Journal of Human Genetics (2019) 64:87–98
https://doi.org/10.1038/s10038-018-0530-z

REVIEW ARTICLE

Carnitine palmitoyltransferase II deficiency with a focus on newborn screening

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Received: 29 June 2018 / Revised: 23 October 2018 / Accepted: 25 October 2018 / Published online: 4 December 2018

Abstract

Carnitine palmitoyltransferase (CPT) II deficiency is one of the most common forms of mitochondrial fatty acid oxidation disorder. Its clinical phenotypes are classified into the muscle, severe infantile, and lethal neonatal forms. Among Caucasians, the muscle form predominates, and the c.338C > T (p.S113L) variant is detected in most cases, whereas among the Japanese, c.1148T > A (p.F383Y) is the variant allele occurring with the highest frequency and can apparently cause symptoms of the severe infantile form. Newborn screening (NBS) for this potentially fatal disease has not been established. We encountered an infantile case of CPT II deficiency not detected in NBS using C16 and C18:1 concentrations as indices, and therefore we adopted the (C16 + C18:1)/C2 ratio as an alternative primary index. As a result, the disease was diagnosed in nine of 31 NBS-positive subjects. The values for (C16 + C18:1)/C2 in the affected newborns partly overlapped with those in unaffected ones. Among several other indices proposed previously, C14/C3 has emerged as a more promising index. Based on these findings, nationwide NBS for CPT II deficiency using both (C16 + C18:1)/C2 and C14/C3 as indices was officially approved and started in April 2018. We diagnosed the disease in four young children presenting with symptoms of the muscle form, whose values for the new indices were not elevated. Although it is still difficult to detect all cases of the muscle form of CPT II deficiency in NBS, our system is expected to save many affected children in Japan with the severe infantile form predominating.

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 (Fig. 1). This pathway was proposed in 1963 [1] and was experimentally supported in 1970 [2]. Further research has revealed that CPT II is ubiquitously expressed and that there are three types of isozymes for CPT I, as follows: IA, the liver type, IB, the muscle type, and IC, the brain type. Detection and chromosomal mapping of the genes responsible for these enzymes are as follows: *CPT2* in 1p32 [3, 4], *CPT1A* in 11q13 [5], *CPT1B* in 22q13 [6], and *CPT1C* in 19q13 [7]. Currently, CPT IA and CPT II deficiencies are described as autosomal recessive human diseases.

Clinical phenotypes

As is characteristic of fatty acid oxidation disorders (FAODs), both CPT IA deficiency and CPT II deficiency can provoke acute metabolic decompensation associated with hypoketotic hypoglycemia. In addition, CPT II

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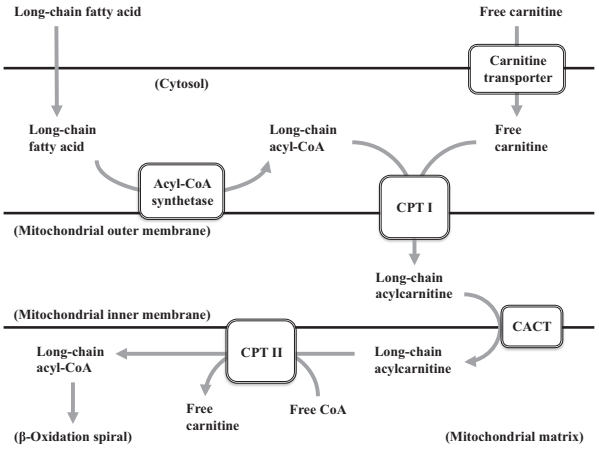

Fig. 1 Schematic representation of the carnitine cycle. CPT carnitine palmitoyltransferase, CACT carnitine-acylcarnitine translocase

deficiency usually shows intermittent myopathic symptoms, including cardiomyopathy in the severest cases, which are not observed in CPT IA deficiency. The first case report of CPT II deficiency appeared in 1973 [8]; the disease was diagnosed in an adult male patient who had exercise-induced muscle cramps and myoglobinuria recurrently over many years. Since then, CPT II deficiency has been clinically classified into the following three phenotypes: (1) an adult-onset muscle form presenting with recurrent rhabdomyolysis in adolescence or later; (2) a severe infantile form which provokes hypoglycemia, Reye-like encephalopathy, and in the worst cases, cardiopulmonary arrest, mainly during infancy and early childhood (the first case was reported by Demaugre et al. [9]); and (3) a lethal neonatal form associated with cardiomyopathy (the first case was reported by Hug et al. [10]). Moreover, increasing data has indicated that not a few patients with the "adult-onset" muscle form have their first episode of myopathic symptoms without hypoglycemia during infancy or early childhood [11].

