ORIGINAL ARTICLE

Three novel mutations in the carnitine–acylcarnitine translocase (*CACT*) gene in patients with *CACT* deficiency and in healthy individuals

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Carnitine–acylcarnitine translocase (*CACT*) and carnitine palmitoyltransferase II (*CPT2*) are key enzymes for transporting long-chain fatty acids into mitochondria. Deficiencies of these enzymes, which are clinically characterized by life-threatening non-ketotic hypoglycemia and rhabdomyolysis, cannot be distinguished by acylcarnitine analysis performed using tandem mass spectrometry. We had previously reported the *CPT2* genetic structure and its role in *CPT2* deficiency. Here, we analyzed the *CACT* gene in 2 patients diagnosed clinically with *CACT* deficiency, 18 patients with non-traumatic rhabdomyolysis and 58 healthy individuals, all of whom were confirmed to have normal *CPT2* genotypes. To facilitate *CACT* genotyping, we used heat-denaturing high-performance liquid chromatography (DHPLC), which helped identify five distinct patterns. The abnormal heteroduplex fragments were subjected to *CACT*-specific DNA sequencing. We found that one patient with *CACT* deficiency, Case 1, carried c.576G>A and c.199-10t>g mutations, whereas Case 2 was heterozygous for c.106-2a>t and c.576G>A. We also found that one patient with non-traumatic rhabdomyolysis and one healthy individual were heterozygous for c.804delG and the synonymous mutation c.516T>C, respectively. In summary, c.576G>A, c.106-2a>t and c.516T>C are novel *CACT* gene mutations. Among the five mutations identified, three were responsible for *CACT* deficiency. We have also demonstrated the successful screening of *CACT* mutations by DHPLC.

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INTRODUCTION

Carnitine–acylcarnitine translocase (*CACT*), as well as carnitine palmitoyltransferase II (*CPT2*), has pivotal roles in the carnitine cycle, which is the β -oxidation pathway of long-chain fatty acids.¹ The *CACT* gene is known to be associated with the *SLC25* gene family, *SLC25A20*. In 1997, the human *CACT* cDNA was cloned and sequenced by Huizing *et al.*² The entire gene, spanning about 42 kb on chromosome 3p21.31, contains 9 exons and codes a 301-amino-acid protein.^{3,4} Cases of *CACT* deficiency are rare, and only 40 cases at most have been reported worldwide.^{5–7} Although defects in *CACT* induce lethal neonatal episodes of coma due to hypoketotic hypoglycemia, cardiomyopathy, cardiac arrhythmia and rhabdomyolysis, quite a few patients exhibit milder phenotypes compatible with longer life spans.^{8,9}

CACT deficiency (OMIM No. 212138) cannot be easily differentiated from severe cases with *CPT2* deficiency (OMIM No. 255110) on the basis of clinical manifestations and blood acylcarnitine profiles analyzed using tandem mass spectrometry;^{10–13} however, genetic

analysis could be utilized to distinguish between the two conditions. *CACT* deficiency is so rare that the characteristics of the *CACT* gene are not yet fully understood. In this study, we analyzed the *CACT* gene in 2 patients diagnosed clinically with *CACT* deficiency, 18 Japanese patients with non-traumatic rhabdomyolysis and 58 healthy Japanese individuals. As little information is available regarding *CACT* polymorphisms and mutations, we introduced heat-denaturing high-performance liquid chromatography (DHPLC) to screen for the *CACT* gene.¹⁴ To confirm that DHPLC would be suitable for screening these gene mutations and variants, the following strategy was carried out. First, we sequenced all fragments showing heteroduplex patterns; second, we sequenced all the exons of the *CACT* gene in randomly selected individuals; third, we included data for two patients who had died of *CACT* deficiency; and fourth, the data were confirmed by family studies.

In this study, we identified mutations responsible for *CACT* deficiency and screened *CACT* mutations with DHPLC. To the best

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of our knowledge, the two infant cases are only the second and third cases wherein the genotypes of Japanese patients with *CACT* deficiency have been fully confirmed.

