

図2 2009～2012年検査時の胸部CT所見

認められた(図2)。現在外来通院にて加療継続中である。

3 考 察

肺ノカルジア症には特徴的な臨床症状はなく、胸部X線所見も肺炎像や結節影、びまん性粒状影、胸水貯留と多彩であると報告されており、本症例の場合は粒状影を伴う気管支拡張像を呈しており、非結核性抗酸菌症との鑑別が重要であった。

本症例で興味深い点は、ノカルジアの持続感染を認める気管支拡張症例で、ノカルジアをコントロールすることにより咳嗽の増悪と気管支拡張の増悪が認められなくなった点である。このことは、気管支拡張症をノカルジアの持続感染が悪化させる可能性があることを示唆する。気管支拡張症の症例で、インフルエンザ桿菌や緑膿菌だけではなく、ノカルジアが増悪に寄与する可能性があることを問題提起してくれた症例であった。

ノカルジアは免疫能が低下した患者の日和見感染の原因菌と考えられてきたが、最近では慢性肺疾患に続発したケースも報告されている。ある報告では、気管支拡張症はノカルジアによるコロナイゼーションの重要なリスクであることが示唆されている¹⁾。発症する原因とし

ては、慢性閉塞性肺疾患、cystic fibrosis、気管支拡張症などの慢性の肺または気道疾患を有する症例において、気道局所の自浄機能低下が考えられている²⁾。慢性呼吸不全患者の肺内常在菌として存在しているノカルジアは、免疫状態が低下したときに発症するとの報告があり³⁾、このことが本症例で咳嗽や陰影の改善と増悪を繰り返した原因の一つと考えることもできる。また、他の文献では、浸潤影や結節影などが出現した、長期間ステロイドを使用しているCOPDや気管支拡張症の患者では、肺ノカルジア症の可能性があり、積極的に精査すべきであると指摘されている¹⁾。当症例でも、気道過敏性を疑い吸入ステロイド治療を開始した後、しばらくして再増悪を呈しており、ステロイド吸入が増悪因子の一つであった可能性が考えられた。

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文 献

- 1) Aidé MA, et al. J Bras Pneumol 2008;34:985-8.
- 2) Martínez Tomás R, et al. Respirology 2007;12:394-400.
- 3) Menéndez R, et al. Eur Respir J 1997;10:1542-6.

Intracellular in vitro probe acylcarnitine assay for identifying deficiencies of carnitine transporter and carnitine palmitoyltransferase-1

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Abstract Mitochondrial fatty acid oxidation (FAO) disorders are caused by defects in one of the FAO enzymes that regulates cellular uptake of fatty acids and free carnitine. An in vitro probe acylcarnitine (IVP) assay using cultured cells and tandem mass spectrometry is a tool to diagnose enzyme defects linked to most FAO disorders. Extracellular acylcarnitine (AC) profiling detects carnitine palmitoyltransferase-2, carnitine acylcarnitine translocase, and other FAO deficiencies. However, the diagnosis of primary carnitine deficiency (PCD) or carnitine palmitoyltransferase-1 (CPT1) deficiency using the conventional IVP assay has been hampered by the

presence of a large amount of free carnitine (C0), a key molecule deregulated by these deficiencies. In the present study, we developed a novel IVP assay for the diagnosis of PCD and CPT1 deficiency by analyzing intracellular ACs. When exogenous C0 was reduced, intracellular C0 and total AC in these deficiencies showed specific profiles clearly distinguishable from other FAO disorders and control cells. Also, the ratio of intracellular to extracellular C0 levels showed a significant difference in cells with these deficiencies compared with control. Hence, intracellular AC profiling using the IVP assay under reduced C0 conditions is a useful method for diagnosing PCD or CPT1 deficiency.

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Introduction

L-Carnitine plays an essential role in the transfer and activation of long-chain fatty acids across the outer and inner mitochondrial membranes during which it is acted upon by enzymes including carnitine transporter (OCTN2), carnitine palmitoyltransferase-1 (CPT1), carnitine palmitoyltransferase-2 (CPT2), and carnitine acylcarnitine translocase (CACT) (Fig. 1) [1, 2]. Carnitine penetrates into cells across the plasma membrane against a high concentration gradient of free carnitine with the aid of the plasma membrane OCTN2 protein encoded by the SLC22A5 gene [3]. Deficiency of OCTN2 causes primary carnitine deficiency (PCD, OMIM 212140), which is characterized by systemic carnitine deficiency in tissues and blood but in concord with increased excretion of free L-carnitine in the urine [4–6]. Clinical symptoms in patients with PCD such as cardiomyopathy,

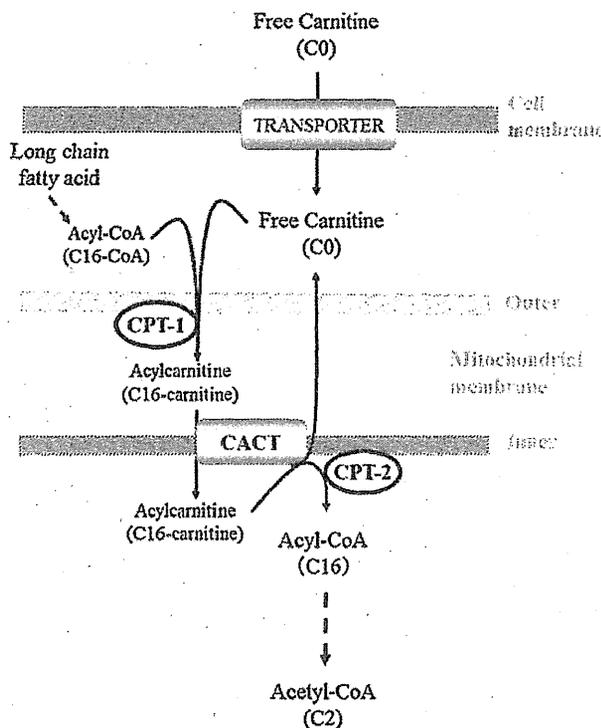


Fig. 1 Pathway for mitochondrial fatty acid beta-oxidation. Transporter: carnitine uptake transporter; *CPT-1*: carnitine palmitoyltransferase-1, *CACT*: carnitine acylcarnitine translocase, *CPT-2*: carnitine palmitoyltransferase-2. Solid arrows indicate single reactions; dashed arrows indicate multiple reactions or steps

encephalopathy, hepatomegaly, myopathy, hypoglycemia, and hyperammonemia, mainly result from low carnitine concentration in the tissues. On the other hand, secondary carnitine deficiency occurs in some conditions such as organic acidemias, renal dialysis, long-term medication (antiepileptic drugs or some antibiotics), and alimentary deficiency of L-carnitine [7–9].

It is necessary to make a differential diagnosis of PCD from the secondary carnitine deficiency or other false-positive cases, and diagnosis is confirmed by demonstrating reduced transport in skin fibroblasts from the patients. Until now, cluster-tray method using radioisotope-labeled substrate was used for the diagnosis of PCD [4, 10–12]. However, such a diagnostic method requires handling of radioactive substrates and focused only on diagnosis of PCD. Gene sequencing in *SLC22A5* is one diagnostic method for PCD. However, it is molecularly heterogeneous, and around 50 different mutations have been identified [6]. After acylcarnitine analysis using tandem MS analysis became available in the worldwide, blood acylcarnitine analysis was used as an initial method for diagnosis of FAO disorders and a detection of FAO disorders has been increased. However, it is necessary to confirm the diagnosis of the diseases with detailed analysis. The *in vitro* probe acylcarnitine (IVP) assay using cultured fibroblasts and tandem mass spectrometry (MS/MS)

has been used to evaluate FAO capacity in the cultured cells and make a diagnosis of FAO disorders [13–15]. However, conventional IVP assay is not feasible to diagnose PCD or *CPT1* deficiency, because excess amount of free carnitine is added to the experimental medium at the beginning. Estimation of free carnitine, which is the key marker for the above diseases, in experimental medium was nonsense for diagnosis of these disorders. We developed a novel functional assay for PCD and *CPT1* deficiency using the IVP assay, with some modifications. This method uses different concentrations of exogenous free carnitine and measures intracellular as well as extracellular acylcarnitine (AC) levels, which overcomes the disadvantage of the conventional IVP assay in the diagnosis of carnitine cycle disorders.