Genetic backgrounds

Caucasian patients

Numerous studies have reported genotype–phenotype correlation in a considerable number of Caucasian patients in whom c.338C > T (p.S113L) emerged as a common variant at an extremely high frequency [11−22]. On the whole, patients homozygous for p.S113L are expected to present with myopathic symptoms in adolescence and adulthood. However, there have also been sporadic reports of myoglobinuria cases during infancy or early childhood [21, 23, 24]. The compound heterozygous genotype of p.S113L and another null variant is also thought to lead to the muscle form of the disease [18, 25], but there was a case report of cardiac arrest in a 6-year-old girl having this genotype, though her blood glucose level was not documented [26]. On the other hand, there were reports of symptomatic patients who had one allele of p.S113L and were judged to be heterozygous carriers of CPT II deficiency based on an enzyme assay and pedigree analysis [16, 21, 27, 28]. The wide variety of clinical findings associated with the p.S113L variant suggests the presence of additional factors lowering the threshold for the onset of symptoms, such as the coexistence of two "thermolabile polymorphisms", c.1102G > A (p.V368I) and c.1939A > G (p.M647V), described further below. However, a straightforward interpretation of their effects seems difficult due to the complexity of the clinical information and experimental data reported thus far [29, 30]. A small number of studies of the severe infantile form and the lethal neonatal form of CPT II deficiency in which the genotypes were documented failed to detect the p.S113L variant allele [29, 31−35]. A similar discussion surrounds another mild variant which occurs with considerable frequency, c.149C > A (p.P50H) [30], although this variant was detected in a case of the severe infantile form in combination with a frame-shift variant [31].

Japanese patients

The rarity of the p.S113L variant allele in Japanese patients constitutes one of the significant differences from the Caucasian population. The first Japanese case report of a patient homozygous for p.S113L, published in 2016, described an adult male who experienced recurrent rhabdomyolysis since his teenage years [36]. Currently, c.1148T > A (p.F383Y) is recognized as the variant having the highest frequency among Japanese patients [37−44]. Previous reports described three symptomatic patients homozygous for p.F383Y; the clinical phenotype of these patients was the severe infantile form and one of them died during a Reye-like episode [37, 39]. The phenotype of the five compound heterozygous patients harboring p.F383Y and another variant was either the severe infantile form (four cases including a case of sudden death) [37, 41, 42, 44] or the muscle form (one case) [44]. Table 1 summarizes the information about these cases, excluding the cases cited in refs. [42−44]. Table 2 summarizes the cases described in refs. [42, 44, 45] (S-01 to S-06), and recent cases we encountered as well (S-07 to S-14). The diagnosis of CPT II deficiency was made in 11 of 14 symptomatic patients (S-01 to S-11) based on an assay of CPT II activity in the lysates of lymphocytes and/or the FAO capability of intact lymphocytes; this assessment was confirmed by genetic analysis.

Table 1 Clinical, biochemical, and genetic characteristics of symptomatic Japanese patients in previous reports

Previous report (Sex)	Blood acylcarnitine (nmol/mL)				CPT II activity (%)[a]	*CPT2* genotype			Clinical symptoms	Phenotype
	Sample	C16	C18:1	C2		Variant	Thermolabile polymorphism[b]			
							p. F352C	p. V368I		
Wataya [37] (M)	NA				6.3 (Ly)	c. [1148T>A];[1148T>A] p.[F383Y];[F383Y]	C/C	I/I	Hypoglycemia at age 9 mo. Older female sibling died of Reye-like syndrome at age 7 mo.	Infantile
Wataya [37] (F)	NA				2.2 (Ly)	c.[621G>A];[1148T>A;1055T>G;1102G>A] p.[E174K];[F383Y;F352C;V368I]			Hypoglycemia at age 9 mo. Younger male sibling also showed hypoglycemia at age 6 mo.	Infantile
Wataya [37] (F)	NA				5.8 (Ly)	c.[621G>A];[621G>A] p.[E174K];[E174K]	F/F	V/V	Recurrent myalgia since age 10 y. Rhabdomyolysis at age 17 y. Older male sibling died of Reye-like syndrome at age 3 y.	Muscle
Kaneoka [38] (M)	NA				2.1 (Fb)	c.[1223_1224delCT]; [1891C>T] p.[S408Yfs;T420*]; [R631C]	NA		Recurrent rhabdomyolysis since age 21 y.	Muscle
Aoki [39] (F)	Serum at stable state at age 21y	(C16 + C18:1)/C2 = 1.16 (reference < 0.48)			2 (Ly) 6 (Sm) 7-8 (Fb)	c. [1148T>A];[1148T>A] p.[F383Y];[F383Y]	C/C	I/I	Hypoglycemia at age 2 y. Rhabdomyolysis at age 19 y. Male sibling died of Reye-like syndrome during infancy. Female sibling also died in infancy.	Infantile
Yasuno [40] (M)	NA				18.6 (Fb)	c.[1148T>A];[1813G>C] p.[F383Y];[V605L]	NA		Reye-like syndrome at age 1mo. Recurrent hypoglycemia and rhabdomyolysis since infancy.	Infantile
Yasuno [40] (F)	Serum (Acute state?)	High	High	Low	14.4 (Fb)	c.[1148T>A];[?] p.[F383Y];[?]	NA		Hypoglycemia and rhabdomyolysis at age 2 y.	Infantile
Yasuno [40] (F)	NA				23.7 (Fb)	c.[1148T>A];[?] p.[F383Y];[?]	NA		Recurrent myalgia since age 19 y.	Muscle
Yasuno [40] (F)	NA				5.6 (Ly)	c.[1148T>A];[?] p.[F383Y];[?]	NA		Recurrent loss of consciousness since age 6 mo. Two male siblings died of Reye-like syndrome.	Infantile
Yasuno [40] (F)	Serum (Acute state?)	High	High	Low	10.3 (Fb)	c.[1148T>A];[?] p.[F383Y];[?]	NA		Hypoglycemia at age 11 y.	Infantile (Late onset)
Yamamoto [41] (M)	Post mortem whole blood	High	High	NA	NT	c.[1148T>A];[1931T>C] p.[F383Y];[L644S]	C/C	I/I	Sudden fatal cardiopulmonary arrest during febrile episode at age 6 mo.	Infantile
		C16/C14:1 = 49.4								
	Newborn DBS	(C16 + C18:1)/C2 = 1.56								
Shima [36] (M)	Serum at stable state at age 46y	0.55	0.59	NA	16 (Fb)	c.[338C>T];[338C>T] p.[S113L];[S113L]	NA		Recurrent rhabdomyolysis since age 14 y.	Muscle

