1 weighed 2952 g when she was born at a gestational age of 39 weeks and 2 days. Abruptio placentae occurred during her birth and she suffered from asphyxia (Apgar score: 3/4). She recovered following hypothermia treatment for hypoxic–ischemic encephalopathy. Cases 2 [21] and 3 [21] had no remarkable delivery events. In these 3 cases, no signs of neurologic complications were evident at 4 months, 5 years, and 7 years old, respectively.

Case 4 was the nonsymptomatic older sister of case 19, who was severely handicapped [16]. She was diagnosed with GA1 by a sibling GC/MS screening in the presymptomatic stage at 2 years old.

Case 5 was hospitalized because of macrocephaly (47.6 cm, +2.5 S.D.) at 6 months. There was no sign of neurologic complications or developmental delay, but cranial CT suggested a subarachnoid cyst and a subdural hematoma. Thereafter, the subarachnoid cyst and subdural hematoma became smaller. At 2.5 years, he was referred to the pediatric department due to progressive macrocephaly (56.5 cm, +3.0 S.D.). Brain CT demonstrated widening of the Sylvian fissures, which in fact had been found by CT at 7 months.

3.2.2. Mild impairment group

Case 6 was treated for initial vomiting and idiopathic hyperbilirubinemia during the neonatal period [18]. Screening by brain echography identified dilated ventricles.

Case 7 was delivered at 27 weeks of dizygotic twin gestation [18]. His birth weight was 998 g. Macrocephaly and convulsions were noticed at 2 and 3 months, respectively. Following treatment, his development caught up.

In case 8, progressive macrocephaly was noticed at 3 months old. Her head circumference was +5.0 S.D. at 7 months old. Her regression and hypotonia, which were accompanied by seizures at 8 months old, improved gradually after treatment.

3.2.3. Severe handicap group

Cases 10, 11, and 13 died. Case 10 displayed a lack of head control at 4 months old [17,18] and irritability and sleeplessness at 5 months old. She died suddenly at 5 years old after developing a common cold. Cases 11 [19] and 13 presented encephalitis-like disease at 5 and 7 months, respectively. Case 11 died suddenly at the age of 3 years. Case 13 died of airway obstruction due to choking after developing an infection at 3 years old.

Similarly, no treatment was effective for the neurological symptoms of the severely handicapped patients that survived, all of whom are bedridden, require tube feeding, and smile spontaneously. Case 9 was born at 35 weeks with an Apgar score of 6/9 by cesarean delivery for premature membrane rupture and breech presentation. His birth weight was 2235 g. He was diagnosed with GA1 at 4 months after an episode of convulsions. He required mechanical ventilation and a tracheostomy for respiratory distress at 10 months old. Case 12 suffered from encephalitis-like symptoms including convulsions, unconsciousness, and rigidity following fever and an upper respiratory tract infection at 5 months old [16]. Case 14 was affected with Kawasaki disease at 5 months old. Intravenous immunoglobulin resulted in rapid defervescence, but his regression, involuntary movement, and irritability accompanied by fever were irreversible. Case 16 was affected by viral encephalitis with hyperpyrexia, consciousness disturbance, and hypertonia at 7 months of age. Case 17 was found to have subependymal pseudocysts and temporal lobe hypoplasia at 1 month. Transient regression was observed at 7 months after gastroenteritis. Thereafter, progressive neurological regression, hypotonia, and rigidity were observed following convulsions associated with pneumonia at 8 months. Case 19 was the younger sister of

Table 1
Clinical manifestations and genetic characteristics of Japanese patients with glutaric acidemia type 1.

