HUMAN MUTATION 11:377–386 (1998)

RESEARCH ARTICLE

Two *CPT2* Mutations in Three Japanese Patients With Carnitine Palmitoyltransferase II Deficiency: Functional Analysis and Association With Polymorphic Haplotypes and Two Clinical Phenotypes

Kaoru Wataya,[1] Jun Akanuma,[1] Patrizia Cavadini,[2] Yoko Aoki,[1] Shigeo Kure,[1] Federica Invernizzi,[2] Ichiro Yoshida,[3] Jun-ichi Kira,[4] Franco Taroni,[2] Yoichi Matsubara,[1]* and Kuniaki Narisawa[1]

Communicated by Henrik Dahl

[1]*Department of Biochemical Genetics, Tohoku University School of Medicine, Sendai, Japan*
[2]*Laboratory of Cellular Pathology, Department of Biochemistry and Genetics, Istituto Nazionale Neurologico C. Besta, Milan, Italy*
[3]*Department of Pediatrics and Child Health, Kurume University School of Medicine, Kurume, Japan*
[4]*Department of Neurology, Kyushu University Faculty of Medicine, Fukuoka, Japan*

Carnitine palmitoyltransferase II (CPT II) deficiency manifests as two different clinical phenotypes: a muscular form and a hepatic form. We have investigated three nonconsanguineous Japanese patients with CPT II deficiency. Molecular analysis revealed two missense mutations, a glutamate (174)-to-lysine substitution (E174K) and a phenylalanine (383)-to-tyrosine substitution (F383Y) in the CPT II cDNA. Transfection experiments in COS-1 cells demonstrated that the two mutations markedly decreased the catalytic activity of mutant CPT II. Case 1 (hepatic form) was homozygous for the F383Y mutation, whereas case 3 (muscular form) was homozygous for the E174K mutation. Case 2 and her brother, who were compound heterozygotes for E174K and F383Y, exhibited the hepatic phenotype. We also identified a novel polymorphism in the *CPT2* gene, a phenylalanine (352)-to-cysteine substitution (F352C), which did not alter CPT II activity in transfected cells. It was present in 21 out of 100 normal alleles in the Japanese population, but absent in Caucasian populations. Genotyping with the F352C polymorphism and the two previously reported polymorphisms, V368I and M647V, allowed normal Japanese alleles to be classified into five haplotypes. In all three families with CPT II deficiency, the E174K mutation resided only on the F1V1M1 allele, whereas the F383Y mutation was observed on the F2V2M1 allele, suggesting a single origin for each mutation. Hum Mutat 11:377–386, 1998.

KEY WORDS: **carnitine palmitoyltransferase II deficiency; missense mutation; polymorphism; fatty-acid β-oxidation**

INTRODUCTION

The β-oxidation of fatty acids in mitochondria is an essential energy-producing pathway during fasting. Whereas short-chain acyl-CoA, an active form of short-chain fatty acid, enters mitochondria freely, the influx of long-chain acyl-CoA is coordinated by the carnitine palmitoyltransferase (CPT) enzyme system in association with carnitine-acylcarnitine translocase (Bieber, 1988; Guzman and Geelen, 1993). The CPT system consists of two distinct enzymes, CPT I and CPT II, both of which are bound to mitochondrial membrane (Woeltje et al., 1990). Although inherited deficiency of either enzyme has been described in humans, CPT II deficiency is by

Received 29 May 1977; accepted 19 October 1997.

*Correspondence to: Yoichi Matsubara, M.D., Department of Biochemical Genetics, Tohoku University School of Medicine, 1-1 Seiryomachi, Aobaku, Sendai 980-8574, Japan. E-mail: ymats@mail.cc.tohoku.ac.jp

The first two authors contributed equally to this study.

Contract grant sponsor: Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports and Science of Japan; Contract grant sponsor: Ministry of Health and Public Welfare of Japan; Contract grant sponsor: Vehicle Racing Commemorative Foundation, Japan; Contract grant sponsor: Telethon-Italia (to F.T.).

far the most common disorder of muscle lipid metabolism (Zierz, 1995).