NT not tested, *NA* data not available, *DBS* dried blood specimen, *Ly* lymphoblast, *Fb* fibroblast, *Sm* skeletal muscle

[a]Various methods were used to measure CPT II activity in these previous reports.

[b]Genotypes of the two thermolabile polymorphisms are as follows:

F/F p.F352 homozygote, *C/C* p.C352 homozygote, *F/C* compound heterozygote of p.F352 and p.C352, *V/V* p.V368 homozygote, *I/I* p.I368 homozygote, *V/I* compound heterozygote of p.V368 and p.I368

Table 2 Clinical, biochemical, and genetic characteristics of Japanese symptomatic patients (cited from our previous report [44] with additional data)

Case, sex, birth year	NBS indices		Blood acylcarnitine (nmol/mL)				CPT II activity (%)[a]	Fatty acid oxidation capability (%)[b]		*CPT2* genotype			Clinical symptoms	Phenotype
	(C16 + C18:1)/C2	C14/C3	Sample	C16	C18:1	C2		$d_1C2/d_{31}C16$	$d_{27}C14/d_{31}C16$	Variant	Thermolabile polymorphism[c]			
											p.F352C	p.V368I		
S-01 (M, 2009)[d]	0.75	0.86	Serum at acute phase at age 7 mo.	3.01	3.92	0.87	13.6	4.1	4.0	c.[481C > T];[1148T > A;1055T > G;1102G > A] p.[R161W];[F383Y;F352C;V368I]			Hypoglycemic encephalopathy at age 7 mo with severe neurological sequelae	Infantile
S-02 (F, 2014)[e]	(Onset before NBS)		DBS at acute phase at age 6 d.	29.9	16.52	2.34	6.6	NT	NT	c.[451C>T];[451C>T] p.[R151W];[R151W]	F/F	I/I	Hypoglycemic encephalopathy without cardiomyopathy at age 1 d	Infantile
S-03 (M, 2013)	(Onset before NBS)		Serum at acute phase at age 3d	11.6	5.43	30.25	NT	9.4	4.4	c.[1148T > A;1055T > G];[1345C > A] p.[F383Y;F352C];[Q449*]	F/C	V/I	Hypoglycemia at age 2 d	Infantile
S-04 (M, 2000)	NT	NT	Serum at stable state at age 17 y	0.96	1.08	13.3	17.3	NT	NT	c.[313C > T];[1891C > T] p.[Q105*];[R631C]	F/F	V/I	Recurrent rhabdomyolysis since age 3 y	Muscle
S-05 (M, 1991)	NT	NT	Serum at stable state at age 25 y	0.94	0.68	4.4	2.8	NT	NT	c.[1148T > A];[1579G > A] p.[F383Y];[E527K]	F/C	I/I	Recurrent myalgia since childhood Rhabdomyolysis at age 25 y	Muscle
S-06 (F, 1953)[f]	NT	NT	Serum at stable state at age 63 y	0.55	0.56	4.3	18.4	NT	NT	c.[338C > T];[641T > C] p.[S113L];[M214T]	F/F	V/I	Recurrent rhabdomyolysis since adolescence	Muscle
S-07 (F, 2013)	0.15	0.24	Serum at acute phase at age 3 y	1.17	1.88	14.04	16.2	NT	NT	c. [1148T > A];[1678C > T] p.[F383Y];[R560W]	C/C	I/I	Recurrent rhabdomyolysis since age 2 y	Muscle
S-08 (M, 2014)[g]	0.25	NA	Serum at stable state at age 3 y	0.48	0.42	7.0	9.5	NT	NT	c.[1678C > T];[1813G > C] p.[R560W];[V605L]	C/C	I/I	Rhabdomyolysis at age 1 y	Muscle
S-09 (M, 2016)[g]	0.31	0.36	Serum at stable state at age 15 mo	0.69	0.65	4.43	15.5	NT	NT	c.[1678C > T];[1813G > C] p.[R560W];[V605L]	C/C	I/I	Rhabdomyolysis at age 1 y	Muscle
S-10 (F, 2014)	0.32	0.22	Serum at stable state at age 4 y	0.64	0.71	6.19	8.1	41.9	33.3	c.[338C > T];[1933G > T] p.[S113L];[E645*]	F/C	V/I	Rhabdomyolysis at age 4 y	Muscle

