**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 <0.35μM | C8/C10 (<3) | HG | SG | Allele 1 | Allele 2 | |
| *Symptomatic group* | | | | | | | | | | | | | | |
| 1 | F | 1y | 1y | – | 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 | 1y 4m | 1y 4m | – | Gastroenteritis, seizures | (+) | (−) | 3.33 | 17.53 | 9.9 | 15.3 | c.449–452delCTGA | c.449–452delCTGA | Severe handicapped |
| 3 [a] | M | 8m | 8m | – | Cardiopulmonary arrest | (n.a) | (+) | 5.97 | 3.49 | 11.1 | 44.5 | c.449–452delCTGA | c.157C>T (R28C) | Developmental delay |
| 4 | F | 1y 1m | 1y 1m | – | Developmental regression | (+) | (+) | 7.00 | 21.00 | 14.7 | 112.2 | del. ex 11–12 | del. ex 11–12 | Developmental delay |
| 5 [a] | F | 2y 2m | 2y 2m | – | Cold, gastroenteritis | (+) | (−) | 1.71 | 15.55 | n.a | n.a | c.449–452delCTGA | c.449–452delCTGA | Developmental delay |
| 6 [a] | F | 1y 3m | 1y 3m | – | 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 | 1y 7m | 1y 7m | – | Unconsciousness, fever | (+) | (+) | 4.12 | 10.05 | 6.1 | 6.4 | c.275C>T (P67L) | c.157C>T (R28C) | Normal |
| *Asymptomatic group* | | | | | | | | | | | | | | |
| 8 [a] * | M | – | 5y 5m | – | Normal | (−) | (−) | 1.37 | 39.14 | n.a | n.a | c.449–452delCTGA | c.449–452delCTGA | Normal |
| 9 [a] | F | – | 5d | + | Normal | (−) | (−) | 5.92 | 11.38 | 12.9 | 14.8 | c.1085G>A (G337E) | c.843A>T (R256S) | Normal |
| 10 | F | – | 5d | + | Normal | (−) | (−) | 5.37 | 12.49 | 6.33 | 39.88 | c.449–452delCTGA | c.157C>A (R28H) | Normal |
| 11 | M | – | 5d | + | Normal | (−) | (−) | 4.82 | 13.03 | 15.3 | 3.8 | IVS3+2T≥C | c.843A>T (R256S) | Normal |
| 12 | F | – | 5d | + | Normal | (−) | (−) | 4.04 | 14.96 | n.a | n.a | c.449–452delCTGA | c.212G≥A (G46D) | Normal |
| 13 [a] | F | – | 5d | + | Normal | (−) | (−) | 2.78 | 15.44 | 11.5 | 5.9 | c.449–452delCTGA | c.134A>G (Q20R) | Normal |
| 14 | F | – | 5d | + | Normal | (−) | (−) | 2.59 | 10.00 | 3.08 | 3.20 | c.1085G≥A (G337E) | c.1184A≥G (K370R) | Normal |
| 15 | M | – | 5d | + | Normal | (−) | (−) | 2.58 | 8.32 | (−) | 1.50 | c.449–452delCTGA | IVS3+5G≥A | Normal |
| 16 [a] | M | – | 5d | + | Normal | (−) | (−) | 0.49 | 3.77 | 9.7 | (−) | c.449–452delCTGA | c.820A>C (M249V) | Normal |
| *Carrier group* | | | | | | | | | | | | | | |
| 17 | M | – | 5d | + | Normal | (−) | (−) | 0.44 | 1.02 | (−) | (−) | c.845C>T (P257L) | n.d | Normal |
| 18 | F | – | 4m | – | 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.

## Acknowledgments

We thank all of the attending physicians for providing clinical information regarding each patient. We are also grateful to Y. Ito, M. Furui, T. Esumi and N. Tomita for their technical assistance. This work was supported by a Grants-in-Aid for scientific research from the Japan Society for the Promotion of Science (J.P., and S.Y.); and a Grant from the Ministry of Education, Science, Technology, Sports and Culture of Japan, and the Ministry of Health, Labour and Welfare of Japan (S.Y).

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Anal Bioanal Chem (2013) 405:1345–1351
DOI 10.1007/s00216-012-6532-3

ORIGINAL PAPER

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

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Received: 24 July 2012 / Revised: 10 October 2012 / Accepted: 30 October 2012 / Published online: 10 November 2012


**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

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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,

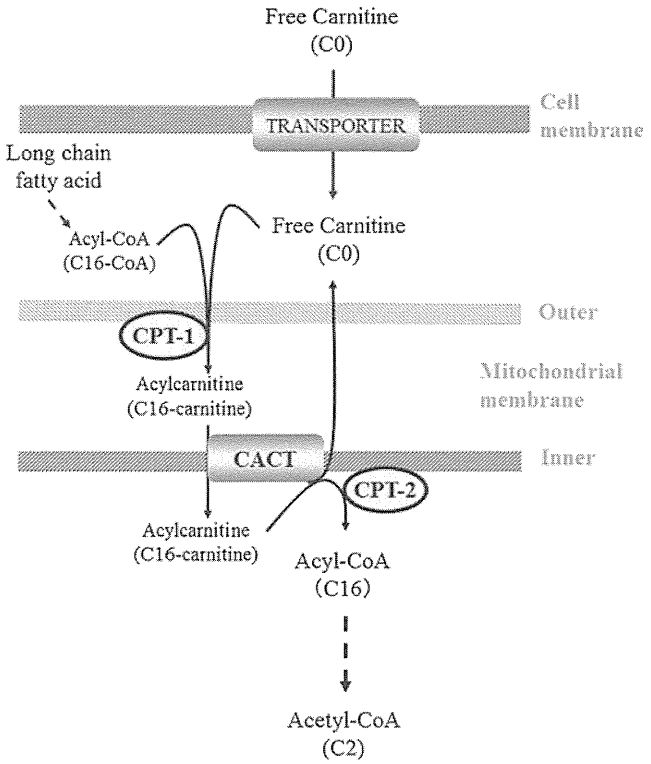


**Fig. 1** Pathway for mitochondrial fatty acid beta-oxidation. Transporter: carnitine uptake transporter; *CPT-1*: carnitine palmitoyltransferase-1, *CACT*: carnitine acylcarnitine translocase, *CPT-2*: carnitine palmitoyltransferase-2. *Solid arrows* indicate single reactions; *dashed arrows* indicate 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, $3\times10^6$ 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).

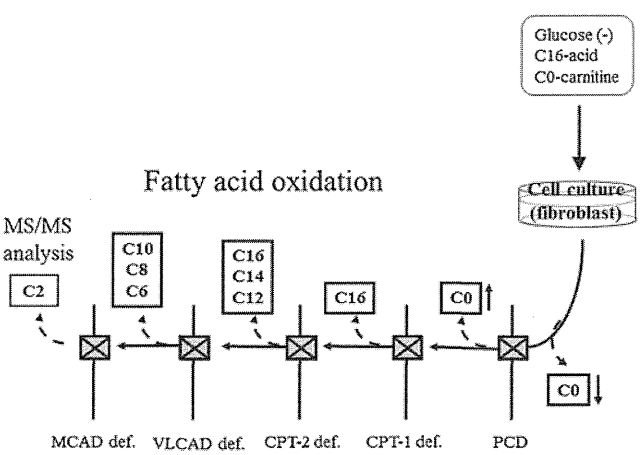


**Fig. 2** Principle of in vitro probe acylcarnitine assay. *C2, C4, C6, C8, C10, C12, C14*, and *C16* represent acylcarnitines

### 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

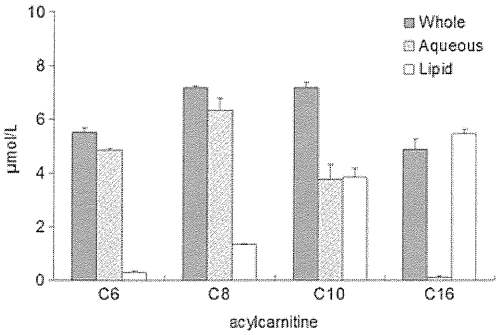


**Fig. 3** Recovery of ACs during extraction using the Folch method. Standard solutions of 10 μmol/L each of C6-, C8-, C10-, and C16-carnitine were used to determine the recovery of ACs in the aqueous and lipid fractions during extraction using the Folch method. *Grey column*: ACs in the whole extract 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).

