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Newborn screening for carnitine palmitoyltransferase II deficiency using (C16 + C18:1)/C2: Evaluation of additional indices for adequate sensitivity and lower false-positivity

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ABSTRACT

Background: Carnitine palmitoyltransferase (CPT) II deficiency is one of the most common forms of mitochondrial fatty acid oxidation disorder (FAOD). However, newborn screening (NBS) for this potentially fatal disease has not been established partly because reliable indices are not available.

Methods: We diagnosed CPT II deficiency in a 7-month-old boy presenting with hypoglycemic encephalopathy, which apparently had been missed in the NBS using C16 and C18:1 concentrations as indices. By referring to his acylcarnitine profile from the NBS, we adopted the (C16 + C18:1)/C2 ratio (cutoff 0.62) and C16 concentration (cutoff 3.0 nmol/mL) as alternative indices for CPT II deficiency such that an analysis of a dried blood specimen collected at postnatal day five retroactively yielded the correct diagnosis. Thereafter, positive cases were

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assessed by measuring (1) the fatty acid oxidation ability of intact lymphocytes and/or (2) CPT II activity in the lysates of lymphocytes. The diagnoses were then further confirmed by genetic analysis.

Results: The disease was diagnosed in seven of 21 newborns suspected of having CPT II deficiency based on NBS. We also analyzed the false-negative patient and five symptomatic patients for comparison. Values for the NBS indices of the false-negative, symptomatic patient were lower than those of the seven affected newborns. Although it was difficult to differentiate the false-negative patient from heterozygous carriers and false-positive subjects, the fatty acid oxidation ability of the lymphocytes and CPT II activity clearly confirmed the diagnosis. Among several other indices proposed previously, C14/C3 completely differentiated the seven NBS-positive patients and the false-negative patient from the heterozygous carriers and the false-positive subjects. Genetic analysis revealed 16 kinds of variant alleles. The most prevalent, detected in ten alleles in nine patients from eight families, was c.1148T > A (p.F383Y), a finding in line with those of several previous reports on Japanese patients.

Conclusions: These findings suggested that CPT II deficiency can be screened by using (C16 + C18:1)/C2 and C16 as indices. An appropriate cutoff level is required to achieve adequate sensitivity albeit at the cost of a considerable increase in the false-positive rate, which might be reduced by using additional indices such as C14/C3.

1. Introduction

Carnitine palmitoyltransferase (CPT) II is an enzyme bound to the mitochondrial inner membrane. Long-chain fatty acids are transported into the mitochondria as acylcarnitines of the corresponding chain-length via the sequential function of acyl-CoA synthetase, CPT I, and carnitine-acylcarnitine translocase (CACT). These long-chain acylcarnitines, represented by palmitoylcarnitine (C16), are then turned back into acyl-CoA by CPT II to supply substrates for the β-oxidation system. Since the first case report on this subject [1], CPT II deficiency has been clinically classified into three phenotypes: 1) a lethal, neonatal form associated with cardiomyopathy; 2) a severe, infantile form which provokes hypoglycemia, Reye-like encephalopathy, and in the worst cases, cardiopulmonary arrest mainly during infancy and young childhood; and 3) an adult-onset form presenting recurrent rhabdomyolysis in adolescence or later. Since the severe, infantile form of CPT II deficiency was identified as the cause of sudden infantile death, this potentially fatal disease has become an important target of tandem mass spectrometry (MS/MS)-based newborn screening (NBS).

MS/MS-based NBS was introduced into Japan in 1997, and pilot studies were begun in several research centers. In Hiroshima, where the first author currently works, screening for CPT II deficiency was initiated in January 2004 using C16 (cutoff 6.3 nmol/mL) and C18:1 (cutoff 3.6 nmol/mL). These cutoff values corresponded to the mean + 4SD when they were set. No positive results were achieved until 2010, when a 7-month-old boy presented with acute encephalopathy associated with hypoketotic hypoglycemia, hyperammonemia, and marked elevation of serum creatine kinase resulting in severe neurological sequelae. The diagnosis of CPT II deficiency was confirmed [2]. The patient had apparently passed the regional pilot study on MS/MS-NBS with C16 and C18:1 at 3.45 nmol/mL and 1.68 nmol/mL in a dried blood specimen (DBS) collected on postnatal day 5, respectively. This "false-negative" case motivated us to revise the screening indices for CPT II deficiency.

2. Methods

2.1. Screening of CPT II deficiency

Blood samples were analyzed by MS/MS (LCMS-8030, Shimadzu, Kyoto, Japan; API 4000 LC/MS/MS system, AB Sciex, Framingham, MA, USA; ACQUITY TQD, Waters, Milford, MA, USA, etc.) following the protocol described in our previous report [3]. For NBS, dried blood specimens were generally collected on postnatal day 4 or 5. This protocol has been used since NBS for phenylketonuria and other amino acid disorders started in 1977. It is widely accepted that earlier sampling of dried blood is desirable for detecting disorders of fatty acid oxidation, but this method is not yet practiced in Japan. To improve the sensitivity for detecting CPT II deficiency, we adopted (C16 + C18:1)/C2, which had previously been proposed for the screening of symptomatic cases using serum or plasma [4]. We set the cutoff value for this ratio at 0.62, which was as high as the 99.9th percentile in healthy control subjects (n = 5914, mean ± SD = 0.282 ± 0.073) and below the value of the "false-negative" patient's newborn DBS (0.75). In order to avoid excessive false-positive results, we decided to retain C16 as the second index but reduced the cutoff value from 6.3 nmol/mL to 3.0 nmol/mL (79.5th percentile; n = 5914, mean ± SD = 2.37 ± 0.87). These alternative indices have been used in NBS in Hiroshima since April 2011 before being adopted in other areas. For selective screening, serum specimens were collected from patients presenting with suggestive clinical symptoms. Patients with elevated serum levels of C16 (cutoff 0.1 nmol/mL) and C18:1 (cutoff 0.1 nmol/mL) were suspected of having CPT II deficiency.