Table 2 (continued)

Case, sex, birth year	NBS indices		Blood acylcarnitine (nmol/mL)				CPT II activity (%)[a]	Fatty acid oxidation capability (%)[b]		*CPT2* genotype			Clinical symptoms	Phenotype
	(C16 + C18:1)/ C2	C14/ C3	Sample	C16	C18:1	C2		$d_1C2/d_{31}C16$	$d_{27}C14/d_{31}C16$	Variant	Thermolabile polymorphism[c]			
											p.F352C	p.V368I		
S-11 (M, 2003)	NT	NT	Serum at acute phase at age 13 y	1.15	1.31	22.3	14.7	NT	NT	c.[365C > T]; [1511C > T] p.[S122F];[P504L]	F/C	V/I	Rhabdomyolysis at age 13 y Older male sibling died of CPT II deficiency at age 1 y	Muscle
S-12 (M, 2017)	0.18	0.18	Serum at acute phase at age 9 mo	0.61	0.79	17.5	45.2	NT	NT	c.[365C > T];[1055T > G;1102G > A] p.[S122F];[F352C;V368I]			Acute encephalopathy (not Reye-like) at age 9 mo	Infantile?
S-13 (F, 2013)	0.36	NA	Serum at acute phase at age 1 y	0.99	1.95	10.77	50.5	NT	NT	c.[365C > T];[=] p.[S122F];[=]	F/F	V/V	CK elevation during febrile episode at age 1 y	Muscle
S-14 (M, 2015)	0.19	NA	Serum at acute phase at age 9 mo	0.59	0.74	30.8	48.7	NT	NT	c.[365C > T];[=] p.[S122F];[=]	F/C	V/I	Rhabdomyolysis at age 9 mo	Muscle

NT not tested, *NA* data not available, *DBS* dried blood specimen

[a]The average value of CPT II activity in 22 normal control subjects (mean ± SD) was 126.3 ± 39.8 pmol/min/10^5 lymphocytes.

[b]The average values of fatty acid oxidation capability in 36 normal subjects (mean ± SD) were 3.39 ± 1.35 for $d_1C2/d_{31}C16$ and 0.273 ± 0.096 for $d_{27}C14/d_{31}C16$.

[c]Genotypes of the two thermolabile polymorphisms are as follows:

F/F p.F352 homozygote, *C/C* p.C352 homozygote, *F/C* compound heterozygote of p.F352 and p.C352, *V/V* p.V368 homozygote, *I/I* p.I368 homozygote, *V/I* compound heterozygote of p.V368 and p.I368

[d]Details of patient S-01 are available in ref. [42].

[e]Details of patient S-02 are available in ref. [45].

[f]Patient S-06 was Caucasian.

[g]Patients S-08 and S-09 were siblings.

Polymorphisms of thermal instability

Three kinds of single nucleotide substitutions in *CPT2* have been described as "thermolabile polymorphisms", as follows: c.1055T > G (p.F352C), c.1102G > A (p.V368I), and c.1939A > G (p.M647V). According to the ExAC database, the frequencies of the minor alleles are 0.4841 for p.V368I, 0.1620 for p.M647V, and 0.02184 for p.F352C. However, as previous reports indicated, p.F352C seems to be specific to the Japanese and perhaps other East Asian populations, whereas p.M647V seems to be specific to the Caucasian population and p.V368I is apparently shared by both groups [37, 46, 47]. Haplotype analysis of 50 healthy Japanese subjects revealed that the frequencies of p.C352, p.I368, and p.V647 were 0.21, 0.70, and 0.04, respectively [37]. In an in vitro expression experiment, the enzymatic activity of p.F352C-CPT II was found to be 58.9% that of the wild-type enzyme, but p.V368I-CPT II and p.M647V-CPT II showed a relative activity as high as 94.6% and 85.7%, respectively. Apart from their own enzymatic activity, these polymorphisms further impaired the activity of other CPT II variants when one (or more) of them lay on the same allele [48]. In addition, co-expression of p.F352C-V368I-V605L-CPT II and the wild-type enzyme resulted in lower activity than expected for the wild-type, suggesting a dominant-negative effect of these polymorphisms [48]. From the clinical perspective, the frequencies of p.F352C and p.V368I were reportedly higher in Japanese patients presenting with influenza-associated encephalopathy (IAE) than in healthy control subjects, suggesting that they were risk factors for IAE and a possible reason for its higher incidence in the Japanese than in the Caucasian population [47−50].