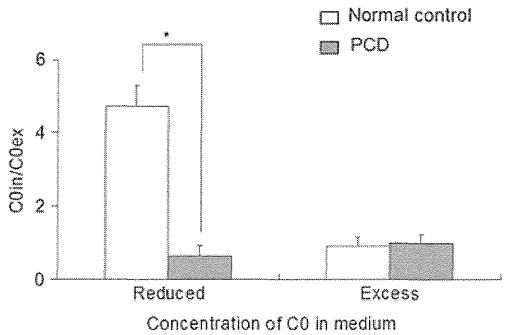


**Fig. 5** Ratio of intracellular C0 to extracellular C0. *Open square*: normal control ($n=6$); *closed square*: PCD ($n=3$). Extra- and intracellular C0 of cells with normal control and PCD were measured in C0-reduced (10 μmol/L) and C0-excess (400 μmol/L) conditions using MS/MS. Data are expressed 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 $*p<0.001$

## 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 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).

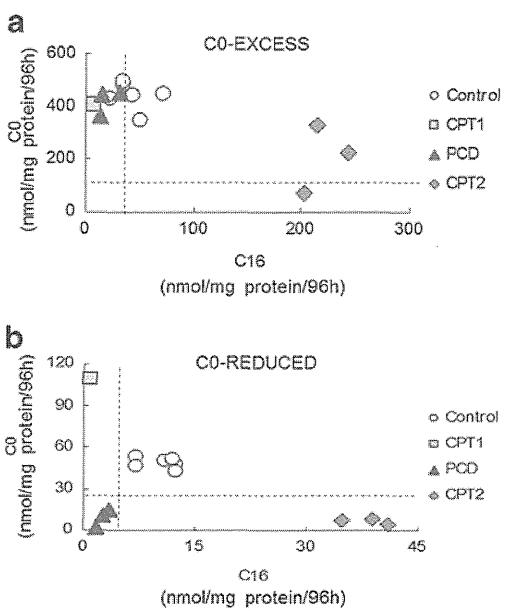


**Fig. 4** Intracellular C0 and C16 correlation in patients with carnitine cycle disorders. **a** C0-excessive condition (−*E*); **b** C0-reduced condition (−*R*). *open circle*: healthy control ($n=6$); *closed triangle*: PCD ($n=3$); *closed square*: CPT1 deficiency ($n=1$); *closed diamond*: CPT2 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)

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.

**Acknowledgments** We thank all the attending physicians for providing clinical information regarding each patient. We are also grateful to Y. Ito, M. Furui, T. Esumi, and N. Tomita for their technical assistance. This work was supported by a Grant-in-Aid for scientific research from the Japan Society for the Promotion of Science (J.P., and S.Y.) and a Grant from the Ministry of Education, Science, Technology, Sports and Culture of Japan and the Ministry of Health, Labour and Welfare of Japan (S.Y).

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Molecular Genetics and Metabolism 107 (2012) 322–329

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

# Effects of supplementation on food intake, body weight and hepatic metabolites in the citrin/mitochondrial glycerol-3-phosphate dehydrogenase double-knockout mouse model of human citrin deficiency

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ARTICLE INFO

*Article history:*
Received 1 June 2012
Received in revised form 24 July 2012
Accepted 24 July 2012
Available online 2 August 2012

*Keywords:*
Dietary supplementation
Body weight
Citrin
Citrin/mGPD double-knockout mice
Food intake
Glycerol 3-phosphate

ABSTRACT

The C57BL/6:*Slc23a13*$^{-/-}$;*Gpd2*$^{-/-}$ double-knockout (a.k.a., citrin/mitochondrial glycerol 3-phosphate dehydrogenase double knockout or Ctrn/mGPD-KO) mouse displays phenotypic attributes of both neonatal intrahepatic cholestasis (NICCD) and adult-onset type II citrullinemia (CTLN2), making it a suitable model of human citrin deficiency. In the present study, we show that when mature Ctrn/mGPD-KO mice are switched from a standard chow diet (CE-2) to a purified maintenance diet (AIN-93M), this resulted in a significant loss of body weight as a result of reduced food intake compared to littermate mGPD-KO mice. However, supplementation of the purified maintenance diet with additional protein (from 14% to 22%; and concomitant reduction or corn starch), or with specific supplementation with alanine, sodium glutamate, sodium pyruvate or medium-chain triglycerides (MCT), led to increased food intake and body weight gain near or back to that on chow diet. No such effect was observed when supplementing the diet with other sources of fat that contain long-chain fatty acids. Furthermore, when these supplements were added to a sucrose solution administered enterally to the mice, which has been shown previously to lead to elevated blood ammonia as well as altered hepatic metabolite levels in Ctrn/mGPP-KO mice, this led to metabolic correction. The elevated hepatic glycerol 3-phosphate and citrulline levels after sucrose administration were suppressed by the administration of sodium pyruvate, alanine, sodium glutamate and MCT, although the effect of MCT was relatively small. Low hepatic citrate and increased lysine levels were only found to be corrected by sodium pyruvate, while alanine and sodium glutamate both corrected hepatic glutamate and aspartate levels. Overall, these results suggest that dietary factors including increased protein content, supplementation of specific amino acids like alanine and sodium glutamate, as well as sodium pyruvate and MCT all show beneficial effects on citrin deficiency by increasing the carbohydrate tolerance of Ctrn/mGPD-KO mice, as observed through increased food intake and maintenance of body weight.



## 1. Introduction

*SLC25A13*, the gene encoding the mitochondrial solute carrier, now known as citrin, was originally found to be the cause of the autosomal recessive disease, adult-onset type II citrullinemia (CTLN2) [1]. Since mutations in the same gene have also been found to cause a form of neonatal intrahepatic cholestasis (NICCD) [2–4], the newly-established disease entity citrin deficiency was established [5]. It is now known that citrin deficiency can also lead to additional consequences

*Abbreviations:* AGC, aspartate–glutamate carrier; ASS, argininosuccinate synthetase; CTLN2, adult-onset type II citrullinemia; Ctrn-KO, Slc25a13 (citrin) knockout; KO, knockout; MCT, medium-chain triglycerides; mGPD, mitochondrial glycerol-3-phosphate dehydrogenase; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; TCA, tricarboxylic acid; wt, wild-type.

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[1] Posthumous.

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http://dx.doi.org/10.1016/j.ymgme.2012.07.021

throughout life including growth retardation and hypoglycemia in infancy, fatty liver, hypertriglyceridemia, pancreatitis and hepatocellular carcinoma [5–17]. Citrin has been identified as the hepatic isoform of the mitochondrial aspartate–glutamate carrier (AGC) [18], known to participate in the cytosolic synthesis of proteins, nucleotides and urea through supplying mitochondrial-derived aspartate. In addition, the AGC plays important roles in gluconeogenesis from lactate (due to its stoichiometric relationship with NADH [19]), and as a member of the malate–aspartate shuttle that, together with the glycerophosphate shuttle, transports cytosolic NADH into the mitochondria for use in ATP synthesis via oxidative phosphorylation.

Several symptoms of citrin deficiency are directly attributable to the loss of citrin function. The hyperammonemia and citrullinemia observed in CTLN2 patients arise from the loss of mitochondrial aspartate as a likely necessary source (under specific physiological conditions) for the condensation reaction with citrulline to form argininosuccinate, catalyzed by the cytosolic urea cycle enzyme, argininosuccinate synthetase (ASS) [20]. Moreover, citrin's role in the malate-aspartate shuttle helps maintain a relatively low cytosolic NADH/$NAD^+$ ratio, promoting aerobic glycolysis as well as leading to the formation of oxaloacetate and malate through the aspartate aminotransferase and malate dehydrogenase reactions, respectively. Loss of citrin function shifts the requirement of the ASS reaction to use cytosolic aspartate derived from oxaloacetate (although other potential sources likely exist), leading to the generation of NADH, an increased cytosolic NADH/$NAD^+$ ratio, and an overall inhibition of cytosolic NADH-dependent reactions (including the glycolytic reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase). It is believed that the increase in the cytosolic NADH/$NAD^+$ ratio likely contributes to such symptoms as hypoglycemia, galactosemia, and possibly the multiple aminoacidemia, observed in NICCD patients. Furthermore, compensatory mechanisms resulting from the increased utilization of cytosolic oxaloacetate, such as pyruvate cycling reactions, may promote fatty acid synthesis and lipogenesis leading to hypertriglyceridemia, fatty liver and pancreatitis in citrin deficient patients.

Despite our understanding of the role citrin plays in intermediate metabolism, and how it contributes to various symptoms observed in patients, there still remain pathophysiologic findings that cannot easily be explained. One such observation in children and adolescents is a failure to thrive leading to the development of thin, lean body habitus, as described by Mutoh et al. [10], Saheki et al. [21] and Song et al. [22,23]. What is known about citrin deficiency, however, is that both simple and complex carbohydrates appear to lead to toxicity: infusion of high concentrations of glucose promotes hyperammonemia, both in humans [24,25] and in our citrin deficiency mouse model [26,27]; infusion of glycerol and fructose to counteract cerebral edema has led to further deterioration of CTLN2 patients [28]; the conventional low-protein, high-carbohydrate diet given to patients with hepatic encephalopathy has also led to worsening of symptoms [29]; and most strikingly, the majority of citrin-deficient patients have been reported to naturally dislike foods rich in carbohydrates, preferring instead foods that are rich in protein and fat [30]. Understanding the role diet plays in the pathophysiology of citrin deficiency, and how patients respond to dietary manipulation or supplementation, will aid in establishing more rational therapies compared to liver transplantation, which remains one of the only effective means to correct the metabolic disturbances in citrin-deficient patients [5,6,31].