SUBJECTS AND METHODS

Patients diagnosed clinically with *CACT* deficiency

Case 1. The patient was the second child of non-consanguineous Japanese parents. His sister was in good health. His mother had pregnancy-induced hypertension. He was born at 36 weeks and 5 days of gestation by cesarean section. He had decreased respiratory functions and experienced asphyxia. At 2 days of age, repeated apnea, muscular hypotonia, irritability, oliguria, hypoglycemia, liver dysfunction and cryptogenic rhabdomyolysis were noted. Although *CPT2* or *CACT* deficiency was suspected on the basis of acylcarnitine profiles of plantar blood spots (Table 1), the subsequent DNA analysis performed at our laboratory showed that his *CPT2* gene was normal (data not shown).¹⁵ The patient was clinically diagnosed with *CACT* deficiency. At the age of 2 years and 9 months, he died of symptoms resembling those of Reye syndrome. (Case 1 is going to be reported in Japanese from a clinical point of view in an upcoming issue of the Journal of the Japan Pediatric Society by Sugiyama, *et al*.)

Case 2. The patient was the second child of non-consanguineous Japanese parents. His elder sister was healthy. The pregnancy was uneventful, and at 37 weeks of gestation, he was born by normal vaginal delivery. At 2 days of age, he suddenly became cyanotic and flaccid and then went into cardiac arrest. He died at 3 days of age. Tandem mass spectrometry analysis of acylcarnitines in a postmortem blood spot card showed increases in C14, C16 and C18 acylcarnitines (Table 1), which led us to consider a defect in either *CACT* or *CPT2*. After the patient died, the physician in charge brought the blood disc of the patient and whole blood from his parents to our laboratory for genetic analysis in order to reach a final diagnosis. Direct DNA sequencing performed on genomic DNA from the parents confirmed the absence of mutations in the *CPT2* gene (data not shown).¹⁵ We highly suspected that the patient had died of *CACT* deficiency.

Patients with non-traumatic rhabdomyolysis

Eighteen patients with non-traumatic rhabdomyolysis (12 men and 6 women; mean age $\pm$ s.d., 51.1 ± 19.6 years) were retrospectively recruited from the outpatient clinic of Fukuoka University Hospital and Clinic, Fukuoka, Japan. The mean $\pm$ s.d. value of their peak serum creatine kinase levels was $22\,522.7 \pm 7\,224.4$ IU l⁻¹. The patients had no history of statin therapy. DNA sequencing carried out at our laboratory indicated that all patients had normal *CPT2* genotypes (data not shown).¹⁵

Healthy volunteers

The 58 individuals in our healthy panel included 47 men and 11 women (mean $\pm$ s.d. age, 28.3 ± 7.1 years). They had no recent history of either heavy sport activities or specific drug usage. The *CPT2* genotypes of these individuals were determined by DNA sequencing and were found to be normal without exception (data not shown).¹⁵

Table 1 Acylcarnitine levels in dried blood spots from Cases 1 and 2

Acylcarnitine	Case 1 (μ M)	Case 2 (μ M)	Upper 95% limit (μ M)
CO	9.37	11.85	60.0
C14	0.66	1.35	0.4
C14:1	0.17	0.56	0.1
C14-OH	0.064	0.24	0.050
C16	4.77	12.07	1.9
C16:1	0.78	1.84	0.3
C18:1	ND	3.07	2.1

Abbreviation: ND, not determined.

Amplification of genomic DNA

Genomic DNA purified from peripheral blood cells was PCR amplified using nine pairs of *CACT*-specific primers (Table 2), as previously described.^{4,16} PCR conditions were as follows: 95 °C for 1 min; 30 cycles each of 95 °C for 1 min, specific annealing temperature for 1 min, and 72 °C for 1 min, followed by a final extension step at 60 °C for 10 min.

DNA sequence analysis

CACT-specific direct DNA sequencing was performed on all PCR products using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and the results were analyzed on the automated ABI Prism 310 Genetic Analyzer (Applied Biosystems), as described by the manufacturer. Cumulative sequences were compared with the *CACT* sequence published in the OMIM database (<http://www.ncbi.nlm.nih.gov/nucore/> 319918843).