Materials and methods

Materials

Hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), and palmitoylcarnitine (C16) were purchased from Sigma–Aldrich (St Louis, MO, USA). Methanol, acetonitrile, and formic acid were purchased from Wako (Osaka, Japan). As an internal standard, a labeled carnitine standard kit (NSK-B), which contains $^2\text{H}_9$ -carnitine, $^2\text{H}_3$ -acetylcarnitine, $^2\text{H}_3$ -propionylcarnitine, $^2\text{H}_3$ -butyrylcarnitine, $^2\text{H}_9$ -isovalerylcarnitine, $^2\text{H}_3$ -octanoylcarnitine, $^2\text{H}_9$ -myristoylcarnitine, and $^2\text{H}_3$ -palmitoylcarnitine, was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Preparation of standard solutions of ACs

Standard solutions containing 1, 10, 25, and 50 $\mu\text{mol/L}$ each of C6, C8, C10, and C16 were used to validate the recovery and determine linear concentration range of ACs after extraction by the Folch method [16]. The ACs were dissolved in methanol (99.8%), and the prepared standard solution was analyzed directly and after extraction by the Folch method.

Subjects

Human skin fibroblasts from six healthy controls (volunteers) and seven patients with various carnitine cycle disorders—three each with PCD and *CPT2* deficiency and one with *CPT1* deficiency—were analyzed. In all cases, diagnoses were confirmed by mass spectrometric analyses (gas chromatography-mass spectrometry and MS/MS), enzyme assay, and protein or mutational analyses. Informed consent was obtained from the patients or their families. This study was approved by the Ethical Committee of the Shimane University School of Medicine.

In vitro probe acylcarnitine (IVP) assay using MS/MS

An IVP assay was performed, as described, with some modifications [13, 15, 17], and principle of IVP assay was shown Fig. 2. Briefly, 3×10^6 cells were seeded in triplicate onto a six-well microplate (35 mm i.d.; Iwaki) and cultured until confluent. After washing twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA), the cells were subsequently cultured for 96 h in 1 ml of a special experimental minimal essential medium (MEM) containing bovine serum albumin (0.4 % essential fatty acid-free BSA; Sigma), two different concentrations of C0 (Sigma)—10 $\mu\text{mol/L}$ (reduced level, lower compared with physiological level) and 400 $\mu\text{mol/L}$ (excess level)—and unlabelled palmitic acid (0.2 mmol/L; Nacalai Tesque). C0 and AC levels in the culture medium (extracellular fraction) and in the intracellular extract were analyzed after a 96-h incubation period using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA), as described [18].

Intracellular acylcarnitine extraction

Intracellular C0 and ACs were extracted using the Folch method, with some modification [16]. Briefly, harvested cells were washed twice with DPBS buffer. The cell pellet was resuspended in 100 μl volume of DPBS buffer and immediately frozen in liquid N_2 . In order to separate phospholipids and cell debris, 250 μl of Folch reagent (chloroform/methanol, 2:1) was added to the resuspended cell pellet. After vigorous mixing using a vortex mixer, the solution was centrifuged for 10 min at 15,000 rpm at 4 $^\circ\text{C}$. The debris layer around the interface between the aqueous and lipid phases was removed, and the extracted aqueous and lipid phases were mixed and thereafter dried under a nitrogen stream at 50 $^\circ\text{C}$. ACs in culture medium supernatants and extracted intracellular ACs lysate were analyzed

using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA). Briefly, methanol (200 μl) including an isotopically labeled internal standard (Cambridge Isotope Laboratories, Kit NSK-A/B, Cambridge, UK) was added to 10 μl of supernatant from culture medium and extracted intracellular ACs, for 30 min. Portions were centrifuged at $1,000 \times g$ for 10 min, and then 150 μl of supernatant was dried under a nitrogen stream and butylated with 50 μl of 3 N *n*-butanol-HCl at 65 $^\circ\text{C}$ for 15 min. The dried butylated sample was dissolved in 100 μl of 80 % acetonitrile/water (4:1 v/v), and then the ACs in 10 μl of the aliquots were determined using MS/MS [18] and quantified using ChemoViewTM software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

Protein concentration and cell viability

Protein concentrations were measured by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) [19]. The percentage of viable cells was determined at 24, 48, 72, and 96 h of incubation using the modified 3-(4,5-dimethyl-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay [20].

Data and statistical analysis

The results are expressed as mean \pm SD from at least three independent experiments for IVP assay in each cultured cell and three intra-assays and three inter-assays for recovery of standard AC solutions, and statistical significance was evaluated using Student's *t* test in Microsoft Excel. The AC concentrations were expressed as nanomoles per milligram protein.

Results

Recovery of ACs during Folch extraction

The AC standards in the aqueous or lipid fraction were analyzed separately using MS/MS, after extraction by the Folch procedure, and compared with direct analysis of the total mixed standard solutions using three inter-assays and three intra-assays of analysis of standard AC solution. As shown in Fig. 3, most of the C6 and C8-carnitines fractionated to the aqueous phase, while almost all C16-carnitine was exclusively retained in the lipid phase. The amount of C10-carnitine was comparable in both aqueous and lipid phases.

To determine the loss of C0 and ACs during Folch extraction, the standard AC solution was analyzed directly after routine sample preparation for MS/MS and compared with that after Folch extraction. The recovery of ACs in the

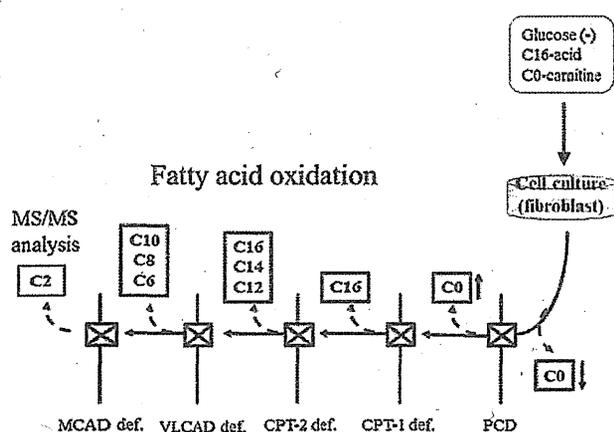


Fig. 2 Principle of in vitro probe acylcarnitine assay. C2, C4, C6, C8, C10, C12, C14, and C16 represent acylcarnitines

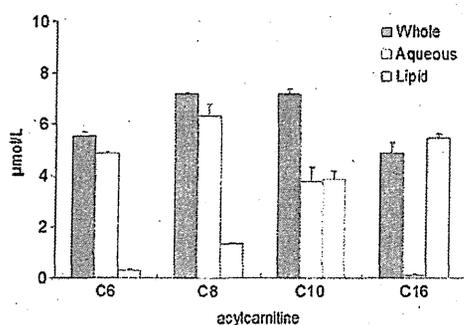


Fig. 3 Recovery of ACs during extraction using the Folch method. Standard solutions of 10 $\mu\text{mol/L}$ each of C6-, C8-, C10-, and C16-carnitine were used to determine the recovery of ACs in the aqueous and lipid fractions during extraction using the Folch method. *Grey column*: ACs in the whole extract after Folch method; *striped column*: ACs in the aqueous fraction of Folch extraction; *open column*: ACs in lipid fraction of Folch extraction. Data are expressed as mean \pm SD (micromoles per liter) from three intra-assays and three inter-assays, and statistical significance was evaluated using Student's *t* test in Microsoft Excel

standard solutions after direct analysis and Folch extraction procedure was analyzed three times by inter-assay. The inter-assay CV of acylcarnitines ranged from 3.21 to 8.33 %. No statistical difference was seen between direct analysis and after Folch extraction.