Case I.D	Sex	Age at onset	Age at diagnosis	Precipitating factor	Clinical symptoms	Macrocephaly	Treatment	Outcome	Urine GA	C5DC (<0.3)	Neuroimaging	Exon or intron affected	Base change	Effect	GCDH activity
Normal development group															
(Newborn screening cases)															
1	F	–	1m	None	Normal development	–	L-carnitine	Normal (4m)	High	1.08	Typical	**Exon9 / Exon10**	**1064 G>A / 1147 C>T**	**R355H/R383C**	N.D
2	F	–	1 m	None	Normal development	–	L-carnitine	Normal (5y4m)	High	2.22	Mild	Exon6 / Exon8	556 A>T / 914 C>T	S186C / S305L	Deficiency
3	F	–	1 m	None	Normal development	–	L-carnitine	Normal (7y6m)	High	1.95	Mild	Exon3 / Exon10	215 G>T / 1237 T>G	R72L /Y413D	Deficiency
(Sibling screening cases)															
a 4	F	–	2 y 0 m	None	Normal development	–	L-carnitine, GABA analogue	Normal (22y)	High	N.D	Mild	Exon8 / Exon9	914C >T / 1015A> G	S305L / M339V	Deficiency
(other cases except for screening)															
5	M	6	2 y 6 m	None	Normal development	+	L-carnitine, vitamin B2	Normal (6y11m)	High	4.4	Typical	Exon5 / ?	416C >T / ?	S139L / ?	Deficiency
Mild impairment group															
6	M	2 m	2 m	None	Enlargement of ventricles	+	L-carnitine, vitamin B2, GABA analogue	Mild (23y)	High	N.D	Typical	**Exon8 / Exon10**	**914C >T / 1147 C>T**	**S305L / R383C**	Deficiency
7	M	2 m	4 m	None	Seizure	+	L-carnitine, GABA analogue	Mild (25y)	High	N.D	Typical	**Exon5 / Exon5**	**413G>A / 416C>T**	**R138K / S139L**	Deficiency
8	F	3 m	8 m	None	Seizure, regression	+	L-carnitine, GABA analogue, antiepileptic	Mild (3y2m)	High	3.36	Typical	**Exon8/ Exon9**	**914C >T / 1081A> G**	**S305L / K361E**	N.D
Severe handicap group															
9	M	4 m	4 m	None	Seizure, regression	+	L-carnitine, vitamin B2, antiepileptic	Severe (1y4m)	High	N.D	Typical	**Intron4 / Exon6**	**IVS4+2T>C / 532G>A**	**Truncated (Splicing) / G178R**	N.D
10	F	4 m	7 m	None	Regression, irritability, sleeplessness, dystonia	–	L-carnitine, vitamin B2, GABA analogue	Severe (5y:died)	High	N.D	Typical	**Exon9 / Exon9**	**1054C > T / 1054C> T**	**Truncated(Q352stop) / Truncated(Q352stop)**	Deficiency
11	M	5 m	6 m	None	Seizure, regression, hypotonia, dystonia	–	L-carnitine, GABA analogue	Severe (3y:died)	High	N.D	Typical	**Exon3 / Exon7**	**226C>T / 730G>A**	**Truncated (Q76stop) / G244S**	Deficiency
12	M	5 m	6 m	Infection, fever	Seizure, dystonic	–	L-carnitine, vitamin B2, GABA analogue	Severe (14y6m)	High	N.D	Typical	Exon9 / Exon9	1064G >A / 1064G >A	R355H / R355H	N.D
13	F	5 m	7 m	Infection	Seizure, regression, hypertonia	–	L-carnitine, GABA analogue	Severe (3y9m: died)	High	N.D	Typical	**Exon9 / Intron10**	**1061G > C / IVS10-2 A>C**	**G354A / Truncated (splicing)**	Deficiency
14	M	5 m	7 m	Kawasaki disease	Regression, dystonia	–	L-carnitine, GABA analogue, antiepileptic	Severe (7y)	High	1.76	Typical	**Exon5 / Exon7**	**416C >T / 769C>T**	**S139L / R257W**	Deficiency
b 15	M	5 m	4 y 3 m	Fever of unknown origin	Regression, dystonia	–	L-carnitine, vitamin B2, GABA analogue	Severe (5y2m)	High	0.38	Typical	**Exon1 / Exon5**	**89 or 90delC / 461A>G**	**Truncated (frame shift) / Y155C**	N.D
16	M	7 m	7 m	Infection, fever	Unconscious, dystonia	–	L-carnitine, GABA analogue	Severe (1y1m)	High	0.57	Typical	**Exon10 / Exon11**	**1144-1145delGC / 1298C>T**	**Truncated (frame shift) / A433V**	N.D
17	M	7 m	12 m	Gastroenteritis	Seizure, regression, dystonia, hypotonia	+	L-carnitine, GABA analogue, antiepileptic	Severe (2y5m)	High	N.D	Typical	**Exon5 / Exon10**	**383G>A/ 1147C>T**	**R128Q / R383C**	Deficiency
b 18	F	8 m	9 m	Polio vaccine, infection, fever	Regression, dystonia	–	L-carnitine, vitamin B2, GABA analogue	Severe (1y8m)	High	0.41	Typical	**Exon1 / Exon5**	**89 or 90delC / 461A>G**	**Truncated (frame shift) / Y155C**	Deficiency
a 19	F	8 m	12 m	Gastroenteritis	Coma, dystonia	–	L-carnitine, vitamin B2, GABA analogue	Severe (20y)	High	N.D	Typical	Exon8 / Exon9	914C >T / 1015A>G	S305L / M339V	Deficiency