Among our symptomatic patients, four had the same variant, p.S122F, in one allele (S-11 to S-14 in Table 2). Patient S-11 presented with rhabdomyolysis at age 13 years and received the diagnosis of CPT II deficiency based on his genotype of p.[S122F];[P504L] and low CPT II activity (14.7%). In comparison, patients S-12 to 14 had p.S122F in one allele without any other allelic variants, and their CPT II activities were 45.2%, 50.5%, and 48.7%, respectively. Despite these results indicating that they were heterozygous carriers of CPT II deficiency, the patients presented clinical symptoms possibly related to the disease. Patient S-12 presented with acute encephalopathy at age 9 months, although neither hypoglycemia nor elevated serum CK was observed. Patient S-13 presented with elevated serum CK (up to 3,700IU/L) without hypoglycemia during a febrile episode at age 1 year. Patient S-14 presented with rhabdomyolysis (maximum serum CK value: 13,000IU/L) during an episode of acute gastroenteritis at age 9 months not associated with hypoglycemic symptoms, although the blood glucose level was not measured. With regard to the thermolabile polymorphisms, S-12 had p.F352C and p.V368I on the other allele; S-13 had neither; S-14 was heterozygous for both, but the haplotype was not analyzed. Taken together, the effect of p.F352C and p.V368I on the function of CPT II in these three patients was vague and difficult to evaluate. Further accumulation of data on the correlation between the clinico-biochemical phenotype and genotype, including the haplotype of the thermolabile polymorphisms, is required to clarify their significance.

Newborn screening

On the whole, FAODs are regarded as good targets for newborn screening (NBS) because affected children usually appear to be healthy, and the onset of catastrophic symptoms can be prevented by frequent feeding and, during sick days, by intravenous administration of glucose. In the 1990s, tandem mass spectrometry (MS/MS)-based acylcarnitine analysis was successfully applied to newborn dried blood specimens (DBSs), enabling NBS for FAODs. However, although CPT II deficiency reportedly has the second highest frequency among FAODS in the Caucasian population [51], it is listed as a primary target disease in NBS in a limited number of countries [52, 53], probably due to the possibility of overlooking the vast majority of patients harboring p.S113L [54]. In comparison, a nationwide Japanese survey of symptomatic FAOD cases diagnosed or reported between 1985 and 2000 revealed that CPT II deficiency occurred in 17 of 64 patients (26.6%), indicating that this disease occurred with the highest frequency. Four of the 17 patients exhibited the severe infantile form [55].

CPT II deficiency was included in a pilot study of MS/MS-based NBS, which was conducted from 2004 to 2012 in several areas of Japan using C16 and C18:1 as indices. For NBS, DBSs were generally collected on postnatal day 4 or 5. The new screening system diagnosed CPT II deficiency in seven of 1,740,387 newborns [44]. The frequency of 1/248,627 was lower than expected in comparison with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (1/108,333) and very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency (1/162,499). We incidentally encountered a case of an infant with CPT II deficiency who presented with acute hypoglycemic encephalopathy but had passed NBS [42].

Based on the acylcarnitine profile of the newborn DBS from this false-negative patient (S-01) and a previous report demonstrating that (C16 + C18:1)/C2 in the serum or plasma can be a sensitive index for high-risk screening of symptomatic patients [56], we adopted (C16 + C18:1)/C2 and C16 as alternative indices for NBS [44]. The data on the NBS indices and the results of the confirmatory tests are summarized in Table 3 (cited from ref. [44] with

Table 3 Clinical, biochemical, and genetic characteristics of the Japanese newborn screening (NBS)-positive subjects (cited from our previous report [44] with additional data)

Case, sex, birth year	NBS indices		Serum acylcarnitine (nmol/mL)		CPT II activity (%)[a]	Fatty acid oxidation capability (%)[b]		*CPT2* genotype			Clinical symptoms
	(C16 + C18:1)/C2	C14/C3	C16	C18:1		d_1C2/ d_{31}C16	d_{27}C14/ d_{31}C16	Variant	Thermolabile polymorphism[c]		
									p. F352C	p. V368I	
Patient N-01 (M, 2014)	3.44	5.65	3.18	2.54	NT	4.4	4.8	c.[451C > T;1102G > A];[1148T > A;1055T > G;1102G > A] p.[R151W;V368I];[F383Y;F352C;V368I]			Sudden death during pyrexia at age 1 y
Patient N-02 (F, 2014)[d]	3.27	8.25	1.47	2.46	12.1	NT	NT	c.[1148T > A];[1148T > A] p.[F383Y];[F383Y]	C/C	I/I	Recurrent elevation of serum CK without any myopathic symptoms since age 4 mo
Patient N-03 (F, 2007)[e]	3.26	4.26	3.02	3.21	NT	2.1	3.3	c.[520G > A];[1148 T> A] p.[E174K];[F383Y]	NA	NA	Recurrent rhabdomyolysis since age 4 y
Patient N-04 (M, 2013)	3.01	NA	3.06	4.24	7.8	NT	NT	c.[1121G > A;1102G > A];[1148T > A;1055T > G] p.[W374*;V368I];[F383Y;F352C]			Sudden death during acute gastroenteritis at age 2 y
Patient N-05 (F, 2008)	1.65	4.56	1.57	1.32	NT	7.7	4.4	c.[1148T > A];[1429C > T] p.[F383Y];[R477W]	NA	NA	Recurrent rhabdomyolysis since age 3 y
Patient N-06 (M, 2004)[e]	1.40	3.18	2.17	2.41	NT	2.7	3.3	c.[520G > A];[1148T > A] p.[E174K];[F383Y]	NA	NA	Hypoglycemia at age 1 y Recurrent rhabdomyolysis since age 4 y
Patient N-07 (F, 2012)	1.10	1.72	0.65	0.88	NT	8.3	4.4	c.[1511C > T];[1813G > C;1055T > G;1102G > A] p.[P504L];[V605L;F352C;V368I]			Rhabdomyolysis during RSV infection at age 3 y
Patient N-08 (F, 2017)	0.67	1.58	1.70	1.70	27.9	6.5	6.2	c.[520G > A];[1813G > C] p.[E174K];[V605L]	F/C	V/I	No symptoms
Patient N-09 (F, 2018)	1.14	2.20	0.84	0.61	4.1	NT	NT	c.[520G > A];[656G > A] p.[E174K];[R219Q]	F/C	V/I	No symptoms