The establishment of a suitable model system can greatly aid in the evaluation of potential therapies for genetic diseases. Although our initial studies of the homologous-recombination-generated *Slc25a13* (Ctrn)-knockout (KO) mouse demonstrated metabolic disturbances in many of the pathways in which citrin was predicted to play a role [32], the mice failed to exhibit an observable phenotype relevant to citrin deficiency. By breeding the Ctrn-KO mice with *Gpd2* (a.k.a., mitochondrial glycerol-3-phosphate dehydrogenase or mGPD)-KO mice, however, we have now established a suitable mouse model [26] that shows perturbations reminiscent of citrin-deficient patients: sustained elevations of plasma citrulline, hyperammonemia under fed conditions that is exasperated by enteral sucrose administration, as well as hypoglycemia and fatty liver under fasted conditions. Using a metabolomics approach, we have recently shown that the Ctrn/mGPD-KO mice exhibit marked increases in hepatic glycerol 3-phosphate, a generalized decrease of hepatic tricarboxylic acid (TCA) intermediates, as well as alterations of hepatic amino acids including marked elevations of citrulline and lysine following enteral sucrose administration [27]. Moreover, these changes could be ameliorated by simultaneous administration of sodium pyruvate with the sucrose [27]. Therefore, the Ctrn/mGPD-KO mouse represents an invaluable model to understand additional pathophysiological mechanisms of citrin deficiency, as well as to test out rational therapies.

In the present study, we describe our evaluation of the effects of dietary protein, as well as specific amino acids, sodium pyruvate and MCT supplementation on food intake, body weight and hepatic metabolite levels of Ctrn/mGPD-KO mice. Switching adult mice from a standard chow diet to a purified maintenance diet resulted in a loss of body weight as well as decreased food intake, both of which could be corrected by the addition of casein to the purified maintenance diet (with concomitant reduction of corn starch). Furthermore, specific supplementation of the purified maintenance diet with alanine, sodium glutamate, sodium pyruvate, or MCT also resulted in increased food intake and body weight gain, similar to that observed for casein. Examination of hepatic glycerol 3-phosphate, citrate, citrulline, lysine, glutamate and aspartate levels following each supplementation of the purified maintenance diet also showed varied corrections to the metabolite changes observed. Overall, our findings suggest that the failure to thrive and thin, lean body habitus of citrin-deficient patients are likely linked to their dietary protein intake and overall carbohydrate tolerance. Furthermore, a higher protein diet, supplementation with specific amino acids like alanine or sodium glutamate, as well as the use of sodium pyruvate or MCT appears to have beneficial effects by improving the observed hepatic metabolite alterations, and increasing the overall carbohydrate tolerance, of the Ctrn/mGPD-KO mice. Therefore, use of dietary supplementation, in conjunction with monitoring but supporting patient's natural predilection for higher protein diet, represents a promising direction for the future treatment of citrin deficiency.

## 2. Methods and materials

### 2.1. Materials

Sodium pyruvate was a gift from Musashino Chemical Co., Tokyo, Japan. MCT oil used for the food intake experiments was purchased from Ultimate Nutrition, Inc. (Farmington, CT, USA), which contains 67% caprylic acid (C8:0) and 33% capric acid (C10:0). Powder MCT used for the enteral administration experiments was obtained from NOF Corporation (Tokyo, Japan), which contained 78.9% MCT consisting of 56% caprylic acid and 44% capric acid. Each ingredient of the AIN-93M diet was from Oriental Yeast Co., Ltd. (Tokyo, Japan). Tryptone was purchased from Becton Dickinson Microbiology Systems (Spark, MD, USA). Sodium hydrogen L(−)-glutamate hydrate and L-alanine were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Enzymes for metabolite determination were from Sigma-Aldrich Corp. (St. Louis, MO, USA) and from Roche Diagnostics (Indianapolis, IN, USA). Additional reagents were from Nacalai Tesque Inc. (Kyoto, Japan).

### 2.2. Animal care

All wild-type (wt), Ctrn-KO, mGPD-KO and Ctrn/mGPD-KO mice used in this study were congenic on the C57BL/6J background. Mice used in the experiments were generated using the breeding scheme described previously by Saheki et al. [26]. Briefly, mGPD-KO and Ctrn/mGPD-KO mice were obtained by mating heterozygous Ctrn-KO;homozygous mGPD-KO (*Slc25a13*$^{+/-}$;*Gpd2*$^{-/-}$) mice, while

wt and Ctrn-KO mice were generated by mating heterozygous Ctrn-KO (i.e., *Slc25a13*$^{+/-}$;*Gpd2*$^{+/+}$) mice. Genotyping was performed with DNA extracted from ear punches using procedures specific for each of the targeted mutations in the Ctrn-KO [32] and mGPD-KO [33] mice, respectively.

Mice were maintained at a constant temperature (23 ± 1 °C) on a 12-hour light (7 am to 7 pm)/dark cycle with free access to water and CE-2 chow (25% protein, 4.6% fat, and 50% carbohydrate providing 343 kcal/100 g; CLEA Japan, Tokyo, Japan). The mice used for the experiments were analyzed between 80 and 160 days of age.

For blood collection to measure ammonia following enteral sucrose and/or tryptone administration, blood was drawn from the heart of mice under somnopentyl anesthesia (64.8 mg sodium pentobarbital/kg) 1 h after the enteral administration. For preparation of liver extracts, mice were sacrificed by cervical dislocation 1 h after enteral administration.

This study was approved by the Committee for Animal Experimentation at Tokushima Bunri University.

### 2.3. Special diet, diet supplementation, food intake and body weight

To examine changes in food intake and body weight in response to dietary changes, female mice were housed individually and provided the AIN-93M [34] diet in powder form, with or without an additional supplement. The modified AIN-93M was prepared by adding the test supplement (e.g., casein, alanine, sodium glutamate, sodium pyruvate, corn oil, soy-bean oil, fish oil, lard or MCT) to the diet while reducing the corn starch component by the same amount. Food intake was monitored daily for 7 days by weighing the food provided, the food remaining, and any spillage that was remarkable. Measurement of food spillage was made possible through use of paper pellet bedding (Paper clean; PEPARLET Co. Japan). Average daily intake for each mouse was calculated over the course of 7 days. Body weight of the mice was measured daily, although the effect of the diet was assessed using the body weight for the last day. All measurements using a Fx-2000iWP balance (A & D Company Ltd, Tokyo, Japan) were taken at the same time each day.

### 2.4. Enteral administration of sucrose and test supplements

A 25% sucrose solution (5 g/kg) was administered enterally via gastric tube to the mice, with or without supplementation, between 9 and 11 am. Fed mice were used for all the experiments except for the experiments shown in Fig. 2 where the mice were fasted for 16 h. All test supplements were administered simultaneously with the sucrose solution at a standard dose of 20 ml/kg and included: tryptone (25% solution; 5 g/kg), 1 M alanine (20 mmol/kg), 0.5 M sodium glutamate (10 mmol/kg), 1 M sodium pyruvate (20 mmol/kg) or 5% MCT (1 g/kg).

### 2.5. Preparation of liver extracts

Livers were quickly removed, freeze-clamped between aluminum tongues, pulverized under liquid nitrogen and homogenized in 3% perchloric acid. Following the centrifugation of the samples at 10,000×*g* for 20 min at 4 °C, the supernatants were neutralized with 1 M sodium bicarbonate and used for enzymatic or amino acid analyses, as described below.

### 2.6. Analytical procedures

Metabolites were assayed as described by Saheki et al. [27]. The hepatic concentrations of glycerol 3-phosphate and citrate were determined using established enzymatic methods [35,36]. Blood ammonia was assayed using the Wako Ammonia test kit (Wako Pure Chemical Industries, Osaka, Japan).

Hepatic amino acids were determined by LC/MS/MS (Acquity UPLC/TQD; Waters, Milford, MA, USA) after solid phase extraction followed by derivatization [37] using the EZ:faast Amino Acid Analysis kit (Phenomenex Ltd., Los Angeles, CA, USA).

