form (juvenile form): patients have intermittent episodic attacks like lethargy, encephalopathy, or even sudden death often onset in infancy or young childhood; 3) mild form (myopathic form): the patients may often show late onset after school ages or adulthood with episodes of hypotonia, myalgia, lethargy, myopathy-like symptoms, or liver dysfunction [6].

In vitro probe acylcarnitine profiling (IVP) assay was developed to evaluate FAO disorders recently [7,8]. Acylcarnitine (AC) profiles in the special culture medium as below after incubating with fatty acids as substrates are determined by MS/MS. Bezafibrate (BEZ) is a hypolipidemic drug, which is an agonist of peroxisome proliferating activator receptor (PPAR), and is claimed to act for induction of several FAO enzymes [9–11].

In this study, the effect of BEZ on various FAO disorders was evaluated using the IVP assay. Furthermore, we report an in vivo trial of BEZ on a boy with the intermediate form of GA2, presenting dramatic improvement with BEZ.

2. Materials and methods

2.1. Subjects and skin fibroblasts

Fibroblasts from 10 Japanese children with FAO disorders, one each of severe and intermediate forms of GA2, 2 each of severe and myopathic (mild) forms of VLCAD deficiency, one each of deficiencies of MCAD, CPT2, CACT, and TFP as well as 6 controls (healthy volunteers, passages 3 to 16) were used. The clinical types and genotypes are shown in Table 1. The child with MCAD deficiency was detected in a newborn mass screening and non-symptomatic, while one with the intermediate form of CPT2 deficiency had liver dysfunction in infancy. The child with the intermediate form of CACT deficiency had two life-threatening episodes in infancy, and after that no episodes were noted with normal development [12]. The child with TFP deficiency had an episode of liver failure in infancy, and then intermittent episodes of myalgia or hypotonia particularly following infection.

The clinical types and genotypes are shown in Table 1. In all cases, at least one allele has missense mutation, although the other alleles had missense or truncated mutations. In CACT deficiency (case 9), a missense mutation in an initiation codon (c.3G>A) in SLC25A29 was detected, but this could harbor a residual activity (Fukao et al., unpublished data).

Table 1
Clinical types and genotypes of patients with mitochondrial fatty acid oxidation disorders investigated.

Disease & case No.	Phenotype	Gene	Genotype, nucleotides (amino acids)	
			Allele 1	Allele 2
GA2				
1 (B)	Severe	*ETFA*	c.799G>A (G267R)	c.7C>T (R3X)
2 (C)	Intermediate	*ETFDH*	c.1217G>A (S406N)	c.1675C>T (R559X)
VLCAD deficiency				
3 (D)	Severe	*ACADV*	c.553G>A (G185S)	IVS9+1g>c
4 (E)	Severe	*ACADV*	c.454G>A (G152S)	c.997insT (A333fsX358)
5 (F)	Myopathic	*ACADV*	c.790A>G (K264E)	c.997insT (A333fsX358)
6 (G)	Myopathic	*ACADV*	c.1144A>C (K382Q)	c.1339G>A (G447R)
MCAD deficiency				
7 (H)	Non-symptomatic	*ACADM*	c.134A>G (Q45R)	c.449delCTGA (T150fsX153)
CPT2 deficiency				
8 (I)	Intermediate	*CPT2*	c.151A>G (R51G)	c.520G>A (E174K)
CACT deficiency				
9 (J)	Intermediate	*SLC25A29*	c.3G>A (M1I)	IVS4+1g>t
TFP deficiency				
10 (K)	Intermediate	*HADHB*	c.739C>T (R247C)	c.817delG (D273fsX292)

Abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase; GA2, glutaric acidemia type 2; VLCAD, very-long-chain acyl-CoA dehydrogenase; CPT2, carnitine palmitoyltransferase-2; TFP, mitochondrial trifunctional protein; CACT, carnitine acylcarnitine translocase. Case 2 (C) is a boy with GA2 who underwent the clinical trial of BEZ. Non-symptomatic case 7 (H) was detected in the newborn mass screening. Severe, intermediate, and myopathic forms are mentioned in the text. (B) to (K) correspond to those of Fig. 1.

2.2. In vitro probe assay with BEZ

Fibroblasts were cultured in 75 cm^2 flasks (Iwaki, Tokyo, Japan) containing modified Eagle's minimal essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 2 mmol/L of L-glutamine (Nacalai Tesque, Kyoto, Japan), 10% FBS (Sigma, St Louis, MO, USA) and 1% penicillin/streptomycin (Sigma) at 37 °C in a humidified 5% CO_2/95% air incubator [13].

Fibroblasts harvested by trypsinization were seeded onto 6-well microplates (35 mm i.d., Iwaki, Japan) with the fresh above medium (2 mL/per well) until they reached confluence. Thereafter, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Carlsbad, CA, USA) and cultured for 96 h in 1 mL of experimental substrate (experimental medium). The experimental medium is MEM containing bovine serum albumin (0.4% essential fatty acid-free BSA; Sigma), L-carnitine (0.4 mmol/L; Sigma), unlabeled palmitic acid (0.2 mmol/L; Nacalai Tesque) and 1% penicillin/streptomycin without L-glutamine, in the presence or absence of BEZ (0.4 mmol/L; Sigma). AC profiles in the culture medium were analyzed after 96 h. The experiments for each case were performed in triplicate.

2.3. Quantitative acylcarnitine analysis

ACs in culture medium supernatants were analyzed using MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA) as described previously [13]. 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 the supernatant from culture medium. The portions were placed on ice for 30 min, and centrifuged at 1000×*g* for 10 min. Then, 150 μL of the supernatant was dried under a nitrogen stream, and butyl-derivatized with 50 μL of 3N n-butanol-HCl at 65 °C for 15 min. The dried butylated sample was dissolved in 100 μL of 80% acetonitrile:water (4:1 v/v). The ACs in 10 μL of the resultant aliquots were analyzed using MS/MS and quantified using ChemoView™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

Protein concentrations were measured by a modification of the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instruction. The AC concentrations are expressed as nmol/mg protein.

2.4. Organic acid analysis using GC/MS

Urinary organic acids were analyzed according to the previous method [14]. Briefly, 40 μg of tropate (IS-2) and 20 μg each of heptadecanoate (IS-1) and tetracosane (C24) as internal standards were added to a urine specimen containing 0.2 mg creatinine. The samples were oxime-derivatized, and solvent extracted with ethylacetate, and trimethylsilylated (TMS-derivatization). The resultant aliquots were subjected to GC/MS (Shimadzu GC/MS QP2010 Plus, Kyoto, Japan), with a DB-5 column of 0.25 mm I.D × 30 m, 1 μm film thickness (J&W, Folsom, CA). The temperature program was from 100 °C to 290 °C at a rate of 4 °C/min).