Table 3 (continued)

Case, sex, birth year	NBS indices		Serum acylcarnitine (nmol/mL)		CPT II activity (%)[a]	Fatty acid oxidation capability (%)[b]		*CPT2* genotype			Clinical symptoms
	(C16 + C18:1)/C2	C14/C3	C16	C18:1		$d_1C2/d_{31}C16$	$d_{27}C14/d_{31}C16$	Variant	Thermolabile polymorphism[c]		
									p. F352C	p. V368I	
N-01−N-09 Mean ± SD	2.10 ± 1.12	3.93 ± 2.27	1.96 ± 0.96	2.15 ± 1.16							
Carrier N-10 (M, 2013)	0.51	0.40	0.23	0.25	31.8	26.0	19.8	c.[1525A > G]; [=] p.[T509A];[=]	F/C	I/I	No symptoms
Carrier N-11 (F, 2017)	0.84	0.17	0.17	0.06	70.8	NT	NT	c.[1634A > C]; [=] p.[E545A]; [=]	F/F	V/I	No symptoms
Suspected carrier ($n = 5$; M × 3, F × 2)	0.80 ± 0.42 (0.48−1.54) ($n = 5$)	0.23 ± 0.10 (0.11 −0.39) ($n = 5$)	0.15 ± 0.09 (0.07 −0.29) ($n = 5$)	0.15 ± 0.06 (0.08 −0.24) ($n = 5$)	51.3 −59.1 ($n = 3$)	46.0, 47.8 ($n = 2$)	52.0, 67.4 ($n = 2$)	*CPT2* ($n = 4$): no variant *CACT* ($n = 3$): no variant	C/C + I/I ($n = 1$) F/C + I/I ($n = 1$) F/F + I/I ($n = 1$) F/F + V/I ($n = 1$)		No symptoms
False-positive subjects ($n = 15$; M × 11, F × 4)	0.58 ± 0.14 (0.40−0.84) ($n = 15$)	0.54 ± 0.69 (0.11 −2.50) ($n = 15$)	0.10 ± 0.03 (0.06 −0.17) ($n = 13$)	0.08 ± 0.03 (0.03 −0.12) ($n = 13$)	51.5 −156.6 ($n = 10$)	67.0−174.9 ($n = 7$)	93.2−195.6 ($n = 7$)	*CPT2* ($n = 4$): no variant *CACT* ($n = 3$): no variant	F/C + I/I ($n = 3$) F/F + V/V ($n = 1$)		No symptoms

NT not tested, *ND* not detected, *NA* data not available

[a]The average value of CPT II activity in 22 normal control subjects (mean ± SD) was 126.3 ± 39.8 pmol/min/10^5 lymphocytes.

[b]The average values of fatty acid oxidation capability in 36 normal subjects (mean ± SD) were 3.39 ± 1.35 for $d_1C2/d_{31}C16$ and 0.273 ± 0.096 for $d_{27}C14/d_{31}C16$.

[c]Genotypes of the two thermolabile polymorphisms are as follows:

F/F p.F352 homozygote, *C/C* p.C352 homozygote, *F/C* compound heterozygote of p.F352 and p.C352, *V/V* p.V368 homozygote, *I/I* p.I368 homozygote, *V/I* compound heterozygote of p.V368 and p.I368

[d]Details of patient N-02 are available in ref. [43].

[e]Patients N-03 and N-06 were siblings.

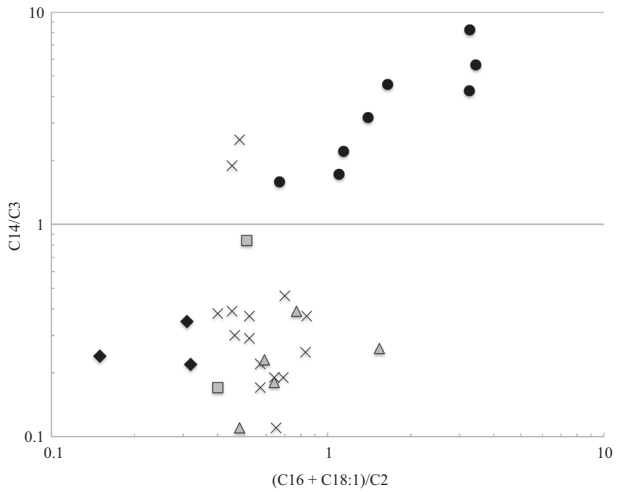

Fig. 2 Distribution of (C16 + C18:1)/C2 and C14/C3 in newborn dried blood specimens. *Black circle* Patients (N-01 to 09 except N-04), *Grey square* Heterozygous carriers (N-10 and N-11), *Grey triangle* Suspected carriers, *Crossmark* False-positive subjects, *Black diamond* False-negative patients (S-07, S-09, and S-10)