### 2.7. Statistical analysis

The difference between two group means was evaluated using the Student's t-test, with a threshold for statistical significance of $p<0.05$. The body weight changes were evaluated using a paired t-test. Differences among multiple group means were initially evaluated using a one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test, with a threshold for statistical significance of $p<0.05$.

## 3. Results

### 3.1. Effects of diet and protein content on body weight and food intake

For the purposes of breeding, the mice are normally maintained on a commercially available pelleted laboratory chow diet (CE-2) recommended for breeding rodents. To enable the assessment of dietary composition on our mouse model of citrin deficiency, the mice were changed from the CE-2 diet to a purified rodent diet (AIN-93M) which has been recommended to be sufficient for mature rodents by the American Institute for Nutrition [34]. Monitoring the mice over a period of 7 days, the change in diet did not affect the body weights of the Ctrn-KO (change in body weight = −1.3 g, or 5.6% body weight; not significant) nor mGPD-KO (−0.7 g or 3.2%; not significant) mice, but did slightly decrease the body weight of wt mice (−1.5 g or 6.6%; $p<0.05$) (Table 1). In contrast, the Ctrn/mGPD-KO mice showed a marked decrease in body weight after 7 days (−5.6 g or 26.7%; $p<0.01$) (Table 1).

The decrease in the body weight of the Ctrn/mGPD-KO mice was accompanied by a decrease in food intake. The Ctrn/mGPD-KO mice had an average food intake that was approximately 50% (actual difference: 41.2% to 53.8%) compared to the average of the other genotypes (Table 1). Interestingly, the Ctrn/mGPD-KO mice were also the only genotype to display a specific behavior associated with the change in diet: there was substantial spillage found in some cages of Ctrn/mGPD-KO mice fed the AIN-93M diet but not observed in the other genotypes using our measuring system.

To further show that the decreased body weight was due to reduced food intake in the Ctrn/mGPD-KO mice, we examined the effect of limiting food intake on body weight. Based on our breeding scheme, the mGPD-KO mice are the only littermates of the Ctrn/mGPD-KO mice and therefore were used as the controls for all subsequent experiments. Limiting the supply of the CE-2 diet to the mGPD-KO mice (2 g/day) caused a similar decrease in the body weight (from 20.5 ± 1.0 g to 16.3 ± 1.3 g, $n=4$; $p<0.01$) to that observed for the Ctrn/mGPD-KO mice when switched to the AIN-93M diet, indicating that a lower food intake is the primary cause of decreased body weight in the Ctrn/mGPD-KO mice. When the amount of CE-2 is not limited, there is no significant difference in food intake between the mGPD-KO and Ctrn/mGPD-KO mice (Fig. 1; CE-2).

**Table 1**
Body weights and food intake of mice following diet change (CE-2 to AIN-93M).

| Genotype | Body weight (g) | | Intake of AIN-93M (g/day) |
|---|---|---|---|
| | CE-2 | AIN-93M | |
| Wild-type | 22.9 ± 0.6 | 21.4 ± 0.6* | 2.9 ± 0.2[b] |
| Ctrn-KO | 23.2 ± 1.6 | 21.9 ± 1.0 | 3.4 ± 0.3[a] |
| mGPD-KO | 22.1 ± 0.9 | 21.4 ± 0.6 | 2.6 ± 0.1[b] |
| Ctrn/mGPD-KO | 21.0 ± 1.1 | 15.4 ± 1.3** | 1.4 ± 0.3[c] |

Mean ± SD. ($n=4$). Differences between diets within each genotype were evaluated using a paired t-test (*$p<0.05$, **$p<0.01$). Differences in food intake among the genotypes were evaluated by ANOVA followed by the Tukey–Kramer test (means with the same character are not statistically different).

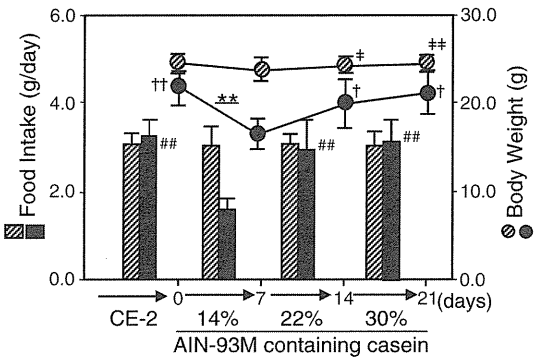


**Fig. 1.** Effects of diet change from CE-2 to AIN-93M, and supplementation of AIN-93M with casein, on body weight and food intake of mice. Female mice were maintained on CE-2 prior to the experiment. On day 0, the diet was changed to AIN-93M containing 14% casein (original AIN-93M formulation) and given for 7 days. The diet was subsequently changed again to the AIN-93M diet containing 22% (by adding 8% casein and reducing the corn starch by 8%) for another 7 days, followed by the AIN-93M diet containing 30% casein (16% added casein with 16% reduction of corn starch) for 7 days. The food intake and body weights of the mice were measured daily. Each value represents mean ± SD (n = 7 for mGPD, and n = 11 for Ctrn/mGPD-KO mice). Mean food intake values were calculated from the average food intake of each mouse over 7 days. Although body weights of the mice were measured daily, mean body weight values were calculated using the body weight of each mouse on day 7. Hatched bars and circles denote mGPD-KO littermate control mice; black bars and circles denote Ctrn/mGPD-KO mice. Differences in food intake for each diet (or supplementation) between genotypes were determined using an unpaired t-test (**$p<0.01$). Differences in food intake between diets within each genotype were determined using a paired t-test (#$p<0.05$ and ##$p<0.01$). Differences in body weight for mGPD-KO mice (‡$p<0.05$ and ‡‡$<0.01$) and Ctrn-KO/mGPD-KO mice (†$p<0.05$ and ††$p<0.01$) comparing the AIN-93M diet versus other diets (or supplementation) were determined using a paired t-test.

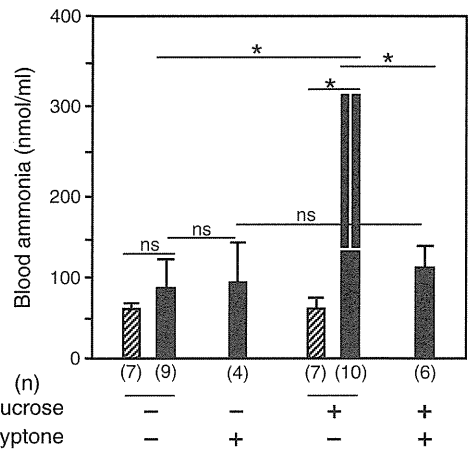


**Fig. 2.** Effect of tryptone (5 g/kg) on hyperammonemia induced by enteral sucrose (5 g/kg body weight) administration in Ctrn/mGPD-KO mice. Solutions of sucrose (25%; 5 g/kg), 25% tryptone (5 g/kg), or 25% sucrose containing 25% tryptone, were administered enterically to 16-hour fasted mice by gastric tube. One hour after administration, blood was taken by cardiac puncture under anesthesia and blood ammonia levels were determined. Hatched bars denote mGPD-KO littermate control mice; black bars denote Ctrn/mGPD-KO mice. Data from each group of mice are expressed as mean ± SD. The numbers of mice used per group are shown in brackets. Differences between treatment groups within each genotype were evaluated using the Student's t-test, where * denotes a statistical difference at $p<0.05$.

A major difference between the CE-2 and AIN-93M diets is the protein content, 25% compared to 14%, respectively. Addition of casein to the AIN-93M diet (14% + 8%; total protein content of 22%), together with a reduction of corn starch from 46.6% to 38.6%, increased the food intake of the Ctrn/mGPD-KO mice to 3.0 ± 0.7 g/day and significantly increased their body weight within 7 days ($p<0.01$; Fig. 1). Further addition of 16% casein (30% total protein) did not further increase the food intake but their average body weight did reach that observed on the CE-2 diet (Fig. 1).

### 3.2. Effect of tryptone on hyperammonemia induced by enteral sucrose administration

Our findings that dietary protein content influenced the food intake and body weight of the Ctrn/mGPD-KO mice prompted us to test whether tryptone, a hydrolysate of casein, would also have a similar effect as sodium pyruvate on lowering the hyperammonemia induced by enteral sucrose administration [27]. As reported previously [26,27], administration of a 25% sucrose solution (5 g/kg body weight) by gastric tube caused an increase in blood ammonia of Ctrn/mGPD-KO mice (Fig. 2), which was reduced to the basal level by the simultaneous administration of 25% tryptone (5 g/kg body weight). Sole administration of tryptone did not change the basal ammonia level in the Ctrn/mGPD-KO mice and no increase in blood ammonia was observed by sucrose administration in the mGPD-KO mice, as reported previously [26].

