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Newborn mass screening and selective screening using electrospray tandem mass spectrometry in Japan

Yosuke Shigematsu[a,*], Satoko Hirano[b], Ikue Hata[b], Yukie Tanaka[c], Masakatsu Sudo[d], Nobuo Sakura[e], Tsuyoshi Tajima[e], Seiji Yamaguchi[f]

[a]*Department of Health Science, School of Nursing, Fukui Medical University, Matsuoka-cho, 910-1193 Fukui, Japan*
[b]*Department of Pediatrics, School of Medicine, Fukui Medical University, Fukui, Japan*
[c]*Central Research Laboratories, Fukui Medical University, Fukui, Japan*
[d]*Fukui Medical University, Fukui, Japan*
[e]*Department of Pediatrics, Faculty of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima, 734-8551, Japan*
[f]*Department of Pediatrics, Shimane Medical University, 89-1 En-ya-cho, Izumo, Shimane, 693-8501, Japan*

Abstract

Electrospray tandem mass spectrometry was applied to detect a series of inherited metabolic disorders during a newborn-screening pilot study and a selective screening in Japan. In our mass screening of 102 200 newborns, five patients with propionic acidemia, two with methylmalonic acidemia, two with medium-chain acyl-CoA dehydrogenase deficiency, three with citrullinemia type II, and one with phenylketonuria were identified. In a selective screening of 164 patients with symptoms mainly related to hypoglycemia and/or hyperammonemia, 12 with fatty acid oxidation disorders and six with other disorders were found. The results indicated the importance of newborn screening using this technology in Japan.

Keywords: Newborn mass screening; Inherited metabolic disorders

1. Introduction

Electrospray tandem mass spectrometry (ESI-MS–MS) has been applied in newborn screening programs around the world in order to detect such inherited metabolic disorders as amino acidopathies, organic acidemias, and fatty acid oxidation disorders [1]. In the United States, phenylketonuria (PKU), and medium-chain acyl-CoA dehydrogenase deficiency (MCAD) were major disorders found in newborn mass screening by ESI-MS–MS [2]. In Japan, on the other hand, the frequency of PKU found in newborn screening was much lower than that reported in Western countries [3]. The occurrences of the other disorders were believed to be even lower, while that of ornithine transcarbamylase (OTC) deficiency was reported to be similar to that of PKU [4].

However, based on the capability of MS–MS to diagnose a large group of inherited metabolic disorders simultaneously, we have been conducting both a pilot study for newborn screening and a selective screening of symptomatic patients using this technology in order to clarify its efficacy in

*Corresponding author. Fax: +81-776-61-8563.
E-mail address: yosuke@fmsrsa.fukui-med.ac.jp (Y. Shigematsu).

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newborn screening in Japan [5]. In this paper, we report a spectrum of the disorders found in our mass screening of 102 200 newborns and the results of a 2-year selective screening.

2. Experimental

2.1. Samples

Some blood samples used in this pilot study were collected on the fifth or sixth day of life of the newborns, with the informed consent of their parents, while the others collected for general neonatal screening were transferred to us with informed consent. The same filter paper (Toyo Roshi, Tokyo, Japan) was used in both cases.

Retrospective analyses were done using newborn blood samples of the patients; the samples were stored in a freezer or refrigerator.

In our selective screening, clinical and laboratory findings of patients were characterized by hypoglycemia, hyperammonemia, elevated creatinine phosphokinase levels, lactic acidemia, high levels of serum transaminases, consciousness disturbance, convulsions, sudden death, or acute life-threatening events in infancy. In some patients, blood samples were not collected during the acute events.

2.2. Materials

Stable isotope-labelled acylcarnitines used in this study were synthesized in our laboratories [6]. Citrulline-3,3,4,-$^{2}H_{3}$ was synthesized using acrylonitrile-2,3,3-$^{2}H_{3}$, following the reported methods [7]. Acrylonitrile-2,3,3-$^{2}H_{3}$ and the other stable isotope-labelled compounds were purchased from CDN Isotopes (Miamisburg, OH, USA) or from Cambridge Isotope Labs. (Andover, MA, USA) [5]. Butanolic HCl (10%), HPLC-grade acetonitrile, methanol, and distilled water were purchased from Nacalai Tesque (Kyoto, Japan).