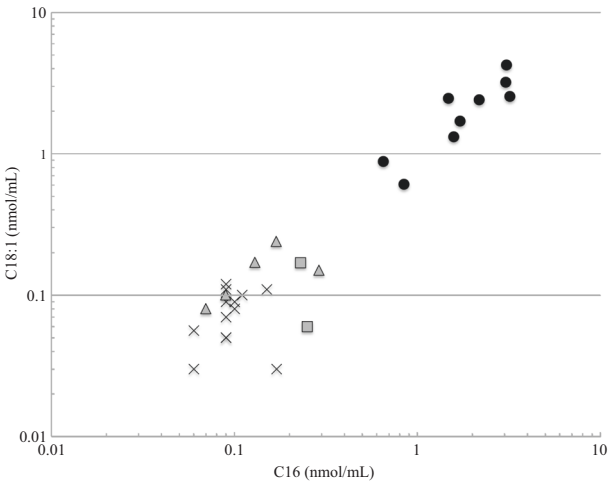

Fig. 3 Distribution of C16 and C18:1 in serum of NBS-positive subjects. *Black circle* Patients (N-01 to 09), *Grey square* Heterozygous carriers (N-10, N-11), *Grey triangle* Suspected carriers, *Crossmark* False-positive subjects

modifications and additional data). Based on CPT II activity in the lysates of lymphocytes and/or the FAO capability of intact lymphocytes, the disease was diagnosed in 9 of 31 NBS-positive subjects (N-01 to N-09). Homozygous or compound heterozygous variants of the *CPT2* gene were detected in all nine patients. As with symptomatic Japanese patients, the variant occurring with the highest frequency among the NBS-positive patients was p.F383Y, which was detected in seven alleles in six patients from five families. Seven newborns showing mild impairment of CPT II activity and/or FAO capability were presumed to be heterozygous carriers, which was later genetically shown to be the case for two of the patients (N-10 and N-11). The other fifteen newborns showed normal levels of CPT II activity and/or FAO capability.

According to our data on NBS conducted in Japan, the (C16 + C18:1)/C2 values in affected newborns partly overlapped with those in unaffected ones. In order to reduce the false-positive rate, we sought new candidates for the NBS index among various long-chain acylcarnitines, such as C16, C16-OH, C18, C18:1, C18-OH, and their ratios to C2 or C3, and found C14/C3 to be the most promising [44]. Based on these data, CPT II deficiency was finally included among the primary target diseases in July 2017, and the nationwide NBS for CPT II deficiency using (C16 + C18:1)/C2 and C14/C3 as indices (both cutoffs 99.9 percentile) began in April 2018. Figure 2 showing a dual axis graph of (C16 + C18:1)/C2 and C14/C3 in newborn DBS, indicates that C14/C3 is superior to (C16 + C18:1)/C2 when discriminating between affected and unaffected newborns. Among recent cases, however, there were two false-positive newborns with a high C14/C3 ratio overlapping the values of some of the affected ones. The false-positive results were evidently caused by very low levels of C3 rather than by elevated C14. Although a certain level of false-positivity appears to be unavoidable using the current NBS system, the serum concentration of C16 and C18:1 can enable a much clearer differentiation between affected and unaffected newborns (Fig. 3).

Problems to be solved

We recently diagnosed CPT II deficiency in four young children who presented with rhabdomyolytic symptoms without hypoglycemia and whose values for (C16 + C18:1)/C2 and C14/C3 in newborn DBS were below the cutoff (S-07 to S-10). The distribution of the NBS indices in these patients indicated that detecting the muscle form of CPT II deficiency by NBS is not infallible (Fig. 2). Nevertheless, our system is expected to save many affected children in Japan, where the proportion of the severe infantile form of the disease is apparently higher than that in Western countries. Sampling of dried blood specimens earlier than postnatal day 4 or 5, which is not yet practiced in Japan, may improve the detection of the muscle form of CPT II deficiency.

Among the nine patients identified via NBS in our study, two died suddenly during an acute infectious episode (N-01 and N-04), which we believe could have been prevented by stricter management. Five other patients showed myopathic findings despite early therapy (N-02, N-03, and N-05 to N-07), and one had hypoglycemia (N-06). In order to offer optimal medical management to patients with diseases

diagnosed via NBS, it is essential to predict their phenotype accurately using objective indices. For this purpose, the correlation between the levels of marker metabolites in the blood, enzymatic function, genotype, and clinical phenotype must be clarified.