### 3.3. Effect of alanine and sodium glutamate on body weight and food intake

The positive effects of increased protein intake on the Ctrn/mGPD-KO mice led us to ask whether these effects may be due to increased protein in general, or whether specific amino acids may be underlying our findings. We choose to examine the effects of alanine and sodium glutamate on food intake and body weight of mGPD-KO and Ctrn/mGPD-KO littermates. The addition of either alanine (5%) or sodium glutamate (5%) to the AIN-93M diet led to increased food intake ($p<0.01$) and body weight ($p<0.05$) in the Ctrn/mGPD-KO mice, while it has no effect on the mGPD-KO mice (Fig. 3). Further addition of alanine (+10%) or sodium glutamate (+10%) did not further increase food intake, but did promote further weight gain in the Ctrn/mGPD-KO mice. Interestingly, the addition of sodium glutamate led to a slight increase in body weight in the mGPD-KO littermates, raising their body weight to that observed on the CE-2 diet (Fig. 3B).

### 3.4. Effect of sodium pyruvate on the body weight and food intake

We have shown previously that sodium pyruvate shows normalizing effects on elevated blood ammonia and hepatic metabolite derangements that are induced by enteral sucrose administration [27]. Fig. 4 shows the effects of sodium pyruvate supplementation on body weight and food intake. Addition of 5% sodium pyruvate to the AIN-93M diet significantly increased the food intake of Ctrn/mGPD-KO mice, but had no significant effect on body weight (Fig. 4). However, addition of 10% sodium pyruvate significantly increased both body weight and food intake, but the body weight still remained lower than that when on the CE-2 diet (Fig. 4).

### 3.5. Effect of various kinds of fat and dose response of medium-chain triglyceride

Next, we tested the effect of various types of fat on body weight and food intake of the Ctrn/mGPD-KO mice. As shown in Fig. 5A, addition of olive oil or lard to the AIN-93M diet had no, or a negative, effect on food intake and body weight of the Ctrn/mGPD-KO mice, but significantly increased the body weight of the mGPD-KO mice without influencing their food intake. Addition of MCT, however, significantly increased the food intake and body weight of the Ctrn/mGPD-KO mice (Fig. 5A). Furthermore, addition of soy-bean oil, corn oil and fish oil also failed to show any effect on food intake and body weight of Ctrn/mGPD-KO mice (data not shown), indicating that only fats containing medium-chain fatty acids were effective.

To examine the effect of different amounts of MCT oil, we performed a dose response experiment. Addition of 2% MCT had little

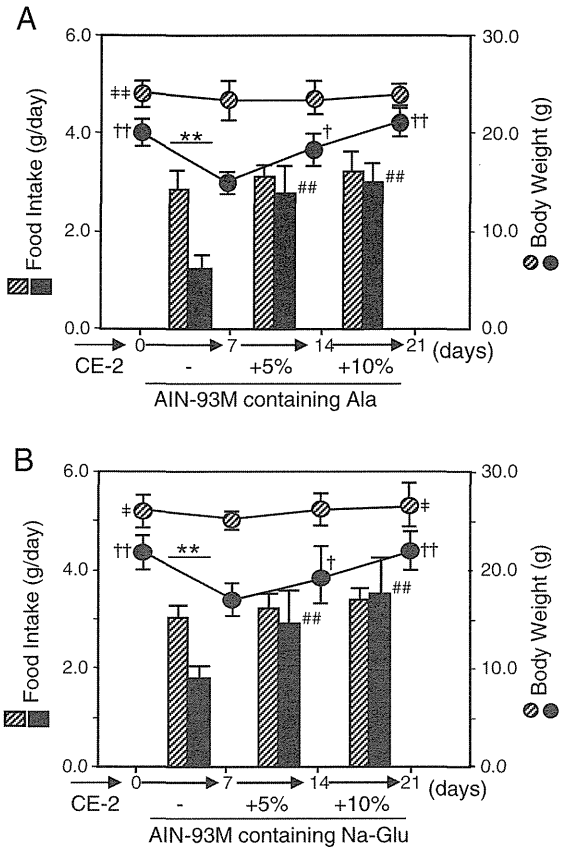


**Fig. 3.** Effect of a single amino acid, alanine or sodium glutamate, on the food intake and body weight of mice. Alanine (Ala) [A] or sodium glutamate (Na-Glu) [B] was added to the AIN-93M diet at doses of 5% and 10%. All other experimental and statistical details are as given in the legend of Fig. 1. The number of mice (n) used for each experiment was 8 and 7 for the Ala experiments, and n=8 and n=6 for the experiments on Na-Glu of mGPD-KO and Ctrn/mGPD-KO mice, respectively.

effect on food intake and body weight, while the addition of 4% and 6% MCT led to similar increased food intake but increasing body weight. Similar to that observed for sodium pyruvate, however, the Ctrn/mGPD-KO mice failed to recover their body weight to that when on the CE-2 diet.

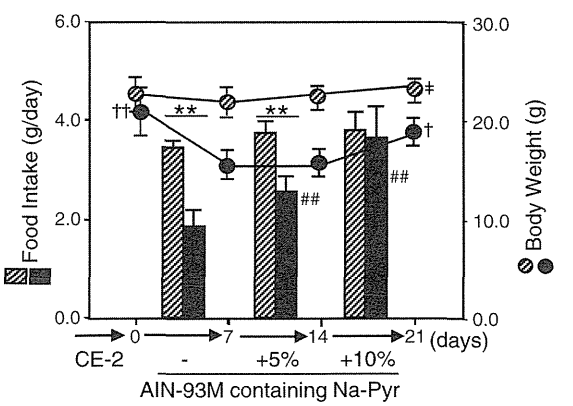


**Fig. 4.** Effect of sodium pyruvate on the food intake and body weight of mice. Sodium pyruvate (Na-Pyr) was added to the AIN-93M diet at doses of 5% and 10%. All other experimental and statistical details are as given in the legend of Fig. 1. The numbers of mice used were 8 and 7 for mGPD-KO and Ctrn/mGPD-KO mice, respectively.

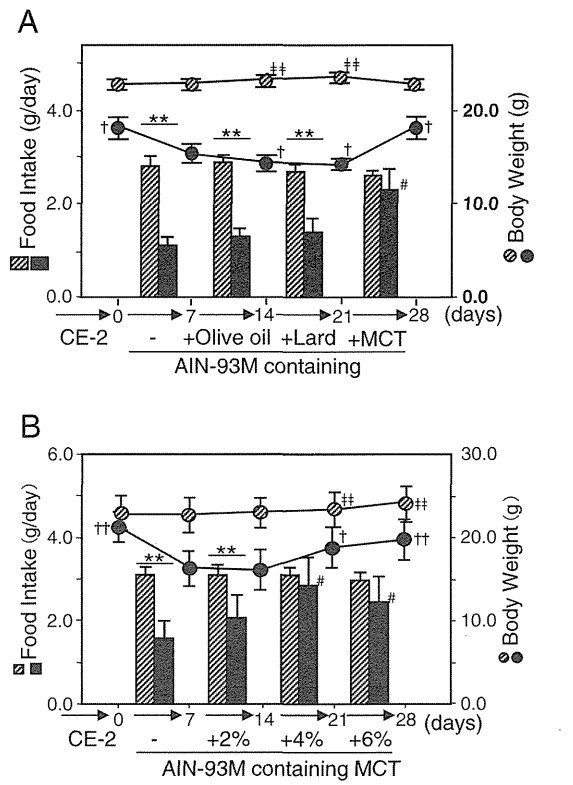


**Fig. 5.** Effect of lipids [A] and dose of MCT [B] on the intake and body weight of mice. In [A], olive oil, lard or medium-chain triglycerides (MCT) were added to the AIN-93M diet at 6%. The number of mice used was 4 for both mGPD-KO and Ctrn/mGPD-KO mice. In [B], dose of MCT was adjusted from 2% to 6%. The numbers of mice used were 6 and 8 for mGPD-KO and Ctrn/mGPD-KO mice, respectively. For [A] and [B], all other experimental and statistical details are as given in the legend of Fig. 1.

### *3.6. Effect of supplementation with enteral sucrose administration on hepatic metabolite levels*

Since sodium pyruvate, the amino acids alanine and sodium glutamate, and MCT supplementation showed beneficial effects on the food intake and body weights of Ctrn-/mGPD-KO mice, we next wanted to examine whether these supplements also corrected the hepatic metabolite disturbances observed previously in the Ctrn/mGPD-KO mice following enteral sucrose administration [27]. In this experiment, we administered 25% sucrose (5 g/kg body weight) by a gastric tube alone or together with sodium pyruvate (20 mmol/kg), sodium glutamate (10 mmol/kg), alanine (20 mmol/kg), or MCT (1 g/kg), and 1 h following enteral administration, each liver was removed using the freeze-clamp method. Some of the results for sodium pyruvate shown in Figs. 6 and 7 have been published previously [27].