The cDNA encoding human CPT II was cloned (Finocchiaro et al., 1991), and the genomic structure of the corresponding gene (*CPT2*) has been characterized. The *CPT2* gene spans ~ 20 kilobases on human chromosome 1p32 and is composed of five exons (Gellera et al., 1994; Verderio et al., 1995). Deficiency of CPT II is typically characterized by recurrent episodes of muscle pain, myoglobinuria, and rhabdomyolysis. These symptoms are triggered by prolonged exercise or viral infection and patients are usually young adults (Zierz, 1995). In addition to this classical muscular type, a hepatic or hepatocardiomuscular form of CPT II deficiency has been reported recently in infants (Demaugre et al., 1988, 1991; Hug et al., 1991; Taroni and Uziel, 1996), although CPT II is believed to be the same gene product in all tissues. Clinical pictures in these children and neonates include hypoketotic hypoglycemia, liver dysfunction, cardiomyopathy, and sudden death. Molecular analysis of Caucasian patients revealed various mutations and molecular heterogeneity of CPT II deficiency (Gellera et al., 1992; Taroni et al., 1992, 1993, 1994; Verderio et al., 1995; Bonnefont et al., 1996). The studies suggested that two clinical forms of the disease appear to result from distinct mutations in these populations. Also, a common mutation associated with the muscular form has been identified (Taroni et al., 1993). However, little is known about molecular defects in the *CPT2* gene among non-Caucasian patients.

In this study, we report the identification and the functional analysis of two mutations and a polymorphism in the CPT II gene in three Japanese patients. We also performed haplotype analysis of the Japanese population using the three polymorphisms (Verderio et al., 1993; this study) observed in the *CPT2* gene. The results indicated an association of a distinct haplotype to each disease-causing mutation.

MATERIALS AND METHODS

Patients

Three patients were examined. Cases 1 and 2 showed clinical pictures of a hepatic form in infancy, and case 3 exhibited a muscular phenotype. There was no consanguinity in any of the families, and the three families were apparently not related.

Case 1

The boy had fever and diarrhea followed by drowsiness at 9 months of age (Morita et al., 1991). Hepatomegaly was noted and laboratory data showed mild liver dysfunction (AST 58 Karmen Units [KU]; ALT 103 KU), metabolic acidosis (pH 7.26, base excess –8.1), and slightly increased lactate (2.6 mmol/l) and pyruvate (0.12 mmol/l). Decreased free carnitine level in serum (free carnitine 3.2 nmol/ml, normal values [n.v.]: 51.7±8.8; acyl carnitine 6.8 nmol/ml, n.v.: 9.7±9.0) and increased acylcarnitine in urine (840 nmol/mg creatinine, n.v.: 288±165) were noted. Urinary ketone bodies were only slightly elevated (1+) when blood glucose was 45 mg/dl. Urinary organic acid analysis revealed prominent dicarboxylic aciduria. Biopsied liver specimen showed swollen hepatocytes and microvesicular-type fatty change. Following intravenous fluid therapy with glucose and bicarbonate, the patient recovered quickly. Although he later developed a similar episode, he is alive and well at age 9 with normal growth and development. He had an elder sister who died of Reye-like syndrome at 7 months of age.

Case 2

The girl showed "hepatic" symptoms at 9 months of age. Clinical pictures of the case have been previously reported in detail (Yamamoto et al., 1996). Her younger brother also had similar symptoms at 6 months of age.

Case 3

This patient experienced myalgia after prolonged exercise or viral infection since she was 10 years old (Taniwaki et al., 1989). At age 17, she had myalgia and pigmenturia provoked by prolonged exercise. Laboratory data showed myoglobinuria with markedly increased serum creatine kinase levels (24,000 IU/l) but normal levels of serum AST and ALT. Electromyography showed myogenic abnormalities. Ischemic-exercise test resulted in normal increase of venous lactate. Fasting test increased plasma ketone bodies, serum creatine kinase, and myoglobin. After the episode, she has been free of symptoms simply by avoiding strenuous exercises. She had an elder brother who died of Reye-like syndrome at 3 years of age.