Acylcarnitine profile in extracellular medium of cultured fibroblasts with excess and reduced concentrations of free carnitine

Using fibroblasts from various carnitine cycle disorders, AC profiles were determined in the extracellular medium with reduced or excess concentration of C0. Reported conventional IVP assay used excess levels of C0 (400 $\mu\text{mol/L}$) [14,

15, 17, 21]. With excess amount of C0 (Table 1, "Medium (C0-excess, 400 μM)"), a selective increase in C16 and a decrease in acetylcarnitine (C2) was observed in cases of CPT2-deficient fibroblasts. AC profiles in media from PCD- and CPT1-deficient fibroblasts were similar to that of healthy controls. In PCD fibroblasts, C2 was 53.1 % of the normal control while C2 in CPT1-deficient fibroblasts was 140 % of the normal control. No statistical difference in C0 level was observed among CPT2-, PCD-, and CPT1-deficient fibroblasts and a healthy control.

In the extracellular medium containing reduced C0, C16 remains higher in cells with CPT2 deficiency, while AC profiles were similar to those observed in C0-excess for PCD- and CPT1-deficient cells and the healthy controls (Table 1, "Medium (C0-reduced, 10 μM)").

Acylcarnitine profile in intracellular lysate with various concentrations of free carnitine

The intracellular C0 and ACs were measured after AC extraction using the Folch method. C16 in the intracellular lysate from CPT2-deficient fibroblasts was significantly elevated in both reduced and excess C0 conditions similar to those in extracellular medium, and diagnostic significant was kept. In the excess C0 condition, CPT1- and PCD-deficient fibroblasts could not be distinguished clearly; based on the C0 levels, even C16 level was relatively low (Fig. 4a). On the other hand, the intracellular C0 under conditions with reduced C0 was 41.78 ± 1.47 and 6.31 ± 2.88 nmol/mg protein/96 h in the normal controls ($n=6$) and patients with PCD ($n=3$), respectively, and the C0 levels of PCD cells were significantly lower ($p<0.001$) as shown in Fig. 4b. This indicated that the C0 uptake was significantly decreased in PCD compared with control in

Table 1 Acylcarnitine profiles of in vitro probe acylcarnitine assay

	Acylcarnitines, nmol/mg protein/96 h						
	C0	C2	C6	C8	C12	C14	C16
Medium (C0 excess, 400 μM)							
Control ($n=6$)	411.74 \pm 23.08	11.80 \pm 1.54	2.60 \pm 0.09	1.70 \pm 0.47	0.79 \pm 0.22	0.34 \pm 0.19	2.06 \pm 0.77
PCD ($n=3$)	432.18 \pm 18.76	6.25 \pm 0.96	2.09 \pm 0.40	0.94 \pm 0.54	0.41 \pm 0.33	0.20 \pm 0.10	1.72 \pm 0.57
CPT-1 ($n=1$)	357.69 \pm 34.16	16.52 \pm 5.60	1.73 \pm 0.87	0.54 \pm 0.94	0.18 \pm 0.14	0.17 \pm 0.16	1.36 \pm 0.98
CPT-2 ($n=3$)	376.56 \pm 42.71	6.88 \pm 0.72	0.94 \pm 0.65	0.41 \pm 0.22	1.70 \pm 0.35	0.80 \pm 0.05	18.73 \pm 1.07
Medium (C0 reduced, 10 μM)							
Control ($n=6$)	9.85 \pm 0.30	1.70 \pm 0.74	0.78 \pm 0.30	0.18 \pm 0.09	0.10 \pm 0.08	0.03 \pm 0.01	0.51 \pm 0.11
PCD ($n=3$)	10.03 \pm 0.71	0.74 \pm 0.33	0.75 \pm 0.31	0.06 \pm 0.04	0.03 \pm 0.01	0.01 \pm 0.01	0.20 \pm 0.08
CPT-1 ($n=1$)	11.06 \pm 0.75	7.56 \pm 3.10	0.98 \pm 0.30	0.55 \pm 0.62	0.09 \pm 0.09	0.08 \pm 0.07	0.01 \pm 0.02
CPT-2 ($n=3$)	9.73 \pm 1.94	0.64 \pm 0.23	0.54 \pm 0.20	0.11 \pm 0.03	0.22 \pm 0.06	0.04 \pm 0.01	2.79 \pm 0.38

The results are expressed as mean \pm SD from three independent experiments with triplication in each cell line. The AC concentration was expressed as nanomoles per milligram protein. C0 free carnitine, C2 acetylcarnitine, C6 hexanoylcarnitine, C8 octanoylcarnitine, C12 dodecanoylcarnitine, C14 myristoylcarnitine, C16 palmitoylcarnitine

C0-reduced condition. Concentration of C16 was also significantly low in PCD in C0-reduced condition. Under the C0-reduced condition, intracellular C0 was much higher, but C16 was much lower in CPT1-deficient fibroblasts, compared with the levels in controls (Fig. 4b).

The ratio of intracellular C0 to extracellular C0 in PCD was significantly lower than that of the controls ($p < 0.001$) in the C0-reduced condition, while that in C0-excessive condition was not significantly different (Fig. 5). Cell viability was measured using the MTT assay under reduced or excess concentrations of C0. The percentage of viable cells cultured in C0-reduced medium was equivalent to that in C0-excess media (data not shown).

Discussion

The present study developed a novel IVP assay for the accurate diagnosis of PCD and CPT1 deficiency. Although previous studies reported that IVP assay was a powerful method for the diagnosis of most FAO disorders [13, 14, 21], this assay turned out to be unable to identify PCD and CPT1 deficiencies. At first, we used a C0-excess experimental medium, which contained 400 $\mu\text{mol/L}$ of C0, according to previous reports [13, 14, 21]. Extracellular

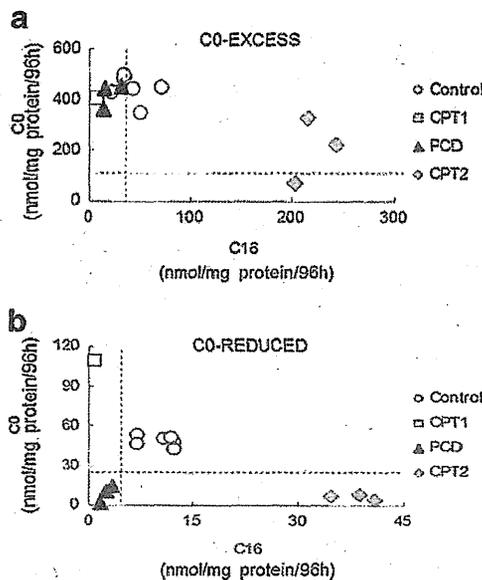


Fig. 4 Intracellular C0 and C16 correlation in patients with carnitine cycle disorders. **a** C0-excessive condition (-E); **b** C0-reduced condition (-R). *open circle*: healthy control ($n=6$); *closed triangle*: PCD ($n=3$); *closed square*: CPT1 deficiency ($n=1$); *closed diamond*: CPT2 deficiency ($n=3$). Cells were incubated in experimental medium with 400 or 10 $\mu\text{mol/L}$ of free carnitine and 200 $\mu\text{mol/L}$ of palmitic acid. After 96-h incubation, cells were harvested, and intracellular free carnitine (C0) and palmitoylcarnitine (C16) were extracted using Folch method and measured using MS/MS. Data of mean values of triplicates are presented (nanomoles per milligram protein per 96 h)

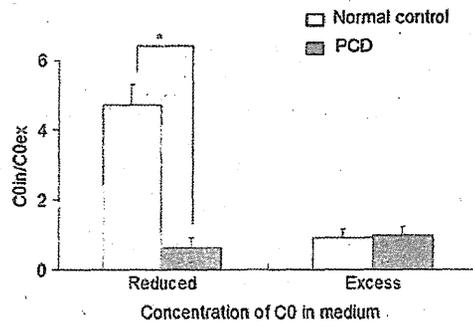


Fig. 5 Ratio of intracellular C0 to extracellular C0. *Open square*: normal control ($n=6$); *closed square*: PCD ($n=3$). Extra- and intracellular C0 of cells with normal control and PCD were measured in C0-reduced (10 $\mu\text{mol/L}$) and C0-excess (400 $\mu\text{mol/L}$) conditions using MS/MS. Data are expressed as mean \pm SD of six normal controls and three patients with PCD. Experiment in each cell line was repeated twice with triplications. Significant differences between normal control and PCD are shown as $*p < 0.001$

AC profiles of patients with PCD and CPT1 deficiency showed a pattern similar to that of normal controls by the conventional assay that contains excessive C0 (400 $\mu\text{mol/L}$) in the culture medium, since C0 moves across the cell membrane down its concentration gradient by passive diffusion. Long-chain fatty acids are transferred across the inner mitochondrial membrane with the assistance of carnitine and carnitine cycle enzymes. The subsequent FAO functions normally even in PCD, and AC profile in PCD is similar to that in normal FAO. Next, we used 50 $\mu\text{mol/L}$ of C0 because the normal range of free carnitine in human plasma was approximately 25 to 50 $\mu\text{mol/L}$ [6]. However, there was no diagnostic difference compare with C0-excess condition, and data are not shown. We analyzed IVP assay in C0-deficient condition (10 $\mu\text{mol/L}$ of C0).