As shown in Fig. 6, hepatic glycerol 3-phosphate was markedly increased by the enteral administration of sucrose [27], but was significantly reduced to lower than the basal (i.e., saline) level in the Ctrn/mGPD-KO mice (and almost to the wt basal level) by the simultaneous administration of sodium pyruvate. Significant effects were also observed for sodium glutamate, alanine and MCT, although the effect was considerably less with MCT. For hepatic citrate, the level was reduced by the enteral administration of sucrose [27], which was significantly ameliorated by simultaneous administration of sodium pyruvate, reaching the wt basal level. All of the other test supplements had no significant effect on the hepatic citrate level.

Enteral sucrose administration had also been shown previously to cause a dramatic increase in hepatic citrulline and lysine levels in Ctrn/mGPD-KO mice [27]. Similar to sodium pyruvate, alanine also completely suppressed hepatic citrulline to near the wt basal level (Fig. 7A). Suppression of hepatic citrulline was observed to lesser

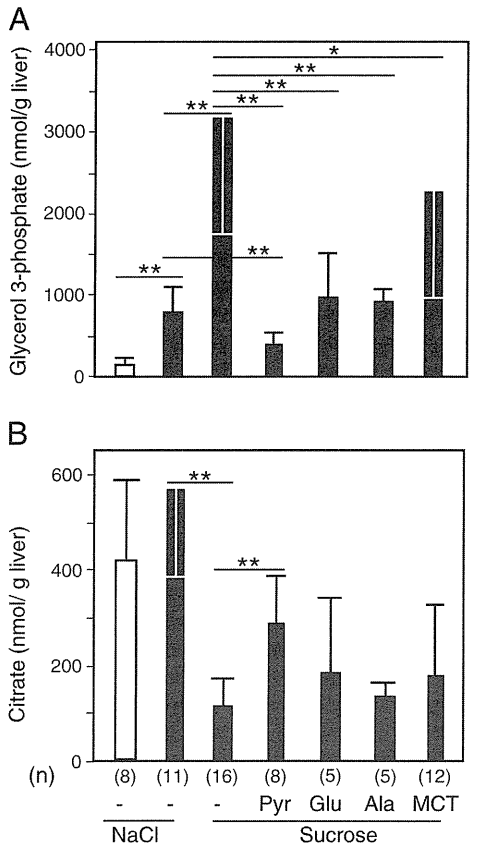


**Fig. 6.** Effect of supplementation on hepatic glycerol 3-phosphate (G3P) [A] and citrate [B] concentrations of Ctrn/mGPD double-KO mice following enteral administration of a 25% sucrose (5 g/kg) solution. A sucrose solution (5 g/kg) was enterally administered alone, or supplemented with sodium pyruvate (Pyr; 20 mmol/kg), sodium glutamate (Glu; 10 mmol/kg), alanine (Ala; 20 mmol/kg) or MCT (1 g/kg). One hour after administration, livers were taken using a freeze-clamp method and extracted as described in the Methods and materials. The data on wt mice and Ctrn/mGPD double-KO mice following sucrose, and sucrose plus Na-Pyr, have been previously published in Saheki et al. [27]. The number of mice used is shown in brackets. Differences between treatment groups were evaluated using the Student's t-test (*$p<0.05$; **$p<0.01$).

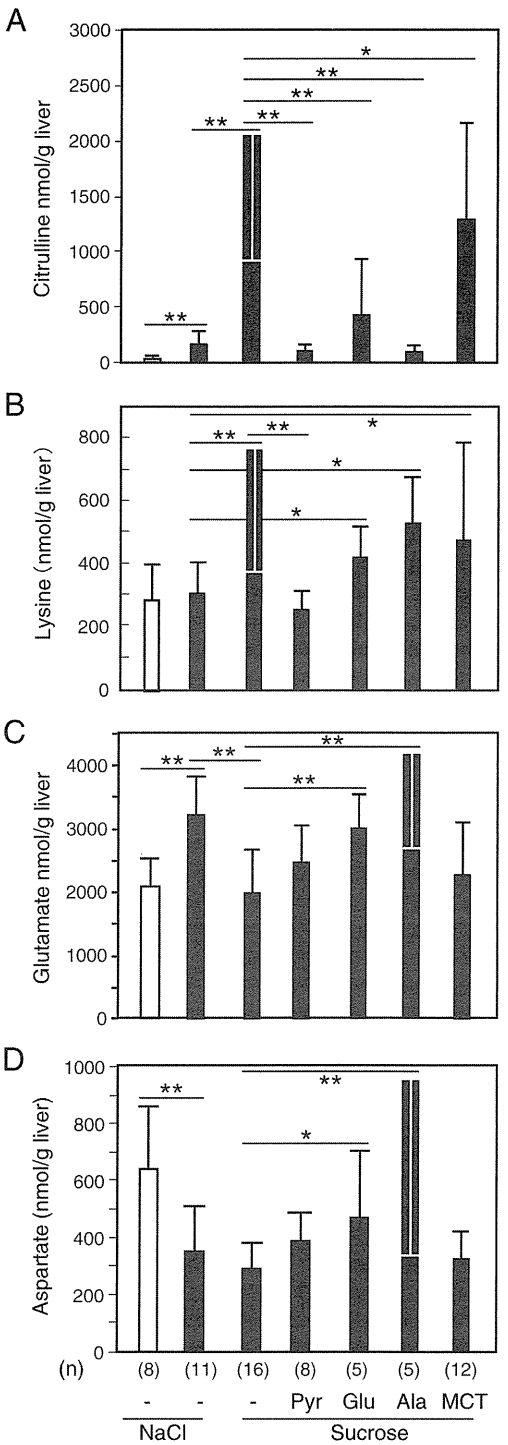


**Fig. 7.** Effect of supplementation on hepatic amino acid concentrations of Ctrn/mGPD double-KO mice following enteral administration of a 25% sucrose (5 g/kg) solution. A sucrose solution (5 g/kg) was enterically administered alone, or supplemented with sodium pyruvate (Pyr; 20 mmol/kg), sodium glutamate (Glu; 10 mmol/kg), alanine (Ala; 20 mmol/kg) or MCT (1 g/kg). One hour after administration, livers were taken using a freeze-clamp method and extracted as described in the Methods and materials. Hepatic citrulline [A], lysine [B], glutamate [C] and aspartate [D] were subsequent assayed in the liver extracts. The data on wt mice and Ctrn/mGPD double-KO following sucrose, and sucrose plus sodium pyruvate, have been previously published in Saheki et al. [27]. The number of mice used is shown in brackets. Differences between treatment groups were evaluated using the Student's t-test (*$p<0.05$; **$p<0.01$).

(albeit still statistically significant) extents for sodium glutamate and MCT (Fig. 7A). Hepatic lysine suppression, similar to hepatic citrate, was only observed when the enteral sucrose administration was supplemented with sodium pyruvate (Fig. 7B).

Lastly, enteral sucrose administration was also shown previously to cause decreased hepatic glutamate and aspartate levels in Ctrn/mGPD-KO mice [27]. Sodium pyruvate supplementation failed to significantly alter hepatic glutamate and aspartate levels (Figs. 7C and D), while alanine and sodium glutamate significantly increased both hepatic amino acids (Figs. 7C and D). Similar to sodium pyruvate, however, MCT had no effect on hepatic glutamate or aspartate levels.

## 4. Discussion

One of the physical characteristics of citrin-deficient patients is a thin, lean body habitus beginning during the so-called adaptive or apparently healthy period; more than 90% of adult patients have a body mass index (BMI) lower than 20, and approximately 40% have a BMI less than 17 [6]. At an earlier age, after resolution of NICCD (in most cases), some patients have developed a failure to thrive with dyslipidemia (FTTDCD) [6,22,23] that has preceded this. Mutoh et al. [10] reported a case of a 13-year-old girl whose BMI stayed at approximately 15 from an age of 6 years old until 13 years old, while the average BMI of aged-matched control Japanese girls increased from 16 to 19 during the same time period. Similar cases with failure to thrive and hypoglycemia have also been observed (Ayano Inui, personal communication). So far, there has been no explanation for this apparent pathophysiologic aspect of citrin deficiency.