Peripheral blood B-lymphocytes from each of the three patients were transformed by Epstein-Barr virus, cultured, and harvested for enzymatic assay and molecular characterization.

Assay of CPT II Activity

CPT II activity was measured in cultured lymphoblasts by detecting palmitoyl-L-[methyl-^{14}C]carnitine formed from L-[methyl-^{14}C]carnitine and palmitoyl-CoA (Demaugre et al., 1988). We used n-octyl-β-D-glucoside as a detergent to extract CPT II activity from the mitochondrial membrane while suppress-

ing CPT I activity (Woeltje et al., 1987). In transfected COS-1 cells, CPT II activity was measured by detecting palmitoyl-L-[methyl-^{3}H]carnitine formed from L-[methyl-^{3}H]carnitine and palmitoyl-CoA (Taroni et al., 1992).

Isolation of mRNA and Genomic DNA

Poly(A)$^+$RNA and genomic DNA were isolated from cultured lymphoblasts (Sambrook et al., 1987). Genomic DNA from family members of the three patients and 50 healthy Japanese volunteers was extracted from either whole-blood specimens or dried blood spots on filter paper by a previously described method (Ogasawara et al., 1994).

Amplification of CPT II cDNA by Polymerase Chain Reaction (PCR)

The isolated mRNA was reverse-transcribed and the entire coding region of CPT II cDNA was amplified by PCR in six overlapping segments, using six sets of primers based on the sequence of human CPT II cDNA (Finocchiaro et al., 1991). The six primer sets were: CPT-EA1/CPT-A2, CPT-3-M13R/CPT-2, CPT-5-M13R/CPT-102, CPT-9-M13R/CPT-6, CPT-13-M13R/CPT-82 and CPT-17-M13R/CPT-104. The primer sequences are given in Table 1.

For direct sequencing of CPT II cDNA, nested form of PCR-amplification was performed. The first PCR reactions were conducted with four sets of outer primers: CPT-EA1/CPT-A2B, CPT-101/CPT-102, CPT-5-M13R/CPT-6B, and CPT-103/CPT-104. Using the first PCR products as templates, second PCR reactions were done with eight sets of inner primers: CPT-N1-M13R/CPT-A2B, CPT-3-M13R/CPT-4B, CPT-7-M13R/CPT-6B, CPT-9-M13R/CPT-6B, CPT-11-M13R/CPT-8B, CPT-15-M13R/CPT-8B, CPT-17-M13R/CPT-10B, and CPT-19-M13R/CPT-10B. Each sense primer carried an M13 reverse sequence at its 5′ end and each antisense primer was biotinylated at its 5′ end to facilitate direct sequencing.

