Title: *Carnitine palmitoyltransferase 2* gene polymorphism is a genetic risk factor for sudden unexpected death in infancy

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Abstract

Rationale: Carnitine palmitoyltransferase (CPT) II is one of a pivotal enzyme in mitochondrial fatty acid oxidation, which is essential for energy production during simultaneous glucose sparing and a requirement for major energy supply, such as prolonged fasting or exercise. When infants require more energy than provided by the glycolytic system, they rely on the mitochondrial fatty acid oxidation pathway. Mutations of the *CPT2* gene have been reported to cause sudden unexpected death in infancy (SUDI). A thermolabile phenotype of a *CPT2* polymorphism (F352C) has been recently reported to reduce CPT II enzyme activity. The F352C variant results in energy crisis at high temperature and is suspected as a risk factor for acute encephalopathy. However, a relationship between *CPT2* gene polymorphism and SUDI has not been described. *Methods:* Single nucleotide polymorphisms of the *CPT2* gene were investigated among 54 SUDI cases and 200 healthy volunteers. *Results:* The frequency of the C allele was significantly higher in the SUDI group than in the control group [25.0% *vs* 16.0%, odds ratio (OR) = 1.75, 95% confidence interval (CI) = 1.05–2.92, *p* = 0.030). The frequency of the F352C homozygote was significantly higher in the SUDI group than in control group (11.1% *vs* 3.5%, OR =

3.45, 95% CI = 1.11–10.73, *p* = 0.036). *Conclusion:* The F352C *CPT2* variant might be a genetic risk factor for SUDI.

Keywords: Carnitine palmitoyltransferase II; Sudden unexpected death in infancy; Polymorphism; Metabolic crisis

1. Introduction

Mitochondrial fatty acid oxidation is essential for energy production during conditions requiring simultaneous glucose sparing and a major energy supply, such as prolonged fasting or exercise. An essential step in fatty acid oxidation is played by the carnitine palmitoyltransferase (CPT) enzyme system, which transfers long-chain fatty acids containing ≥16 carbons (C16, C18:1, C18:2, and C18) from the cytosolic compartment to the mitochondrial matrix, where β-oxidation takes place. The CPT enzyme system consists of several mitochondrial membrane-bound enzymes: CPT I, CPT II (EC 2.3.1.21), and carnitine-acylcarnitine translocase. CPT II is located on the inner aspect of the inner mitochondrial membrane and converts long-chain acylcarnitines to long-chain acyl-CoAs [1–3].

Carnitine palmitoyltransferase II deficiency is one of the most common inherited metabolic disorders and is categorized into three forms: neonatal (OMIM 608836) [5], infantile (OMIM 600649) [5], and adult (OMIM 255110) [6]. The infantile form usually manifests at 6–24 months of age as recurrent attacks of hypoketotic hypoglycemia, resulting in coma and seizures, liver failure, and transient hepatomegaly. About half of cases have heart involvement with cardiomyopathy and arrhythmia, and some result in sudden unexpected death in infancy (SUDI). The neonatal form is more severe

than the infantile form. Many sudden death cases are reported, most often during the first month of life [1–3,7,8].

Various mutations of the *CPT2* gene have been reported and some of the more severe mutations are fatal [1–3]. *Carnitine palmitoyltransferase 2* gene polymorphisms have not been reported to affect enzyme activity, although a thermolabile phenotype of the *CPT2* polymorphism (F352C) has been recently reported to reduce enzyme activity at high temperature [9–11]. Shinohara et al. have reported that the F352C variant causes energy crisis at high temperature and is associated with acute encephalopathy [12]. The F352C variant is therefore a genetic risk factor for acute encephalopathy.

Various environmental and genetic risk factors have been associated with SUDI. Serotonin transporter, cardiac ion channel, autonomic nervous system, and complement or interleukin polymorphisms have been reported as genetic risk factors, while smoking, prone or side sleeping, soft bedding, and prematurity are recognized as environmental risk factors [13,14]. However, to our knowledge, a relationship between *CPT2* gene polymorphism and SUDI has not been described.

Under heat stress, fasting, acidosis, or seizures, moderately reduced CPT II activity due to the F352C variant may accelerate the disease process of acute encephalopathy [11]. Therefore, infants who have the F352C variant may be more

vulnerable to energy crisis than those without the polymorphism, leading to a hypothetical risk of SUDI.

In the present study, we conducted a single nucleotide polymorphism (SNP) analysis of the *CPT2* gene among the SUDI group in order to determine whether the F352C variant might be a genetic risk factor for SUDI or not.