2.2. Measurement of fatty acid oxidation (FAO) by intact cells

Lymphocytes collected from heparinized whole blood using the Ficoll-Paque solution method were suspended in 1 mL of Dulbecco's phosphate-buffered saline (D-PBS) and incubated at 37 °C for 2 h after adding D-PBS containing L-carnitine and a fatty acid solution containing deuterium-labeled palmitate (d_{31}-palmitate: 0.5 mg/mL in 3% fatty acid-free bovine serum albumin solution). The washed lymphocytes were homogenized in methanol, and the supernatant, spiked with stable isotope-labeled acylcarnitines as internal standards, was analyzed by flow-injection electrospray-ionization tandem mass spectrometry using API 4000 LC/MS/MS system (AB Sciex). Fatty acid oxidation was assessed by the ratio of d_1-acetylcarnitine (d_1C2) to d_{31}-palmitoylcarnitine (d_{31}C16) while the CPT II activity was assessed by the ratio of d_{27}-tetradecanoylcarnitine (d_{27}C14) to d_{31}C16.

2.3. Measurement of CPT II activity

As the revised indices for CPT II deficiency raised the number of positive cases, we developed a simple and rapid enzymatic assay as another confirmatory test. In brief, the production of palmitoyl-CoA from palmitoyl-L-carnitine (C16AC; Sigma Chemical, St. Louis, MO) and coenzyme A trilithium salt ($CoALi_3$; Kohjin, Tokyo, Japan) catalyzed by a crude lysate of peripheral lymphocytes was detected by high-performance liquid chromatography (HPLC). Lymphocytes were sonicated in 1% octyl glucoside (Sigma Chemical, St. Louis, MO) solution so as to abolish the activity of CPT I [5]. The final concentration of each reagent in the reaction mixture was as follows: 100 mmol/L Tris-HCl (pH 7.4), 10 mmol/L C16AC, 10 mmol/L $CoALi_3$, and lysate of 4×10^5 lymphocytes. The mixture was incubated at 37 °C for 10 min, and the reaction was terminated by the addition of acetonitrile. After centrifugation, the supernatant was introduced into an HPLC system

(Shimadzu, Kyoto, Japan) equipped with a reverse-phase octadecylsilane column of 150 mm × 6.0 mm (STR-ODS-II; Shinwa Chemical Industries, Kyoto, Japan). The mobile phase was composed of 100 mmol/L NaH_2PO_4 (pH 4.0) and 49% (v/v) acetonitrile and was pumped at a flow rate of 1.5 mL/min. Palmitoyl-CoA formation was quantified according to ultraviolet absorbance at 260 nm, and the calculation of CPT II activity was based on picomoles of palmitoyl-CoA/min/10^5 lymphocytes and evaluated as a percentage of the average of normal control values.

2.4. Sequence analysis of the CPT2 gene and the CACT gene

In cases where FAO ability or CPT II activity was judged to be impaired, the results were further tested by genetic analysis after informed consent was obtained. Genomic DNA was extracted from peripheral white blood cells. All exons and flanking intron regions comprising the *CPT2* gene were PCR-amplified, and the products were sequenced directly using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 310 genetic analyzer (Applied Biosystems). As the biochemical characteristics of CPT II deficiency are similar to CACT deficiency, sequencing of the *CACT* gene was also done for some of the cases.

3. Results

3.1. Confirmatory diagnosis of the NBS-positive subjects

Twenty-one screened newborns were included in this study and listed in the order of (C16 + C18:1)/C2 value (Table 1; cases N-01 to 21). The diagnoses were confirmed by the assay of FAO ability in 12 subjects, by the assay of CPT II activity in seven subjects, or by the both in two subjects (Table 2). As a result, five subjects showed definite impairment of FAO ability (N-01, 03, 05 to 07), with $d_1C2/d_{31}C16$ (mean ± SD = 3.39 ± 1.35, n = 36) ranging from 0.07 to 0.28 and $d_{27}C14/d_{31}C16$ (mean ± SD = 0.273 ± 0.096, n = 36) from 0.009 to 0.013, and two subjects showed CPT II activity as low as 12.1% (N-02) and 7.8% (N-04). In their newborn DBS, (C16 + C18:1)/C2 ranged from 1.10 to 3.44 and C16 ranged from 3.37 to 13.07 nmol/mL. Concentrations of C16 and C18:1 in their serum, collected around two to four weeks after birth, ranged from 0.65 to 3.18 nmol/mL and from 0.88 to 4.24 nmol/mL, respectively. Biallelic variants of the *CPT2* gene were detected in all of these patients.

Milder impairment of CPT II activity was observed in two subjects at 70.8% (N-08) and 31.8% (N-20). The latter subject was also evaluated with the FAO ability assay and showed $d_1C2/d_{31}C16$ and $d_{27}C14/d_{31}C16$ as low as 0.88 and 0.058, respectively. The indices (C16 + C18:1)/C2 and C16 in their newborn DBS were 0.84 and 6.11 nmol/mL (N-08), and 0.51 and 3.58 nmol/mL (N-20), respectively. Subject N-20 was assessed due to a decreased level of free carnitine (8.41 nmol/mL) in the newborn dried blood specimen. Serum C16 and C18:1 were 0.17 nmol/mL and 0.06 nmol/mL (N-08 on postnatal day 9), and 0.12 nmol/mL and 0.10 nmol/mL (N-20 on postnatal day 12), respectively. During the follow-up of N-20, C16 and C18:1 in serum in a stable state reached 0.25 nmol/mL and 0.23 nmol/mL, respectively. As the subjects harbored one variant *CPT2* allele each, we concluded that they were heterozygous carriers.

Two other cases had milder impairment of FAO ability; their $d_1C2/d_{31}C16$ and $d_{27}C14/d_{31}C16$ were 1.62 and 0.142 (N-11) and 1.56 and

Table 1
Acylcarnitine profile in the false negative patient (S-01) and NBS-positive subjects.