TABLE 1. **Nucleotide Sequences of PCR Primers Used in the Study**

Primer	Sequence[a]
CPT-101	5-GGCCTTGTGTTTAGACTCCA-3
CPT-102	5-GCTGCTGTCTGAGAGAATGT-3
CPT-103	5-TCCTGGTCCTAAGGAAAGGA-3
CPT-104	5-TCCCAGTTTTCATGATGAGGA-3
CPT-EA1	5-CTTGTGTTTAGACTCCAGAACTCCC-3
CPT-A2	5-CCAATCCCATTTTCAAAACTCTTGCA-3
CPT-2	5-TTCAGAACAACGGAGTCTCG-3
CPT-6	5-AGTAGAGTCAGTGGTAGCTG-3
CPT-82	5-TGCCAGATGCCGCAGAGCAA-3
CPT-A2-B	5-(Biotin)-CCAATCCCATTTTCAAAACTCTTGCA-3
CPT-4B	5-(Biotin)-AGATGTGCCTGGATTTCCGA-3
CPT-6B	5-(Biotin)-AGTAGAGTCAGTGGTAGCTG-3
CPT-8B	5-(Biotin)-TTCTTTGGTCAGCTGGCCAT-3
CPT-10B	5-(Biotin)-GGAAGTGATGGTAGCTTTTCA-3
CPT-N1-M13R	5-AGGAAACAGCTATGACACTTGCCGCGTTCTCGCCG-3
CPT-3-M13R	5-AGGAAACAGCTATGACAGGCTGCCTATTCCCAAACT-3
CPT-5-M13R	5-AGGAAACAGCTATGACGGGACCCTGGTTTGATATGT-3
CPT-7-M13R	5-AGGAAACAGCTATGACAGTGACACTATCACCTTCAAG-3
CPT-9-M13R	5-AGGAAACAGCTATGACTCTTTGATGTCCTGGATCAAG-3
CPT-11-M13R	5-AGGAAACAGCTATGACAAAGTGGACTCGGCAGTGTT-3
CPT-13-M13R	5-AGGAAACAGCTATGACTAAAGACAGCACTCAGACCC-3
CPT-15-M13R	5-AGGAAACAGCTATGACGAGGAGGCAAAGAATTCCTG-3
CPT-17-M13R	5-AGGAAACAGCTATGACGTGAGCTTCAGCAGATGATG-3
CPT-19-M13R	5-AGGAAACAGCTATGACAACCACAATGTCCTGTCCAC-3
CPT-F383Y-ApoI	5-TGGTGTGGCAGTGCTCAAAT-3
CPT-E174K-MboII	5-GTCACTTTTTGCAGGGTTCAAGTGGAACTCTT-3
CPT-F352C-SgrAI	5-GATGGCACAAACCGCCGGT-3
CPT-7-XbaI	5-GCTCTAGAGTGACACTATCACCTTCAAG-3
CPT-10-BamHI	5-GCGGATCCGGAAGTGATGGTAGCTTTTCA-3
INS1	5-GGCATACCTGACCAGTGAGAACCGA-3
D2.1	5-GCTACAGGACTCGTAGGTGGCCAC-3
GGS	5-GGAGCCCCCAGTCGGCCCCTCAGCGCCGG-3
C2	5-AATGAGGTTAAAGGATTTATCAAACCA-3
K174S	5-TCTGGAGCCAAAAGTGTTCCACTTGAAC-3
K174AS	5-GTTCAAGTGGAACACTTTTGGCTCCAGA-3

[a]Single-underlined sequence is an M13 reverse primer sequence used for direct sequencing. Double-underlined nucleotide indicates an artificial mismatched residue introduced for generating a diagnostic restriction site in the PCR fragment. Nucleotide with a dotted underline indicates the G-1036-A substitution.

Sequence Analysis

Amplified PCR fragments were subcloned into ddT-tailed pBluescript II KS(+) (Stratagene, La Jolla, CA), which was prepared by a previously described method (Holton and Graham, 1991). At least five clones of each fragment were isolated, and the plasmids were sequenced on an A.L.F. automated laser fluorescent sequencer (Pharmacia Biotech, Uppsala, Sweden). Direct sequencing of the amplified cDNA fragments was also carried out: the second PCR products were immobilized onto Dynabeads M-280 streptavidin (Dynal, Oslo, Norway), denatured in 0.1 M NaOH to produce single-strand DNA, and subjected to sequencing analysis using an M13 reverse primer. Sequencing of genomic DNA was performed as previously described (Verderio et al., 1995).

Detection of Nucleotide Substitutions by Restriction-Site-Generating PCR

To facilitate DNA analysis for the two mutations and the polymorphism identified in this study, we performed restriction-site-generating PCR. Three sets of PCR primers were used: CPT-5-M13R/CPT-E174K-MboII for detecting E174K, CPT-F383Y-ApoI/CPT-6 for F383Y, and CPT-F352C-SgrAI/CPT-6 for F352C. Since none of the three substitutions generate any restriction site change, mismatch residues were introduced in the PCR primers to create restriction enzyme recognition sequence at each substitution site (Table 1). The amplified PCR products were digested with either *Mbo*II, *Apo*I (New England Biolabs, Beverly, MA) or *Sgr*AI (Boehringer-Mannheim, Mannheim, Germany), electrophoresed on a 6% agarose gel and stained with ethidium bromide.