Homoduplex or heteroduplex formation

CACT-specific PCR products were purified using Microcon (Millipore Corp., Billerica, MA, USA). PCR product of each test was mixed with the comparable PCR product from a normal donor. The mixtures were denatured at 95 °C for 10 min and then slowly cooled down to 65 °C at a rate of 1 °C min⁻¹. Following 5-min incubation at 65 °C, the samples were cooled to 4 °C at a rate of 1 °C every 5 s.

DHPLC analysis

PCR-amplified products were analyzed using the Nucleic Acid Fragment Analysis System (Transgenomic, Inc., San Jose, CA, USA). The results have been presented in the form of chromatographic peaks by using the Navigator software. Complete sequence-matched hybridization produced a single homoduplex peak, whereas hybridization with sequences containing mutation(s) resulted in heteroduplexes and aberrant peaks with different retention times. PCR products that showed heteroduplex patterns by DHPLC were subjected to *CACT*-specific direct sequencing using PCR primers as described above.

Ethical consideration

Written informed consent for genetic investigations was obtained from all patients and family members, as well as from volunteers. The Fukuoka University Ethics Committee investigated and approved this research project (12-12 [08-77]).

Table 2 Primers for amplifying and sequencing the *CACT* gene

product	Exon	Primer	Sequence (5'–3')	Size (bp)	T _m (°C)
CACT 1	1	1F	GGTCGAGAAGTGCAGACGGAG	247	70
		1R	ACACATGCCCTCTCTGCCAG		
CACT 2	2	2F	CAGGCAGTTCTGATTCTGGT	194	60
		2R	AACCCCGTGAATGTGTTCTG		
CACT 3	3	3F	AAAGGTGGTGGTGTCTGTAAC	228	64
		3R	GTCACGCTACCAAGCACAAAC		
CACT 4	4	4F	CTCGGTGGTTAGTCACAGG	251	66
		4R	GCCACTGCACCCAGTCTCTGA		
CACT 5	5	5F	CCTGCTGGGTCTGTGACTCTGA	224	70
		5R	CCACTTCAGGTGACCTCCCCA		
CACT 6	6	6F	TGGCGAAGAGTTTATGTACTTT	233	60
		6R	CAGACATGGAGCCAAGAACA		
CACT 7	7	7F	GCCACTCTCAGAGCTTTGTCTA	237	70
		7R	TATGAGCTTTGCACCCAGGATTA		
CACT 8	8	8F	GACTTAACGCTAGTTTCTCCT	255	62
		8R	GGAACAAGCAAAAGTCAAACCA		
CACT 9	9	9F	AATAGCCTATGAATAGTTATTCC	187	66
		9R	TTACTACTCTCTCTCAACGA		

Abbreviation: *CACT*, carnitine-acylcarnitine translocase.

RESULTS

DHPLC analysis and subsequent DNA sequencing of the *CACT* gene

We used DHPLC to facilitate the screening of unknown mutations in the *CACT* gene. PCR-amplified fragments of all nine *CACT* exons from a randomly chosen healthy volunteer were directly sequenced and were found to have no mutations. On using autologous DHPLC analysis, no heteroduplex pattern was found in any exon (Figure 1); therefore, this DNA fragment was used as the reference for further DHPLC studies.

We performed DHPLC analysis for nine exons of the *CACT* gene amplified from Case 1, Case 2, and their parents. As expected, we found heteroduplex patterns at exons 3 and 6 (namely, *CACT* 3 and *CACT* 6, respectively) in Case 1, as well as exon 2 (*CACT* 2) and *CACT* 6 in Case 2 (Figure 2). The segregations of *CACT* heteroduplexes were subsequently confirmed by family studies.

Our results from direct DNA sequencing of the *CACT* gene showed that Case 1 had the c.576G>A and c.199-10t>g mutations. The former mutation, which has never been described elsewhere, introduces a premature stop codon at the amino-acid residue 192, tryptophan, whereas the latter is a previously reported splicing acceptor mutation at the intron 1/exon 2 junction and results in the skipping of both exons 3 and 4 or only exon 3.¹⁷ The genetic segregation was subsequently confirmed by family studies using genomic DNA from his parents (data not shown). Based on these genetic analyses, we confirmed that Case 1 had *CACT* deficiency.