Acylcarnitine index	Newborn dried blood (nmol/mL)									Serum (nmol/mL)		
	(C16 + C18:1)/C2	C16	C18:1	C18	C16-OH	C14	C3	C2	C0	Age	C16	C18:1
Revised cutoff	0.62	3.0	–	–	–	–	–	–	–		0.1	0.1
Previous cutoff	–	6.3	3.6									
Case (sex, birth year)												
S-01[a] (M, 2009)	0.75	3.45	1.68	1.47	0.041	0.57	0.66	6.81	25.31	7 months[b]	3.01	3.92
N-01 (M, 2014)	3.44	9.93	5.25	3.27	0.06	1.75	0.31	4.4	18.5	12 d	3.18	2.54
N-02[c] (F, 2014)	3.27	4.98	3.22	1.96	0.04	0.66	0.08	2.51	11.55	21 d	1.47	2.46
N-03[d] (F, 2007)	3.26	12.20	6.05	4.13	0.08	1.32	0.31	5.6	14.9	19 d	3.02	3.21
N-04 (M, 2013)	3.01	10.6	4.45	1.4	0.1	NT	0.2	5	12	1 month	3.06	4.24
N-05 (F, 2008)	1.65	5.07	1.54	1.24	0.08	0.41	0.09	4.01	10.89	16 d	1.57	1.32
N-06[d] (M, 2004)	1.40	13.07	5.92	4.41	0.25	1.05	0.33	13.6	19.5	14 d	2.17	2.41
N-07 (F, 2012)	1.10	3.37	2.31	1.1	0.031	0.74	0.43	5.16	8.32	32 d	0.65	0.88
N-08[e] (F, 2017)	0.84	6.11	1.20	1.29	0.04	0.26	1.56	8.67	24.51	9 d	0.17	0.06
N-09 (F, 2011)	0.84	5.18	1.93	1.16	0.019	0.24	0.65	8.46	24.13	16 d	0.09	0.07
N-10 (M, 2013)	0.83	3.11	1.39	0.95	0.01	0.17	0.67	5.42	21.31	20 d	0.09	0.12
N-11 (F, 2012)	0.77	3.51	1.18	1.33	0.025	0.15	0.38	6.09	13.27	18 d	0.29	0.15
N-12 (M, 2015)	0.69	2.64[i]	1.27	0.70	0.013	0.13	0.67	5.7	20.45	NT	NT	NT
N-13 (F, 2013)	0.65	4.44	1.55	1.18	0.02	0.2	1.82	9.26	27.27	15 d	0.09	0.09
N-14 (M, 2012)	0.64	5.33	1.59	1.13	0.024	0.24	1.24	10.81	33.41	14 d	0.09	0.11
N-15 (M, 2017)	0.64	3.41	2.00	1.47	0.02	0.23	1.3	8.52	16.15	17 d	0.17	0.24
N-16 (M, 2014)	0.57[g]	6.79	2.55	1.68	0.02	0.36	2.13	16.3	28.27	9 d	0.09	0.05
N-17 (F, 2015)	0.57[g]	3.74	2.35	1.23	0.021	0.21	0.96	10.79	14.253	NT	NT	NT
N-18 (M, 2016)	0.52[g]	6.06	2.10	1.37	0.038	0.4	1.38	15.6	23.1	15 d	0.06	0.06
N-19 (M, 2015)	0.52[g]	4.04	1.75	1.28	0.02	0.26	0.7	11.18	24.92	10 d	0.16	0.13
N-20 (M, 2013)	0.51[f]	3.58	1.20	1.1	0.03	0.3	0.76	9.85	8.41	12 d	0.12	0.10
N-21 (M, 2011)	0.46[h]	9.67	3.92	2.3	0.033	0.45	1.49	29.54	33.26	67 d	0.11	0.10

NT: not tested.
[a] Details of patient S-01 are available in Kobayashi et al. [2].
[b] This analysis was applied to the serum collected during the acute symptomatic period.
[c] Details of patient N-02 are available in Yamada et al. [25].
[d] Patients N-03 and N-06 were siblings.
[e] Newborn dried blood specimen from subject N-08 was collected on postnatal day 3.
[f] Subject N-20 was assessed due to a decreased level of free carnitine (8.41 nmol/mL) in the newborn dried blood specimen.
[g] These indices were tentatively changed to (C16 + C18:1)/C2 ≥ 0.50 and C16 ≥ 3.0 nmol/mL in October 2013 after subject N-20 was suspected of having a mild case of the disease.
[h] Subject N-21 was detected using the previous cutoffs for C16 (≥ 6.3) and C18:1 (≥ 3.6).
[i] Subject N-12 was assessed because this was the first case that showed (C16 + C18:1)/C2 higher than the cutoff in an area.

Table 2
Results of confirmatory tests and clinical findings in the false negative patient (S-01) and NBS-positive subjects.