Abbreviations: a,b, siblings; M, male; F, female; Age, y (years); m (months); Treatment, except for dietary restriction ; GA, glutaric acid; C5DC, glutarylcarnitine in dried spots (nmol/ml) when the patient was diagnosed; GCDH, glutaryl-CoA dehydrogenase.
Novel mutations are underlined. The mutations highlighted in bold were identified in this study; Deficiency: GCDH activity ≤5%.
N.D: not determined.

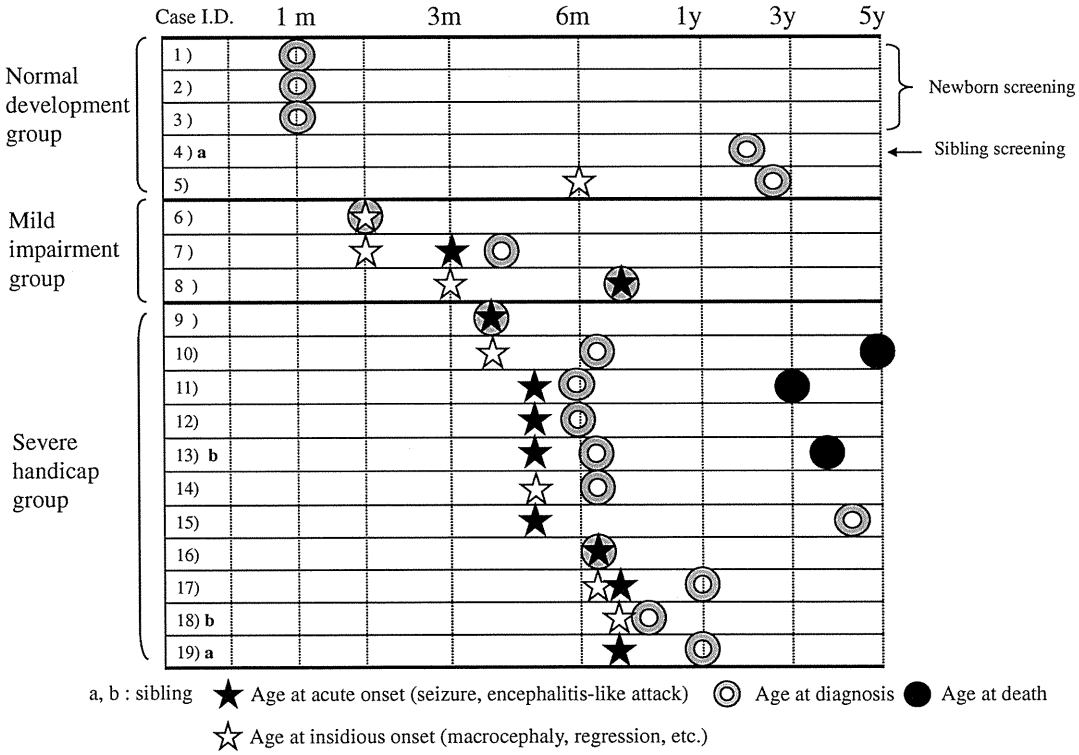

Fig. 1. Age at onset and diagnosis in three groups with different outcomes. The mild impairment group was diagnosed earlier than the severe handicap group {4.7 m (2–8 m) vs. 11.6 m (4–51 m)}. Three cases (cases 10, 11, and 13) died.

case 4. At 8 months old, she suffered an encephalopathic crisis after gastroenteritis, which lasted for several days [16]. Cases 15 and 18 were siblings. Case 15, the older brother, was hospitalized for fever of unknown origin at 5 months of age and treated with antibiotics for 10 days. In addition to hypotonia, which appeared at the time of discharge, his regression, rigidity, and involuntary movement worsened every month that he suffered from fever. Although idiopathic encephalopathy was initially suspected, a diagnosis of GA1 was made in a sibling screening program by GC/MS, and treatment was initiated at 4 years and 3 months. Case 18 suffered from fever after polio vaccination at 8 months. Thereafter, she became unable to support her head and roll over. Her neurological skills deteriorated every month that she suffered from fever. The diagnosis of GA1 was made by GC/MS at 9 months of age.