For Case 2, at first only genomic DNA samples from the parents were sequenced, because the only samples that were available for Case 2 were a few residual blood spots obtained for neonatal screening. We found that the father had c.106-2a>t at the splice acceptor site of intron 1, which was another novel mutation

identified in this study, whereas the mother had the c.576G>A mutation in exon 6, the same novel mutation shared by Case 1. As Case 2 was found to have a normal *CPT2* gene, we highly suspected that he had *CACT* deficiency. Next, exons 2 and 6 of Case 2 were sequenced to determine the presence of these mutations. The DHPLC and partial sequencing results described above, together with full-length exon sequencing and DHPLC of the *CACT* gene of his parents, confirmed the presence of the c.106-2a>t mutation at the junction of intron 1 and exon 2, as well as c.576G>A in exon 6. Based on these genetic analyses, Case 2 was finally diagnosed with *CACT* deficiency. To the best of our knowledge, the c.576G>A and c.106-2a>t genotypes have never been reported in the literature. It is also worth noting that c.576G>A was seen in both Cases 1 and 2.

In parallel, we investigated *CACT* mutations in 18 patients with non-traumatic rhabdomyolysis and 58 healthy volunteers by using DHPLC. Among the 18 patients, only 1 heteroduplex was found in exon 8 (*CACT* 8; Figure 2). The mutation, identified as c.804delG by DNA sequencing, resulted in a frameshift at codon 269. This patient, Case 3, was a 74-year-old Japanese man with hypertension and previously undetected rhabdomyolysis (peak serum creatine kinase level, 4832 IU ml⁻¹) with renal injury (serum creatinine level, 2.2 mg dl⁻¹). The *CACT* enzymatic activity in the fibroblasts was determined on a later day and found to be within the normal range (101 pmol · min⁻¹ · mg⁻¹ vs the normal range, 59–308 pmol · min⁻¹ · mg⁻¹; data from Dr Ronald J. A. Wanders).¹⁸

Among the 58 healthy individuals, 1 heteroduplex was found at exon 5 of the *CACT* gene (*CACT* 5). This individual was a 33-year-old man, namely, Case 4. This exon 5 mutation was subsequently confirmed by DNA sequencing as c.516T>C, which resulted in the synonymous mutation T172T (Figure 2). This genetic alteration is the third novel *CACT* mutation identified in this report.

Figure 1 DHPLC patterns of homoduplexes obtained with normal *CACT* exons. Complete sequence-matched hybridization between two normal or autologous sequences produced a single peak for each exon.

Figure 2 Aberrant DHPLC patterns of heteroduplexes and corresponding DNA sequences. Hybridization of unmatched normal and mutated sequences generated heteroduplexes, thereby resulting in aberrant peaks with different retention times. Red arrows indicate positions of mutations identified by direct DNA sequencing.

To confirm the reliability of DHPLC, 13 healthy individuals without any DHPLC heteroduplex were randomly selected, and their DNA was subjected to full-length DNA sequencing. No mutations were found in any exon of their *CACT* gene (data not shown).

DISCUSSION

CACT, as well as *CPT2*, is one of the key enzymes that participate in the intake of long-chain fatty acids into the mitochondrial matrix; therefore, defective activities of these enzymes result in impaired β -oxidation of fatty acids and lack of indispensable energy. *CPT2* deficiency and *CACT* deficiency are known to induce a wide range of clinical manifestations, from lethal neonatal episodes of coma due to

hypoketotic hypoglycemia, cardiomyopathy and cardiac arrhythmia to the adult-type of non-traumatic rhabdomyolysis.^{5,8,19} *CPT2* deficiency consists of a lethal neonatal form, an infantile (hepatic) form and an adult-onset (muscular) form. The genetic characteristics of *CPT2* deficiency have been well analyzed. We have previously described gene mutations in seven patients with definitive *CPT2* deficiency, and by determining their genotypes, we found Japanese patient-specific mutations; however, we failed to find any relationship between their genotypes and clinical manifestations.^{15,20,21} On the other hand, the genetic characteristics of *CACT* deficiency have not been well described, due to the scarcity of the disease. To the best of our knowledge, only 40 patients at most have been reported