Fatty acid oxidation			CPT II activity (%)	Genetic analysis		Clinical symptoms
Index	$d_1C2/d_{31}C16$	$d_{27}C14/d_{31}C16$				
Mean ± SD (n = 36)	3.39 ± 1.35	0.273 ± 0.096	SD = 31.5%[a]	*CPT2*	*CACT*	
Case (sex, birth year)						
S-01[b] (M, 2009)	0.14	0.011	13.6	c.[481C > T]; [1148T > A] p.[R161W];[F383Y]	NT	Hypoglycemic encephalopathy at age 7 months with severe neurological sequelae
N-01 (M, 2014)	0.15	0.013	NT	c.[451C > T]; [1148T > A] p.[R151W];[F383Y]	NT	Sudden death during pyrexia at age 1 yr
N-02[c] (F, 2014)	NT	NT	12.1	c.[1148T > A]; [1148T > A] p.[F383Y];[F383Y]	NT	Recurrent elevation of serum CK without any myopathic symptoms since age 4 months
N-03[d] (F, 2007)	0.07	0.009	NT	c.[520G > A]; [1148T > A] p.[E174K];[F383Y]	NT	Recurrent rhabdomyolysis since age 4 yr
N-04 (M, 2013)	NT	NT	7.8	c.[1121G > A]; [1148T > A] p.[W374*];[F383Y]	NT	Sudden death during acute gastroenteritis at age 2 yr
N-05 (F, 2008)	0.26	0.012	NT	c.[1148T > A]; [1429C > T] p.[F383Y];[R477W]	NT	Recurrent rhabdomyolysis since age 3 yr
N-06[d] (M, 2004)	0.09	0.009	NT	c.[520G > A]; [1148T > A] p.[E174K];[F383Y]	NT	Hypoglycemia at age 1 yr; recurrent rhabdomyolysis since age 4 yr
N-07 (F, 2012)	0.28	0.012	NT	c.[1511C > T]; [1813G > C] p.[P504L];[V605L]	NT	Rhabdomyolysis during RSV infection at age 3 yr
N-08 (F, 2017)	NT	NT	70.8	c.[1634A > C];[=] p.[E545A];[=]	NT	No symptoms
N-09 (F, 2011)	2.63	0.271	NT	ND	ND	No symptoms
N-10 (M, 2013)	4.73	0.344	NT	NT	NT	No symptoms
N-11 (F, 2012)	1.62	0.142	NT	ND	ND	No symptoms
N-12 (M, 2015)	NT	NT	108.1	NT	NT	No symptoms
N-13 (F, 2013)	2.27	0.311	NT	ND	ND	No symptoms
N-14 (M, 2012)	5.93	0.534	NT	NT	NT	No symptoms
N-15 (M, 2017)	1.56	0.184	NT	ND	ND	No symptoms
N-16 (M, 2014)	NT	NT	152.7	NT	NT	No symptoms
N-17 (F, 2015)	NT	NT	122.0	NT	NT	No symptoms
N-18 (M, 2016)	NT	NT	128.0	NT	NT	No symptoms
N-19 (M, 2015)	4.59	0.407	116.9	NT	NT	No symptoms
N-20 (M, 2013)	0.88	0.058	31.8	c.[1525A > G];[=] p.[T509A];[=]	NT	No symptoms; highest serum C16 and C18:1 values at age 3 months were 0.25 and 0.23, respectively
N-21 (M, 2011)	3.60	0.430	NT	ND	ND	No symptoms

NT: not tested, ND: not detected.
[a] The average value of CPT II activity in 22 normal control subjects was 126.3 ± 39.8 pmol/min/10^5 cells (mean ± SD).
[b] Details of patient S-01 are available in Kobayashi et al. [2].
[c] Details of patient N-02 are available in Yamada et al. [25].
[d] Patient N-03 and N-06 were siblings.

0.184 (N-15), respectively. Compared with the previously mentioned two carriers, they showed similar levels of (C16 + C18:1)/C2 and C16 in the newborn DBS, at 0.77 and 3.51 nmol/mL (N-11) and 0.64 and 3.41 nmol/mL (N-15), respectively. C16 and C18:1 in their serum appeared to be higher than in that of either N-08 or N-20 at 0.29 nmol/mL and 0.15 nmol/mL (N-11) and 0.17 nmol/mL and 0.24 nmol/mL (N-15), respectively. In spite of these findings, no variants were detected in the *CPT2* or *CACT* gene in either subject. We concluded that they did not have CPT II deficiency, but there was still a possibility that they might be heterozygous for the allele, which carried a large deletion or a deep-intronic splice variant.

The remaining ten subjects were judged to have normal CPT II and CACT enzymes. The assay of FAO ability was used for six subjects (N-09, 10, 13, 14, 19, 21) and showed $d_1C2/d_{31}C16$ and $d_{27}C14/d_{31}C16$ ranging from 2.27 to 5.93 and from 0.271 to 0.534, respectively. The CPT II activity in five subjects (N-12, 16 to 19) ranged from 108.1% to 152.7%. The indices (C16 + C18:1)/C2 and C16 in their newborn DBS ranged from 0.46 to 0.84 and from 2.64 to 9.67 nmol/mL, respectively. Their serum concentrations of C16 and C18:1 ranged from 0.06 to 0.16 nmol/mL and from 0.05 to 0.13 nmol/mL, respectively. Serum C16 = 0.16 nmol/mL and C18:1 = 0.13 nmol/mL were observed in N-19, who proved to have normal FAO ability and CPT II activity. N-09 and N-13 showed a slightly lower value of $d_1C2/d_{31}C16$ (2.63 and 2.27), and N-21 showed C16 = 9.67 nmol/mL and C18:1 = 3.92 nmol/mL in the newborn DBS, which met the previous criteria. No variants were detected either in the *CPT2* or *CACT* gene of N-09, 13, 21.

Compared with these results, CPT II enzymatic activity in the false-negative patient (patient S-01 in Tables 1 and 2) was similarly impaired as in the seven affected newborns; $d_1C2/d_{31}C16$ and $d_{27}C14/d_{31}C16$ were 0.14 and 0.011, and the CPT II activity was 13.6%, respectively. However, it was difficult to differentiate clearly the (C16 + C18:1)/C2 = 0.75, C16 = 3.45 values of S-01 from those of the carriers and false-positive subjects.

Although the total number of screened newborns or the true- and false-positive rate in the areas where one or more cases included in this study were detected was not available, such data as we were able to

obtain in Hiroshima are summarized in Table 3. From the start of CPT II deficiency screening in January 2004 to March 2011 when C16 and C18:1 were adopted as indices, only one false-positive case (N-21) in 185,211 newborns (0.0005%) was detected, and S-01 failed to be detected. After these indices were substituted by (C16 + C18:1)/C2 ≥ 0.62 and C16 ≥ 3.0 nmol/mL in April 2011, four false-positive subjects (N-09 to 11, 14) and a heterozygous carrier (N-20) in 65,239 newborns (0.0077%) were detected. After our experience with N-20, we tentatively changed the inclusion criteria in October 2013 to (C16 + C18:1)/C2 ≥ 0.50 and C16 ≥ 3.0 nmol/mL so as to minimize the risk of false-negative results. In June 2017, four false-positive cases (N-13, 15, 16, 19) were detected in 90,635 newborns (0.0044%). The frequency of CPT II deficiency in the Hiroshima area was 1/341,085 live births.