2.3. Sample preparation and mass spectrometry

A microplate sample process was carried out, using principally the previously reported methods [5,8]. To the blood-spot punch in each well, we added a methanol solution (110 μl) containing known concentrations of stable isotope-labelled standards of the following compounds: 2 nmol of glycine-$^{2}H_{2}$, 1.5 nmol each of alanine-$^{2}H_{3}$, valine and leucine-$^{2}H_{10}$, 0.5 nmol each of methionine-$^{2}H_{3}$, phenylalanine-$^{2}H_{5}$ and arginine-$^{13}C_{6}$, 0.8 nmol of tyrosine-$^{2}H_{2}$, 0.2 nmol of citrulline-$^{2}H_{3}$, 3 nmol of glutamine-$^{2}H_{5}$, 100 pmol of carnitine-$^{2}H_{3}$, 100/3 pmol of acetylcarnitine-$^{2}H_{3}$, 50/3 pmol each of propionylcarnitine-$^{2}H_{3}$ and glutarylcarnitine-$^{2}H_{9}$, 10 pmol of butyrylcarnitine-$^{2}H_{3}$, and 20/3 pmol of octanoylcarnitine-$^{2}H_{3}$ and palmitoylcarnitine-$^{2}H_{9}$.

ESI-MS–MS analysis was done using a Model TSQ7000 triple-stage mass spectrometer (ThermoQuest, Tokyo, Japan) equipped with a Model LC10 HPLC system and a Model SIL-10ADVP autoinjector (Shimadzu, Kyoto, Japan) [5], with some modifications. Using the autoinjector, derivatized samples (13 μl) were injected at 1.9-min intervals into a flow (20 μl/min) of 50% aqueous acetonitrile. The resolution on both mass spectrometers was tuned to 0.7 automatically. The data were collected in the multiple-channel acquisition mode for 1.2 min after every sample injection. The modified scanning functions for the analysis of acylcarnitines and amino acids were performed in the order listed in Table 1 and cycled. The tube lens voltage in each function was set to 90.0 V.

For the parameter detecting PA and MMA, a peak height ratio of m/z 274 (propionylcarnitine) to m/z 260 (acetylcarnitine) in precursor ion scanning (C3/C2) was used [8], and an upper cutoff value of 0.25 (mean+6SD) was tested.

The medium-chain acyl-CoA dehydrogenase activities in leukocytes were measured using the reported methods [9,10].

3. Results

In our newborn screening, which took place from April 1997 to July 2001, 102 200 newborns were tested, and five patients with propionic acidemia (PA), two with methylmalonic acidemia (MMA), two with MCAD, two with citrullinemia type II (CTLN2), 1 with PKU were identified. No consanguinities were shown in any patient.

Table 1
Scan functions in ESI-MS–MS analysis of butyrated acylcarnitines and amino acids in the blood spots of the newborns

Order	Scan function	Collision energy (eV)	Scan range (m/z)	Scan time (s)	Target analytes
1	Precursor ions of m/z 85	−30	210–505	2.0	Acylcarnitines
2	Product ions of m/z 459	−30	144	0.1	Argininosuccinic acid
3	Product ions of m/z 459	−30	172	0.1	Argininosuccinic acid
4	Neutral loss of m/z 161	−26	231	0.1	Arginine
5	Neutral loss of m/z 163	−26	237	0.1	Arginine-$^{13}C_6$
6	Neutral loss of m/z 119	−22	232	0.1	Citrulline
7	Neutral loss of m/z 119	−22	235	0.1	Citrulline-$^{2}H_3$
8	Neutral loss of m/z 56	−12	132	0.1	Glycine
9	Neutral loss of m/z 56	−12	134	0.1	Glycine-$^{2}H_2$
10	Neutral loss of m/z 102	−14	146	0.1	Alanine
11	Neutral loss of m/z 102	−14	149	0.1	Alanine-$^{2}H_3$
12	Neutral loss of m/z 102	−16	174	0.1	Valine
13	Neutral loss of m/z 102	−16	182	0.1	Valine-$^{2}H_8$
14	Neutral loss of m/z 102	−16	188	0.1	Leucine
15	Neutral loss of m/z 102	−16	198	0.1	Leucine-$^{2}H_{10}$
16	Neutral loss of m/z 102	−18	203	0.1	Glutamine
17	Neutral loss of m/z 102	−18	208	0.1	Glutamine-$^{2}H_5$
18	Neutral loss of m/z 102	−20	222	0.1	Phenylalanine
19	Neutral loss of m/z 102	−20	227	0.1	Phenylalanine-$^{2}H_5$
20	Neutral loss of m/z 102	−20	238	0.1	Tyrosine
21	Neutral loss of m/z 102	−20	240	0.1	Tyrosine-$^{2}H_2$

