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Ⅳ. 研究成果の刊行物・別刷

研究成果の刊行に関する一覧表

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雑誌　　（謝辞に本研究補助金の記載のあったもののみを抜粋）

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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.

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.

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.

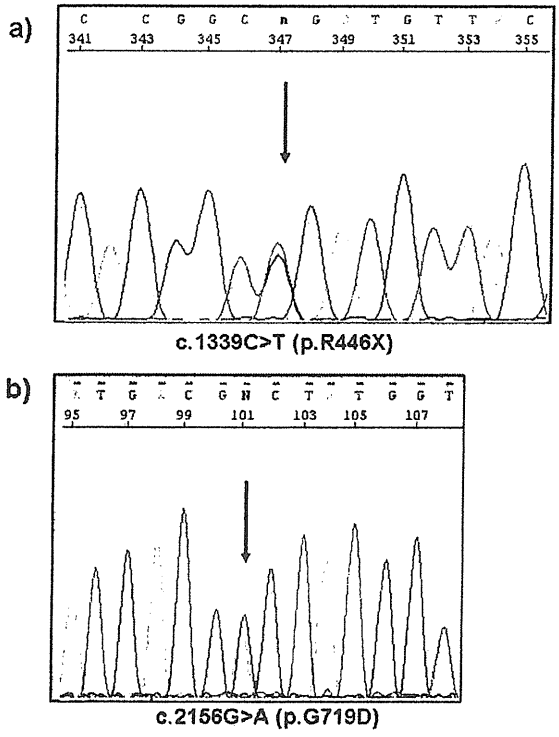

Fig. 1. (a) A C-to-T substitution at c.1139 in exon 11 was detected in a heterozygous pattern. This c.1339C > T substitution created a stop codon (p.R446X). (b) A G-to-A substitution at c.2156 in exon 18 was found in a heterozygous pattern. The c.2156G > A substitution changed the codon of glycine at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

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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NEWBORN SCREENING

Useful second-tier tests in expanded newborn screening of isovaleric acidemia and methylmalonic aciduria

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Abstract Common use of pivalate-generating antibiotics in newborns in Japan and low cutoff value of C5-acylcarnitine (C5) to detect mild forms of isovaleric acidemia (IVA) led to 1,065 positive results from IVA screening among 146,000 newborns tested by tandem mass spectrometry over the last 3 years. Using our method to determine isovalerylglycine (IVG) levels in dried blood spots (DBS) as a second-tier test with IVG cutoff value of 0.5 nmol/ml in DBS, one patient with severe IVA was identified, and no recall of the second DBS was needed. Retrospective analysis revealed that most patients with moderate to severe forms of IVA have decreased free-carnitine levels shortly after birth and higher levels of IVG than those of C5, which suggests that this method is useful in evaluating the severity of IVA. Another second-tier test, to measure methylmalonic acid (MMA) levels in DBS by gas chromatography/mass spectrometry (GC/MS), has been developed to overcome difficulties in screening methylmalonic aciduria (MMAU) and propionic acidemia. Methanol extract from DBS was dried and derivatized using N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide. GC/MS was performed using splitless injection, electron-impact ionization, and selected ion monitoring for data recording. MMAU patients had much higher DBS concentrations of MMA (24.2–321.9 nmol/ml) than control newborns (0.34±0.11 nmol/ml). MMA measurement in DBS was thought to provide useful information about the severity of MMAU, as MMAU patients with high levels of MMA had decreased levels of free carnitine and mildly increased levels of propionylcarnitine.

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Abbreviations

C2	Acetylcarnitine
C3	Propionylcarnitine
C5	Acylcarnitine with 5-carbon-atom acyl group
DBS	Dried blood spot
GC/MS	Gas chromatography-mass spectrometry
IVA	Isovaleric acidemia
IVG	Isovalerylglycine
LC	Liquid chromatography
MS/MS	Tandem mass spectrometry
MMA	Methylmalonic acid
MMAU	Methylmalonic aciduria
PA	Propionic acidemia
PGA	Pivalate-generating antibiotics
SIM	Selected ion monitoring