It is known that fibroblasts and muscle and cardiac cells have a high-affinity, low-capacity transporter system [22], and carnitine concentrations in the tissues are much higher than those in serum [23]. Analysis of intracellular C0 and ACs is more relevant for the diagnosis of PCD and CPT1 deficiency because it was shown that C0 was decreased in PCD and increased in CPT1 deficiency in those tissues. When we analyzed cell lysates with MS/MS after direct sonication, artificial peaks of ACs were detected, and the background peaks of mass spectrum were high and hampered the subsequent analyses (data not shown). Hence, we extracted intracellular ACs using a modified Folch method and analyzed both the intracellular lysate and the extracellular medium. This allowed visualization of clear peaks of C0 and ACs in the intracellular lysate, validating that the Folch extraction can be used for simultaneous quantitation of intracellular C0 and a wide range of ACs (short- to long-chain AC).

Uptake of C0 and abnormalities in ACs were associated with the concentration of C0 in culture medium. In the C0-excess condition, it was hard to differentiate PCD from control

cells. Levels of C0 and C16 were overlapped with those of normal control. On the other hand, in the C0-reduced condition, intracellular C0 was significantly decreased in PCD while being increased in CPT1 deficiency, compared with that in normal control. C0-reduced medium was changed after fibroblasts equilibrated in MEM, and normal control could force to uptake free carnitine in C0-deficient condition while cells with PCD could not uptake sufficiently in that condition. Furthermore, the following fatty acid oxidation cycle interrupted, and C16 also decreased in PCD. This correlation of C0 and C16 in the C0-reduced condition is more informative for the diagnosis of carnitine cycle disorders (Fig. 4b). Since cells with PCD cannot uptake C0 via the cell membrane, the finding of reduction of both C0 and C16 is specific for PCD. In case of CPT1 deficiency, C0 uptake is normal, but it cannot bind acyl-CoA ester, resulting in reduced long-chain acylcarnitine production, and FAO is disturbed. Therefore, the stored intracellular ACs were consumed by FAO, and intracellular C16 as well as total ACs were decreased, and C0 was accumulated in intracellular lysate. In contrast, the AC profile of low level of C0 and high level of C16 is diagnostic for CPT2 deficiency. In this disease, normally transferred long-chain AC cannot be converted back from ACs to acyl-CoA esters and C0, the substrate for FAO. Additionally, the ratio of intracellular and extracellular C0s can sensitively distinguish PCD from control in the C0-reduced medium because carnitine transporter of normal cells was forced to uptake C0 up to physiological level in C0-reduced condition while cells with PCD failed for it. In excessive C0 condition, ratio of intracellular and extracellular C0 was similar to that in normal control and PCD since C0 transfer by passive diffusion across the cell membrane.

In conclusion, the simultaneous analysis of intracellular and extracellular C0 and ACs under the various concentrations of free carnitine in the culture medium is useful for diagnosis of FAO, especially carnitine cycle disorders. This study confirms that the newly modified IVP assay is an easy and safe method to diagnose PCD and CPT1 deficiency.

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References

- McGarry JD, Brown NF (1997) The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur J Biochem* 244:1–14
- Sim KG, Hammond J, Wilcken B (2002) Strategies for the diagnosis of mitochondrial fatty acid beta-oxidation disorders. *Clin Chim Acta* 323:37–58
- Tamai I, Ohashi R, Nezu J, Yabuuchi H, Oku A et al (1998) Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. *J Biol Chem* 273:20378–20382
- Treem WR, Stanley CA, Finegold DN, Hale DE, Coates PM (1988) Primary carnitine deficiency due to a failure of carnitine transport in kidney, muscle, and fibroblasts. *N Engl J Med* 319:1331–1336
- Eriksson BO, Gustafson B, Lindstedt S, Nordin I (1989) Transport of carnitine into cells in hereditary carnitine deficiency. *J Inher Metab Dis* 12:108–111
- Longo N, di San A, Filippo C, Pasquali M (2006) Disorders of carnitine transport and the carnitine cycle. *Am J Med Genet C Semin Med Genet* 142C:77–85
- Nakajima Y, Ito T, Maeda Y, Ichiki S, Sugiyama N et al (2010) Detection of pivaloylcarnitine in pediatric patients with hypocarnitinemia after long-term administration of pivalate-containing antibiotics. *Tohoku J Exp Med* 221:309–313
- Hori T, Fukao T, Kobayashi H, Teramoto T, Takayanagi M et al (2010) Carnitine palmitoyltransferase 2 deficiency: the time-course of blood and urinary acylcarnitine levels during initial L-carnitine supplementation. *Tohoku J Exp Med* 221:191–195
- Tein I, DiMauro S, Xie ZW, De Vivo DC (1993) Valproic acid impairs carnitine uptake in cultured human skin fibroblasts. An in vitro model for the pathogenesis of valproic acid-associated carnitine deficiency. *Pediatr Res* 34:281–287
- Pons R, Carozzo R, Tein I, Walker WF, Addonizio LJ et al (1997) Deficient muscle carnitine transport in primary carnitine deficiency. *Pediatr Res* 42:583–587
- Scaglia F, Wang Y, Longo N (1999) Functional characterization of the carnitine transporter defective in primary carnitine deficiency. *Arch Biochem Biophys* 364:99–106
- Wang Y, Ye J, Ganapathy V, Longo N (1999) Mutations in the organic cation/carnitine transporter OCTN2 in primary carnitine deficiency. *Proc Natl Acad Sci U S A* 96:2356–2360
- Endo M, Hasegawa Y, Fukuda S, Kobayashi H, Yotsumoto Y et al (2010) In vitro probe acylcarnitine profiling assay using cultured fibroblasts and electrospray ionization tandem mass spectrometry predicts severity of patients with glutaric aciduria type 2. *J Chromatogr B Analyt Technol Biomed Life Sci* 878:1673–1676
- Law LK, Tang NL, Hui J, Ho CS, Ruiten J et al (2007) A novel functional assay for simultaneous determination of total fatty acid beta-oxidation flux and acylcarnitine profiling in human skin fibroblasts using (2)H(31)-palmitate by isotope ratio mass spectrometry and electrospray tandem mass spectrometry. *Clin Chim Acta* 382:25–30
- Okun JG, Kolker S, Schulze A, Kohlmuller D, Olgemoller K et al (2002) A method for quantitative acylcarnitine profiling in human skin fibroblasts using unlabelled palmitic acid: diagnosis of fatty acid oxidation disorders and differentiation between biochemical phenotypes of MCAD deficiency. *Biochim Biophys Acta* 1584:91–98
- Jauregui O, Sierra AY, Carrasco P, Gratacos E, Hegardt FG et al (2007) A new LC-ESI-MS/MS method to measure long-chain acylcarnitine levels in cultured cells. *Anal Chim Acta* 599:1–6
- Ventura FV, Costa CG, Struys EA, Ruiten J, Allers P et al (1999) Quantitative acylcarnitine profiling in fibroblasts using [U-13C] palmitic acid: an improved tool for the diagnosis of fatty acid oxidation defects. *Clin Chim Acta* 281:1–17
- Li H, Fukuda S, Hasegawa Y, Kobayashi H, Purevsuren J et al (2010) Effect of heat stress and bezafibrate on mitochondrial beta-oxidation: comparison between cultured cells from normal and mitochondrial fatty acid oxidation disorder children using in vitro probe acylcarnitine profiling assay. *Brain Dev* 32:362–370

19. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
20. Honma Y, Ishii Y, Yamamoto-Yamaguchi Y, Sassa T, Asahi K (2003) Cotylenin A, a differentiation-inducing agent, and IFN- α cooperatively induce apoptosis and have an antitumor effect on human non-small cell lung carcinoma cells in nude mice. *Cancer Res* 63:3659–3666
21. Schulze-Bergkamen A, Okun JG, Spiekerkotter U, Lindner M, Haas D et al (2005) Quantitative acylcarnitine profiling in peripheral blood mononuclear cells using in vitro loading with palmitic and 2-oxoadipic acids: biochemical confirmation of fatty acid oxidation and organic acid disorders. *Pediatr Res* 58:873–880
22. Tein I, De Vivo DC, Bierman F, Pulver P, De Meirleir LJ et al (1990) Impaired skin fibroblast carnitine uptake in primary systemic carnitine deficiency manifested by childhood carnitine-responsive cardiomyopathy. *Pediatr Res* 28:247–255
23. Stanley CA (1987) New genetic defects in mitochondrial fatty acid oxidation and carnitine deficiency. *Adv Pediatr Infect Dis* 34:59–88

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Coagulopathy in Patients With Late-Onset Ornithine Transcarbamylase Deficiency in Remission State: A Previously Unrecognized Complication

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KEY WORDS

coagulopathy, late-onset type, ornithine transcarbamylase deficiency

ABBREVIATIONS

ALT—alanine aminotransferase
AST—aspartate aminotransferase
OTC—ornithine transcarbamylase
PIVKA-II—des- γ -carboxyprothrombin
PT—prothrombin time
VK—vitamin K

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The late-onset type of ornithine transcarbamylase (OTC) deficiency is almost asymptomatic before an abrupt onset of metabolic crisis in adolescence. This study focused on coagulopathy in OTC deficiency. We collected laboratory data regarding coagulation from OTC-deficient patients in Kyushu University Hospital in Japan or from cases reported from previous articles. Five patients with late-onset OTC deficiency, admitted to Kyushu University Hospital at the first metabolic attack or who presented at the outpatient clinic in the hospital, were analyzed, and 3 additional cases of OTC deficiency with coagulopathy in previous articles were included. As a result, the blood ammonia levels in these patients were remarkably high at the time of the metabolic attack, and prothrombin times were far below the normal level. The prothrombin times remained significantly abnormal on remission, despite almost normal levels of blood ammonia, serum aspartate aminotransferase, and alanine aminotransferase. Coagulation abnormality is a previously unidentified complication of OTC deficiency in remission state. This information will aid in the identification of patients with OTC deficiency before a lethal metabolic crisis occurs during adolescence. *Pediatrics* 2013;131:e1–e4

Ornithine transcarbamylase (OTC) deficiency, an X-linked disorder, is the most frequent urea cycle disorder characterized by an acute clinical manifestation of hyperammonemia, accounting for >60% of all urea cycle disorders.¹ The classic type of OTC deficiency demonstrates a severe hyperammonemia during infancy and sometimes proves fatal, whereas the late-onset type is almost asymptomatic before an abrupt onset of metabolic crisis in adolescence.²⁻⁴ The early diagnosis of the late-onset type is sometimes difficult because the first crisis usually appears in either adolescent or adult patients who have exhibited normal growth and development. Specific findings of abnormal metabolites in urine such as orotic acid or uracil are critical for making an accurate diagnosis of OTC deficiency.⁵ In contrast, the biochemical markers in routine examinations are usually within the normal ranges. Consequently, the identification of specific markers in a routine biochemical analysis would be clinically beneficial for patients with OTC deficiency. Coagulopathy in OTC deficiency is usually accompanied by acute liver failure, and slight changes in the coagulation data in a remission state have been overlooked as nonspecific findings. Hence, coagulopathy is not recognized as a consequence of OTC deficiency by itself. This report focused on the presence of any underlying coagulation abnormality in OTC deficiency during a metabolically compensated state. We retrospectively collected laboratory data on coagulation from the clinical records of OTC-deficient patients of Kyushu University Hospital in Japan. A bibliographic search was also conducted to determine whether any coagulopathy had previously been noticed before the abrupt onset of metabolic attack in cases of OTC deficiency.

METHODS

Patients with OTC deficiency, admitted to Kyushu University Hospital at the first

metabolic attack or who presented at the outpatient clinic in the hospital from January 1993 to December 2010, were enrolled in this study. A systematic search of the PubMed database was conducted for all articles from 1990 through 2012 with the terms "OTC deficiency," "coagulopathy," "coagulation," or "prothrombin time" in the title, abstract, or key words to determine whether any articles described coagulation abnormality before and after the onset of metabolic attack in OTC-deficient patients.

RESULTS

Five patients were identified at our hospital as follows: patient 1 was a 10-year-old boy with OTC deficiency underlying asymptomatic coagulopathy of unknown etiology before the onset of metabolic crisis. Two patients (patients 2 and 4) were diagnosed during the asymptomatic period because the brother of patient 2 and the maternal uncle of patient 4 died of OTC deficiency at the first metabolic attack. Patients 3 and 5 experienced the first attack during infancy (10 months) and childhood (10 years), respectively. The database search identified 3 publications, and 3 cases of OTC deficiency from these articles with the description of the coagulation at remission state are shown in Table 1 (patients 6-8).⁶⁻⁸

Six of these 8 patients were male. Five patients were successfully treated and maintained normal growth and development for years. One patient died a sudden death at 19 years of age. The 2 patients described in the literature developed a metabolic attack at 24 years of age and died at the first attack. The levels of blood ammonia of these patients were remarkably high during the metabolic attack, whereas other laboratory data such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were indistinctive.

In contrast, prothrombin times (PTs) were below the normal level at the metabolic attack and were also considerably abnormal during remission. The blood ammonia, serum AST, ALT, or albumin levels were close to normal ranges while the patients were being managed by using either mild restriction of protein intake or oral arginine supplementation (Table 1).

DISCUSSION

Coagulation abnormalities have rarely been recognized in association with OTC deficiency. Coagulopathy may occur if the metabolic attacks cause severe liver damage or disseminated intravascular coagulation, but such critical conditions were not found in any of these patients. These cases also demonstrated the presence of abnormalities in coagulation even during remission. The findings suggest that coagulopathy may be a useful sign for detecting an underlying OTC deficiency, especially in boys with nonspecific clinical symptoms such as cyclic vomiting or psychological problems. To the best of our knowledge, no common genetic coagulopathy has yet been identified in the Japanese population; therefore, it is likely that this coagulation abnormality may be specific to OTC deficiency.