The propionylcarnitine levels and the C3/C2 ratios in patients with PA and MMA are shown in Fig. 1. The values of three patients with PA who were identified in a newborn screening by gas chromatography–mass spectrometry (GC–MS) using urine samples [11] were included in the retrospective analysis. The conditions were discriminated using the above-mentioned cutoff value both in prospective

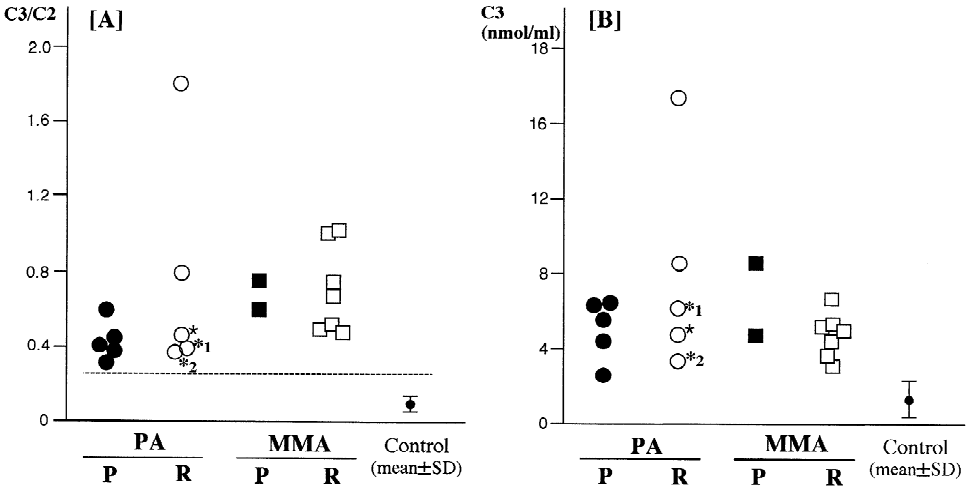

Fig. 1. The peak height ratios of m/z 274 (propionylcarnitine) to m/z 260 (acetylcarnitine) (A) and propionylcarnitine levels (B) in the prospective screening (P) and retrospective analysis (R) of newborn blood spots of patients with propionic acidemia (PA) and methylmalonic acidemia (MMA). See the text for the cutoff value indicated by a broken line. *; Patients identified in GC–MS newborn screening using urine samples. *1 and *2; Identical twins.

Table 2
Characterization of patients with propionic acidemia found in newborn screening

Patient	C3/C2	Urinary methylcitrate* (mmol/mol creatinine)	Residual PCC activity (%)
1	0.59	75	2.4
2	0.38	28	7.5
3	0.42	35	4.1
4	0.30	14	7.5
5	0.35	45	10.1
Controls	<0.25	<7	100

Abbreviations: C3/C2, peak height ratio of propionylcarnitine to acetylcarnitine; C3, propionylcarnitine; PCC, propionyl-CoA carboxylase.
*Collected at 18–25 days of age.

and retrospective analyses of newborn blood spots. The recall rate under this cutoff value was 0.06%. In Table 2, the characteristics of the five patients with PA who were identified in our screening are shown. They are undergoing therapies of mild to moderate restrictions of dietary protein and L-carnitine administration. They have not experienced any acute metabolic crises and have showed normal developmental milestones. The brother of patient No. 3 is a symptom-free PA patient confirmed by propionyl-CoA carboxylase (PCC) assay. Two patients with MMA presented acute symptoms before the sample were collected.