3.2. Confirmatory diagnosis of the symptomatic patients

We applied the FAO assay or the CPT II activity assay to five symptomatic patients (Table 4; S-02 to 06). Patient S-02 presented with hypoglycemic encephalopathy on postnatal day 1. The concentrations of C16 and C18:1 in the DBS from the acute phase collected on postnatal day 6 were as high as 29.9 nmol/mL and 16.52 nmol/mL, respectively [6]. The CPT II activity in this patient was 6.6%. Patient S-03 presented with hypoglycemia on postnatal day 2. The concentrations of C16 and C18:1 in serum collected on postnatal day 3 were 11.6 nmol/mL and 5.43 nmol/mL, respectively. The diagnosis of this patient was confirmed by the FAO assay, which disclosed a $d_1C2/d_{31}C16$ and $d_{27}C14/d_{31}C16$ of 0.32 and 0.012, respectively. As patients S-02 and S-03 did not present with cardiomyopathy in spite of neonatal onset, we classified the cases as the severe, infantile form of CPT II deficiency.

The phenotypes of the three other symptomatic patients (S-04 to 06) were classified as the myopathic form. The concentrations of C16 and C18:1 in their serum collected under stable condition were around 1 nmol/mL or lower, reflecting the attenuated severity of their disease. The residual enzymatic activity in patients S-04 and S-06 at 17.3% and 18.4%, respectively, was consistent with their disease. However, the value of 2.8% observed in patient S-05 seemed too low for the patient's clinical picture. Although he had presented with only myopathic symptoms since early childhood, the potential of hypoglycemic attack was suspected.

3.3. Genetic analysis

The results of the biochemical and enzymatic evaluation were further confirmed by genetic analysis (Tables 2 and 4). *CPT2* gene sequencing was applied to cases diagnosed on the basis of impaired CPT II activity and/or FAO ability including the false-negative patient (S-01), the five symptomatic patients (S-02 to 06), and the seven NBS-positive patients (N-01 to 07). As a result, biallelic variants were detected in all thirteen cases tested. Subject N-08, who showed 70.8% CPT II activity, was apparently a heterozygous carrier of a variant that had not yet been characterized. Subject N-20, who showed 31.8% CPT II activity, a known variant was detected in an allele which neither of the parents harbored. Both parents also showed normal CPT II activity (117% and 119%). Therefore we concluded that subject N-20 was a heterozygous carrier of a de novo variant allele.

In line with previous reports on Japanese patients [7,8,9], the variant with the highest frequency was c.1148T > A (p.F383Y) detected in ten alleles in nine patients from eight families, followed by c.451C > T (p.R151W) [6] detected in three alleles in two patients. Among the other sporadic variants, c.338C > T (p.S113L) [10], c.481C > T (p.R161W) [11], c.520G > A (p.E174K) [7], c.641T > C (p.M214T) [12], c.1511C > T (p.P504L) [13], c.1813G > C (p.V605L) [9], and c.1891C > T (p.R631C) [14] are known while c.313C > T (p.Q105*), c.1121G > A (p.W374*), c.1345C > A (p.Q449*), c.1429C > T (p.R477W), c.1525A > G (p.T509A), c.1579G > A (p.E527K), and c.1634A > C (p.E545K) have not yet been actually reported. The variant c.338C > T (p.S113L), known to be most prevalent among Caucasian patients, usually causes the myopathic form of the disease [10,15] and was detected in a Swiss patient (S-06) in this study, who suffered from recurrent rhabdomyolysis since adolescence. Our analysis of each variant, together with the reference SNP ID, allele frequency (ExAC database), clinical significance (ClinVar), and in silico predictions (PolyPhen-2), is summarized in Table 5.

4. Discussion

Of the various types of mitochondrial fatty acid oxidation disorder (FAOD), CPT II deficiency is one of the most common. Before MS/MS-NBS was introduced, the number of patients with CPT II deficiency was reportedly second only to that of patients with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency among Caucasians [16]. A nationwide Japanese survey of symptomatic FAOD cases diagnosed or reported between 1985 and 2000 revealed that most patients had CPT II deficiency [17]. Nevertheless, it has been listed as a primary target disease in NBS in a limited number of countries [18,19] probably because of the excessively large number of false-positive cases or the considerable risk of false-negative cases in the MS/MS analysis of dried blood specimens.

For high-risk screening of symptomatic patients, (C16 + C18:1)/C2 in the serum or plasma was proposed as a sensitive index [4]. In contrast, another report showed that this ratio was hardly able to differentiate between confirmed adult patients and healthy control subjects when dried blood specimens were used [20].

However, few reports have evaluated the reliability of the NBS for CPT II deficiency. A recent case report described a 4-year-old girl presenting with rhabdomyolysis whose condition had been missed by NBS [21]. The indices for CPT II deficiency in her dried blood specimen collected on postnatal day 2 were 3.44 nmol/mL of C16 (cutoff 10) and 1 nmol/mL of C18:1 (cutoff 3), respectively, but (C16 + C18:1)/C2 was not assessed. The ratio was 0.34, which was deemed to be non-informative because the value fell between the 5th percentile for affected subjects and the 99th percentile for normal subjects, according to a previous study on the clinical validation of cutoff target ranges [22].

Table 3
Summary of newborn screening in the Hiroshima area.

Period	Number of newborns screened	Indices for CPT II deficiency	True positive	False positive (and carrier)			False negative
				Number	(%)	Case ID	
Jan 2004–Mar 2011	185,211	C16 ≥ 6.3 nmol/mL and C18:1 ≥ 3.6 nmol/mL	0	1	0.0005	N-21	1 (S-01)
Apr 2011–Sep 2013	65,239	(C16 + C18:1)/C2 ≥ 0.62 and C16 ≥ 3.0 nmol/mL	0	5	0.0077	N-09, 10, 11, 14 N-20 (carrier)	0
Oct 2013–Jun 2017	90,635	(C16 + C18:1)/C2 ≥ 0.50 and C16 ≥ 3.0 nmol/mL	0	4	0.0044	N-13, 15, 16, 19	0
Total	341,085		0	10			1

Table 4
Clinical, biochemical, and genetic characteristics of the symptomatic patients.