Haplotype Analysis of 50 Healthy Japanese Individuals by Restriction Enzyme Digestion

Haplotype analysis of 50 healthy Japanese individuals and three patients were performed for the previously identified polymorphisms (V368I and M647V) and the newly identified polymorphism, F352C. V368I and M647V polymorphic alleles, designated as V1/V2 and M1/M2 where 1 indicates no substitution and 2 indicates the presence of substitution, were detected by PCR amplification and restriction digestion as described previously (Verderio et al., 1993). F352C alleles, designated as F1/F2, were detected by the method described above.

When the independent analysis of each polymorphism did not allow to assess it, the haplotype for the three polymorphisms was determined as follows. DNA fragments encompassing the two polymorphic sites F352C and V368I were PCR-amplified with primers CPT-6 and CPT-11-M13R and subcloned into ddT-tailed pBluescript. Haplotype of each allele was then determined by analysing multiple plasmid clones. Similarly, 3.8 kbp-fragments containing both the F352C and the M647V sites were amplified with CPT-7-XbaI and CPT-10-BamHI, digested with *Xba*I and *Bam*HI, subcloned into *Xba*I/*Bam*HI-digested pBluescript, and examined for the two polymorphisms.

Expression Studies in Mammalian Cells

Plasmid pG7C1-YCI (F383Y/F352C/V368I triple mutant) and plasmid pG7C1-FCI (F352C/V368I double mutant) contained the T-1664-A transversion and were derived from wild-type plasmid pG7C1-WT (Taroni et al., 1992). A 607-bp fragment encompassing the putative disease-causing mutation T-1664-A and the two polymorphic variants, T-1571-G and G-1618-A, was amplified with the sense primer INS1 and the antisense primer D2.1 (Taroni et al., 1993) using genomic DNA from the patient's father as template. Plasmids pG7C1-YCI and pG7C1-FCI were generated by replacing the 330-bp *Asp*I fragment (nt 1518-1847) of pG7C1-WT with the corresponding fragment obtained from the parent-derived 607-bp PCR product. Plasmid pG7C1-E174K contained the G-1036-A transition and was also derived from pG7C1-WT by PCR-primer-directed mutagenesis. Two partially overlapping fragments of 483 and 568 bp, both containing the G-1036-A substitution, were amplified using primer GG-S (Verderio et al., 1995) along with the mutagenic primer K174-AS and primer K174-S in conjunction with primer C2 (Taroni et al., 1992), respectively. The PCR products were then used as megaprimers/templates in a final amplification reaction in the presence of the sense primer GG-S and the antisense primer C2. The final 1023-bp fragment was digested with *Pst*I and *Asp*I and the resulting 894-bp (nt 624-1517) fragment was used to replace the corresponding wild-type fragment in *Pst*I/*Asp*I-digested plasmid pG7C1-WT. The mutant expression clones pcDC1-YCI, pcDC1-FCI, and pcDC1-E174K were constructed as previously reported for the normal CPT II cDNA (pcDC1-WT) (Taroni et al., 1992). The mutations and the integrity of the inserts were confirmed by sequence analysis.

COS-1 cells were cultured, pulse-labeled with L-[^{35}S]methionine, and immunoprecipitated as previously described (Taroni and Rosenberg, 1991; Taroni et al., 1992, 1993). Anti-ornithine transcarbamoylase antibodies were a generous gift of F. Kalousek (Yale University). CPT II cDNA expression constructs were introduced into COS-1 cells by electroporation

and transfection experiments were performed essentially as described previously (Taroni et al., 1992, 1993). Plasmid pSV-βGal (Promega) or plasmid pODb, an expression vector containing the human ornithine transcarbamoylase cDNA (gift of A. Horwich, Yale University), was cotransfected as an internal standard. Mock transfection was carried out with control plasmid alone (pSV-βGal or pODb). Enzyme activity and protein were determined as described (Taroni et al., 1992).