Table 3 Mutations reported in this study

	Subject	Final diagnosis	Mutation	Location	Genetic defect
1	Case 1	CACT deficiency	c.199-10t>g	Intron 2	Skipping of exon 3 or exons 3 and 4
	Cases 1 and 2		c.576G>A ^a	Exon 6	Premature stop codon W192ter
2	Case 2	CACT deficiency	c.106-2a>t ^a	Intron 1/exon 2 junction	Splicing acceptor mutation
3	Case 3	Non-traumatic rhabdomyolysis (a possible carrier of CACT deficiency)	c.804delG	Exon 8	Frameshift from codon 269
4	Case 4	Healthy	c.516T>C ^a	Exon 5	Synonymous mutation T172T

Abbreviation: CACT, carnitine-acylcarnitine translocase.

^aNewly identified mutations in this study.

worldwide in the literature, and among them, the partial genotype of one Japanese patient was briefly described by a researcher from our group (TF).^{6,7,22,23} In many cases, CACT deficiency was seen during the neonatal period, with a few cases seen in their infantile period. The rarity of the disease may be explained in part by miscarriage and stillbirth.

Because of the resemblance of clinical features, including the acylcarnitine profiles determined by tandem mass spectrometry, between CACT deficiency and the neonatal form of CPT2 deficiency, genotyping is expected to be a pivotal tool for differentially diagnosing these two disorders. Here, we report the genotypes of two cases with CACT deficiency. Two *CACT* mutations, c.576G>A and c.106-2a>t, have never been reported in the literature; the former mutation was detected in both the affected babies and could be Japanese patient-specific. The c.576G>A mutation changes the tryptophan at the amino-acid residue 192 to a stop signal. The three-dimensional structure of rat CACT indicates that such a mutation might result in an incomplete binding-site structure for cytosomal fatty acids and therefore lead to impaired enzymatic functions.²⁴ The importance of the splicing mutation c.106-2a>t has been emphasized by Korman *et al.*²⁵ Another identified mutation, c.199-10t>g, was the commonest one among the patients reported, especially the Chinese population.⁷

Among the patients with non-traumatic rhabdomyolysis, we found the c.804delG mutation in the *CACT* gene, which has been reported to be pathogenetic in a Cape Indian individual and a Caucasian individual.⁷ Although heterozygous CPT2 deficiency has also been reported to cause rhabdomyolysis,²⁶ we propose that our patient bearing this mutation was a heterozygous carrier of CACT deficiency, because the CACT enzymatic activity of his fibroblasts was maintained within the normal range. Acylcarnitine analysis was not performed for these patients; hence, we could not completely exclude the possibility that they were affected by other β -oxidation defects, such as very long chain acyl-CoA dehydrogenase (VLCAD) deficiency. However, the incidence of VLCAD deficiency is believed to be less than that of CPT2 deficiency in the Japanese population.²⁷ c.516T>C was found to be a synonymous variant in the case of the healthy volunteers. Table 3 summarizes all the genetic variations found in this study.

CACT deficiency needs to be diagnosed genetically as early as possible because of the following reasons: (1) patients with the disorder die during the neonatal period, (2) tests for measurement of the enzyme activity are not necessarily available worldwide, (3) differential diagnosis between CPT2 and CACT deficiency can be achieved only by genetic methods, (4) immediate induction of adequate therapies is required, and (5) genetic analysis can distinguish patients with CACT deficiency-like diseases but without genetic mutations.^{28,29} However, due to the rarity of the disease, the structure and characteristics of the *CACT* gene are still not well

understood. Recently, DHPLC has been developed to screen mutations in these types of rare diseases, as it is particularly useful for detecting unknown polymorphisms and mutations. In this study, we demonstrate that all the PCR products producing heteroduplexes during DHPLC indeed contained mutated sequences, whereas normal sequences did not cause heteroduplex formation. These findings were further confirmed by family studies of each CACT-deficiency patient.

In conclusion, we identified five mutations in the *CACT* gene, three of which were responsible for CACT deficiency. We have also demonstrated the successful screening of unknown *CACT* gene mutations with DHPLC.

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