Introduction

In newborn screening by tandem mass spectrometry (MS/MS), screening markers are not always specific to the target

diseases, and confirmation tests, such as urinary organic acid analysis, are needed. Second-tier tests using the first dried blood spots (DBS), which provide diagnostic information more specific to the designated disorders, are thought to contribute to reduced recall rates and useful for therapy of patients with early-onset diseases. In newborn screening for isovaleric acidemia (IVA, OMIM #243500), the level of C5-acylcarnitines (C5) is the primary screening marker, although the isomers of isovalerylcarnitine as an indicator of IVA and pivaloylcarnitine, which is derived from pivalate-generating antibiotics, are not determined separately. In Japan, common use of these antibiotics to newborns resulted in relatively high false-positive rates (0.5–1%) for IVA (Shigematsu et al. 2007). In addition, patients with a mild biochemical form of this disease had C5 levels <1.0 nmol/ml (Ensenauer et al. 2004), and low cutoff value in order to detect this form may cause the increase of false-positive rates. Thus, we developed a second-tier test in which isovalerylglycine (IVG) levels in DBS is measured and tested its usefulness in our pilot study of newborn screening.

In newborn screening for methylmalonic acidurias (MMAUs)—including methylmalonyl-CoA mutase deficiency (OMIM #25100), cobalamin disorders, and propionic acidemia (PA, OMIM #25100)—the level of propionylcarnitine (C3) is the primary marker. Because of the unfavorable balance between specificity and sensitivity of C3, ratios of C3 to various other acylcarnitine species, such as C3/acetylcarnitine (C3/C2), have been used as secondary or primary parameters (Lindner et al. 2008). Despite the use of these parameters, further differential diagnosis among the disorders mentioned above was not possible, and a liquid chromatography (LC)-MS/MS method was used to identify methylmalonic acid (MMA) and 3-hydroxypropionic acid in DBS as a second-tier test (la Marca et al. 2007). We developed a sensitive method using GC/MS to measure MMA in DBS and tested its usefulness.

Methods

Samples

The DBS used in our pilot study of newborn screening by MS/MS were collected on the newborns' fifth or sixth day of life with the informed consent of their parents. Positive results were based on C3 levels >3.6 nmol/ml combined with the ratios of C3/C2 >0.25 for screening for MMAU and PA and on C5 levels >0.6 nmol/ml for IVA. IVG and MMA measurements using positive DBS were started in 2005 and 2008, respectively, in our laboratory. If MMA levels were not high, urinary organic analysis by GC/MS was performed on newborns with positive results in the second DBS and those with C3 levels >6.0 nmol/ml in the first DBS to identify PA. DBS tested in selective screening of symptomatic patients (Shigematsu et al. 2002) was evaluated using the same marker levels as in newborn screening. Retrospective analyses were performed using DBS; samples were stored in a freezer.

Acylcarnitine analysis by MS/MS

The acylcarnitines in DBS were measured according to the methods reported previously (Shigematsu et al. 2002), except that derivatization was not used. One punch (3.1 mm in diameter) of DBS was extracted with methanol solution (99%), with deuterium-labeled acylcarnitines as internal standards. Methanol extracts were introduced into LC flow of methanol:acetonitril:water (4:4:2) with 0.1% formic acid using a model SIL-20 AC autoinjector (Shimadzu, Kyoto, Japan), and MS/MS analysis was performed using a model API 4000 LC/MS/MS system (Applied Biosystems, Tokyo, Japan).

MMA analysis

MMA concentrations in DBS were determined by stable-isotope-dilution methods. To a DBS punch (3.1 mm in diameter) in each well of a 96-well microplate, 120 μl of methanol solution (99%) containing 0.05 nmol of MMA (methyl-$^2H_3$) (CDN Isotopes, Pointe-Claire, Canada) was added, and the plate was agitated gently at room temperature for 30 min. Three punches were used for each individual. The extract solution collected from three wells for each individual was transferred to a glass test tube and dried under a nitrogen stream. The dried residue was derivatized using N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (Shigematsu et al. 2005). GC/MS analysis was performed on a model DSQ GC/MS (ThermoFisher, Tokyo, Japan) using a capillary column, SPB-50 (30 m × 0.25 mm inner diameter, film thickness 0.25 mm) (Supelco, Tokyo, Japan). The processed samples were injected using the splitless mode, and the oven temperature was programmed to rise from 80°C to 290°C at a rate of 10°C/min. The injection-to-injection time using an autoinjector was about 27 min. The mass spectrometer was operated under a positive electron-impact ionization mode, and the intensities of the ion $[M-57]^+$, m/z 289 for MMA and m/z 292 for labeled MMA, were recorded using a selected ion monitoring (SIM) mode. Linearity was evaluated by analyzing DBS prepared using control blood samples spiked with MMA at concentrations of 0.45, 10, 50, 100, and 300 nmol/ml.