The Ctrn/mGPD-KO mice have been shown previously to have a slower growth rate than mGPD-KO or mGPD-KO heterozygous for Ctrn (i.e., *Slc25a13*$^{+/-}$;*Gpd2*$^{-/-}$) mice post-weaning [26], but thereafter appear to catch up, reaching a similar or slightly lower body weight compared to littermate controls, as represented in Figs. 1, 3–5. Our previous observations, however, had been made while the mice were being maintained on a standard laboratory chow (CE-2) used for breeding. When mature female mice (aged between 90 and 160 days) were now switched from CE-2 to a purified maintenance diet (AIN-93M), reported to be sufficient for mature rodents by the American Institute for Nutrition [34], the Ctrn/mGPD-KO mice failed to maintain their body weight due primarily to a reduction in food intake. Since the major difference between the CE-2 and AIN-93M diets was in the protein content, 25% versus 14% respectively, we modified the AIN-93M diet by increasing the amount of casein to 22%, which resulted in an increased food intake and body weight gain in the Ctrn/mGPD-KO mice to a level similar to that observed on the CE-2 diet within 7 days. In actuality, the increased food intake of the Ctrn/mGPD-KO mice after modifying the protein content of the AIN-93M diet also increased the total carbohydrate intake of Ctrn/mGPD-KO mice from $0.75 \pm 0.11$ g/day to $1.14 \pm 0.27$ g/day ($p < 0.01$), based on the dietary intake and the calculated carbohydrate content of the original and modified AIN-93M diets (46.6% and 38.6%, respectively). This latter observation is noteworthy as we have shown previously that carbohydrates have a toxic effect on our citrin-deficient mouse model [13,14], and by increasing the protein content of the AIN-93M diet, we appear to have increased the overall carbohydrate tolerance of the mice. This is consistent with a case reported by Dimmock et al. [9]; a citrin-deficient baby with growth retardation and bleeding diathesis showed a dramatic improvement (including an increase in growth velocity) when the baby was placed on a high-protein, low-carbohydrate diet.

To further test the possibility that protein can alter the carbohydrate tolerance of the Ctrn/mGPD-KO mice by ameliorating its toxic effect, we tested whether tryptone, a hydrolysate of casein, could improve the hyperammonemia induced by enteral sucrose administration. As shown in Fig. 2, enteral administration of tryptone alone did not affect the blood ammonia level of Ctrn/mGPD-KO mice, while its simultaneous administration with sucrose suppressed the increased blood ammonia. This finding appears counterintuitive at first, as ammonia is derived mainly from amino acids and is therefore avoided in patients with hyperammonemia. However, working under the hypothesis that specific amino acids or peptides present in the tryptone may be responsible for its ameliorating effect, we used two additional methods to assay for these effects: decreasing the hepatic glycerol 3-phosphate content which is also increased by enteral sucrose administration [27], and voluntary intake of a 20% sucrose solution in which the amount of the 20% sucrose solution ingested was measured when the mice had free access to both water and the sucrose solution (with or without a test substance). So far, we have found a number of amino acids that appear to have similar positive effects using these assays (data not shown), and here have simply shown the activity of alanine and sodium glutamate on the food intake, body weight and hepatic metabolite levels. One possible indirect effect of amino acids on carbohydrate tolerance in the Ctrn/mGPD-KO mice could be the effect that amino acids have on increasing glucose uptake in muscle by enhancing insulin action [38]. Although this effect has been mainly attributed to the branched-chain amino acids leucine and isoleucine, it is conceivable that other amino acids have a similar effect by increasing glucose uptake by muscle and therefore reducing the amount of glucose taken up by the liver to undergo glycolysis, hence reducing the inhibitory effect of an elevated NADH/NAD$^+$ ratio on urea cycle function and glycerol 3-phosphate accumulation. Further studies such as isotope labeling experiments are needed to show if the effect of amino acids on Ctrn/mGPD-KO mice is due to this indirect effect, or whether there are more direct effects on the liver to provide limiting substrate for urea cycle function.

Sodium pyruvate supplementation also had a positive effect on food intake and body weight maintenance in the Ctrn/mGPD-KO mice (Fig. 4), having already been shown previously to normalize the hyperammonemia as well as the abnormal hepatic metabolite patterns induced by enteral sucrose administration [27]. Since the effects of pyruvate are most likely occurring within the liver by reducing the NADH/NAD$^+$ ratio, these direct effects on the liver further support the notion that carbohydrate tolerance is influencing the food intake and body weight maintenance of the Ctrn/mGPD-KO mice.

Fat-rich foods are also a dietary preference of citrin-deficient patients [30]. Although MCT also had a positive effect on food intake and body weight maintenance in the Ctrn/mGPD-KO mice, surprisingly, none of long-chain fatty acid-containing triglycerides tested including corn oil, soy-bean oil, olive oil, fish oil or lard showed a positive effect. The preferential effect of MCT may be due to the fact that the liver can utilize MCT at any time, even under conditions where long-chain fatty acid oxidation may be suppressed. In addition, MCT has also been used as a therapy of NICCD patients [6,7,39,40], but in these cases, MCT was provided as a digestible fat in place of long-chain fats to aid in their absorption and digestion under acholic conditions.

We also examined whether the amino acids alanine and sodium glutamate, as well as MCT would exhibit similar effects on hepatic metabolite levels similar to sodium pyruvate. As shown in Figs. 6 and 7, both alanine and sodium glutamate suppressed the increased glycerol 3-phosphate and citrulline levels that are also caused by enteral sucrose administration, while MCT had a significant but relatively smaller effect. Again, the effect of the amino acids could be indirect, through increasing glucose uptake in muscle versus liver, while the underlying mechanism for the effect of MCT is not immediately clear. The altered levels of hepatic citrate and lysine, however, were not corrected by alanine, sodium glutamate or MCT and indicate that they are not having a significant effect on increasing TCA cycle intermediate levels [41]. Concerning the hepatic amino acid levels of glutamate and aspartate, administration of amino acids, but especially alanine, substantially increased levels of both, while sodium pyruvate and MCT had no significant effect. The effect of alanine on aspartate and glutamate levels may simply be due to transamination or deamination reactions occurring in the liver, but the effect appears to be more than simply providing an alternative source of pyruvate and altering the NADH/NAD$^+$ ratio.

Lastly, our present study using the Ctrn/mGPD mouse model of citrin deficiency suggests that the underlying mechanism for the failure to thrive and thin, lean body habitus of citrin deficient patients is related to dietary composition and likely carbohydrate intolerance. More fundamentally though, it is not clear what the physiological triggers influencing the Ctrn/mGPD-KO mice to restrict their food intake leading to loss in body weight are, and whether these are the same triggers that may be influencing citrin-deficient patients to naturally prefer high protein and fat foods while disliking high carbohydrate foods [30]. Possible possible candidates could include blood ammonia levels that could directly affect the brain to suppress intake, or hepatic glycerol 3-phosphate levels that could lead to discomfort and decisions of food avoidance. What is clear though is that the supplements tested in the present study, sodium pyruvate, amino acids alanine and sodium glutamate, and MCT, all appear to have overlapping beneficial effects on citrin deficiency, so that combinations of these supplements may mutually potentiate a significant beneficial effect on patients suffering from symptoms. One important consideration, however, is that patients with low hepatic ASS activity (as observed in many CTLN2 patients) may not tolerate a higher protein diet nor amino acid supplementation, although administration of sodium pyruvate and/or MCT could still be considered.

## Acknowledgments

This study was supported in part by KAKENHI (C 21591337 and 24591516), JSPS Asia Africa Science Platform Program, Adaptable and Seamless Technology Transfer Program through Target-driven

R&D (A-STEP) from Japan Science and Technology, and grants from the Ministry of Health, Labor and Welfare in Japan.

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Molecular Genetics and Metabolism 105 (2012) 553–558

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

# Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in *SLC25A13*

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ARTICLE INFO

*Article history:*
Received 13 November 2011
Received in revised form 29 December 2011
Accepted 30 December 2011
Available online 8 January 2012

*Keywords:*
Citrin deficiency
Genetic diagnosis
Rapid diagnosis
Expanded newborn screening
*SLC25A13*

ABSTRACT

Citrin deficiency is an autosomal recessive disorder caused by mutations in the *SLC25A13* gene and has two disease outcomes: adult-onset type II citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency. The clinical appearance of these diseases is variable, ranging from almost no symptoms to coma, brain edema, and severe liver failure. Genetic testing for *SLC25A13* mutations is essential for the diagnosis of citrin deficiency because chemical diagnoses are prohibitively difficult. Eleven *SLC25A13* mutations account for 95% of the mutant alleles in Japanese patients with citrin deficiency. Therefore, a simple test for these mutations is desirable. We established a 1-hour, closed-tube assay for the 11 *SLC25A13* mutations using real-time PCR. Each mutation site was amplified by PCR followed by a melting-curve analysis with adjacent hybridization probes (HybProbe, Roche). The 11 prevalent mutations were detected in seven PCR reactions. Six reactions were used to detect a single mutation each, and one reaction was used to detect five mutations that are clustered in a 21-bp region in exon 17. To test the reliability, we used this method to genotype blind DNA samples from 50 patients with citrin deficiency. Our results were in complete agreement those obtained using previously established methods. Furthermore, the mutations could be detected without difficulty using dried blood samples collected on filter paper. Therefore, this assay could be used for newborn screening and for facilitating the genetic diagnosis of citrin deficiency, especially in East Asian populations.