The pathogenesis of such a coagulation abnormality is unclear. This coagulopathy does not seem similar to that in fulminant hepatic failure with the collapse of protein synthesis in the liver. The coagulopathy in OTC deficiency might be under the same mechanism that occurs with vitamin K (VK) deficiency because elevation of serum des- γ -carboxyprothrombin (PIVKA-II) levels was sometimes detected, and VK administration seemed effective for these patients. The coagulation factors II, VII, IX, and X are activated in a VK-dependent manner during carboxylation at the γ -terminus-glutamate in

TABLE 1 Clinical, Molecular, and Laboratory Data of the Patients

Variable	RR	Patient No.							
		1	2	3	4	5	6 (Thurlow et al, ⁷ 2010)	7 (Schimanski et al, ⁶ 1996)	8 (Zammarchi et al, ⁸ 1996)
Gender		M	M	M	M	M	M	F	F
Proband		Himself	Brother ^a	Himself	Maternal uncle ^a	Himself	Himself	Herself	Herself
Transmission type		ND	Maternal	Maternal	Maternal	ND	Maternal	ND	ND
OTC gene mutation		R40H	R40H	IVS2-1G>A	R40H	R40H	R40C	Deletion	ND
Diagnosis									
Age		10 y	PP	8 mo	1 mo	10 y	24 y	24 y	11 mo
Method		BDx	GDx	BDx	BDx	BDx	BDx	BDx	BDx
Age at first attack		10 y	12 y	7 d	None	10 y	24 y	24 y ^c	None
Representative data at attack									
AST/ALT, U/L	13–33/6–30	31/23	16/14	86/126 ^d	—	67/154 ^d	114 (AST) ^d	52/47 ^d	—
PT, % [INR]	≥70% [0.90–1.10]	44 [1.59] ^d	56 [1.37] ^d	11 ^d	—	17.5% ^{d,e}	ND [2.7] ^d	ND [3.0] ^d	—
APTT, s	26.0–41.0	38.3	38.4	64.0 ^d	—	60.7 ^d	ND	ND	—
Ammonia, μmol/L	7–39	175 ^d	399 ^d	112 ^d	—	214 ^d	348 ^d	380 ^d	—
Representative data at remission, age		15 y	17 y	23 y	16 y	12 y	24 y	24 y	11 m
AST/ALT, U/L	13–33/6–30	21/21	12/11	25/34 ^d	16/8	29/18	Normal	32/32 ^d	223/103 ^d
PT, % [INR]	≥70 [0.90–1.10]	58 [1.40] ^d	68 [1.20] ^d	69 [1.22] ^d	82 [1.11] ^d	64.1 ^{d,e}	ND [2.2] ^d	ND [3.0] ^d	50 ^d
APTT, s	26.0–41.0	42.1 ^d	40.6	44.0 ^d	32.0	30.3	47.0 ^d (RR: 29–37)	Normal	42 (RR: 30–45)
Ammonia, μmol/L	7–39	19	27	41	9	19	Normal	23.5	74 ^d
PIVKA-II, mAU/mL	<40	32	27	37	56 ^d	ND	ND	ND	ND
Serum albumin, g/dL	4.0–5.0	4.1	4.2	3.9	5.0	4.0	ND	ND	ND
Frequency of severe attack		Once	3 times	3 times	None	3 times	Once	Once	None
Long-term treatment		A, P	A	A, P	A	A, B	—	—	B, P, C
Outcome (age)		Healthy adolescent (16 y)	Healthy adolescent (17 y)	Healthy adult (24 y)	Healthy adolescent (17 y)	Dead (19 y)	Dead (24 y)	Dead (24 y)	Healthy (ND)

A, oral arginine supplement; APTT, activated partial thromboplastin time; B, oral sodium benzoate treatment; BDx, biochemical diagnosis based on the hypersecretion of orotic acid and uracil in urine without elevation of any specific amino acid in blood; C, oral citrulline treatment; GDx, genetic diagnosis by the analysis of the *OTC* gene; INR, international normalized ratio; ND, not described or not available; P, intake protein restriction; PP, during the prenatal period; RR, reference range.

^a Dead.

^b Deletion, T892del,G893del in exon 9.

^c In pregnancy.

^d The laboratory data out of RRs.

^e Hepaplastin test (normal range: 67%–138%).

their N-terminal domains, whereas in the absence of VK or in the presence of VK antagonists, hepatic VK-dependent carboxylase activity is inhibited, and PIVKA-II is released into the blood.⁹ It is possible that abnormal metabolites in association with OTC deficiency, such as orotic acid, might inhibit the hepatic VK-dependent carboxylase. In fact, hyperornithinemia-hyperammonemia-homocitrullinuria syndrome, which is a congenital error in the metabolism of ornithine accompanied with substantial elevations of orotic acid, is also associated with coagulopathy.^{10,11} In addition, a large amount of polyunsaturated fatty acids reduced the expression

of γ -glutamyl carboxylase in apolipoprotein E knock-out mice.¹² Taken together, orotic acid or other unknown abnormal products in common with hyperornithinemia-hyperammonemia-homocitrullinuria syndrome may affect the lipid metabolism and reduce the γ -glutamyl carboxylase activity and, consequently, cause coagulopathy. Further investigation is needed to understand the pathophysiology of coagulation in OTC deficiency. Moreover, the discovery of novel factor-specific inhibitors may provide valuable information for the design of new anticoagulation drugs with mechanisms of action distinct

from warfarin or novel oral anticoagulants.¹³

CONCLUSIONS

The current results suggest that coagulation abnormality is a previously unidentified complication of late-onset OTC deficiency in a metabolically compensated state. This information would be beneficial for undiagnosed patients to avoid lethal metabolic crises during adolescence.

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REFERENCES

1. Kido J, Nakamura K, Mitsubuchi H, et al. Long-term outcome and intervention of urea cycle disorders in Japan. *J Inherit Metab Dis*. 2012;35(5):777–785
2. Gilchrist JM, Coleman RA. Ornithine transcarbamylase deficiency: adult onset of severe symptoms. *Ann Intern Med*. 1987;106(4):556–558
3. Finkelstein JE, Hauser ER, Leonard CO, Brusilow SW. Late-onset ornithine transcarbamylase deficiency in male patients. *J Pediatr*. 1990;117(6):897–902
4. Yoshino M, Nishiyori J, Yamashita F, et al. Ornithine transcarbamylase deficiency in male adolescence and adulthood. *Enzyme*. 1990;43(3):160–168
5. Grompe M, Jones SN, Caskey CT. Molecular detection and correction of ornithine transcarbamylase deficiency. *Trends Genet*. 1990;6(10):335–339
6. Schimanski U, Krieger D, Horn M, Stremmel W, Wermuth B, Theilmann L. A novel two-nucleotide deletion in the ornithine transcarbamylase gene causing fatal hyperammonia in early pregnancy. *Hepatology*. 1996;24(6):1413–1415
7. Thurlow VR, Asafu-Adjaye M, Agalou S, Rahman Y. Fatal ammonia toxicity in an adult due to an undiagnosed urea cycle defect: under-recognition of ornithine transcarbamylase deficiency. *Ann Clin Biochem*. 2010;47(pt 3):279–281
8. Zammarchi E, Donati MA, Filippi L, Resti M. Cryptogenic hepatitis masking the diagnosis of ornithine transcarbamylase deficiency. *J Pediatr Gastroenterol Nutr*. 1996;22(4):380–383
9. Shearer MJ. Vitamin K metabolism and nutrition. *Blood Rev*. 1992;6(2):92–104
10. Fecarotta S, Parenti G, Vajro P, et al. HHH syndrome (hyperornithinaemia, hyperammonaemia, homocitrullinuria), with fulminant hepatitis-like presentation. *J Inherit Metab Dis*. 2006;29(1):186–189
11. Smith L, Lambert MA, Brochu P, Jasmin G, Qureshi IA, Seidman EG. Hyperornithinemia, hyperammonemia, homocitrullinuria (HHH) syndrome: presentation as acute liver disease with coagulopathy. *J Pediatr Gastroenterol Nutr*. 1992;15(4):431–436
12. Vanschoonbeek K, Wouters K, van der Meijden PE, et al. Anticoagulant effect of dietary fish oil in hyperlipidemia: a study of hepatic gene expression in APOE2 knock-in mice. *Arterioscler Thromb Vasc Biol*. 2008;28(11):2023–2029
13. Schulman S, Crowther MA. How I treat with anticoagulants in 2012: new and old anticoagulants, and when and how to switch. *Blood*. 2012;119(13):3016–3023

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Expanded newborn mass screening with MS/MS and medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in Japan

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Abstract

Expanded newborn mass screening (NBS) with tandem mass spectrometry (MS/MS) and medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in Japan are described. Prognosis of patients with inborn metabolic disease was compared between groups detected in the symptomatic and pre-symptomatic stages. Furthermore, clinical, biochemical and genetic findings of Japanese children with MCAD deficiency, which is a most important target of MS/MS screening, was investigated. Our study concluded as follows: 1) the detection incidence in MS/MS screening in Japan is totally about 1 in 9,000, which might be smaller than that of the other countries; 2) Outcomes of patients detected by NBS (pre-symptomatic stage) is more favorable than that of cases detected after symptomatic onset; 3) the incidence of MCAD deficiency in the Japanese population is 1 in 110 thousands, which is approximately 10 times smaller than that in Caucasian; 4) Japanese patients with MCAD deficiency have a common mutation, c.449-452delCTGA, which covers about 45% of alleles of MCAD gene, but not have 985A>G, which is a common mutation of Caucasians patients; 5) genotype/phenotype correlation was not observed in MCAD deficiency; 6) prognosis of the non-symptomatic group is much more favorable than that of the symptomatic group. In conclusion, it is indicated that detection of inborn metabolic disease in the pre-symptomatic stage by NBS is essential to prevent children affected with target diseases of NBS including MCAD deficiency from neurological impairments or infant death.