The concentrations of carbon-number 8 (C8) acylcarnitine in the blood spots of two patients with MCAD were 0.62 and 4.12 nmol/ml, respectively, using an upper cutoff value of 0.3 nmol/ml [12]. The diagnosis of the former patient, a Peruvian boy of Japanese descent, however, was missed, probably because of an erroneous manual data interpretation process, and he experienced a hypoglycemic episode with a serum C8-acylcarnitine level of 5.92 nmol/ml at the age of 7 months. The latter patient, a Japanese girl, has been treated with low-fat formula and L-carnitine and has not experienced any hypoglycemic episodes. The medium-chain acyl-CoA dehydrogenase activities of both patients decreased to 39.4% and 35.9% of those of normal controls, respectively. The precursor ion mass spectra of newborn blood spots of all patients with MCAD diagnosed in Japan are shown in Fig. 2. In the second patient, C6 and C10:1 acylcarnitines were also elevated. The third patient was identified in our selective screening process during a hypoglycemic crisis at the age of 15 months; the C8-acylcarnitine level in her stored newborn blood spot was 2.52 nmol/ml, and the medium-chain acyl-CoA dehydrogenase activity decreased to 35.4% of those of the controls.

The citrulline levels in the two patients with CTLN2 identified in our pilot study are listed in Table 3. The first patient had a markedly high citrulline level similar to those in patients with classic citrullinemia. Combined with the prospective and retrospective analyses, two patients with heterozygous mutations in the citrin gene had citrulline levels in their newborn blood spots lower than the tentative upper-cutoff value of 50 nmol/ml (mean+6SD).

The overall recall rate in our newborn screening was 0.58%; the false-positive rate in the diagnosis of isovaleric acidemia due to the use of pivalic acid-containing antibiotics was 0.39%.

In a selective screening of 164 patients with the symptoms mentioned above, we found five patients with very long-chain acyl-CoA dehydrogenase deficiency (VLCAD), two with long-chain hydroxy acyl-CoA dehydrogenase deficiency (LCHAD), two with glutaric aciduria type II (GA-II), two with OTC deficiency, and one patient with each of the following disorders; MCAD, short-chain acyl-CoA dehydrogenase deficiency (SCAD), carnitine palmitoyltransferase-2 (CPT-2) deficiency, multiple carboxylase deficiency, 3-hydroxy-3-methylglutaryl-CoA lyase deficiency, MMA, CTLN2. No consanguinities were shown in families of the patients listed