3.3. Gene mutations in GCDH

Nineteen mutations were identified in 13 cases, and 8 of them were novel. These included four missense mutations (Y155C, G244S, G354A, and K361E), a nonsense change (Q352X), a splice site alteration (IVS4+2T>C), and frame shift mutations (89 or 90delC, and 1144-1145delGC). These novel mutations were not detected in 100 chromosomes from unaffected Japanese individuals.

All mutations are summarized in Table 1 and Supplemental Fig. 1, together with information on 6 cases whose genetic alterations were reported previously ([16] and Japanese domestic journal). Only two unrelated patients out of 19 cases had homozygous mutations (Q352X, R355H). In 34 independent alleles, the frequency of S305L was 12.1% (4/34 alleles), S139L, R355H, and R383C had frequencies of 8.8% (3/34 alleles), respectively and Q352X were found in 2 alleles (5.8%) each. Another 19 mutations were only found in a single allele.

4. Discussion

Since it has been remaining unknown whether there are common mutations and a phenotype/genotype correlation in Japanese GA1 cases, we investigated the relationship between clinical and mutational spectrums of 19 Japanese patients with GA1. Japanese are relatively homogenous ethnic population on islands isolated from other countries. We found a few common mutations distinct from other nations. We also found that mutations in Japanese cases are different from what have been reported in the Caucasian cases, indicating specific genetic information unique for Japanese cases are crucial for their diagnosis in the future. The current study also indicates that earlier detection of the disease followed by appropriate medicare is crucial for the better outcome than the genotype, reinforcing the importance of neonatal screening for GA1. This is a first report that studied the largest cohort of Japanese patients with GA1.

In this study, we identified 19 mutations in 24 independent alleles including eight novel mutations. The amino acids affected by these new mutations are highly conserved among different species (Pan troglodytes, mice, Xenopus, and Bordetella parapepertusis) including humans, suggesting that the region plays an important functional role in GCDH activity. It is highly likely that Q352X, 89 or 90 delC, 1144-1145delGC, and IVS4+2T C abolish GCDH activity, because these mutations result in truncation of the peptide. G354S and Y155H, which affect the same positions as G354A and Y155C, respectively, were reported to have no enzymatic activity [6,7]. The homology of the peptide's structure indicates that G244, G354, and K361 are conserved in the acyl-CoA dehydrogenase group [22]. These findings suggested that all 8 novel mutations in this study have little GCDH activity. In the 19 Japanese cases of GA1 including 6 previously reported patients, Q352X and R355H were homozygous mutations and found in 2 alleles. The frequency of S305L was 12.1% (4/34 alleles), suggesting that this mutation is common in Japanese, in contrast to the very few reports of this mutation from other countries. S139L, R355H, and R383C were also found on 8.8% (3/34 alleles), respectively, implicating that these mutations may be also common, respectively. Additionally, mutations in exon 9 were found more frequently in Japanese GA1 compared with the report by the HGMD (http://www.hgmd.cf.ac.uk/ac/index.php). It is highly likely that understanding common mutations

will facilitate rapid and accurate diagnosis of Japanese cases with GA1. Furthermore, this information may be useful for other Asian countries as well, since some of them are shared with patients from other Asian countries. Newborn screening using MS/MS is becoming popular, and the number of patients will become larger in Asian countries [23] as well as the other countries [24–26]. R402W, the most common mutation in Caucasians, in whom it shows an allele frequency of 12–25% [6,8], was not found in our Japanese cases. IVS10-2A C, a common mutation in China (30%, 3/10 alleles) [10] and Taiwan (66.7%, 4/6 alleles) [13], was also only found in a single allele in our study. Collectively, these findings suggest that Japanese GA1 patients show allelic heterogeneity and have different genetic backgrounds to GA1 patients from other countries. However, S139L, R355H and G178R, in addition to IVS10-2A C, may be common mutations among oriental populations, since S139L have been discovered in 2 of 4 alleles in Korean cases [27], and R355H and G178R were detected in one allele in Chinese case, respectively [10].