Isovalerylglycine analysis

IVG concentrations in DBS were determined by isotope-dilution methods reported previously (Shigematsu et al.

2007). Briefly, the dried methanol extract from DBS was derivatized using hydrochloric acid (HCl)/butanol. The processed sample was analyzed by API 4000 LC/MS/MS system using flow-injection mode. The injection-to-injection time using an autoinjector was about 0.9 min.

Results

Typical SIM chromatograms of MMA in DBS samples of a control and a patient with MMAU are shown in Fig. 1. Quantification was performed using peak areas of the designated ion on SIM chromatograms. The linearity of the calibration curve for MMA was observed over the concentration range 0.45 to 300 nmol/ml in DBS: slope = 0.96, intercept = 0.008, and r^2=0.998. The variabilities were as follows;:0.43±0.032 nmol/ml (CV:7.4%) and 48.1± 1.89 nmol/ml (CV: 3.9%) in the intraday assay (n=6), and 0.42±0.039 nmol/ml (CV: 9.2%) and 48.9±3.19 nmol/ml (CV: 6.5%) in the interday assay (n=6), respectively.

Concentrations of MMA, C3, and free carnitine in newborn DBS of patients with MMAU or PA are shown in Table 1. From September 2008 to December 2009, about 65,000 DBS samples were tested, and 16 were positive based on C3 and C3/C2 values and thus subjected to MMA measurement. If MMA level was not high, a second DBS was requested. Two patients with MMAU (MMAU1 and MMAU2) and two with PA (PA1 and PA2) were identified. MMAU1 was born at 34 gestational weeks with birth weight of 1.6 kg and had clinical symptoms at 8 days, when the first DBS was collected. Mutation analysis indicated methylmalonyl-coenzyme A (CoA) mutase deficiency, and he died at 3 months of age despite aggressive therapies. MMAU2 was born at 32 gestational weeks, with birth weight of 1.3 kg, and was asymptomatic before a high MMA level in the first DBS collected at 5 days was observed. After therapy with a large dose of vitamin B_{12}, serum and urinary MMA levels were decreased but not normalized. PA1 and PA2 were classified as having a mild form based on the results of mutation analysis (Yorifuji et