RESULTS

CPT II Activity in Patient Cells

CPT II activities in lymphoblasts from cases 1, 2, and 3 were 0.087, 0.031 and 0.081 nmol/min per mg of protein, respectively, whereas the normal value was 1.39±0.50 (mean±SD, n=9). Residual CPT II activity in case 1 (6.3% of control mean) was similar to that (7.8%; 0.11 nmol of L-[methyl-^{14}C]carnitine formed/min per mg of protein; control, 1.41±0.42 [mean±SD]) previously observed in fibroblasts from the infant (Morita et al., 1991).

Sequencing Analysis of CPT II cDNA and Genomic DNA in Three Patients

Sequencing of CPT II cDNAs from lymphoblasts of three patients revealed three nucleotide substitutions: a G-to-A transition at nucleotide position 1036, a T-to-G change at nucleotide position 1571, and a T-to-A change at position 1664 (data not shown). These substitutions resulted in amino acid changes of glutamate at position 174 to lysine (E174K), phenylalanine at position 352 to cysteine (F352C) and phenylalanine at position 383 to tyrosine (F383Y), respectively. Sequencing analysis of genomic DNA indicated that case 1 was homozygous for F383Y and case 3 was homozygous for E174K, whereas case 2 was a compound heterozygote for F383Y and E174K. We found that alleles with F383Y also carried the F352C substitution in both cases 1 and 2. In addition, on these alleles, F383Y and F352C were associated with a previously reported polymorphism, a G-to-A change at nucleotide position 1618, which leads to valine-368-to-isoleucine substitution (V368I) (Taroni et al., 1992).

In Vitro Expression Study of Mutant CPT II cDNAs Carrying E174K, F352C, or F383Y

To study the effect of the identified mutations on the activity of human CPT II, the substitutions were introduced into the CPT II cDNA by PCR-primer-directed mutagenesis and four CPT II cDNAs were transiently expressed in COS-1 cells: a wild-type cDNA (pcDC1-WT), a triple mutant containing the putative disease-causing mutation F383Y along with the polymorphic variants F352C and V368I (pcDC1-YCI), a double mutant containing the polymorphic variants F352C and V368I (pcDC1-FCI), and a mutant containing the putative disease-causing mutation E174K (pcDC1-E174K). The results of the transfection experiments are shown in Table 2. The activity in cells transfected with pcDC1-YCI was significantly lower (4.29±2.0% [mean±SD; n=9; $P \leq 10^{-4}$]) than the activity in pcDC1-WT-transfected cells. By contrast, COS-1 cells transfected with the polymorphism-containing plasmid pcDC1-FCI exhibited only slightly reduced CPT II activity (69.27±8.4% [mean±SD; n=7; $P \leq 10^{-3}$]), whereas COS-1 cells cotransfected with both the polymorphism-containing plasmid pcDC1-FCI and the mutant plasmid pcDC1-YCI exhibited intermediate CPT II activity (34.27±6.4% [mean±SD; n=6; $P \leq 10^{-3}$]). CPT II activity in cells transfected with mutant plasmid pcDC1-E174K was also significantly lower (10.8±2.1% [mean±SD; n=4; $P \leq 10^{-4}$]) than the activity in pcDC1-WT-transfected cells. Finally, COS-1 cells cotransfected with both mutant plasmids pcDC1-YCI and pcDC1-E174K exhibited very low CPT II activity (9.8% and 11.2% in two experiments).

To rule out the possibility that the reduction of CPT II activity in the mutant-transfected cells was caused by reduced enzyme synthesis, transfected cells were radiolabeled and immunoprecipitated with anti-CPT II antibody and anti-ornithine transcarbamoylase antibody as a transfection internal control (Fig. 1a). Radiolabeled CPT II was present at substantially similar levels in cells transfected with the normal (lane 2) and the mutant cDNAs (lanes 3 and 4), thus demonstrating that both the F383Y and the E174K substitutions are the crucial disease-causing mutations.