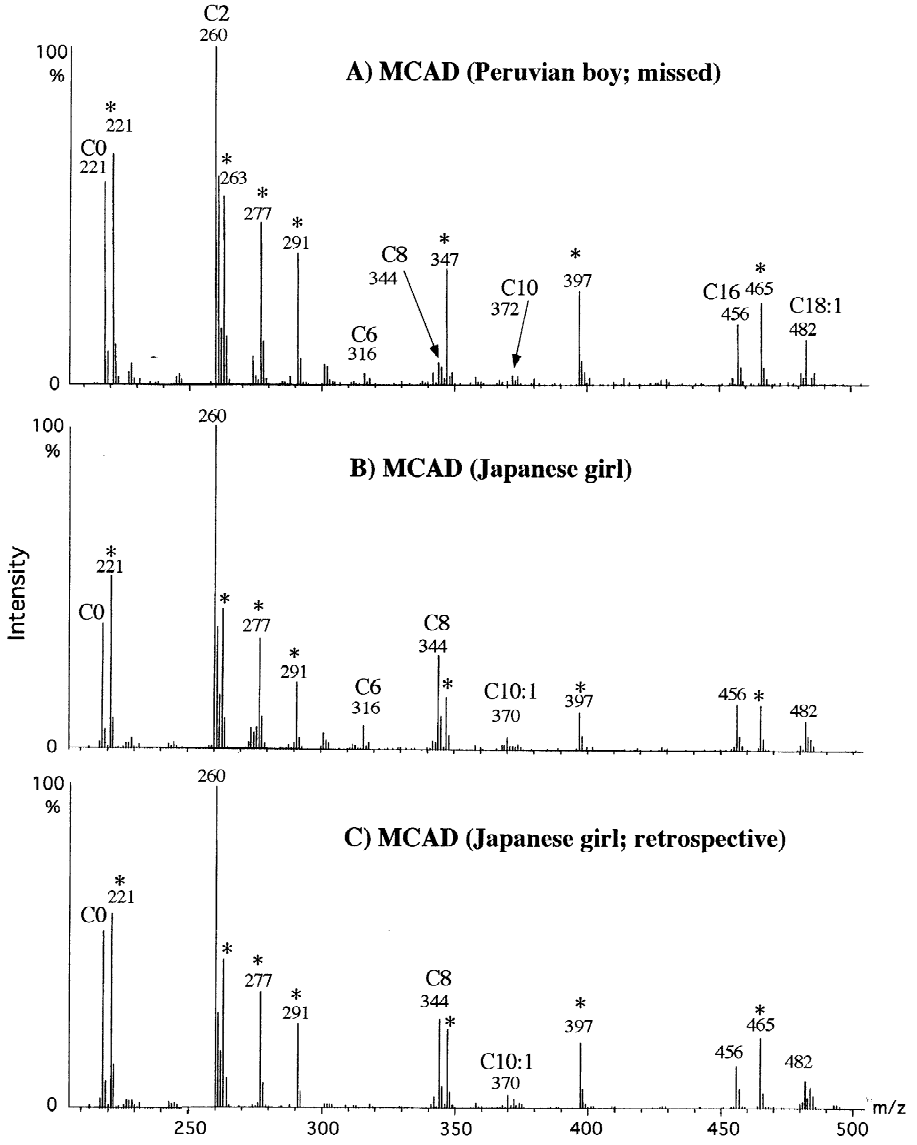

Fig. 2. Newborn blood spot acylcarnitine profiles obtained by ESI-MS–MS analysis with precursor ion scanning of m/z 85. See the text about the levels of C8-acylcarnitine and clinical features of the three patients with MCAD. The ion peaks indicate the molecular ions of the butyrated acylcarnitines. Their masses are as follows: free carnitine (C0, 218), acetyl (C2, 260), C6-acyl (C6, 316), C8-acyl (C8, 344), C10:1-acyl (C10:1, 370), C10-acyl (C10, 372), C16-acyl (C16, 456), C18:1-acyl (C18:1, 482). The ion peaks highlighted with an asterisk indicate the stable isotope-labelled internal standards. Their masses are as follows: free carnitine-$^2H_3$ (221), acetylcarnitine-$^2H_3$ (263), propionylcarnitine-$^2H_3$ (277), butyrylcarnitine-$^2H_3$ (291), octanoylcarnitine-$^2H_3$ (347), glutarylcarnitine-$^2H_9$ (397), and palmitoylcarnitine-$^2H_9$ (465).

above. The patients with OTC deficiency were screened by the low values of citrulline (below 4 nmol/ml) and increased levels of glutamine in blood spots, and the diagnosis was confirmed by enzyme assay.

Acylcarnitine profiles in sera or blood spots of

Table 3
Prospective and retrospective analysis of amino acids in the blood spots of patients with citrullinemia type II

Patient No.	Sampling date	Gene mutation#	Amino acid (nmol/ml)				
			Citrulline	Arginine	Methionine	Tyrosine	Galactose
1§	5 days	IVS11+1G>A IVS11+1G>A	1022	163	402	205	(+)
	3 weeks		236	138	1026	881	NM
2§	5 days	IVS11+1G>A IVS11+1G>A	214	19	102	468	(+)
3§§	5 days	IVS11+1G>A 851del4	49	32	33	153	(−)
	4 months		273	114	160	193	NM
4	5 days*	IVS11+1G>A 851del4	39	52	44	130	(+)
	3 weeks		323	223	386	148	NM
5	5 days**	851del4 851del4	241	16	34	145	(−)
Control newborns (mean±SD)			17±5	37±14	22±15	84±33	

§, Identified in our newborn screening.
§§, Measured in our newborn screening.
*, Stored in a freezer for 4 months.
**, Stored in a refrigerator for 3 years.
#, See Ref. [22].
(+), Galactose levels above the cutoff value in general mass screening.
NM, Not measured.

patients with several conditions in which elevated C14:1 acylcarnitine may be featured are shown in Figs. 3 and 4.