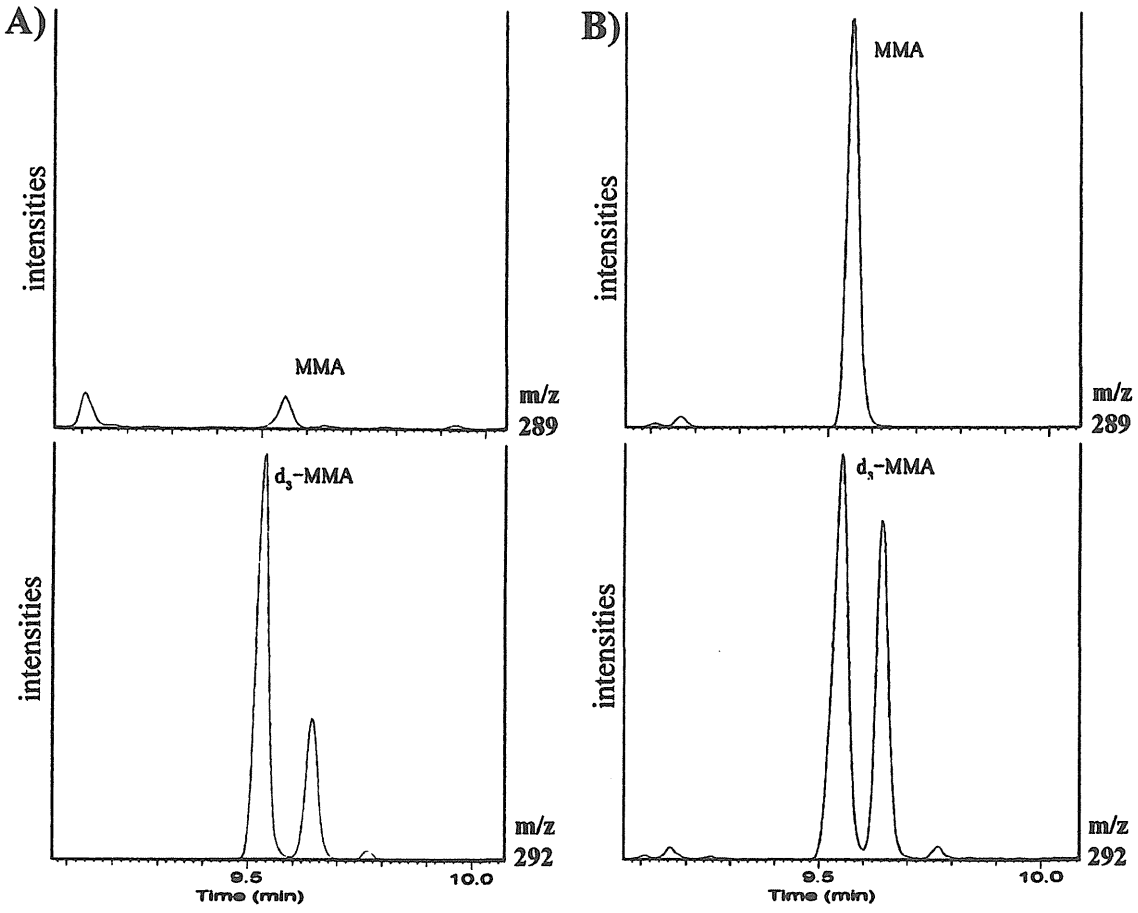

Fig. 1 Selected ion chromatograms of methylmalonic acid (MMA) in dried blood spots (DBS) of a control (**A**) and patient with methylmalonic aciduria (MMAU) (**B**). Intensities of the panels are not shown by absolute values, nor in an exactly proportional manner, due to the limitation of drawing software of the instrument. The MMA concentrations for control and patient were 0.56 and 67.7 nmol/ml, respectively

Table 1 *Methylmalonic acid (MMA) and carnitine levels in dried blood spots (DBS) of patients with methylmalonic aciduria (MMAU) and propionic acidemia (PA)*

	MMA (nmol/ml)	Carnitine (nmol/ml)		
		C3	C3/C2	Free carnitine
DBS samples (newborn screening)				
MMAU1[a]	321.9	11.4	0.81	14.8
MMAU2[b]	238.3	10.0	0.61	18.3
MMAU3[a]	219.7	7.1	0.52	12.3
MMAU4[b]	24.5	7.1	0.53	24.4
MMAU5[b]	24.2	6.5	0.68	39.1
PA1	0.43	5.6	0.65	27.0
PA2	0.32	4.0	0.29	23.3
False-positive samples (12)	0.38±0.12	6.2±1.5	0.30±0.05	36.9±12.4
Control newborns (n=46)	0.34±0.11	2.7±1.2	<0.25	35.2±11.5
MMAU (n=5): mean (range) (selective screening)	121.2 (34.2–252.4)	8.6 (4.5–13.3)	0.82 (0.70–0.99)	19.5 (6.2–31.4)
PA (n=3) (selective screening)	0.28, 0.41, 0.31	12.2, 12.5, 22.0	1.16, 2.47, 0.85	12.2, 6.2, 18.6

MMA levels in patients MMAU3, MMAU4, and MMAU5 were obtained by retrospective analysis

MMAU methylmalonic aciduria patient, *MMA* methylmalonic acid, *PA* propionic acidemia patient, *C3* propionylcarnitine, *C2* acetylcarnitine

[a] Methylmalonyl-CoA mutase deficiency

[b] Vitamin B_{12}-responsive form

al. 2002). Three other patients with MMAU (MMAU3, MMAU4, and MMAU5) were identified in our laboratory before the start of DBS MMA analysis. MMAU3 with mutase deficiency, who was born at full term with birth weight of 3.1 kg and had severe acidosis with hypoglycemia on day 1, survived due to aggressive therapy and received liver transplantation at 4 years of age. MMAU4 and MMAU5 were asymptomatic during the newborn period and classified clinically as having the vitamin B_{12}-responsive form. Among the five patients with MMAU, those with the severe form had decreased levels of free carnitine in DBS, and DBS concentrations of MMA were not apparently correlated with those of C3.

Results of selective screening for MMAU or PA using DBS of sick infants are included in Table 1. MMA concentrations were distributed across the range similar to that in newborn screening. Some patients with MMAU had decreased levels of free carnitine, and the DBS concentrations of MMA were not correlated with those of C3.