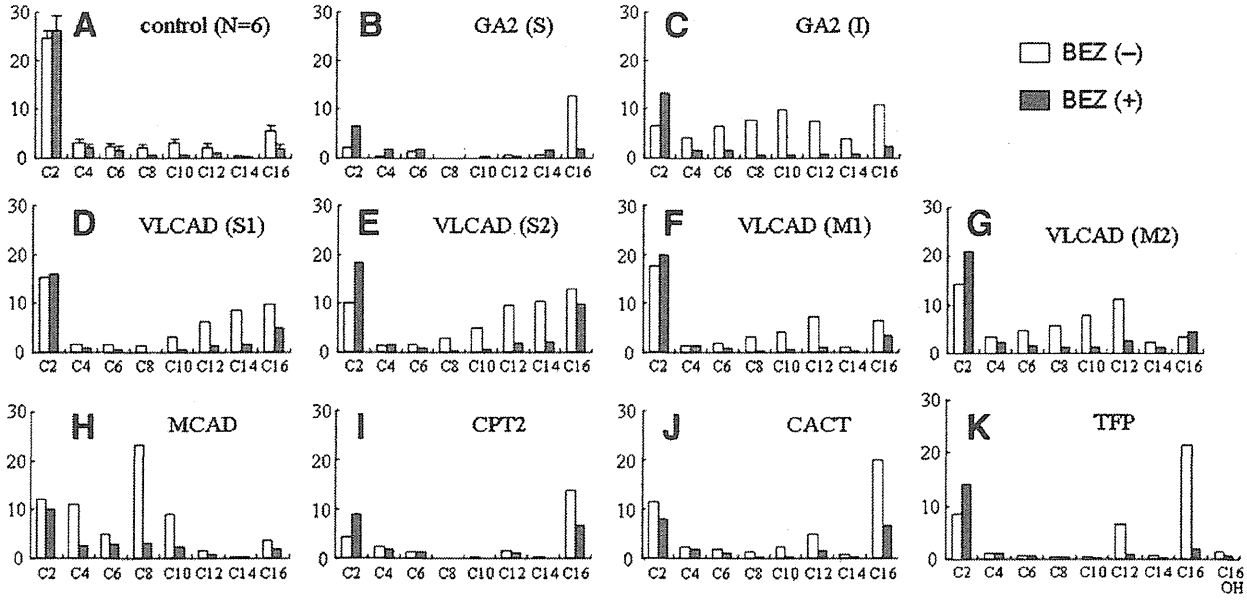

Fig. 1. Acylcarnitine profiles of in vitro probe assay in the presence and absence of bezafibrate. A, normal control; B, severe form of GA2; C, intermediate form of GA2 (the boy who underwent the clinical trial) (S and I, the clinically severe and intermittent form, respectively); D and E, severe form of VLCAD deficiency (S1 and S2, two cases, respectively); F and G: myopathic (mild) form of VLCAD deficiency (M1 and M2, two cases, respectively); H, I, J, and K: deficiencies of MCAD, CPT, CACT, and TFP, respectively. Unit of vertical lines, nmol /mg protein of acylcarnitines; the horizontal lines represent acylcarnitines from C2, C4, C6, C8, C10, C12, C14, C16, and C16-OH. The experiments for each were performed in triplicate, and the mean values of ACs are illustrated with bars. In control (A), the mean plus SD values of 6 controls are shown.

2.5. BEZ trial on a child with the intermittent form of GA2

A Japanese boy with GA2 was detected in the newborn mass screening using MS/MS, and had no special symptoms in infancy with therapies of special formula and carnitine (approximately100 mg/kg/day, div. 3). After 1 year of age, however, he sometimes experienced episodes of hypotonia or lethargy following infection, and muscle weakness, often falling. At the age 2 years and 1 month, he was hospitalized for 2 and a half months, because of infection and lethargy, receiving treatments including artificial respiration to repeated aspiration pneumonia and unconsciousness in intensive care unit (ICU). At discharge, he could not walk alone, and could speak only a few words. So, his family consulted us, and strongly expressed a desire for any new therapies that might help their son.

Thereafter, under the approval by the ethical committee of Shimane University, we started a clinical trial of BEZ, continuing the dietary and carnitine therapies as before, since 2 years and 9 months of his age. His body weight ranged from 12 to 14 kg during the treatment, and 200 to 300 mg/day (approximately 17 to 25 mg/kg/day, div. 3) of BEZ was used in the trial. BEZ was purchased from Kissei Co Ltd, Tokyo, Japan. The study had no potential conflicts of interest (COI) to the authors.

3. Results

3.1. Effects of BEZ on FAO disorders by IVP assay

The AC profiles in the culture medium of fibroblasts from various FAO disorders in the presence and absence of BEZ are illustrated in Fig. 1. In control cells, C2 (acetylcarnitine) is the only prominent peak, and many of ACs further decreased in the presence of BEZ (Fig. 1A).

In the severe form of GA2 (Fig. 1B/S), C16 was apparently decreased, and C2 increased in the presence of BEZ, while C16 was extremely high before BEZ addition. The increase of C2 may indicate the acceleration of FAO, namely an increase of acetyl-CoA production. In the intermediate form of GA2 (Fig. 1C/I), all elevated ACs clearly decreased and normalized in the presence of BEZ, although broad ranges of ACs from C4 to C16 were extremely high before adding BEZ. This patient is the case 3 in Table 1, who underwent the clinical trial of BEZ treatment as illustrated in Fig. 2.

In 2 cases of the severe form of VLCAD deficiency (Figs. 1D/S1, and 1E/S2), elevation of C14 and C16 was larger, compared with that in 2 cases of the mild form (Figs. 1F/M1, and 1G/M2). The elevated ACs

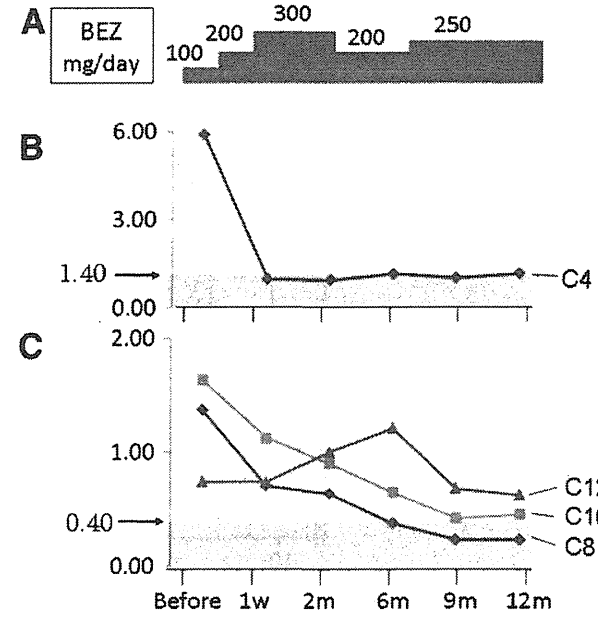

Fig. 2. Bezafibrate administration and changes in blood acylcarnitines. A, dose of bezafibrate, mg/day (approximately 17 to 25 mg/kg/day, div. 3); B, change of C4 acylcarnitine; C, changes in C8, C10, and C12. Arrows with the 1.40 and 0.40 indicate the cutoff values of blood acylcarnitines. Unit of acylcarnitine is nmol/mg protein.

Table 2
Time course of biochemical findings after initiation of bezafibrate administration.

	(Unit)	Before	After the start of BEZ treatment					Reference value*
			1w	2 m	6 m	9 m	12 m	
AST	(IU/L)	47	35	44	43	26	42	10–38
ALT	(IU/L)	27	17	22	24	20	21	5–40
LDH	(IU/L)	448	426	392	384	341	371	100–215
CK	(IU/L)	496	185	187	324	174	207	36–216
TChol	(mg/dL)	161	127	117	141	127	140	150–219

* : used in Shimane University Hospital. Abbreviations: AST, aspartate amino transferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; and TChol, total cholesterol.

such as C10, C12, C14, or C16 in both the severe and mild forms apparently decreased in the presence of BEZ.

In MCAD deficiency (Fig. 1H), the AC peaks of C4 to C10 were significant, but in the presence of BEZ, these AC peaks were almost normalized. In cases of CPT2 deficiency (Fig. 1I), CACT deficiency (Fig. 1J) and TFP deficiency (Fig. 1K), the extremely high AC peaks of C16 and/or C12 apparently decreased to an almost normal level, in the presence of BEZ.

3.2. Clinical trial of BEZ to a GA2 patient

Since the start of BEZ treatment, his motor and social development, and languages remarkably improved, and no metabolic episodes were noted. He became able to walk alone, showed improved muscle strength, and could speak markedly more words in a few weeks. Furthermore, several months later, he could ride a kid's tricycle by himself, although his intellectual ability was on the borderline for entrance into a kindergarten. For at least 1 year of the administration, no adverse effects of BEZ such as hypolipidemia or rhabdomyolysis have been observed.

The routine laboratory data such as blood AST, ALT, LDH or CK were in normal or subnormal ranges as shown in Table 2, showing stable levels of each test, although these laboratory data had sometimes fluctuated, in particular, when his condition was unstable before the initiation of BEZ. For example, during the stay in the ICU at the age of 2 years, the maximum levels of AST, ALT, LDH or CK were 1450 IU/L, 825 IU/L, 5200 IU/L, or 10,750 IU/L, respectively. The maximum level of blood ammonia at the ICU was 126 μg/dL, while no significant elevation was observed after that. Hypoglycemic attacks have not been noted.

BEZ is a hypolipidemic drug, and we have paid attention to the blood level of Cholesterol (TChol), because of the potential adverse effects. The dose of BEZ was 100 mg/day for the first 3 days, 200 mg/day for 4 days, and 300 mg/day for 2 months, respectively, as shown in Fig. 2A. At 2 months after starting BEZ of 300 mg/day, TChol level was a bit low, 117 mg/dL. Since then the dose has been lowered to 200 or 250 mg/day, and the TChol level has ranged between around 130 to 150 mg/mL, as shown in Table 2.