4. Discussion

The results of our MS–MS newborn screening in Japan revealed a different spectrum of prevalent inherited metabolic disorders from those reported in other countries. In the United State, Naylor and Chace reported the following frequencies in a prospective screening of 600 000–750 000 newborns: PKU, 1/20 200; MSUD, 1/83 000; MCAD, 1/15 400; glutaric aciduria type I (GA-I), 1/76 400; 3-methylcrotonyl-CoA carboxylase deficiency (3MCC), 1/76 400; and PA, 1/137 500 [2]. Marsden et al. reported the following results in 100 000 newborns: MCAD, 1/25 000; SCAD, 1/25 000; PA, 1/50 000 [13]. In Germany, Roscher et al. described the following frequencies in 166 000 screened newborns: MCAD, 1/12 800; 3MCC, 1/33 200; VLCAD, 1/83 000; GA-II, 1/83 000 [14]. In Australia, Wilcken et al. reported the following frequencies in 190 000 newborns: PKU, 1/7900; MCAD, 1/47 500 [15]. In Saudi Arabia, Rashed showed the following frequencies in 27 600 newborns: PKU, 1/9200; MSUD, 1/18 400; MMA, 1/6900; GA-I, 1/13 800; PA, 1/27 600 [16].

The prevalence of PA in Japan has been estimated as 1/465 000, based on a national survey of patients with organic acidemias in the last 10 years. Our pilot study revealed that the rate is 1/20 000, which is even higher than that of PKU (1/80 500) in Japan [3]. All five patients are assumed to have a mild form of this disease based on their residual PCC activities. It is noteworthy that a PA patient has an asymtomatic sibling with PA. Nevertheless, it seems very important to remember that some PA patients with residual PCC activities that are 5% of those of the control can present with a pure neurological disease without acute crises [17] and that a PA patient with residual activities of 11% of those of control have developed a fatal necrosis of the basal