All 19 cases demonstrated a high-excretor phenotype in urinary organic acid analysis by GC/MS, suggesting that their mutations resulted in lower enzyme activity (≤5%) [6]. In fact, an enzyme assay confirmed 0–5% residual GCDH activity in 11 cases (Cases 2–7, 10–11, 13–14, and 19) [19,21,28,29]. Furthermore, an *in vitro* probe assay using cultured fibroblasts and MS/MS demonstrated a deficiency of GCDH in 10 cases (cases 4, 6, 7, 10, 11, 13–14, and 17–19) [30]. Although all 19 cases were assumed to have barely detectable enzyme activity, their clinical outcomes were diverse, ranging from normal development, through mild impairment, to severe handicap. This study suggests that the phenotypes of Japanese GA1 patients are not associated with a specific genotype. A previous study also showed that there is no clear correlation between genotype, biochemical phenotype, and the clinical severity of GA1 [6,24]. Frequency (31.6%: 6/19 cases) of macrocephaly of this study is lower than other reports (65–75%) [2,31]. This may represent unique phenotype in Japanese patients with GA1, which have genetic backgrounds distinct from other nations. However, additional case studies are warranted to validate whether this is indeed the cases.

All symptomatic cases except for case 5 had mild impairment or severe handicap indicating that the neurological sequelae of symptomatic cases are poor in Japanese GA1 patients, as reported in previous cases [24,31–33]. With respect to the grounds for the neurological manifestation, we were not able to completely rule out hypoxic–ischemic encephalopathy, hyperbilirubinemia, prematurity, very low birth weight, or encephalitis. However, since there was no sign of neurologic complications or developmental delay before the onset in any cases, we suspect that the neurological symptoms are not a consequence of these conditions. Importantly, the mild impairment group was diagnosed earlier than the severe handicap group {4.7 ± 2.5 m (2–8 m) vs. 11.6 m ± 12.7 m (4–51 m)}, suggesting that a better outcome was induced by early diagnosis. The reason for the better outcome seen in the patients who were diagnosed younger age was considered that early diagnosis led to an earlier initiation of the treatment and/or intervention in a timely manner for any medical conditions, which in turn prevented patients from neurological impairment. The frequency of macrocephaly was higher in the mild impairment group (3/3 cases) than in the severe impairment group (2/11 cases), making it likely that macrocephaly led to an early diagnosis of GA1. Furthermore, there was a notable difference in the phenotypes of siblings with the same mutations: case 4 showed normal development, whereas case 19 showed severe retardation (Supplemental Fig. 2), indicating that genotype does not predict clinical outcome. Taken together, these findings strongly suggest that early diagnosis and treatment but not genotype are associated with a better patient outcome.

Because the diagnosis was made by newborn screening only in 15.8% (3/19 cases), there is no direct evidence that newborn screening has neuroprotective effect for the patients with GA1 in this study. However, our study indicates that genotype does not necessarily predict clinical outcome and that early diagnosis and treatment are critical for a better outcome. While indirect findings, these observations strongly suggest that earliest diagnosis by the newborn screening will also be beneficial for a better outcome. In this regard, it is very important to expand newborn screening by MS/MS to improve the outcome of Japanese GA1 patients.

Supplementary materials related to this article can be found online at doi:10.1016/j.ymgme.2010.11.159.

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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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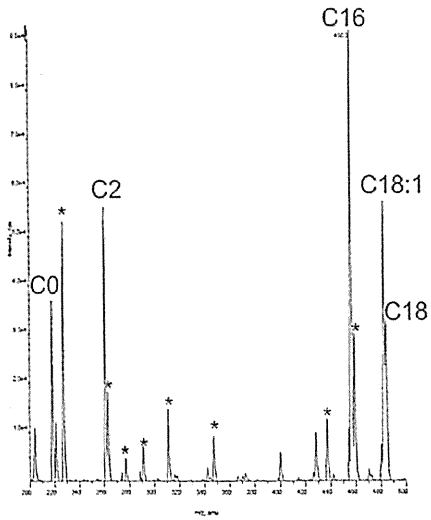