Case (sex, birth year)	Serum acylcarnitine (nmol/mL)				CPT II activity (%)	Fatty acid oxidation (mean ± SD, n = 36)		*CPT2* variant	Clinical symptoms
	Sample	C16	C18:1	C2		$d_1C2/d_{31}C16$	$d_{27}C14/d_{31}C16$		
		0.1	0.1	–	SD = 31.5%[a]	3.39 ± 1.35	0.273 ± 0.096		
S-02[b] (F, 2014)	DBS[c] of acute phase at age 6 d	29.9	16.52	2.34	6.6	NT	NT	c.[451C > T]; [451C > T] p.[R151W]; [R151W]	Hypoglycemic encephalopathy without cardiomyopathy at age 1 d
S-03 (M, 2013)	Serum of acute phase at age 3 d	11.6	5.43	30.25	NT	0.32	0.012	c.[1148T > A]; [1345C > A] p.[F383Y];[Q449*]	Hypoglycemia at age 2 d
S-04 (M, 2000)	Serum of stable state at age 17 yr	0.96	1.08	13.3	17.3	NT	NT	c.[313C > T]; [1891C > T] p.[Q105*];[R631C]	Recurrent rhabdomyolysis since age 3 yr
S-05 (M, 1991)	Serum of stable state at age 25 yr	0.94	0.68	4.4	2.8	NT	NT	c.[1148T > A]; [1579G > A] p.[F383Y];[E527K]	Recurrent myalgia since childhood; rhabdomyolysis at age 25 yr
S-06 (F, 1953)	Serum of stable state at age 63 yr	0.55	0.56	4.3	18.4	NT	NT	c.[338C > T]; [641T > C] p.[S113L]; [M214T]	Recurrent rhabdomyolysis since adolescence

NT: not tested.
[a] The average value of CPT II activity in 22 normal control subjects was 126.3 ± 39.8 pmol/min/10^5 cells (mean ± SD).
[b] Details of case S-02 are available in Ikeda et al. [6].
[c] Serum of acute phase was not analyzed.

The patient was found to be homozygous for c.338C > T (p.S113L). According to another previous report, the disease frequency based on NBS carried out in Australia, Germany, and the United States ranged from 1/380,000 to 1/2,000,000 newborns [23], suggesting that the frequency of the severe, infantile form of CPT II deficiency was higher in Japan. In the pilot study of MS/MS-NBS, which we conducted from 2004 to 2012 in several areas of Japan including Hiroshima, CPT II deficiency was diagnosed in six (including S-01, N-03, and 05 to 07 in the current study) of 1,740,387 newborns, resulting in a frequency of 1/290,065.

Recently an adult Japanese patient who had suffered since adolescence from recurrent rhabdomyolysis provoked by exercise or infection turned out to be homozygous for c.338C > T (p.S113L) [24]. CPT II activity in his fibroblasts was reportedly 16% of the normal control value. Although the assay used was different from ours, the residual enzymatic activity of 13.6% in the lymphocytes of our false-negative patient (S-01) suggested that he had a marginal risk for the severe infantile form of the disease. In order to detect such patients by NBS, the cutoff for (C16 + C18:1)/C2 must be lowered at the cost of incurring a higher false-positive rate.

The above-cited report suggested that the ratio of several long-chain acylcarnitines to propionylcarnitine (C3) in newborn DBS such as (C16 + C18:1)/C3, C18/C3, C16/C3, C16-OH/C3, and C14/C3 can better serve as indices in CPT II deficiency screening [21]. We added the C18, C16-OH, C14, C3, C2, and C0 values for the newborn DBS of our NBS-positive subjects to Table 1 and evaluated their utility (Fig. 1).

Table 5
Information on *CPT2* variants detected in this study.

Variant cDNA (Amino acid)	Reference SNP ID	Allele frequency (ExAC)	Clinical significance (ClinVar)	PolyPhen-2	Estimate of allele-specific activity	Reference
c.313C > T (p.Q105*)	–	–	–	–	Abolished[a]	–
c.338C > T (p.S113L)	rs74315294	0.001271	Pathogenic	Probably damaging	16%[b]	[10,15,24]
c.451C > T (p.R151W)	rs200080591	0.00001648	–	Probably damaging	5–10%[a]	[6]
c.481C > T (p.R161W)	–	0.000008242	–	Probably damaging	10–15%[a]	[11]
c.520G > A (p.E174K)	rs28936674	0.000008243	Pathogenic	Probably damaging	Mostly abolished[c]	[7]
c.641T > C (p.M214T)	rs515726174	–	Pathogenic	Probably damaging	15–20%[d]	[12]
c.1121G > A (p.W374*)	–	0.000008244	–	–	Abolished[a]	–
c.1148T > A (p.F383Y)	rs74315295	0.00003299	Pathogenic/likely pathogenic	Benign	10–15%[a]	[7,8,9,25]
c.1345C > A (p.Q449*)	–	–	–	–	Abolished[a]	–
cf. c.1345C > T (p.Q449*)	rs1057517492	–	Likely pathogenic	–	–	–
c.1429C > T (p.R477W)	–	0.000008361	–	Probably damaging	(Not measured)	–
c.1511C > T (p.P504L)	rs368311455	0.00003596	–	Probably damaging	(Not measured)	[13]
c.1525A > G (p.T509A)	–	–	–	Possibly damaging	Mostly abolished[a]	–
c.1579G > A (p.E527K)	–	–	–	Benign	Mostly abolished[a]	–
c.1634A > C (p.E545A)	rs17848485	0.001068	Pathogenic	Possibly damaging	30–50%[a]	–
c.1813G > C (p.V605L)	rs53679103	0.00002471	–	Possibly damaging	(Not measured)	[9]
c.1891C > T (p.R631C)	rs74315293	0.00002471	Pathogenic	Benign	30–40%[a]	[14]

[a] These estimates are based on the results of this study and expressed as the percentage of the wild-type enzyme activity.
[b] Data from Shima et al. [24].
[c] This estimate is based on the results of this study and data from Yamamoto et al. [7].
[d] This estimate is based on the results of this study and data from Shima et al. [24].