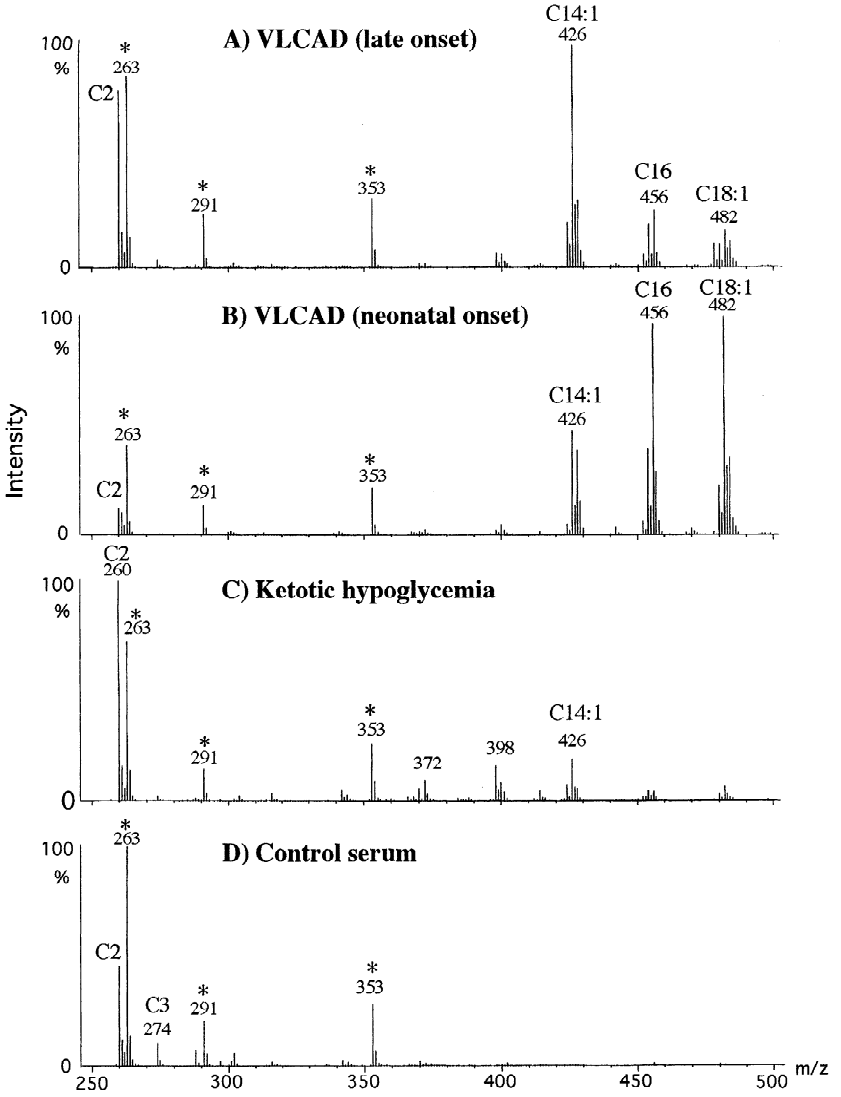

Fig. 3. Serum acylcarnitine profiles obtained by ESI-MS–MS analysis with precursor ion scanning of m/z 85. (A) A VLCAD patient, presenting muscle weakness during a febrile illness at the age of 3 years; the acylcarnitine profile in the newborn blood spot was typical for this disorder, and decreased activities of VLCAD in cultured skin fibroblasts were confirmed. (B) A VLCAD patient who died of hypoglycemia and cardiac arrest at the age of 3 months; the acylcarnitine profile in newborn blood spot was typical, and VLCAD activities were decreased. (C) A patient aged 4 years, presenting several episodes of ketotic hypoglycemia; VLCAD activities in cultured skin fibroblasts were normal and urinary organic acid profile was atypical for GA-II. All blood samples were collected when the patients were symptomatic. The masses of butyrated acylcarnitines are as follows: C12-acyl (398), C14:1-acyl (C14:1, 426), octanoylcarnitine-$^2H_9$ (*, 353).

ganglia [18]. Since the urinary excretion of methylcitrate in patients with this form of PA may not be large enough, as shown in Table 2, it is possible that the diagnosis of PA was missed. Thus, it seems important to include blood propionylcarnitine levels for the chemical diagnosis of this form of PA. Considering that the entire clinical picture of the mild form of PA has not been clarified, we should carefully follow patients and provide specific therapies.