The changes in the AC levels of C4, C8, C10, and C12 are illustrated in Figs. 2B and C, respectively. All the increased ACs returned to approximately normal levels with the administration of BEZ after several months. In particular, C4 decreased to the normal range within a few weeks. Urinary organic acid analysis showed remarkable increases of ethylmalonate, methylsuccinate, adipate, 2-hydroxyglutarate, hexanoylglycine, suberate, and suberylglycine, before the BEZ treatment as shown in Fig. 3. The abnormalities in urinary organic acids were markedly corrected as early as 2 weeks after the initiation of BEZ therapy. The profile was almost normal but for a slight increase of ethylmalonate, and/or hexanoylglycine as illustrated in Fig. 3B.

4. Discussion

The treatments for FAO disorders have generally been described as follows: 1) avoiding a "long fasting": it prevents the increased requirement of fuel from FAO; 2) early infusion of glucose: it should be performed during the metabolic stress resulting from infection, diarrhea or overexercise, to prevent hypercatabolism; 3) carnitine therapy: it may be effective in many cases, although controversy

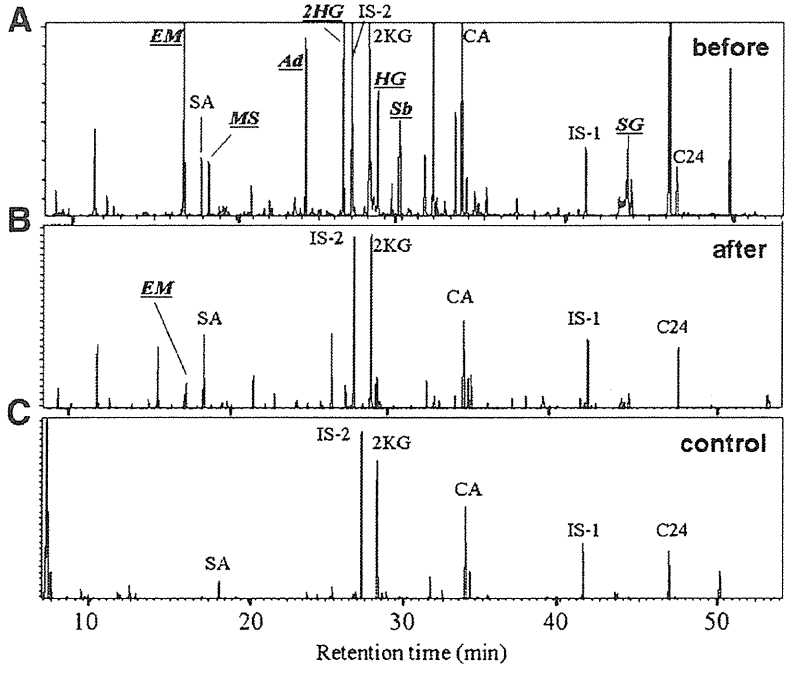

Fig. 3. Urinary organic acid profiles before and after bezafibrate administration. A, The total ion chromatogram (GC/MS) of urinary organic acids just before the start of BEZ; B, One year after the treatment; C, Normal control. Abbreviations: IS-2, IS-1 and C24 are tropate, heptadecanoate, and tetracosane, respectively, as internal standards; EM, ethylmalonate; SA, succinate; MS, methylsuccinate; Ad, adipate; 2HG, 2-hydroxyglutarate; 2KG, 2-ketoglutarate; HG, hexanoylglycine; Sb, suberate; CA, citrate; SG, suberylglycine. Metabolites judged as abnormal are shown in bold letters underlined.

remains in some cases; and 4) dietary therapy, including high carbohydrate/low lipid diet: Dietary restriction in FAO disorders may be less strict [15–18].

In this study, we demonstrated the effect of BEZ on various FAO disorders at both in vitro and in vivo levels. It was indicated by the IVP assay that FAO capacity was corrected by BEZ in various FAO disorders, and a clinical trial of BEZ in a boy with the intermediate form of GA2 showed a favorable consequence. Bastin, Djourdi and their colleagues reported the potential effect of BEZ for FAO disorders showing the increase of enzyme activity and mRNA production in several FAO enzymes from normal individuals, or reduced ACs in cells from VLCAD deficiency by the IVP assay using stable isotope-labeled palmitate [19]. Furthermore, they are performing a clinical trial on adult cases of mild form of CPT2 deficiency [20,21]. We should continue to pay attention to potential adverse effects of BEZ, including hypolipidemia or rhabdomyolysis, although such signs have never seen up to now.

We used the IVP assay to investigate the effect of BEZ in the other FAO disorders including GA2, deficiencies of MCAD, CACT, and TFP as well as CPT2 or VLCAD deficiencies. The beneficial effect of BEZ was clearly demonstrated in all these cases tested in this study, which included the clinically intermediate or severe forms as well as the mild form, having missense mutation of at least one allele. However, it is not yet clear whether the effect of BEZ is due to induction of mutant enzyme itself, or due to stimulation of the other FAO enzymes. If the effect is due to the latter mechanism, BEZ could potentially induce a "high pressure" on the FAO pathway, even resulting in devastating outcomes. We should further investigate the effect on the other severe forms of FAO disorders, the relation with the genotypes, or the dose dependency.

BEZ is an agonist of PPAR, which facilitates transcription of genes encoding FAO enzymes, and subsequently induces FAO enzyme production. Eventually, it can be considered to correct the FAO capacity in FAO disorders. Recently, it was reported that resveratrol which is a natural polyphenol and an activator of Sirtuin 1, is also expected to be a novel treatment option for FAO disorders [22]. The effect of resveratrol on FAO capacity can also be evaluated by the IVP assay like this study.

In conclusion, BEZ could be a new promising treatment option for FAO disorders. Many of patients with FAO disorders, particularly children with the milder form or adult cases, are intellectually normal, and their life prognosis is favorable if they can be prevented from severe episodes like encephalopathy. Symptoms or severity of FAO disorders are very heterogeneous depending on the disease, genetic background or lifestyle. Additional clinical studies of BEZ treatment will be essential for confirmation of its safety and practical utility.