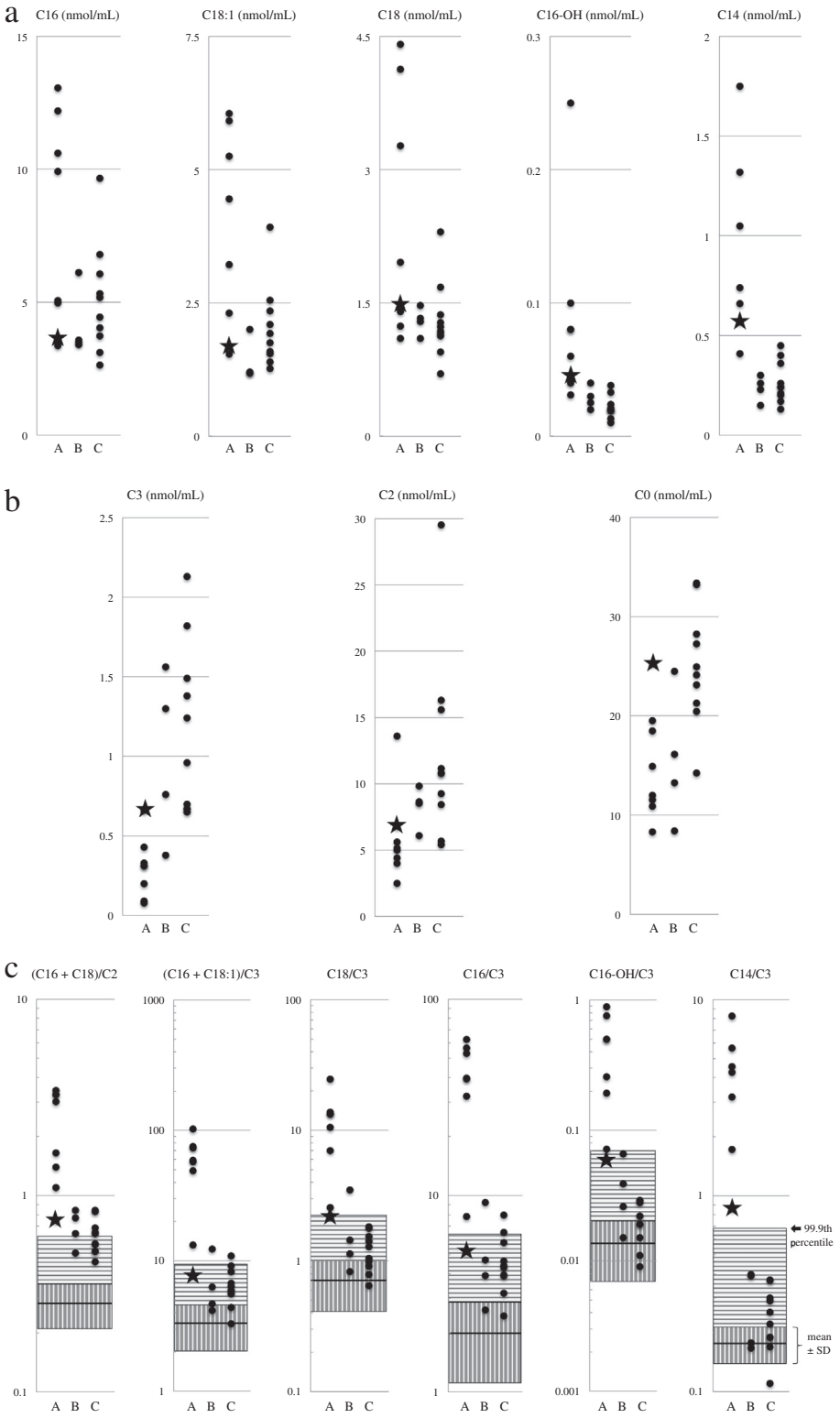

Fig. 1. Comparative evaluation of several additional indices for CPT II deficiency.
In order to reduce the false-positive rate in NBS based on (C16 + C18:1)/C2 and C16, several additional indices were evaluated. (a) None of the single long-chain acylcarnitines (C16, C18:1, C18, C16-OH, or C14) could separate patients with CPT II deficiency (Group A; patients N-01 to 07, and S-01 indicated as ★) from heterozygous carriers or those with mild impairment of FAO ability (Group B; N-08, 11, 15, 20) and false-positive subjects (Group C; N-09, 10, 12 to 14, 16 to 19, 21). Minimal overlap was observed between Group A and Groups B and C in terms of C14. (b) As a denominator for long-chain acylcarnitines, the distribution of C3, C2, and C0 concentrations were compared among the three groups. C3 showed better separation between Group A and Groups B and C than C2 or C0. (c) (C16 + C18:1)/C2 was compared with five kinds of ratio using C3 as the denominator: (C16 + C18:1)/C3, C18/C3, C16/C3, C16-OH/C3, and C14/C3. As expected from the data shown in (a) and (b), C14/C3 was able to differentiate Group A from Groups B and C perfectly.

Although none of the single long-chain acylcarnitines (C16, C18:1, C18, C16-OH, or C14) was able to distinguish the patients with CPT II deficiency (Fig. 1a; Group A including N-01 to 07 and S-01) from the carriers (Group B; N-08, 11, 15, 20) and the false-positive subjects (Group C; N-09, 10, 12 to 14, 16 to 19, 21), C14 appeared to be promising with the smallest overlap between Group A and the other groups. With regard to the denominator, the C3 values in the patients were apparently lower than in the carriers and the false-positive subjects (Fig. 1b). As a result, of (C16 + C18:1)/C2 and the five kinds of ratio using C3 as the denominator, we found that C14/C3 was able to differentiate the patients from the carriers and the false-positive subjects perfectly (Table 6 and Fig. 1c). Although the biochemical mechanism is unclear, as an additional index, C14/C3 is expected simultaneously to improve the sensitivity of CPT II deficiency screening and reduce the false-positive rate.