However, the genotypes encountered in the present case series were complicated by the presence of p.F352C and p.V368I, whose effects on enzymatic function are difficult to gauge. To understand how substantial their effects are in vivo, it is necessary to analyze genotypes of the parents to determine the haplotypes of these polymorphisms and other variants in as many cases as possible. Another problem is that accurate evaluation of enzymatic function is reportedly difficult for CPT II. Although a variety of methods have been proposed for measuring CPT II activity using cell lysates, the correlation between the values derived from these methods and disease severity has been questioned [4, 12, 30, 46, 57, 58]. However, the long-chain fatty acid oxidation capability of intact cells was found to correlate well with disease severity [30, 32, 59]. Our data appear to agree with those of previous reports. The distribution of CPT II activity in the lymphocytes of patients presenting with the infantile form of the disease ranged from 6.6% to 13.6% of the average value of normal controls (S-01 and S-02 in Table 2 and N-04 in Table 3), whereas that of the patients presenting with the muscle form of the disease ranged from 2.8% to 18.4% (S-04 to S-11 in Table 2 and N-02 in Table 3). In addition, the data from patient S-10 in Table 2 provides useful information. This female patient had rhabdomyolysis at age 4 years and was identified as a compound heterozygote for p.S113L and p.E645*. Her CPT II activity (8.1%) seemed to be too low for the muscle form of the disease and the p.S113L variant enzyme. On the other hand, her FAO capability was 41.9% for $d_1C2/d_{31}C16$ and 33.3% for $d_{27}C14/d_{31}/C16$, thus possibly better reflecting her phenotype and genotype. As the number of cases of CPT II deficiency confirmed on the basis of both CPT II activity and FAO capability is small, we must continue to accumulate more data.

In view of the fact that NBS for CPT II deficiency has yet to be adopted internationally, we hope that our initiative will contribute greatly to increasing interest in the methods suggested for detecting this potentially fatal disease.

Acknowledgements The authors thank Dr. Miyuki Tsumura, Dr. Reiko Kagawa, and Dr. Satoshi Okada (Department of Pediatrics, Hiroshima University Graduate School of Biomedical & Health Sciences) for helping with the genetic analysis, Dr. Nobuo Sakura (Nursing House for Severe Motor and Intellectual Severities, Suzugamine) for assistance with the enzymatic assay, Dr. Ikue Hata and Dr. Yosuke Shigematsu (Department of Pediatrics, School of Medical Sciences, University of Fukui) for assistance with the acylcarnitine analysis and the measurement of fatty acid oxidation capability, and Dr. Seiji Yamaguchi (Department of Pediatrics, Faculty of Medicine, Shimane University) for managing the pilot study on tandem mass spectrometry-based newborn screening in Japan. The authors also thank Ms. Chiyoko Yoshii, Ms. Chiyomi Morioka, and Ms. Saki Fujihara (Hiroshima City Medical Association Clinical Laboratory) for analyzing the dried blood specimens of newborns in Hiroshima prefecture. This study was supported in part by the Health and Labour Sciences Research Grants for (1) Health Research on Children, Youth and Families (Chief Investigator: Go Tajima) and (2) the Research on Rare and Intractable Diseases (Chief Investigator: Kimitoshi Nakamura), by the Practical Research Project for Rare/Intractable Diseases from the Japan Agency for Medical Research and Development, AMED, Grant Number JP18ek0109276 (Chief Investigator: Toshiyuki Fukao), by a Grant-in-Aid for Young Scientists, No. 18K15663, from the Japan Society for the Promotion of Science (Chief Investigator: Miori Yuasa), and by a grant from The Morinaga Hoshi-Kai Foundation. This study was carried out in part at the Analysis Center of Life Science, Hiroshima University, the Institute for Clinical Research at the National Hospital Organization Kure Medical Center and Chugoku Cancer Center.

Funding Go Tajima received Health and Labour Sciences Research Grants (Health Research on Children, Youth and Families; Chief Investigator: Go Tajima, and Research on Rare and Intractable Diseases; Chief Investigator: Kimitoshi Nakamura) from the Ministry of Health, Labour and Welfare of Japan, and a Practical Research Project for Rare/Intractable Diseases Grant from the Japan Agency for Medical Research and Development, AMED (Grant number JP18ek0109276; Chief Investigator: Toshiyuki Fukao), and a grant from The Morinaga Hoshi-Kai Foundation. Miori Yuasa received a Grant-in-Aid for Young Scientists, No. 18K15663 from the Japan Society for the Promotion of Science. Keiichi Hara did not receive any funding for this study.

Author contributions Go Tajima developed the assay of CPT II activity, carried out the enzymatic assays for all cases in this study, managed the entire project, and wrote this paper. Keiichi Hara performed the analysis of the *CPT2* and *CACT* genes. Miori Yuasa analyzed the acylcarnitine profiles and measured fatty acid oxidation capability using tandem mass spectrometry.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Approval for the biochemical, enzymatic, and genetic studies was obtained from the ethics committees of the National Center for Child Health and Development, Hiroshima University, University of Fukui, National Hospital Organization Kure Medical Center and Chugoku Cancer Center. All procedures were carried out in accordance with the ethical standards of the relevant committees 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.

The guarantor for the article Go Tajima.

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