2. Materials and methods

This study was approved by the Ethics Committee of the Nagasaki University Graduate School of Medicine and Osaka University Graduate School of Medicine.

2.1. Study population

We retrospectively reviewed 54 SUDI cases at ages ranging from 1 day to 10 months (30 male; 24 female), whose causes of death were unexplained after thorough autopsies. We excluded apparent congenital abnormality and traumatic death cases.

2.2 Extraction of genomic DNA and polymorphism analysis

Genomic DNA was purified from frozen blood samples with the PureLink™ Genomic DNA kit (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. Exon 4 of the *CPT2* gene was then amplified, followed by polymerase chain reaction (PCR) reactions performed in a 25-μL volume containing 12.5 μL of PrimeSTAR Max Premix (2×) (Takara, Otsu, Japan), 0.4 μM each of the primers (Forward; 5'-CAGTGTTCTGTCTCTGCCTA-3', Reverse; 5'-GCCTCCTCTCTGAAACTGGA-3'), and 200 ng of template DNA under the following conditions: 98.0˚C for 1 min, 30 cycles of 98.0˚C for 10 sec, 54.0˚C for 5 sec, and 72.0˚C for 30 sec, and finally 72.0˚C for 5 min. PCR products were sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems) according to the manufacturer's instructions. Sequences from the 5' ends were confirmed by comparison with those from the 3' ends at least twice independently and each sequence was compared with the standard sequence (GenBank accession number: *CPT2*: NM_000098.2).

2.3 Haplotype analysis of the CPT2 *gene*

When two heterozygous genotypes (c.1055T>G [p.F352C] and c.1102G>A [p.V368I]) in *CPT2* exon 4 were recognized, cloning was performed. The PCR products were amplified with a pair of primers. Next, we performed reconditioning PCR in order to avoid heteroduplexes from mixed-template PCR products [15]. After the PCR products were obtained, they were diluted 10-fold into a fresh reaction mixture of the same composition and cycled three times. The PCR products were purified from agarose gel and cloned into the pCR®-Blunt II-TOPO® vector (Invitrogen), and the resulting constructs were transformed into Competent high DH5α cells (Toyobo, Osaka, Japan). Colonies were selected and analyzed on kanamycin agar and the plasmid inserts were sequenced using the M13 Forward primer and the M13 Reverse primer (Invitrogen).

*2.4 Genotyping of healthy individual*s

Two hundred healthy Japanese volunteers (Non-SUDI control group, whose ages were over 20 years old) were subjected to genotyping by direct sequencing in the same manner as described above.

2.5 Statistical analysis

Allele frequencies were derived from gene counts, and differences between the SUDI and the control groups were evaluated by χ^2-tests or the Fisher's exact test. Significant differences were defined as $p < 0.05$ in conditional analysis. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to evaluate the effects of the different genotypes.

3. Results

Two *CPT2* polymorphisms (c.1055T>G [p.F352C] and c.1102G>A [p.V368I]) of the *CPT2* gene were found in the SUDI and the control groups. Chen et al. have previously detected three polymorphisms (F352C, V368I, and M647V), which were classified into nine genotypes [9]. M647V is irrelevant to enzyme activity and we did not investigate exon 5 where it is located. Six genotypes were therefore classified in the present study and their distribution among the SUDI and the control groups is shown in Table 1. F352C is the Asian-specific variant and has not been reported in Caucasians and several genetic distribution has been reported so far [12,16]. The

genotypic distribution among controls in the present study was not significantly different (p = 0.704) from that for one of the previously reported data [12] and is consistent with the general genotypic distribution among the Japanese population. The most frequent genotype was FV–FI (type C) in both the SUDI and the control groups. Genotypic distribution was not significantly different (p = 0.266) between the SUDI and the control groups (Table 1).

The allelic frequencies of F352C and V368I are shown in Table 2. The frequency of the C allele was higher in the SUDI group compared to the control group (25.0% vs 16.0%). The frequency of F352C was significantly higher in the SUDI group compared to the control group (OR = 1.75, 95% CI = 1.05–2.92, p = 0.030), while the frequency of V368I was not significantly different between the SUDI and the control groups (OR = 0.97, 95% CI = 0.61–1.55, p = 0.898).

The numbers of CI alleles in the SUDI and the control groups is shown in Table 3. The frequency of having a CI allele was not significantly different between the SUDI and the control groups (p = 0.060).

The frequency of wild/heterozygocity or homozygocity for F352C is shown in Table 4. The frequency of the F352C homozygote was significantly higher in the SUDI group compared to the control group (OR = 3.45, 95% CI = 1.11–10.73, p = 0.036).