Our experience and data indicated that reliable NBS for CPT II

Table 6
Comparative data of (C16 + C18:1)/C2 and ratios of various long-chain acylcarnitine to propionylcarnitine (C3).

Acylcarnitine ratios in newborn DBS		(C16 + C18:1)/C2	(C16 + C18:1)/C3	C18/C3	C16/C3	C16-OH/C3	C14/C3
(n = 5914)	99.9th percentile	0.62	9.40	2.24	6.47	0.070	0.68
	mean ± SD	0.282 ± 0.073	3.28 ± 1.27	0.71 ± 0.30	2.02 ± 0.89	0.0135 ± 0.0066	0.175 ± 0.037
Case	Diagnosis						
S-01	Patient	0.75	7.77	2.23	5.23	0.06	0.86
N-01	Patient	3.44	48.968	10.548	32.03	0.194	5.645
N-02	Patient	3.27	102.50	24.50	62.30	0.50	8.25
N-03	Patient	3.26	58.871	13.322	39.35	0.258	4.258
N-04	Patient	3.01	75.25	7.00	53.00	0.50	No data
N-05	Patient	1.65	73.444	13.778	56.33	0.889	4.556
N-06	Patient	1.40	57.545	13.364	39.61	0.758	3.182
N-07	Patient	1.10	13.209	2.558	7.837	0.072	1.721
N-08	Carrier	0.84	4.686	0.827	3.92	0.026	0.167
N-09	False positive	0.84	10.94	1.78	7.97	0.029	0.37
N-10	False positive	0.83	6.716	1.418	4.64	0.015	0.254
N-11	False positive (or carrier?)	0.77	12.34	3.5	9.24	0.066	0.39
N-12	False positive	0.686	5.862	1.046	3.96	0.019	0.189
N-13	False positive	0.65	3.291	0.648	2.44	0.011	0.11
N-14	False positive	0.64	5.58	0.91	4.30	0.019	0.19
N-15	False positive (or carrier?)	0.64	4.162	1.131	2.62	0.015	0.177
N-16	False positive	0.57	4.385	0.789	3.19	0.009	0.169
N-17	False positive	0.565	6.357	1.288	3.91	0.022	0.221
N-18	False positive	0.52	5.913	0.993	4.39	0.028	0.29
N-19	False positive	0.52	8.271	1.829	5.77	0.029	0.371
N-20	Carrier	0.51	6.289	1.447	4.71	0.039	0.395
N-21	False positive	0.46	9.12	1.54	6.49	0.022	0.3

deficiency can be realized by setting appropriate indices and cutoff values. Unfortunately, until recently CPT II deficiency was overlooked by the local government in several regions in Japan where it was considered to be a secondary NBS target. Our recent survey revealed that at least 13 patients in those areas who could have been saved by NBS died of acute metabolic failure [unpublished data]. When this fact came to light, it was finally decided in July 2017 that CPT II deficiency should be included among the primary target diseases. Although most of the affected subjects in the present study identified through NBS repeatedly showed myopathic symptoms (N-03, 05 to 07) or an elevation of serum CK without myopathic symptoms (N-02) [25] despite early therapy, we believe that patients N-01 and N-04 could have been saved from sudden death by stricter management of their illness.

5. Conclusion

The findings of the present study suggest that the substitution of "(C16 + C18:1)/C2 and C16" for "C16 and C18:1" as NBS indices for CPT II deficiency will significantly reduce the risk of overlooking affected newborns. The considerable increase in the false-positive rate we experienced might be reduced by using additional indices such as C14/C3. Although determining the cutoff value for these new indices may require that the conditions prevailing in each laboratory including the schedule of dried blood sampling, methods of sample preparation, types of MS/MS apparatus used, and so on, be carefully considered, our successful detection of seven affected newborns and two heterozygous carriers from many false-positive subjects and the evaluation of several additional indices based on definite confirmatory diagnoses will serve as a good source of information for those who hope to design a better NBS system for CPT II deficiency.

Author contributions

Go Tajima developed the HPLC-based assay of CPT II activity under the instruction of Nobuo Sakura, carried out the enzymatic evaluation of all cases included in this study, managed the entire project, and wrote this paper. Keiichi Hara, Miyuki Tsumura, and Reiko Kagawa performed the analysis of the *CPT2* and *CACT* genes, and Satoshi Okada evaluated the significance of the variants detected. Shinsuke Maruyama, Atsuko Noguchi, Tomonari Awaya, Mika Ishige, Ikuma Musha, Sayaka Ajihara, Akira Ohtake, Etsuo Naito, Yusuke Hamada, Tomotaka Kono, Tomoko Asada, Ryosuke Bo, Kenji Yamada, Hironori Kobayashi, and Yuki Hasegawa clinically managed the patients. Etsuo Naito also analyzed the *CPT2* gene of two patients. Ryosuke Bo, Kenji Yamada, Hironori Kobayashi, and Yuki Hasegawa also analyzed the acylcarnitine profiles and the *CPT2* gene of several patients. Nobuyuki Ishige analyzed the acylcarnitine profiles of several patients. Hideo Sasai, Toshiyuki Fukao, Ryoji Fujiki, and Osamu Ohara established a system of gene panel analysis for newborn screening target diseases and applied it to one patient. Seiji Yamaguchi and Masaki Takayanagi surveyed the symptomatic patients missed in newborn screening who suffered serious outcomes. Ikue Hata and Yosuke Shigematsu analyzed the acylcarnitine profiles and measured fatty acid oxidation using tandem mass spectrometry. Masao Kobayashi supervised the project as the head of the laboratory.

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Statement of competing interests

None to declare.

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Ethical approval

Approval for the enzymatic and genetic studies was obtained from the ethics committee of Hiroshima University. All procedures were carried out in accordance with the ethical standards of the relevant committee on human experimentation (institutional and national) and the Helsinki Declaration of 1975 as revised in 2000.

Informed consent

Informed consent was obtained from all families enrolled in the study.

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