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References

[1] L.L. McCabe, E.R.B. McCabe, Expanded newborn screening: implications for genomic medicine, Annu. Rev. Med. 59 (2008) 163–175.
[2] B. Wilcken, M. Haas, P. Joy, V. Wiley, F. Bowling, K. Carpenter, J. Christodoulou, D. Cowley, C. Ellaway, J. Fletcher, E.P. Kirk, B. Lewis, J. McGill, H. Peters, J. Pitt, E. Ranieri, J. Yaplito-Lee, A. Boneh, Expanded newborn screening: outcome in screened and unscreened patients at age 6 years, Pediatrics 124 (2009) e241–e248.
[3] P. Rinaldo, D. Matern, M.J. Bennett, Fatty acid oxidation disorders, Annu. Rev. Physiol. 64 (2002) 477–502.
[4] M. Kompare, W.B. Rizzo, Mitochondrial fatty-acid oxidation disorders, Semin. Pediatr. Neurol. 15 (2008) 140–149.
[5] M.J. Bennett, Pathophysiology of fatty acid oxidation disorders, J. Inherit. Metab. Dis. 33 (2010) 533–537.
[6] B.S. Andresen, S. Olpin, B.J. Poorthuis, H.R. Scholte, C. Vianey-Saban, R. Wanders, L. Ijlst, A. Morris, M. Pourfarzam, K. Bartlett, E.R. Baumgartner, J.B. deKlerk, L.D. Schroeder, T.J. Corydon, H. Lund, V. Winter, P. Bross, L. Bolund, N. Gregersen, Clear correlation of genotype with disease phenotype in very-long-chain acyl-CoA dehydrogenase deficiency, Am. J. Hum. Genet. 64 (1999) 479–494.
[7] J.G. Okun, S. Kolker, A. Schulze, D. Kohlmuller, K. Olgemoller, M. Linder, G.F. Hoffmann, R.J.A. Wanders, E. Mayatepek, 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 (2002) 91–98.
[8] K.G. Sim, K. Carpenter, J. Hammond, J. Christodoulou, B. Wilcken, Quantitative fibroblast acylcarnitine profiles in mitochondrial fatty acid beta-oxidation defects: phenotype/metabolite correlations, Mol. Genet. Metab. 76 (2002) 327–334.
[9] T. Aoyama, J.M. Peters, N. Iritani, T. Nakajima, K. Furihata, T. Hashimoto, F.J. Gonzalez, Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor alpha (PPARalpha), J. Biol. Chem. 273 (1998) 5678–5684.
[10] F. Djouadi, J.P. Bonnefont, L. Thuillier, V. Droin, N. Khadom, A. Munnich, J. Bastin, Correction of fatty acid oxidation I carnitine palmitoyl transferase 2-deficient cultured skin fibroblasts by bezafibrate, Pediatr. Res. 54 (2003) 446–451.
[11] S. Gobin-Limballe, F. Djouadi, F. Aubey, S. Olpin, B.S. Andresen, S. Yamaguchi, H. Mandel, T. Fukao, J.P. Ruiter, R.J. Wanders, R. McAndrew, J.J. Kim, J. Bastin, Genetic basis for correction of very-long-chain acyl-coenzyme A dehydrogenase deficiency by bezafibrate in patient fibroblasts: toward a genotype-based therapy, Am. J. Hum. Genet. 81 (2007) 1133–1143.
[12] E. Lopriore, R.J. Gemke, N.M. Verhoeven, C. Jakobs, R.J. Wanders, A.B. Roeleveld-Versteeg, B.T. Poll-The, Carnitine-acylcarnitine translocase deficiency: phenotype, residual enzyme activity and outcome, Eur. J. Pediatr. 160 (2001) 101–104.
[13] H. Li, S. Fukuda, Y. Hasegawa, H. Kobayashi, J. Purevsuren, Y. Mushimoto, S. Yamaguchi, Effect of heat stress and bezafibrate on mitochondrial β-oxidation: comparison between cultured cells from normal and mitochondrial fatty acid oxidation disorder children using in vitro probe acylcarnitine profiling assay, Brain Dev. 32 (2010) 362–370.
[14] S. Yamaguchi, M. Iga, M. Kimura, Y. Suzuki, N. Shimozawa, T. Fukao, N. Kondo, Y. Tazawa, T. Orii, Urinary organic acids in peroxisomal disorders: a simple screening method, J. Chromatogr. B 758 (2001) 81–86.
[15] U. Spiekerkoetter, M. Lindner, R. Santer, M. Grotzke, M.R. Baumgartner, H. Boehles, A. Das, C. Haase, J.B. Hennermann, D. Karall, H. de Klerk, I. Knerr, H.G. Koch, B. Plecko, W. Röschinger, K.O. Schwab, D. Scheible, F.A. Wijburg, J. Zschocke, E. Mayatepek, U. Wendel, Management and outcome in 75 individuals with long-chain fatty acid oxidation defects: results from a workshop, J. Inherit. Metab. Dis. 32 (2009) 488–497.
[16] U. Spiekerkoetter, J. Bastin, M. Gillingham, A. Morris, F. Wijburg, B. Wilcken, Current issues regarding treatment of mitochondrial fatty acid oxidation disorders, J. Inherit. Metab. Dis. 33 (2010) 555–561.
[17] P. Laforêt, C. Vianey-Saban, Disorders of muscle lipid metabolism: diagnostic and therapeutic challenges, Neuromuscul. Disord. 20 (2010) 693–700.
[18] J. Vockley, D.A. Whiteman, Defects of mitochondrial beta-oxidation: a growing group of disorders, Neuromuscul. Disord. 12 (2002) 235–246.
[19] F. Djouadi, F. Aubey, D. Schlemmer, J.P. Ruiter, R.J. Wanders, A.W. Strauss, J. Bastin, Bezafibrate increases very-long-chain acyl-CoA dehydrogenase protein and mRNA expression in deficient fibroblasts and is a potential therapy for fatty acid oxidation disorders, Hum. Mol. Genet. 14 (2005) 2695–2703.
[20] J.P. Bonnefont, J. Bastin, P. Laforet, F. Aubey, A. Mogenet, S. Romano, D. Ricquier, S. Gobin-Limballe, A. Vassault, A. Behin, B. Eymard, J.L. Bresson, F. Djouadi, Long-term follow-up of bezafibrate treatment in patients with the myopathic form of carnitine palmitoyltransferase 2 deficiency, Clin. Pharmacol. Ther. 88 (2010) 101–108.
[21] J.P. Bonnefont, J. Bastin, A. Behin, F. Djouadi, Bezafibrate for treatment of an inborn mitochondrial b-oxidation defect, N. Engl. J. Med. 360 (2009) 838–840.
[22] J. Bastin, A. Lopes-Costa, F. Djouadi, Exposure to resveratrol triggers pharmacological correction of fatty acid utilization in human fatty acid oxidation-deficient fibroblasts, Hum. Mol. Genet. 20 (2011) 2048–2057.

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Brief Communication

Clinical and molecular aspects of Japanese children with medium chain acyl-CoA dehydrogenase deficiency

Jamiyan Purevsuren [a,*], Yuki Hasegawa [a], Seiji Fukuda [a], Hironori Kobayashi [a], Yuichi Mushimoto [a], Kenji Yamada [a], Tomoo Takahashi [a], Toshiyuki Fukao [b,c], Seiji Yamaguchi [a]

[a] Department of Pediatrics, Shimane University Faculty of Medicine, Izumo 693-8501, Japan
[b] Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Gifu 501-1194, Japan
[c] Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu, Gifu 501-1194, Japan

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ABSTRACT

We report the outcome of 16 Japanese patients with medium chain acyl-CoA dehydrogenase deficiency. Of them, 7 patients were diagnosed after metabolic crisis, while 9 were detected in the asymptomatic condition. Of the 7 symptomatic cases, 1 died suddenly, and 4 cases had delayed development. All 9 patients identified by neonatal or sibling screening remained healthy. Of 14 mutations identified, 10 were unique for Japanese, and 4 were previously reported in other nationalities. Presymptomatic detection including neonatal screening obviously improves quality of life of Japanese patients, probably regardless of the genotypes.

1. Introduction

Medium chain acyl-CoA dehydrogenase deficiency (MCADD) (MIM #201450) is an autosomal recessive inherited metabolic disorder of mitochondrial fatty acid oxidation. The number of MCADD patients has recently become larger in Japan with the spread of acylcarnitine analysis using tandem mass spectrometry (MS/MS). The disease frequency was estimated to be approximately 1:100,000 in Japan according to a newborn screening pilot study of 1.57 millions babies (unpublished report). Clinical symptoms of MCADD are heterogeneous, ranging from asymptomatic to severe handicaps followed by metabolic crisis or sudden unexpected death (SUD) [1,2]. Approximately 20% of previously undiagnosed patients die during their first metabolic decompensation [3–7]. Blood acylcarnitine, urinary organic acid analyses, MCAD activity and mutation analyses are major tools for diagnosis of MCADD. A common c.985A>G mutation has been reported in 80–90% of Caucasian patients [8–16] while c.449–452delCTGA mutation was identified in 45% of mutant alleles in Japanese patients with MCADD [17]. In recent years, the detection incidence of the presymptomatic patients with MCADD has increased since the neonatal mass screening was expanded in Japan. However, there are few reports of the outcomes of the Japanese patients. Herein, we report the relation of clinical onsets, genotypes and outcomes of 16 Japanese children with MCADD, and 4 heterozygote carriers, which were analyzed in Shimane University.

2. Subjects and methods

2.1. Subjects

Sixteen Japanese patients with MCADD from 15 unrelated families, including previously reported 9 cases [17], and 4 carriers were studied (Table 1). The patients were analyzed for confirmation of diagnosis in Shimane University from 2001 to 2011. Of them, 8 (cases 8 to 16) were identified by neonatal mass screening, 7 (cases 1 to 7) were diagnosed after metabolic crisis, and 1 was detected by sibling screening. Cases 2 and 8 were siblings, and cases 19 and 20 were parents of case 16. Diagnosis of the patients was confirmed by urinary organic acid, blood acylcarnitine and mutation analyses.

2.2. Mass spectrometric analysis

Acylcarnitines in blood spots on filter paper were analyzed by a method standardized for neonatal mass screening using MS/MS, an API 3000 instrument (Applied Biosystems, Foster City, CA, USA) [8,18]. Urinary organic acids were analyzed using the solvent extraction method by the QP 2010 capillary GC/MS system (Shimadzu Co., Ltd., Kyoto, Japan) [19]. The determination of test values was assessed using reference values set at the Shimane University.

* Corresponding author at: Department of Pediatrics, Shimane University Faculty of Medicine, 89-1 Enya, Izumo, Shimane 693-8501, Japan. Fax: +81 853 20 2215.
E-mail address: jamiyan@med.shimane-u.ac.jp (J. Purevsuren).