4. Discussion

It is well known that some mutations of the *CPT2* gene decrease CPT II activity and cause CPT II deficiency. While *CPT2* gene polymorphisms do not decrease CPT II activity in normal physiological situations, the F352C variant alone has been reported to reduce CPT II activity by about 50% under certain conditions such as heat stress, fasting, acidosis, and seizures [9–11,17]. Shinohara et al. has reported that the F352C variant is a statistically significant risk factor in patients with acute encephalopathy [12].

Sudden unexpected death in infancy is defined as sudden unexpected death occurring before 12 months of age. The common causes of SUDI are infection, cardiovascular anomaly, child abuse, and metabolic disorders. However, any background genetic risk factors for SUDI currently remain unknown.

In the present study, we found that the F352C allele was significantly higher among the SUDI group compared to the control group. Haplotype analysis revealed no difference, but the frequency of the F352C homozygote was significantly higher in the SUDI group than in the control group.

The *in vitro* expression of the F352C variant in COS-7 cells is <30% of the wild type at high temperature [9]. A congenital or acquired abnormality of mitochondrial fatty

acid oxidation causes the accumulation of mini-plasmin in the cerebral capillaries and the proteolytical destruction of the blood–brain barrier in mice after influenza virus infection [9,18]. Intracellular ATP production is reduced by about 50% for the F352C variant compared to the wild type under severe conditions [9–11,17]. The suppression of intracellular ATP production weakens interactions between tight junction proteins of cerebral microvascular endothelial cells, leading to the destruction of brain homeostasis and brain edema [10]. Thus, under severe conditions such as high fever and respiratory or metabolic acidosis, decreased CPT II activity with the F352C variant might lead to energy crisis, acute brain edema, and sudden death.

We have previously performed metabolic autopsy among SUDI cases and found some inherited metabolic disorders [19]. However, in some SUDI cases, even after thorough investigations, we could sometimes find only negligible pathological abnormalities. In these cases, if they had a genetic risk factor, even a negligible abnormality might trigger an energy crisis and might lead to death. In fact, we had experienced one girl patient with the F352C variant, whose long-chain acylcarnitines had been distinctly increased [19]. We cannot confirm the reason of the increase, but the F352C variant possibly might have affected it.

In conclusion, we found that the F352C variant was statistically higher among the SUDI group compared to the control group in our case-control study. The F352C

variant has been associated with acute encephalopathy after viral infection [12]. In the present study, we suggest that the F352C variant is also related to SUDI. The F352C *CPT2* variant would therefore appear to be a genetic risk factor for SUDI. Further studies are needed to confirm our results.

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Table 1

Genotypic distribution of *CPT 2* gene polymorphisms.

Type	Genotypes		Alleles	No. (%)	
	F352C	V368I		SUDI (n = 54)	Control (n = 200)
A	FF	VV	FV–FV	4 (7.4)	15 (7.5)
B	FF	II	FI–FI	11 (20.4)	60 (30.0)
C	FF	VI	FV–FI	18 (33.3)	68 (34.0)
D	CC	II	CI–CI	6 (11.1)	7 (3.5)
E	FC	II	FI–CI	9 (16.7)	32 (16.0)
F	FC	VI	FV–CI	6 (11.1)	18 (9.0)

Table 2

F352C and V368I allelic frequency.

Polymorphism	Allele	No. (%)		OR (95% CI)	p value
		SUDI (n = 54)	Control (n = 200)		
F352C	F	81 (75.0)	336 (84.0)	1.75 (1.05-2.92)	0.030
	C	27 (25.0)	64 (16.0)		
V368I	V	32 (29.6)	116 (29.0)	0.97 (0.61-1.55)	0.898
	I	76 (70.4)	284 (71.0)		

Abbreviations: CI, confidence interval; OR, odds ratio.

Table 3

CI haplotypes among the SUDI and the control groups.

CI	No. (%)	
	SUDI (n = 54)	Control (n = 200)
2	6 (11.1)	7 (3.5)
1	15 (27.8)	50 (25.0)
0	33 (61.1)	143 (71.5)

Table 4

F352 wild/Hetero- and homozygocity among the SUDI and the control groups.

F352C	No. (%)		OR (95% CI)	p value
zygocity	SUDI (n = 54)	Control (n = 200)		
Wild/Hetero	48 (88.9)	193 (96.5)	3.45 (1.11-10.73)	0.036
Homo	6 (11.1)	7 (3.5)		

Abbreviations: CI, confidence interval; OR, odds ratio.