The concentrations of IVG, C5, and free carnitine in newborn DBS of patients with IVA are shown in Table 2. From January 2007 to December 2009, about 146,000 DBS samples were tested, and 1,065 positive samples based on C5 levels were subjected to IVG determination with cutoff value of 0.5 nmol/ml. During this period, one patient with IVA (IVA1) was identified based on a high IVG level in the first DBS collected at age 6 days. On examination, she did not have acute symptoms, except for slightly elevated blood ammonia levels and markedly increased urinary excretion of IVG. Treatment using leucine-free formula and oral carnitine was started promptly, and she has not experienced any acute crises for 18 months. Diagnosis was confirmed by enzyme assay (Tajima G et al. 2005). No cases other than IVA1 had positive results from IVG measurement. Another patient with IVA (IVA2) was identified based on a mildly elevated DBS C5 level in a different screening laboratory and has been asymptomatic without treatment. Four other patients became symptomatic at age 4 days to 5 months before this analysis was started, and the analyses

Table 2 *Isovalerylglycine (IVG), C5-acylcarnitine, and free carnitine levels in dried blood spots (DBS) tested in newborn screening*

	IVG (nmol/ml)	C5-acylcarnitine (nmol/ml)	Free carnitine (nmol/ml)
DBS samples			
IVA1	41.1	11.9	9.8
IVA 2	1.3	2.0	34.9
IVA 3	68.5	9.1	17.9
IVA 4	15.5	10.3	10.1
IVA 5	18. 1	8.9	11.2
IVA 6	80.0	12.7	7.0
False-positive samples (n=1064)	0.20±0.04	2.4±1.8	27.3±10.9
Control newborns (n=273)	0.18±0.03	0.13±0.05	34.2±9.7

IVA isovaleric acidemia patient

IVG levels in IVA2-6 were obtained by retrospective analysis

of IVG and acylcarnitine using the stored newborn DBS samples were performed retrospectively. Diagnosis was made by urinary organic acid analysis and enzyme assay. Most patients with moderate to severe disease had low levels of free carnitine.

Discussion

The data in our pilot study of MS/MS newborn screening showed that our second-tier tests were useful for identifying patients with mild to severe forms of IVA and MMAU and for reducing false positive rates. In our pilot study, screening of IVA was performed using C5 cutoff value of 0.6 nmol/ml, as the lowest level of newborn DBS C5 in IVA patients with a mild biochemical form was reported to be 0.8 nmol/ml (Ensenauer et al. 2004). Using IVG measurement, a patient with a mild form could be identified, and the recall rate became zero despite high false-positive rate of 0.7%. On the other hand, it is reported that newborns with a mild form of IVA, who had 0.8–6 nmol/ml of C5 DBS concentrations, remained asymptomatic during episodes of febrile illnesses, even without dietary therapy (Vockley and Ensenauer 2006). It is reasonable to argue that one should avoid detecting such newborns with a mild phenotype and that a cutoff value for C5 much higher than 0.6 nmol/ml should be considered. Although our data suggest that a cutoff value as high as 6 nmol/ml for C5 may be acceptable to identify only moderate to severe forms of IVA, further investigation is be needed to determine suitable cutoff value to identify IVA patients, because overlapping urinary IVG and serum C5 levels was reported among mild and moderate forms (Ensenauer et al. 2004), and C5 levels may vary due to carnitine deficiency, as shown in this study. Otherwise, IVG measurement seems effective in estimating the severity of this disease. In most patients with moderate to severe forms of IVA, free carnitine levels in DBS were decreased, and IVG levels were markedly higher than C5 levels. Interestingly, the serum free-carnitine levels of a patient with a severe form of IVA, who had been diagnosed prenatally, remained <10 nmol/ml before carnitine therapy was started at age 8 days, and IVG levels at age 2 days were much higher than C5 levels (Shigematsu et al. 2007).