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Table 1
Clinical and genetic characteristics of Japanese patients with MCAD deficiency.

Patient	Sex	Age at onset	Age at diagnosis	Neonatal screening	Primary clinical symptoms	Hypoglycemia	Hyperammonemia	Tandem MS		GC/MS (RPA%)		Genotype		Outcome
								C8 <0.35 μM	C8/C10 (<3)	HG	SG	Allele 1	Allele 2	
Symptomatic group														
1	F	1 y	1 y	–	Cardiopulmonary arrest, dyspnea, poor feeding	(+)	(−)	4.52	8.69	n.a	n.a	IVS4+1G≥A	c.422A≥T (Q116L)	Sudden death
2 [a] [*]	M	1 y 4 m	1 y 4 m	–	Gastroenteritis, seizures	(+)	(−)	3.33	17.53	9.9	15.3	c.449–452delCTGA	c.449–452delCTGA	Severe handicapped
3 [a]	M	8 m	8 m	–	Cardiopulmonary arrest	(n.a)	(+)	5.97	3.49	11.1	44.5	c.449–452delCTGA	c.157C>T (R28C)	Developmental delay
4	F	1 y 1 m	1 y 1 m	–	Developmental regression	(+)	(+)	7.00	21.00	14.7	112.2	del. ex 11–12	del. ex 11–12	Developmental delay
5 [a]	F	2 y 2 m	2 y 2 m	–	Cold, gastroenteritis	(+)	(−)	1.71	15.55	n.a	n.a	c.449–452delCTGA	c.449–452delCTGA	Developmental delay
6 [a]	F	1 y 3 m	1 y 3 m	–	Unconsciousness, apnea, vomiting	(n.a)	(−)	n.a	n.a	n.a	n.a	del. ex 11–12	del. ex 11–12	Normal
7 [a]	F	1 y 7 m	1 y 7 m	–	Unconsciousness, fever	(+)	(+)	4.12	10.05	6.1	6.4	c.275C>T (P67L)	c.157C>T (R28C)	Normal
Asymptomatic group														
8 [a] [*]	M	–	5 y 5 m	–	Normal	(−)	(−)	1.37	39.14	n.a	n.a	c.449–452delCTGA	c.449–452delCTGA	Normal
9 [a]	F	–	5 d	+	Normal	(−)	(−)	5.92	11.38	12.9	14.8	c.1085G>A (G337E)	c.843A>T (R256S)	Normal
10	F	–	5 d	+	Normal	(−)	(−)	5.37	12.49	6.33	39.88	c.449–452delCTGA	c.157C>A (R28H)	Normal
11	M	–	5 d	+	Normal	(−)	(−)	4.82	13.03	15.3	3.8	IVS3+2T≥C	c.843A>T (R256S)	Normal
12	F	–	5 d	+	Normal	(−)	(−)	4.04	14.96	n.a	n.a	c.449–452delCTGA	c.212G≥A (G46D)	Normal
13 [a]	F	–	5 d	+	Normal	(−)	(−)	2.78	15.44	11.5	5.9	c.449–452delCTGA	c.134A>G (Q20R)	Normal
14	F	–	5 d	+	Normal	(−)	(−)	2.59	10.00	3.08	3.20	c.1085G≥A (G337E)	c.1184A≥G (K370R)	Normal
15	M	–	5 d	+	Normal	(−)	(−)	2.58	8.32	(−)	1.50	c.449–452delCTGA	IVS3+5G≥A	Normal
16 [a]	M	–	5 d	+	Normal	(−)	(−)	0.49	3.77	9.7	(−)	c.449–452delCTGA	c.820A>C (M249V)	Normal
Carrier group														
17	M	–	5 d	+	Normal	(−)	(−)	0.44	1.02	(−)	(−)	c.845C>T (P257L)	n.d	Normal
18	F	–	4 m	–	Eczema	(−)	(−)	0.51	0.88	(−)	(−)	c.843A>T (R256S)	n.d	Normal
19	M	–		–	Normal	(−)	(−)	0.37	1.00	n.a	n.a	c.449–452delCTGA	n.d	Normal
20	F	–		–	Normal	(−)	(−)	0.20	0.95	n.a	n.a	c.820A>C (M249V)	n.d	Normal

[a]: Purevsuren et al. [17] reported; [*]: siblings; sex: M, male; F, female; age: y, year; m, month; d, day; +, involved to neonatal mass screening; (−), not detected; n.a, not available; RPA%, relative peak area percentage; HG, hexanoylglycine; SG, suberylglycine; novel mutations are underlined.

2.3. DNA sequencing of gene, acyl-CoA dehydrogenase, medium chain (ACADM)

Genomic DNA was purified from the patients' fibroblasts or blood filter papers using the QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany). Mutation analysis on genomic DNA was performed by PCR for each exon and its intron boundaries followed by direct sequencing [17].

Informed consent to perform DNA analysis was obtained from the parents of the patients. This study was approved by the Ethical Committee of the Shimane University Faculty of Medicine.

3. Results

3.1. Clinical features of patients

The clinical features of 16 Japanese patients with MCADD and 4 carriers (9 males and 11 females) are summarized in Table 1, including previously reported cases [17]. All 7 patients that were diagnosed after metabolic crisis were born before the initiation of newborn screening in their local area. The mean age at onset of the symptomatic cases was 1 y 3 m (range: 8 m to 2 y 2 m). The symptomatic patients were all in good general health with normal development until metabolic crisis. Metabolic crises were triggered by common cold or gastroenteritis in 5 cases. One of them died of SUD. Four cases had mild to severe handicaps, and 2 cases developed normally. The patients who were identified by neonatal screening remain healthy at this time.

3.2. Biochemical results of patients

The results of mass spectrometric analysis are shown in Table 1. Blood acylcarnitine analysis was available in 15 of the 16 patients. Octanoylcarnitine (C8) and octanoyl:decanoylcarnitine (C8/C10) ratio were assessed for detection of MCADD. Marked elevation of C8 and C8/C10 was observed in 14 cases (1.37–7 μmol/L), and slight elevation of C8 and C8/C10 (0.49 μmol/L and 3.77) was found in one case (case 16). The level of C8 was also mildly elevated in 3 (0.44, 0.51 and 0.37 μmol/L, respectively) of the 4 carriers while C8/C10 value was under cut-off (1.02, 0.88 and 1.00). Case 20, who is a mother of case 16, showed no abnormal findings.

Urinary organic acids were analyzed in 11 cases with MCADD and 4 carriers. Both hexanoylglycine and suberylglycine were elevated in 9 patients, and hexanoylglycine or suberylglycine was increased in one case each. However, neither hexanoylglycine nor suberylglycine was identified in the carriers.

3.3. Mutations in acyl-CoA dehydrogenase, medium chain (ACADM) gene

Fourteen types of mutations were identified in 30 independent alleles, 7 of which were novel. These included three types of splice site alterations (IVS3+2T>C, IVS3+5G>A and IVS4+1G>A), and four missense mutations (G46D, Q116L, G337E and K395R). These novel mutations were not detected in 120 alleles from unaffected Japanese individuals. All mutations are summarized in Table 1, together with previously reported cases (cases 2, 3, 5–9, 13 and 16) [17]. A c.449–452delCTGA [20,21] was detected in 10 (33.3%) of 30 independent alleles (2 cases with homozygous and 6 cases with compound heterozygous). A homozygous large deletion including exons 11 and 12 [22] was identified in 4 (13.3%) alleles. R28C (2/30 alleles), R256S (2/30 alleles), P67L (1/30 alleles), M249V (1/30 alleles) and G337E (1/30 alleles) were also observed (Table 1) [9,17,22].

4. Discussion

We investigated the relationship between clinical and molecular spectrums of 16 Japanese patients with MCADD. While symptomatic patients remained undiagnosed until metabolic crisis, asymptomatic patients were identified by neonatal mass screening (8 cases), or by sibling screening (1 case). Most of the symptomatic cases developed metabolic crisis associated with hypoglycemia triggered by common infection and prolonged fasting [3,4]. Those patients had poor outcomes such as mild to severe impairments or SUD. However, expansion of blood acylcarnitine analysis using MS/MS for neonatal mass screening in Japan allowed earlier detection of MCADD in the asymptomatic/presymptomatic stage. Subsequent prophylactic management for those children was conducted in a more appropriate and timely manner during metabolic stress such as fever, viral infection and other medical procedures.