## 1. Introduction

Citrin deficiency is an autosomal recessive disorder that results from mutations in the *SLC25A13* gene [1] and causes two diseases: adult-onset type II citrullinemia (CTLN2; OMIM #603471) and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; OMIM#605814) [1–4]. The clinical appearance of these diseases is variable and ranges from almost no symptoms to coma, brain edema, and severe liver failure requiring transplantation [5–8]. In a study of patients with NICCD, only 40% of individuals were identified by newborn screenings to have abnormalities, such as hypergalactosemia, hypermethioninemia, and hyperphenylalaninemia [9]. Other patients were referred to hospitals with suspected neonatal hepatitis or biliary atresia, due to jaundice or discolored stool [9]. Hypercitrullinemia was not observed in all patients [9]. Mutation analysis of *SLC25A13* is indispensable because of the difficulties associated with the chemical diagnosis of citrin deficiency. The *SLC25A13* mutation spectrum in citrin deficiency is heterogeneous, and more than 31 mutations of *SLC25A13* have been identified to date [1,10–18]. However, there are several predominant mutations in patients from East Asia. As shown in Table 1, 6 prevalent mutations account for 91% of the mutant alleles in the Japanese population [12,19]. Five additional mutations also occur within a 21-bp cluster in exon 17 (Table 1 and Fig. 1D). The six prevalent mutations, together with the five mutations in exon 17, account for 95% of the mutant alleles in Japan [12,19].

Several different methods, such as direct sequencing, PCR restriction fragment length polymorphism (PCR-RFLP), and denaturing high performance liquid chromatography (DHPLC), are currently used for the detection of mutations in *SLC25A13* [1,10–14,19]. However, these methods are too complex for clinical use. Direct sequencing is a standard but cumbersome method. The PCR-RFLP method is

*Abbreviations:* CTLN2, adult-onset type II citrullinemia; FRET, fluorescence resonance energy transfer; HRM, high resolution melting; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; Tm, melting temperature.

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doi:10.1016/j.ymgme.2011.12.024

**Table 1**
Seven primer/probe sets and 11 targeted mutations of *SLC25A13*.

| Primer/probe set | Mutation | | Location | Nucleotide change | Effects of mutations | Allele frequency*[19] | References |
|---|---|---|---|---|---|---|---|
| A | Mutation [I] | :851del4 | exon 9 | c.851_854delGTAT | p.R284fs(286X) | 33.2% | [1] |
| B | Mutation [II] | :g.IVS11+1G>A | intron 11 | c.1019_1177del | p.340_392del | 37.6% | [1] |
| C | Mutation [III] | :1638ins23 | exon 16 | c.1638_1660dup | p.A554fs(570X) | 3.4% | [1] |
| D | Mutation [IV] | :S225X | exon 7 | c.675C>A | p.S225X | 5.3% | [1] |
| E | Mutation [V] | :g.IVS13+1G>A | intron 13 | c.1231_1311del | p.411_437del | 8.2% | [1] |
| F | Mutation [XIX] | :IVS16ins3kb | intron 16 | c. aberrant RNA | p.A584fs(585X) | 4.6% | [19] |
| G | Mutation [VI] | :1800ins1 | exon 17 | c.1799_1800insA | p.Y600X | 1.3% | [10] |
| | Mutation [VII] | :R605X | exon 17 | c.1813C>T | p.R605X | 0.90% | [10] |
| | Mutation [VIII] | :E601X | exon 17 | c.1801G>T | p.E601X | 1.2% | [11] |
| | Mutation [IX] | :E601K | exon 17 | c.1801G>A | p.E601K | 0.30% | [11] |
| | Mutation [XXI] | :L598R | exon 17 | c.1793T>G | p.L598R | 0% | [15] |
| | | | | | | Total 95.1% | |

* The frequency of each mutant allele among Japanese patients with citrin deficiency.

complicated and can lead to genotyping errors, due to incomplete digestion by the restriction enzymes. DHPLC is time-consuming and requires expensive equipment. Thus, there is a strong need for the development of a simple test for these mutations.

The goal of this study was to establish a rapid and simple test for the detection of the 11 most common *SLC25A13* mutations. We adopted the HybProbe format (Roche) for the detection of the mutations using real-time PCR followed by a melting-curve analysis with adjacent hybridization probes [20,21]. This assay can be completed in less than 1 h and has the advantage of being a closed-tube assay. The fundamental process for detecting point mutations using the HybProbe assay is presented in Fig. 1A. The 11 prevalent mutations contain not only point mutations but also include a 4-bp deletion and insertions of 1-bp, 23-bp and 3-kb genomic fragments (Table 1 and Fig. 1). Careful design of the PCR primers and HybProbes enabled us to test for these various *SLC25A13* mutations.

## 2. Methods

### 2.1. Subjects

CTLN2 and NICCD were diagnosed, as previously described [9,10,19,22–24]. Genomic DNA of the patients was obtained from peripheral blood leukocytes using the DNeasy blood kit (Qiagen Inc., Valencia, CA, USA). Genomic DNA was purified from filter paper blood samples using the ReadyAmp Genomic DNA Purification System (Promega, Madison, WI, USA). Mutations in these DNA samples were analyzed at Kagoshima University using a combination of PCR with or without restriction enzyme digestion or by direct sequencing, as previously described [1,10–14,19]. Another set of samples was obtained from 420 healthy volunteers (mainly from Miyagi prefecture in the northeastern region of Japan) at Tohoku University. Genomic DNA from leukocytes was extracted, as described above.

### 2.2. Detection of seven prevalent mutations in SLC25A13 using the HybProbe assay

HybProbe probes comprise a pair of donor and acceptor oligonucleotide probes designed to hybridize adjacent to their target sites in an amplified DNA fragment [20,21]. The donor probes are labeled at their 3′ end with fluorescein isothiocyanate (FITC), whereas the acceptor probes are labeled at their 5′ end with LC Red640; these acceptor probes are phosphorylated at their 3′ end to prevent extension by the DNA polymerase. When two probes hybridize to the amplicon, the fluorescent dyes are located within 5 bases of each other, which allows fluorescence resonance energy transfer (FRET) between the excited FITC and the LC Red640; this process emits light that can be quantified by real-time PCR. Following PCR amplification, a melting-peak analysis is performed. The melting peak is produced by the reporter probe, which has a lower melting temperature (Tm) than the other probe, called the anchor probe. As the reporter melts from the target, the fluorophores are separated, and the FRET ceases. The Tm of the reporter probe determines the reaction

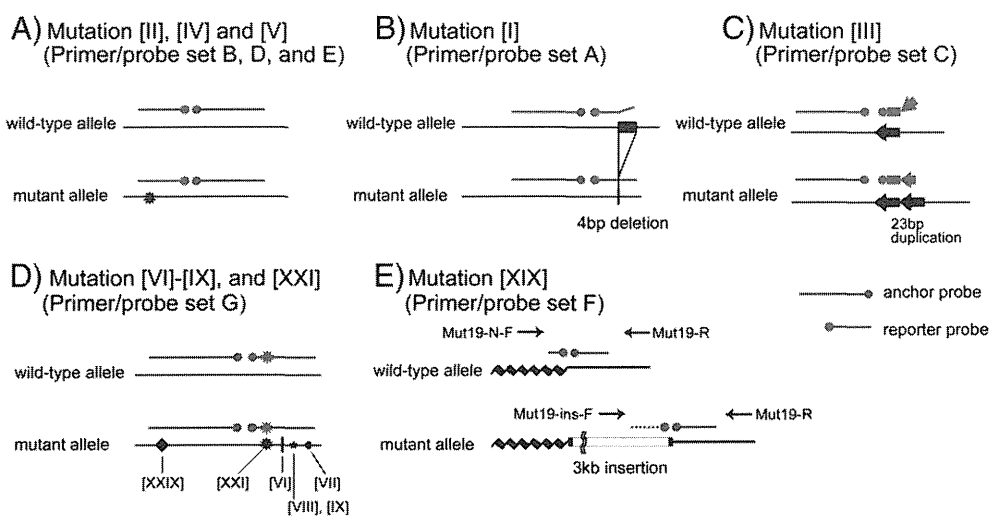


**Fig. 1.** Principle of *SLC25A13* mutation detection by melting-curve analysis with the HybProbe assay. In primer/probe sets A–E, and G, PCR was performed with a pair of primers, whereas in primer/probe set F, two forward primers and one common reverse primer were used for the amplification of both wild-type and mutant alleles. Note that mutation [XXIX], located on the anchor probe of primer/probe set G, is a non-target mutation.