Our method of determining MMA concentration in DBS using GC/MS was sensitive enough to identify MMAU patients. We recommend three punches for a single determination to securely quantify MMA levels in control newborns. In our study, MMA concentrations in MMAU patients ranged from 24.2 to 321.9 nmol/ml, whereas those in PA patients and control newborns were lower than 1.0 nmol/ml. In our retrospective study of the benign form of MMAU (Sniderman et al. 1999), MMA levels of 5–10 nmol/ml were observed in DBS of two patients who were identified in newborn screening by GC/MS using urine (Kuhara et al. 1999), whereas urinary levels of methylcitric acid and DBS levels of C3 were not elevated. Detection of neonatal vitamin B_{12} deficiency by MS/MS newborn screening has been reported in newborns whose mothers had vitamin B_{12} deficiency based on elevated C3, although MMA levels in DBS were not mentioned (Wiley et al. 1999, Campbell et al. 2005, Marble et al. 2008). In our study, it is not clear whether newborns with false-positive results had vitamin B_{12} deficiency or not, as their serum vitamin B_{12} concentrations were not determined.

In our pilot study for MMA measurement in DBS, the GC/MS method was used partly because the reported LC-MS/MS method (la Marca et al. 2007) did not seem sensitive enough to determine MMA levels in controls. In MS/MS newborn screening, urinary organic analysis by GC/MS is needed for positive cases of organic acidemia in order to obtain quick diagnosis, and we recommend GC/MS measurement of MMA in DBS as an option for screening laboratories where GC/MS instrument is set up. Our second-tier tests using the first DBS were also useful to provide quick diagnosis to patients with moderate to severe forms of MMAU and IVA. In our study, due to carnitine deficiency, the levels of such primary screening markers as C3 and C5 were not always markedly elevated, and high levels of MMA and IVG were thought to provide a better estimate of disease severity for most of these patients. In addition, some of them had clinical symptoms due to acidosis or hyperammonemia during the newborn period, and our diagnostic information was thought to be of much value for prompt and pertinent therapy.

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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	3mM Methylmalonyl-CoA	Adenosyl-cobalamin 0.5mM	Tris sulfate (pH 7.5) 100mM	1×10⁶ (#1)	0.3N $HClO_4$	NaH_2PO_4 (pH 2.5) 100mM Methanol 12%	Succinyl-CoA
PCC	0.5mM Propionyl-CoA	$NaHCO_3$ 30mM ATP 3mM $MgCl_2$ 5mM	K_2HPO_4 (pH 7.0) 80mM	1×10⁶	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 (pH 8.2) 100mM	1×10⁶	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 (pH 7.5) 50mM EDTA 0.2mM	2×10⁶ (#2)	0.3N $HClO_4$	NaH_2PO_4 (pH 4.0) 100mM Acetonitrile 15%	Isovaleryl-CoA
IVDH	1mM Isovaleryl-CoA	PMS 2mM FAD 0.1mM	K_2HPO_4 (pH 7.0) 80mM	1×10⁶	0.3N $HClO_4$	NaH_2PO_4 (pH 4.0) 100mM Acetonitrile 13%	3-Methylcrotonyl-CoA
GDH	3mM Glutaryl-CoA	PMS 2mM FAD 0.1mM	K_2HPO_4 (pH 7.0) 80mM	2×10⁶	0.3N $HClO_4$	NaH_2PO_4 (pH 2.5) 100mM Methanol 15.5%	3-Hydroxybutyryl-CoA
MCAD	2mM n-Octanoyl-CoA	$FcPF_6$ 1mM	K_2HPO_4 (pH 7.0) 80mM	1×10⁶	Acetonitrile	NaH_2PO_4 (pH 4.0) 100mM Acetonitrile 28%	2-Octenoyl-CoA
VLCAD	2mM Palmitoyl-CoA	PMS 2mM FAD 0.1mM	K_2HPO_4 (pH 7.0) 80mM	1×10⁶ (#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）。

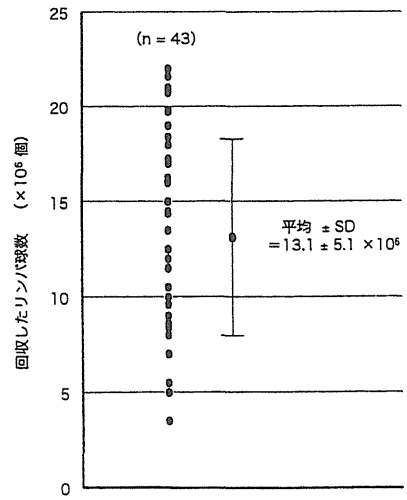

図1　タンデムマス・スクリーニング陽性となった新生児の血液検体から回収したリンパ球数

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。