Key words

expanded newborn mass screening, MS/MS, MCAD deficiency, genotype/phenotype correlation, prevention of neurological impairment

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1. Introduction

The expanded newborn screening (NBS) using tandem mass spectrometry (MS/MS) is becoming popular worldwide. In Japan, a pilot screening was initiated at Fukui University in 1997 (1), and the national

project of MS/MS screening (Principle Investigator, Dr. Seiji Yamaguchi, Shimane University) funded by Grant-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare was started in 2004. For the pilot screening, about 10 laboratories in Japan joined up to 2012. The Japanese government issued notice urging implementation of MS/MS to NBS in 2011. Eventually, the MS/MS screening is becoming spread from 2012 to 2013 (2), and will initiate officially nationwide in next year (2014).

In the expanded NBS, it is considered that medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is a most important screening target, particularly in Caucasian people, because the incidence is high (1 in 10,000), and MCAD deficiency is a causative disease of sudden infant death, and preventable by detection in NBS (3). In this paper, clinical and genetic aspects of MCAD deficiency as well as the results of the pilot MS/MS screening in Japan are described.

2. Pilot MS/MS Screening in Japan

a) Results of pilot MS/MS screening in Japan

A total number of babies screened by MS/MS during the period between 1997 and 2012 was 1,949,987 (about 1.95 million), and 217 cases affected with disease were found as shown in Table 1. The detection incidence of Japanese babies was calculated to be 1 in 8,986, while that in USA was estimated to be 1 in 4,353 (4). The prevalence of Japanese is likely smaller than that of the other countries.

b) The detection prevalence of each disease group

The incidence of amino acidemias was totally 1 in 27 thousands; that of organic

acid disorders, 1 in 22 thousands; and that of fatty acid disorders, 1 in 34 thousands. The most common disease in Japan was propionic acidemia, which was found in 1 in 45,349 (about 45 thousands); and phenylketonuria, 1 in 52,702 (53 thousands); followed by methylmalonic acidemia and MCAD deficiency, each of which in 1 in 108,333 (110 thousands). A considerable number of Japanese patients with propionic acidemia show a mild phenotype with a common mutation, 1304T>C (Y435C) in *PCCB* gene (5).

c) Comparison of outcomes between cases detected by NBS and by tests after symptomatic onset

Outcomes of cases with organic and fatty acid disorders detected in NBS were compared with those detected after symptomatic onset by MS/MS, GC/MS and/or molecular investigation in Shimane University. As shown in Table 2, in organic acid disorder, normal development was achieved in 58 of 70 cases (83%) of the NBS group, as of at least 1 year of age. In contrast, normal development was gotten in only 28 of 114 cases (19%) in the "symptomatic" group.

In fatty acid disorder, normal development was achieved in 40 of 45 cases (89%) in the NBS group, although that was in 25 of 52 cases (48%) in the symptomatic group. Hence, beneficial effect of NBS using MS/MS was indicated. Through the results of the pilot study, we proposed 16 kinds of disease which should be screened as primary targets as marked with black circle in Table 1, in consideration of the false negative rate, complexity of diagnostic approaches, or the benefit of detection by NBS.

Table 1. Results of the pilot screening using MS/MS in Japan (1997 to 2012)

Disease	No. of Cases	Incidence (Japan)	Estimated in USA*
AMINO ACIDEMIA	72	(1:27 K)	(1:15 K)
● Phenylketonuria	37	1: 53 K	1: 19 K
● MSUD	1	1: 1,950 K	1: 159 K
● Homocystinuria	3	1: 650 K	1: 38 K
● Citrulinemia type I	6	1: 330 K	1: 173 K
● Argininosuccinic A	2	1: 980 K	1: 591 K
● Citrin deficiency	23	1: 85 K	n.a.
ORGANIC ACIDEMIA	87	(1: 22 K)	(1:16 K)
● Methylmalonic acidemia	18	1: 110 K	1: 67 K
● Propionic acidemia	43	1: 45 K	1: 276 K
● Isovaleric acidemia	3	1: 650 K	1: 129 K
● MCD	3	1: 650 K	1: 1,380K
● Methylcrotonylglycinuria	13	1: 150 K	1: 44 K
● HMGL deficiency	—	—	1: 1,380K
● Glutaric acidemia type 1	7	1: 280 K	1: 109 K
● β -ketothiolase deficiency	—	—	1: 591 K
FATTY ACID DISORDER	58	(1: 34 K)	(1:10 K)
● CPT1 deficiency	5	1: 390 K	n.a.
● VLCAD deficiency	12	1: 160 K	1: 69 K
● MCAD deficiency	18	1: 110 K	1: 17 K
● TFP/LCHAD deficiency	2	1: 980 K	1: 276 K
● CPT2 deficiency	7	1: 280 K	n.a.
● CACT deficiency	—	—	n.a.
● Glutaric acidemia type 2	6	1: 330 K	n.a.
● Carnitine uptake defect	7	1: 280 K	1: 49 K
● SCHAD deficiency	1	1: 1,950 K	n.a.
TOTAL	Affected cases 217 Screened babies 1,949,987	1: 8,986	1: 4,353

●, primary target disease in the MS/MS screening in Japan. "K" means thousands. * Estimated incidence in USA, based on live birth for 2006 (n=4,138,349) (4). Abbreviation: —, not detected; n.a., not applicable; MCD, multiple carboxylase deficiency; HMGL, 3-hydroxy-3-methylglutaryl-CoA lyase; CPT1 and CPT2, carnitine palmitoyl transferase-I and -II, respectively; VLCAD and MCAD, very-long-, and medium-chain acyl-CoA dehydrogenase, respectively; TFP, mitochondrial trifunctional protein; CACT, carnitine acylcarnitine translocase; LCHAD and SCHAD, long- and short-chain 3-hydroxyacyl-CoA dehydrogenase, respectively.

3. Clinical, Biochemical and Genetic Investigation of Japanese MCAD Deficiency

a) Outline of MCAD deficiency

MCAD deficiency was first discovered in children with sudden infant death syn-

drome-like illness in 1982, and has been found at an incidence of 1 in 10,000. A common mutation, 985A>G, covering about 90% of the alleles in this disease was seen among Caucasian population. Acute symptoms of MCAD deficiency include acute

Table 2. Comparison of outcomes between presymptomatic and symptomatic detection cases in Japanese children with organic academia and fatty acid disorder.

Disease	NBS (MS/MS screening)	Symptomatic (Shimane)
No. of cases	115	196
ORGANIC ACID DISORDER	70	144
Normal development	58 (83%)	28 (19%)
Handicaps or death	12 (17%)	116 (81%)
FATTY ACID DISORDER	45	52
Normal development	40 (89%)	25 (48%)
Handicaps or death	5 (11%)	27 (52%)

NBS, newborn mass screening, MS/MS screening, cases detected by the pilot study between 2004 and 2011. Symptomatic, cases after symptomatic onset and detected by MS/MS, GC/MS or molecular tests in Shimane University from 2001 to 2011.

encephalopathy-like symptoms, or even sudden death (6), following after long fasting or infection, although the patients have no symptoms in the stable condition. Concerning the prognosis, it has been claimed that as many as 35% of MCAD deficiency patients are asymptomatic lifelong, but that over 25% of the symptomatic cases die suddenly during the first episodic attack (3).