C0 14.16 (下限カットオフ値10.0)
C2 9.7 (下限カットオフ値5.0)

C16 6.64 (上限カットオフ値7.0)
C18:1 2.64 (上限カットオフ値3.2)
C18 2.08 (上限カットオフ値3.2)

C18:1+C16／C2 0.96
(上限カットオフ値0.35)

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2.64μmol/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

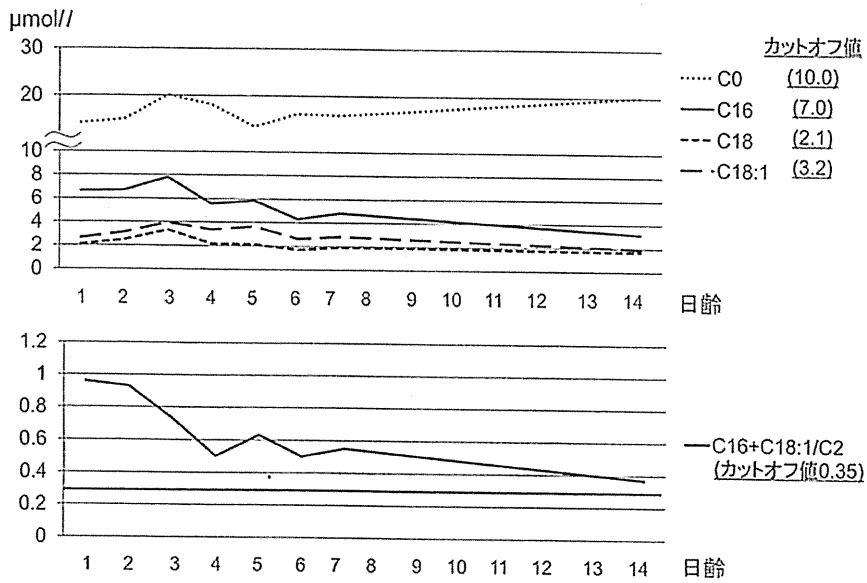

図2　血液ろ紙アシルカルニチンプロファイルの推移
上段の図では横軸は日齢，縦軸は各アシルカルニチン量．括弧内に上限または下限カットオフ値を示した．C16，C18，C18：1はいずれも日齢3にピークとなり，その後漸減した．遊離カルニチンは明らかな低値を認めない．下段の図は（C16＋C18：1)/C2を縦軸に示した．図中にカットオフ値を直線で示した．出生直後が最も高く，日齢14まで上限値を超えている．

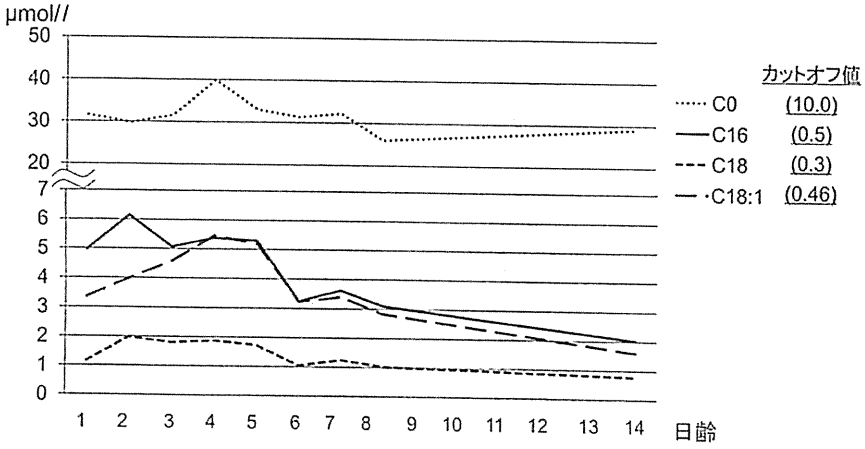

図3　血清アシルカルニチンプロファイルの推移
横軸は日齢，縦軸は各アシルカルニチン量．括弧内に上限または下限カットオフ値を示した．日齢1より明らかな長鎖アシルカルニチンの増加を認める．C16，C18，C18：1は日齢4にピークとなりその後低下した．遊離カルニチンは明らかな低値を認めない．

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.