Fourteen mutations were identified in 30 independent alleles including seven novel mutations. The amino acids affected by the novel missense mutations (G46D, Q116L, G337E and K395R) are highly conserved among different species (*Pan Troglodytes*, *Rattus norvegicus*, *Xenopus laevis* and *Danio rerio*), suggesting that these amino acids play an important role in medium acyl-CoA dehydrogenase activity. There are also splice site alterations such as IVS3+2T>C, IVS3+5G>A and IVS4+1G>A positioned at a 5′ donor splice site. Shapiro and Senapathy 5′ splice site scores [23] of altered sites changed from 76.4 to 58.6 for IVS3+2T>C, from 76.4 to 62.4 for IVS3+5G>A, and from 86.3 to 68.1 for IVS4+1G>A, respectively, suggesting that these changes are likely responsible for aberrant mRNA splicing. It is reported that point mutations in donor splice site produced exon skipping or aberrant 5′ donor splice site activation [24]. Since these changes likely resulted in aberrant splicing and premature truncation, non-sense mediated mRNA decay [25] or translation into shorter proteins with unlikely residual activity would result.

Most of the mutations detected in Japanese patients were unique, but Q20R, R28C, R256S and c.449–452delCTGA were previously reported in other nationalities [9,22,26,27]. The Japanese patient with compound heterozygous of R28C was one quarter of Caucasian. In contrast, a common missense mutation c.985A>G (80–90%) of Caucasian [8,15,28–30] was not detected in any Japanese patients in this study.

Our study demonstrates that detection in the asymptomatic/presymptomatic stage is essential to achieve favorable outcomes of patients with MCADD. Neonatal mass screening is absolutely a beneficial system to improve the quality of life of patients with MCADD. Genetic background of Japanese patients with MCADD is different from those in Caucasians. It is likely that there is no correlation between genotype and phenotype in Japanese patients with MCADD, and a specific genotype does not predict the clinical outcome.

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References

[1] L. Waddell, V. Wiley, K. Carpenter, B. Bennetts, L. Angel, B.S. Andresen, B. Wilcken, Medium-chain acyl-CoA dehydrogenase deficiency: genotype-biochemical phenotype correlations, Mol. Genet. Metab. 87 (2006) 32–39.
[2] B. Wilcken, M. Haas, P. Joy, V. Wiley, M. Chaplin, C. Black, J. Fletcher, J. McGill, A. Boneh, Outcome of neonatal screening for medium-chain acyl-CoA dehydrogenase deficiency in Australia: a cohort study, Lancet 369 (2007) 37–42.
[3] A.K. Iafolla, R.J. Thompson Jr., C.R. Roe, Medium-chain acyl-coenzyme A dehydrogenase deficiency: clinical course in 120 affected children, J. Pediatr. 124 (1994) 409–415.
[4] R.J. Pollitt, J.V. Leonard, Prospective surveillance study of medium chain acyl-CoA dehydrogenase deficiency in the UK, Arch. Dis. Child. 79 (1998) 116–119.
[5] B. Wilcken, J. Hammond, M. Silink, Morbidity and mortality in medium chain acyl coenzyme A dehydrogenase deficiency, Arch. Dis. Child. 70 (1994) 410–412.

[6] E.H. Touma, C. Charpentier, Medium chain acyl-CoA dehydrogenase deficiency, Arch. Dis. Child. 67 (1992) 142–145.
[7] T.G. Derks, D.J. Reijngoud, H.R. Waterham, W.J. Gerver, M.P. van den Berg, P.J. Sauer, G.P. Smit, The natural history of medium-chain acyl CoA dehydrogenase deficiency in the Netherlands: clinical presentation and outcome, J. Pediatr. 148 (2006) 665–670.
[8] T.H. Zytkovicz, E.F. Fitzgerald, D. Marsden, C.A. Larson, V.E. Shih, D.M. Johnson, A.W. Strauss, A.M. Comeau, R.B. Eaton, G.F. Grady, Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: a two-year summary from the New England Newborn Screening Program, Clin. Chem. 47 (2001) 1945–1955.
[9] K. Tanaka, I. Yokota, P.M. Coates, A.W. Strauss, D.P. Kelly, Z. Zhang, N. Gregersen, B.S. Andresen, Y. Matsubara, D. Curtis, et al., Mutations in the medium chain acyl-CoA dehydrogenase (MCAD) gene, Hum. Mutat. 1 (1992) 271–279.
[10] Y. Matsubara, K. Narisawa, S. Miyabayashi, K. Tada, P.M. Coates, Molecular lesion in patients with medium-chain acyl-CoA dehydrogenase deficiency, Lancet 335 (1990) 1589.
[11] Y. Matsubara, K. Narisawa, S. Miyabayashi, K. Tada, P.M. Coates, C. Bachmann, L.J. Elsas II, R.J. Pollitt, W.J. Rhead, C.R. Roe, Identification of a common mutation in patients with medium-chain acyl-CoA dehydrogenase deficiency, Biochem. Biophys. Res. Commun. 171 (1990) 498–505.
[12] J.H. Ding, C.R. Roe, A.K. Iafolla, Y.T. Chen, Medium-chain acyl-coenzyme A dehydrogenase deficiency and sudden infant death, N. Engl. J. Med. 325 (1991) 61–62.
[13] N. Gregersen, A.I. Blakemore, V. Winter, B. Andresen, S. Kolvraa, L. Bolund, D. Curtis, P.C. Engel, Specific diagnosis of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in dried blood spots by a polymerase chain reaction (PCR) assay detecting a point-mutation (G985) in the MCAD gene, Clin. Chim. Acta 203 (1991) 23–34.
[14] S. Giroux, A. Dube-Linteau, G. Cardinal, Y. Labelle, N. Laflamme, Y. Giguere, F. Rousseau, Assessment of the prevalence of the 985A>G MCAD mutation in the French-Canadian population using allele-specific PCR, Clin. Genet. 71 (2007) 569–575.
[15] G.A. Horvath, A.G. Davidson, S.G. Stockler-Ipsiroglu, Y.P. Lillquist, P.J. Waters, S. Olpin, B.S. Andresen, J. Palaty, J. Nelson, H. Vallance, Newborn screening for MCAD deficiency: experience of the first three years in British Columbia, Canada, Can. J. Public Health 99 (2008) 276–280.
[16] I. Yokota, P.M. Coates, D.E. Hale, P. Rinaldo, K. Tanaka, Molecular survey of a prevalent mutation, 985A-to-G transition, and identification of five infrequent mutations in the medium-chain Acyl-CoA dehydrogenase (MCAD) gene in 55 patients with MCAD deficiency, Am. J. Hum. Genet. 49 (1991) 1280–1291.
[17] J. Purevsuren, H. Kobayashi, Y. Hasegawa, Y. Mushimoto, H. Li, S. Fukuda, Y. Shigematsu, T. Fukao, S. Yamaguchi, A novel molecular aspect of Japanese patients with medium-chain acyl-CoA dehydrogenase deficiency (MCADD): c.449-452delCTGA is a common mutation in Japanese patients with MCADD, Mol. Genet. Metab. 96 (2009) 77–79.
[18] B. Wilcken, V. Wiley, J. Hammond, K. Carpenter, Screening newborns for inborn errors of metabolism by tandem mass spectrometry, N. Engl. J. Med. 348 (2003) 2304–2312.
[19] M. Kimura, T. Yamamoto, S. Yamaguchi, Automated metabolic profiling and interpretation of GC/MS data for organic acidemia screening: a personal computer-based system, Tohoku J. Exp. Med. 188 (1999) 317–334.
[20] G. Tajima, N. Sakura, H. Yofune, Y. Nishimura, H. Ono, Y. Hasegawa, I. Hata, M. Kimura, S. Yamaguchi, Y. Shigematsu, M. Kobayashi, Enzymatic diagnosis of medium-chain acyl-CoA dehydrogenase deficiency by detecting 2-octenoyl-CoA production using high-performance liquid chromatography: a practical confirmatory test for tandem mass spectrometry newborn screening in Japan, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 823 (2005) 122–130.
[21] K. Yokoi, T. Ito, Y. Maeda, Y. Nakajima, A. Ueta, T. Nomura, N. Koyama, I. Kato, S. Suzuki, Y. Kurono, N. Sugiyama, H. Togari, Acylcarnitine profiles during carnitine loading and fasting tests in a Japanese patient with medium-chain acyl-CoA dehydrogenase deficiency, Tohoku J. Exp. Med. 213 (2007) 351–359.
[22] R. Ensenauer, J.L. Winters, P.A. Parton, D.F. Kronn, J.W. Kim, D. Matern, P. Rinaldo, S.H. Hahn, Genotypic differences of MCAD deficiency in the Asian population: novel genotype and clinical symptoms preceding newborn screening notification, Genet. Med. 7 (2005) 339–343.
[23] M.B. Shapiro, P. Senapathy, RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression, Nucleic Acids Res. 15 (1987) 7155–7174.
[24] E. Buratti, M. Chivers, J. Kralovicova, M. Romano, M. Baralle, A.R. Krainer, I. Vorechovsky, Aberrant 5′ splice sites in human disease genes: mutation pattern, nucleotide structure and comparison of computational tools that predict their utilization, Nucleic Acids Res. 35 (2007) 4250–4263.
[25] P.A. Frischmeyer, H.C. Dietz, Nonsense-mediated mRNA decay in health and disease, Hum. Mol. Genet. 8 (1999) 1893–1900.
[26] M.J. Nichols, C.A. Saavedra-Matiz, K.A. Pass, M. Caggana, Novel mutations causing medium chain acyl-CoA dehydrogenase deficiency: under-representation of the common c.985 A>G mutation in the New York state population, Am. J. Med. Genet. A 146A (2008) 610–619.
[27] H.I. Woo, H.D. Park, Y.W. Lee, D.H. Lee, C.S. Ki, S.Y. Lee, J.W. Kim, Clinical, biochemical and genetic analyses in two Korean patients with medium-chain acyl-CoA dehydrogenase deficiency, Korean J. Lab. Med. 31 (2011) 54–60.
[28] K. Carpenter, V. Wiley, K.G. Sim, D. Heath, B. Wilcken, Evaluation of newborn screening for medium chain acyl-CoA dehydrogenase deficiency in 275 000 babies, Arch. Dis. Child. Fetal Neonatal Ed. 85 (2001) F105–F109.
[29] H.R. Seddon, A. Green, R.G. Gray, J.V. Leonard, R.J. Pollitt, Regional variations in medium-chain acyl-CoA dehydrogenase deficiency, Lancet 345 (1995) 135–136.
[30] T.G. Derks, T.S. Boer, A. van Assen, T. Bos, J. Ruiter, H.R. Waterham, K.E. Niezen-Koning, R.J. Wanders, J.M. Rondeel, J.G. Loeber, L.P. Ten Kate, G.P. Smit, D.J. Reijngoud, Neonatal screening for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in The Netherlands: the importance of enzyme analysis to ascertain true MCAD deficiency, J. Inherit. Metab. Dis. 31 (2008) 88–96.