Abnormal laboratory tests in the acute condition include hypoglycemia or hyperammonemia. Biochemical markers in MS/MS analysis of blood acylcarnitines are elevation of C8, C6 or C10, or C8/C10. Elevation of hexanoylglycine (HG) and suberylglycine (SG) as well as dicarboxylic acids is often observed by GC/MS analysis of urinary organic acids.

b) Prevalence of MCAD deficiency in Japan

According to the results of pilot screening using MS/MS, MCAD deficiency was found at the incidence of 1 in 110 thousands, and was most common among fatty acid disorders in the pilot study of Japan, as shown in Table 1. However, the incidence is

about 10 times smaller than that of Caucasian whose incidence is 1 in 10 thousands (3).

c) Subjects of MCAD deficiency

Ages at onset, and diagnosis, clinical, biochemical and genetic findings of a total 20 Japanese cases whose blood C8 acylcarnitine, a diagnostic marker of MCAD deficiency, was over the cut off value (0.3 nmol/mL) in MS/MS analysis were investigated (Table 3). Cases 1 through 9 were identified after symptomatic onset (symptomatic group), while cases 10 through 18 were detected in the non-symptomatic or pre-symptomatic stage (non-symptomatic group). Cases 10 through 17 were detected by NBS while Case 18 was by the sibling screening using MS/MS. Acylcarnitine analysis was performed at Shimane University or the other laboratories. GC/MS analysis (7) and gene analysis (8) were performed at Shimane University. The final diagnosis was confirmed by gene analysis, which revealed that cases 19 and 20 were heterozygotes (carrier group). Clinical information was surveyed using questionnaire.

Table 3. Clinical, biochemical and genetic profiles of Japanese cases of MCAD deficiency

No.	Age at onset	Age at diag.	NBS	MS/MS		GC/MS		Genotype		Outcome
				C8 (<0.3)		HG	SG	Allele 1	Allele 2	
Symptomatic group										
1	8m	8m	-	5.97		11.1	44.5	c.449-452del	c.157C>T	impair
2	1y0m	1y0m	-	4.52		n.a	n.a	IVS4+1G>A	c.422 A>T	SID
3 ^a	1y0m	8y10m	-	1.57		45.4	29.6	c.449-452del	c.449-452del	impair
4	1y1m	1y1m	-	7.00		14.7	112.2	del. ex 11-12	del. ex 11-12	impair
5	1y3m	1y3m	-	high*		n.a	n.a	del. ex 11-12	del. ex 11-12	impair
6 ^b	1y4m	1y4m	-	3.33		9.9	15.3	c.449-452del	c.449-452del	impair
7	1y7m	1y7m	-	4.12		6.1	6.4	c.275C>T	c.157C>T	impair
8 ^a	1y8m	1y8m	-	4.75		69.3	1.2	c.449-452del	c.449-452del	SID
9	2y2m	2y2m	-	1.71		n.a	n.a	c.449-452del	c.449-452del	normal
Non-symptomatic group										
10	-	5d	+	5.92		12.9	14.8	c.1085G>A	c.843A>T	normal
11	-	5d	+	5.37		6.3	39.9	c.449-452del	c.154A>G	normal
12	-	5d	+	4.82		15.3	3.8	IVS3+2T>C	c.843 A>T	normal
13	-	5d	+	4.04		n.a	n.a	c.449-452del	c.212 G>A	normal
14	-	5d	+	2.78		11.5	5.9	c.449-452del	c.134 A>G	normal
15	-	5d	+	2.59		3.1	(-)	c.1085G>A	c.1184A>G	normal
16	-	5d	+	2.58		(-)	3.2	c.449-452del	IVS3+5G>A	normal
17	-	5d	+	0.49		9.7	1.5	c.449-452del	c.820 A>C	normal
18 ^b	-	5y5m	-	1.37		n.a	n.a	c.449-452del	c.449-452del	normal
Carrier group										
19	-	5d	+	0.44		(-)	(-)	c.845C>T	(-)	normal
20	-	4m	-	0.51		(-)	(-)	c.843A>T	(-)	normal

Cases 3 and 8 (a-a), and cases 6 and 18 (b-b) are sibling cases, respectively. Abbreviation: diag, diagnosis; NBS, newborn mass screening; -, none; +, NBS received; MS/MS, blood acylcarnitine analysis; GC/MS, urinary organic acid analysis; HG and SG, hexanoylglycine and suberylglycine, respectively; high*, elevated but detailed value not available. n.a, data not available in Shimane University; (-), not detected; SID, sudden infant death; c.449-452del, 4 base deletion of CTGA. impair, neurological impairments as sequellae. Unit: C8, nmol/mL (cut off, <0.3); HG and SG, peak area ratio to IS (%) (7) on GC/MS (normal, both undetectable).

d) Comparison between Symptomatic and non-symptomatic groups of MCAD deficiency

1) Ages at onset and diagnosis: In the symptomatic group, the ages at onset was 8 mo to 2 yr 2 mo. Cases 3 and 8, and cases 6 and 18, were siblings. In 9 cases of the non-symptomatic group, 8 cases were detected on 5 day after birth by NBS, and the other one (case 18) was identified at the age of 5 yr 5 mo by sibling screening.

2) Clinical findings of symptomatic case: In the symptomatic group, all 9 cases had acute encephalopathy or sudden death-like illness in the acute stage. Hypoglycemia was observed in all 7 cases tested, while hyperammonemia was seen in 4 of the 9 cases.

3) Biochemical findings: As shown in Table 2, C8 (cut off, <0.3 nmol/mL) ranged between 1.57 and 7.00 in the symptomatic group, while C8 did between 0.49 and 5.92

in the non-symptomatic group. No significant difference between these two groups was seen in the level of C8. The C8 value of the carrier group (cases 19 and 20) was 0.44 and 0.51, respectively, which was lower compared to those of the 18 affected cases. No significant difference was seen in the urinary excretion amounts of HG or SG between these two groups (Table 3).

4) Gene mutation: c.449-452delCTGA (c.449del4) was identified in 16 of 36 alleles (44%) in 18 Japanese patients with MCAD deficiency. The homozygote of c.449del4 mutants were observed in 4 and 1 cases in the symptomatic and non-symptomatic groups, respectively. A common mutation, 985A>G, found in Caucasian population was never identified in Japanese cases (8). On the other hand, it was reported that the c.449del4 mutation was in 3 of 5 alleles of 3 Korean MCAD deficiency cases (9). It would be interesting to investigate and compare the genotypes of Japanese MCAD deficiency with those of the other Asian countries and the other ethnic groups.

5) Outcomes: With respect to the outcomes, 8 of 9 cases of the symptomatic group resulted in severe handicaps or sudden death, whereas all 9 cases of the non-symptomatic group showed normal development and growth (Table 3). It was likely that there were no genotype/phenotype correlation, although existence of the correlation is not clear enough in the present point (10). These findings indicate that pre-symptomatic detection is important for the favorable outcome in MCAD deficiency. Namely, NBS is essential (11, 12). Furthermore, our data suggests no clear genotype/phenotype in MCAD deficiency.

4. Conclusion

Our study indicated that: 1) the detection incidence in MS/MS screening is totally about 1 in 9,000 in Japan, which might be 2 times smaller than that of other countries; 2) the outcomes of patients detected by NBS is more favorable compared with that of cases detected after symptomatic onset; 3) the incidence of MCAD deficiency is 1 in 110 thousands in Japanese population. This is approximately 10 times smaller than that in Caucasian population; 4) 45% of alleles of *MCAD* gene in Japanese patients have a common mutation, c.449-452delCTGA. The genetic background of Japanese cases is likely the same with Korean patients, but different from those in Caucasians with MCAD; 5) clinical severity of MCAD deficiency may be similar despite the different genetic mutations, suggesting that genotype does not necessarily predict phenotype in MCAD deficiency; 6) prognosis of the symptomatic cases with MCAD deficiency was poor, whereas that of the non-symptomatic group was excellent, indicating "pre-symptomatic detection" is essential to prevent children affected with MCAD deficiency from impairments or sudden death.

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References

- 1) Shigematsu Y, Hirano S, Hata I,