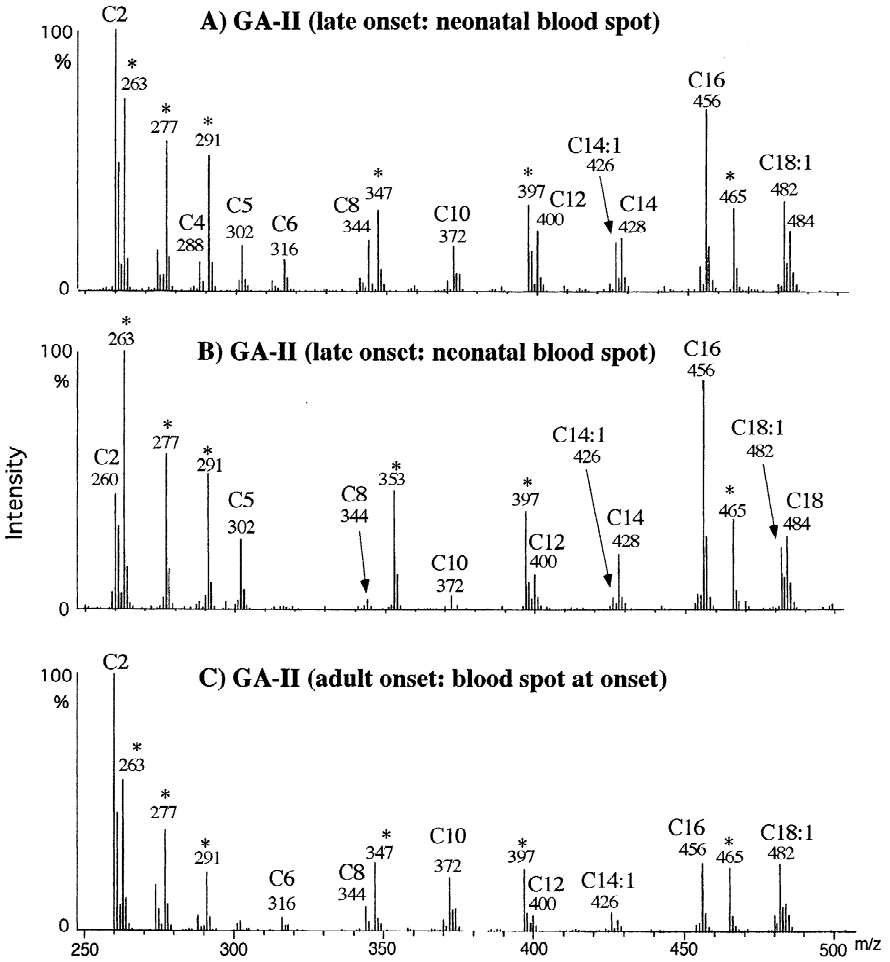

Fig. 4. Blood spot acylcarnitine profiles obtained by ESI-MS–MS analysis with precursor ion scanning of m/z 85. (A) The newborn blood spot of a GA-II patient whose brother died suddenly during a febrile illness at the age of 8 months; a typical acylcarnitine profile for this disorder characterized by increased levels of C4 to C16 and C5 acylcarnitines. (B) The newborn blood spot of a GA-II patient presenting cardiac failure without hypoglycemia at the age of 8 months; an atypical acylcarnitine profile characterized by increased levels of C10 to C16 and C5 acylcarnitines. (C) The acute-phase blood spot of an adult-onset GA-II patient presenting several episodes of rhabdomyolysis; an atypical acylcarnitine profile characterized by increased levels of C6 to C14:1 acylcarnitines with the most prominent level of C10 acylcarnitine but without the increased C5 acylcarnitine. See Figs. 2 and 3 about the masses of butyrated acylcarnitines.

The results of the present study suggest that the prevalence of MCAD in Japan is not much less than that in Western countries. This is surprising, since no patient with MCAD had been identified before the start of our screening project. These unexpected results may be due partly to the small number of screened newborns in the restricted areas. A nationwide MS–MS newborn screening should be performed in order to clarify the true prevalence of these disorders in Japan. In our newborn screening, the Peruvian MCAD patient had a C8-acylcarnitine level lower than an upper cutoff value of 1.2 nmol/ml proposed in the UK study using MS–MS [19]. The upper cutoff value for C8-acylcarnitine should be further tested.

The relatively high recall rate in our newborn screening was mainly due to the use of pivalic acid-containing antibiotics [5]. In addition, it was partly because we adopted the upper-cutoff value for the C3/C2 ratio of 0.25 in order to detect the

patients with PA and MMA. It has been reported that the cutoff value of C3/C2 ratio of 0.4 (99.5 percentile) resulted in missing some MMA cases [1], and that an appropriate cutoff for propionylcarnitine has not been established because of the effect of cobalamine deficiency in newborns [2].

The results in our selective screening suggest that VLCAD and GA-II may be relatively common disorders among the fatty acid oxidation disorders in Japan. Our retrospective analyses showed that some of these patients could be screened in their newborn period by the increased levels of C14:1 acylcarnitine. Although Vianey-Saban et al. reported that C14:1 acylcarnitine is diagnostic for VLCAD [20], additional tests are necessary to confirm the diagnosis in some patients with atypical acylcarnitine profiles as shown in Figs. 3 and 4.

In Japan, the blood samples for newborn screening are collected on day 5 or 6, which may cause low levels of C8-acylcarnitine or the other acylcarnitines specific to the respective fatty acid oxidation disorder [21]. Earlier collection of blood samples and improvement in the performance of the MS–MS machine may be necessary for the sensitive screening of fatty acid oxidation disorders.

CTLN2 is an adult-onset type of citrullinemia that is frequent in Japan and is caused by a deficiency of citrin, which is encoded by the SLC25A13 gene [22]. Some patients with this disorder exhibit transient infantile cholestatic jaundice and increased levels of blood galactose in neonatal period [23,24]. ESI-MS–MS screening enables us to differentiate this disorder from classic inherited galactosemias. Although the specific therapy for CTLN2 to prevent the appearance of neurological deterioration with hyperammonemia later in adulthood is not known at present, the pathophysiology of this disorder is being unveiled [25]. The identification of patients with CTLN2 in newborn screening or in early infancy may help in clarifying the clinical course of this disorder.

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