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ORIGINAL PAPER

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

Jamiyan Purevsuren · Hironori Kobayashi · Yuki Hasegawa · Kenji Yamada · Tomoo Takahashi · Masaki Takayanagi · Toshiyuki Fukao · Seiji Fukuda · Seiji Yamaguchi

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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.

Keywords Fatty acid oxidation · Carnitine cycle disorder · Acylcarnitine profile · ESI-MS/MS

J. Purevsuren · H. Kobayashi · Y. Hasegawa · K. Yamada · T. Takahashi · S. Fukuda · S. Yamaguchi (✉)
Department of Pediatrics, Shimane University School of Medicine, 89-1 Enya,
Izumo, Shimane 693-8501, Japan
e-mail: seijiyam@med.shimane-u.ac.jp

M. Takayanagi
Division of Metabolism, Chiba Children's Hospital,
Chiba 266-0007, Japan

T. Fukao
Department of Pediatrics, Graduate School of Medicine,
Gifu University,
Gifu, Gifu 501-1194, Japan

T. Fukao
Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences,
Gifu University,
Gifu, Gifu 501-1194, Japan

J. Purevsuren
Medical Genetics Laboratory,
National Center for Maternal and Child Health,
Khuvisgalchdyn street, Bayangol district,
Ulaanbaatar 210624, Mongolia

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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,

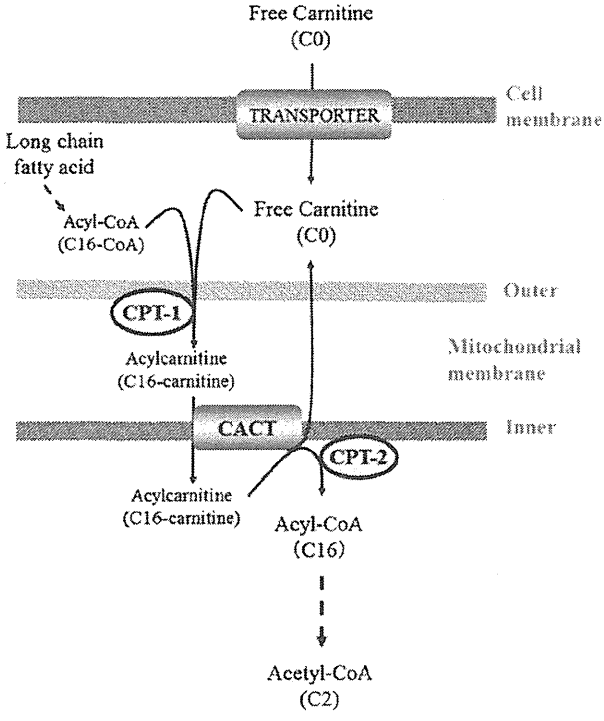

Fig. 1 Pathway for mitochondrial fatty acid beta-oxidation. Transporter: carnitine uptake transporter; *CPT-1*: carnitine palmitoyltransferase-1, *CACT*: carnitine acylcarnitine translocase, *CPT-2*: carnitine palmitoyltransferase-2. *Solid arrows* indicate single reactions; *dashed arrows* indicate 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 heterogenous, 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 $^{2}[H]_{9}$-carnitine, $^{2}[H]_{3}$-acetylcarnitine, $^{2}[H]_{3}$-propionylcarnitine, $^{2}[H]_{3}$-butyrylcarnitine, $^{2}[H]_{9}$-isovalerylcarnitine, $^{2}[H]_{3}$-octanoylcarnitine, $^{2}[H]_{9}$-myristoylcarnitine, and $^{2}[H]_{3}$-palmitoylcarnitine, was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Preparation of standard solutions of ACs

Standard solutions containing 1, 10, 25, and 50 μmol/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 μmol/L (reduced level, lower compared with physiological level) and 400 μmol/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 °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 °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×*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 °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 Chemo-View™ software (Applied Biosystems/MDS SCIEX, Toronto, Canada).

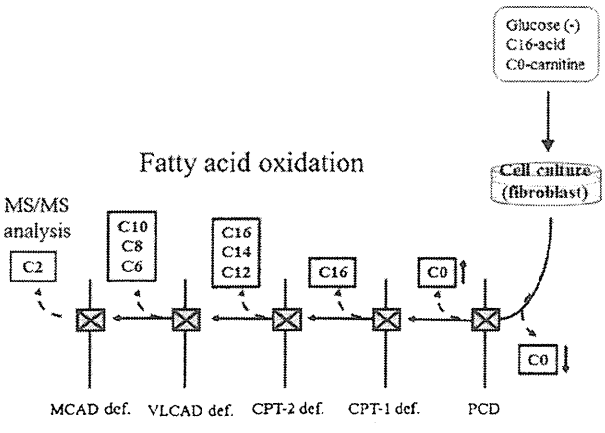

Fig. 2 Principle of in vitro probe acylcarnitine assay. *C2, C4, C6, C8, C10, C12, C14*, and *C16* represent acylcarnitines

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±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

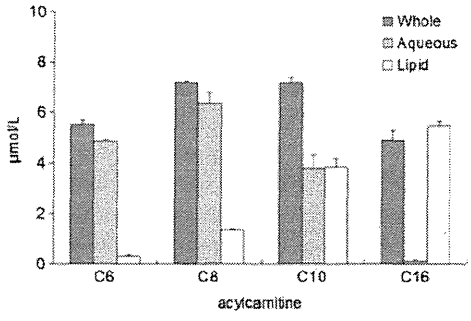

Fig. 3 Recovery of ACs during extraction using the Folch method. Standard solutions of 10 μmol/L each of C6-, C8-, C10-, and C16-carnitine were used to determine the recovery of ACs in the aqueous and lipid fractions during extraction using the Folch method. *Grey column*: ACs in the whole extract after 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±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 μmol/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±23.08	11.80±1.54	2.60±0.09	1.70±0.47	0.79±0.22	0.34±0.19	2.06±0.77
PCD (n=3)	432.18±18.76	6.25±0.96	2.09±0.40	0.94±0.54	0.41±0.33	0.20±0.10	1.72±0.57
CPT-1 (n=1)	357.69±34.16	16.52±5.60	1.73±0.87	0.54±0.94	0.18±0.14	0.17±0.16	1.36±0.98
CPT-2 (n=3)	376.56±42.71	6.88±0.72	0.94±0.65	0.41±0.22	1.70±0.35	0.80±0.05	18.73±1.07
Medium (C0 reduced, 10 μM)							
Control (n=6)	9.85±0.30	1.70±0.74	0.78±0.30	0.18±0.09	0.10±0.08	0.03±0.01	0.51±0.11
PCD (n=3)	10.03±0.71	0.74 ±0.33	0.75±0.31	0.06±0.04	0.03±0.01	0.01±0.01	0.20±0.08
CPT-1 (n=1)	11.06±0.75	7.56±3.10	0.98±0.30	0.55±0.62	0.09±0.09	0.08 ±0.07	0.01 ±0.02
CPT-2 (n=3)	9.73±1.94	0.64±0.23	0.54±0.20	0.11±0.03	0.22±0.06	0.04±0.01	2.79±0.38

The results are expressed as mean±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 μmol/L of C0, according to previous reports [13, 14, 21]. Extracellular

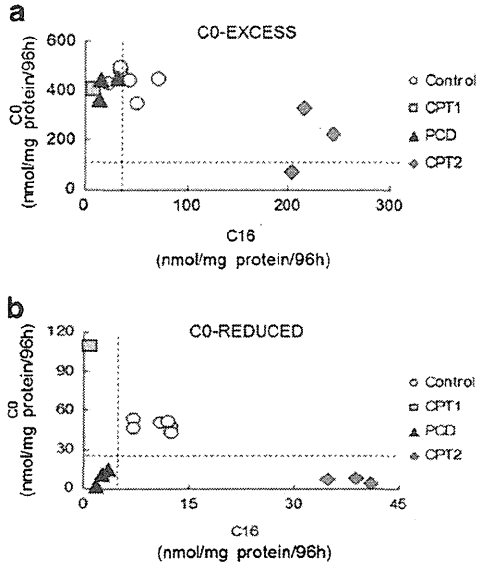

Fig. 4 Intracellular C0 and C16 correlation in patients with carnitine cycle disorders. **a** C0-excessive condition (−*E*); **b** C0-reduced condition (−*R*). *open circle*: healthy control ($n=6$); *closed triangle*: PCD ($n=3$); *closed square*: CPT1 deficiency ($n=1$); *closed diamond*: CPT2 deficiency ($n=3$). Cells were incubated in experimental medium with 400 or 10 μmol/L of free carnitine and 200 μmol/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)

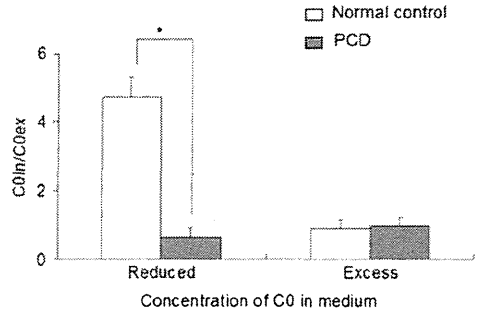

Fig. 5 Ratio of intracellular C0 to extracellular C0. *Open square*: normal control ($n=6$); *closed square*: PCD ($n=3$). Extra- and intracellular C0 of cells with normal control and PCD were measured in C0-reduced (10 μmol/L) and C0-excess (400 μmol/L) conditions using MS/MS. Data are expressed as mean±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 μmol/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 μmol/L of C0 because the normal range of free carnitine in human plasma was approximately 25 to 50 μmol/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 μmol/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

1. McGarry JD, Brown NF (1997) The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem 244:1–14
2. Sim KG, Hammond J, Wilcken B (2002) Strategies for the diagnosis of mitochondrial fatty acid beta-oxidation disorders. Clin Chim Acta 323:37–58
3. 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
4. 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
5. Eriksson BO, Gustafson B, Lindstedt S, Nordin I (1989) Transport of carnitine into cells in hereditary carnitine deficiency. J Inherit Metab Dis 12:108–111
6. 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
7. 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
8. 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
9. 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
10. Pons R, Carrozzo R, Tein I, Walker WF, Addonizio LJ et al (1997) Deficient muscle carnitine transport in primary carnitine deficiency. Pediatr Res 42:583–587
11. Scaglia F, Wang Y, Longo N (1999) Functional characterization of the carnitine transporter defective in primary carnitine deficiency. Arch Biochem Biophys 364:99–106
12. 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
13. 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
14. Law LK, Tang NL, Hui J, Ho CS, Ruiter 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
15. 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
16. 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
17. Ventura FV, Costa CG, Struys EA, Ruiter 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
18. 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-alpha 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

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

AUTHORS: Kenji Ihara, MD, PhD,[a] Makoto Yoshino, MD, PhD,[b] Takayuki Hoshina, MD, PhD,[a] Nawomi Harada, PhD,[b] Kanako Kojima-Ishii, MD, PhD,[a] Mika Makimura, MD, PhD,[a] Yuki Hasegawa, MD, PhD,[c] Yoriko Watanabe, MD, PhD,[b] Seiji Yamaguchi, MD, PhD,[c] and Toshiro Hara, MD, PhD[a]

[a]Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; [b]Department of Pediatrics and Child Health, Kurume University School of Medicine; and [c]Department of Pediatrics, Shimane University School of Medicine

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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Address correspondence to Kenji Ihara, MD, PhD, Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan. E-mail: k-ihara@pediatr.med.kyushu-u.ac.jp

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abstract

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 nutriture. *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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診療

タンデムマス法を導入した新生児マススクリーニングの現状

山口 清次（やまぐち せいじ）*

要旨　新生児マススクリーニングにタンデムマス法が世界的に普及しつつある．わが国でも2004年以来厚生労働省の研究班で検討され，厚生労働省から課長通達が2011年に出され，全国に広がりつつある．これまでの研究成果を踏まえて，タンデムマス法導入の現状といくつかの課題について述べた．タンデムマス法による疾患の発見頻度は全体では約1万人に1人で，費用対効果は良好，また発症前診断による臨床的効果も良好なことが確認された．今後の検討課題として，患者の長期追跡体制，カウンセリング体制の充実，検査施設の適正配置，患者家族のQOLの向上などがあげられている．

はじめに

新生児マススクリーニング（mass-screening：MS）は，知らずに放置すると障害の起こるような先天性疾患を発症前に見つけて，障害を予防（または軽減）する事業である．1950年代にフェニルケトン尿症の治療ミルクが開発され，1960年代にガスリーテストが発明されて以来，新生児MSの概念が急速に広まった[1)~3)]．

日本では1977年から新生児MSが全国的に開始されて，これまでに4,300万人以上が新生児MSを受け，1万人以上の小児が障害から救われたといわれている．かくして新生児MSによる小児の障害予防効果は広く認識され，最近タンデムマス法の導入による対象疾患の拡大（拡大スクリーニング）がトピックになっている[4)5)]．2004年からタンデムマス法導入に関連した厚生労働省科学研究班が組織され，2011年には厚生労働省課長通達が出され，全国自治体に広がりつつある．そこで，わが国のタンデムマス法導入による新生児MSの現状を述べたい．

表1　これまでの新生児マススクリーニングの対象疾患と発見頻度

疾患	頻度	費用便益
1）フェニルケトン尿症	1：7万	○
2）メープルシロップ尿症	1：50万	△
3）ホモシスチン尿症	1：80万	△
4）ガラクトース血症（全体）	1：3万*	
（1型）	（1：80万）	△
（2型）	（1：60万）	
5）先天性甲状腺機能低下症	1：3,000	◎
6）先天性副腎過形成	1：2万	○

*：ガラクトース高値の多くは酵素欠損でなく，門脈奇形やシトリン欠損症などの2次性のもので，真の先天性ガラクトース血症はきわめてまれである．6疾患全体での発見頻度（約1,600人に1人の頻度）
◎：費用便益がきわめて良好，○：良好，△：あまり良くない

I　これまでのわが国の新生児マススクリーニングの状況

これまでわが国では6疾患を対象に新生児MSが行われてきた（表1）．日本人における各

* 島根大学医学部小児科
〒693-8501 島根県出雲市塩冶町89-1