TABLE 2. In Vitro Expression of Mutant cDNA in COS-1 Cells[a]

Plasmid used for transfection	% of normal CPT II activity (mean±SD)
pcDC-1 with F383Y, V368I & F352C	4.29 ±2.0 (n=9)
pcDC-1 with V368I & F352C	69.27 ±8.4 (n=7)
pcDC-1 with V368I	88.25 ±12.7 (n=6)
pcDC-1 with F383Y, V368I & F352C and pcDC-1 with V368I & F352C	34.27 ±6.4 (n=6)
pcDC-1 with E174K	10.80 ±2.1 (n=4)
pcDC-1 with F383Y, V368I & F352C and pcDC-1 with E174K	9.8; 11.2

[a]Results are expressed as percentage of the CPT II activity determined in the cells transfected with the normal CPT II cDNA following subtraction of the endogenous activity (Taroni et al., 1992). Endogenous CPT II activity and CPT II activity after transfection of the wild-type cDNA were 30.7±3.8 (mean±SD, n=23) and 105.8±39.6 (mean±SD, n=10) nmol L-[methyl-^{3}H]carnitine released/min per mg of protein, respectively.

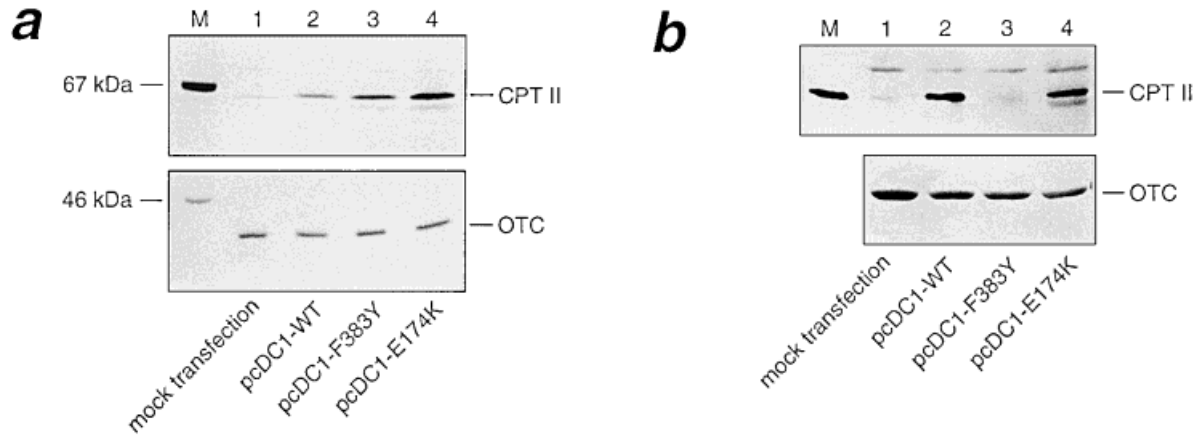

FIGURE 1. **CPT II expression in COS-1 cells transfected with the wild-type and the mutant CPT II cDNAs. (a)** SDS-PAGE analysis of CPT II and ornithine transcarbamoylase (OTC) immunoprecipitated from transfected cells pulse-labeled with L-[^{35}S]methionine. **(b)** Western blot analysis of CPT II and OTC in transfected cells. Cells were transfected with the control vector pODb alone, indicated as mock transfection (lane 1), the control vector + pcDC1-WT, indicated as pcDC1-WT (lane 2), the control vector + pcDC1-YCI, indicated as pcDC1-F383Y (lane 3), or the control vector + pcDC1-E174K, indicated as pcDC1-E174K (lane 4). Lane M, molecular weight markers (a) or purified porcine CPT II (b).

The steady-state levels of normal and mutant CPT II in transfected COS-1 cells were also analysed by immunoblot with anti-CPT II and anti-ornithine transcarbamoylase antibodies (Fig. 1b). In cells transfected with the F383Y-containing cDNA, CPT II was almost undetectable (lane 3), suggesting that the F383Y mutation renders the mutant CPT II much less stable than the normal counterpart (lane 2). By contrast, the apparently normal steady-state level of the E174K mutant CPT II (lane 4) would suggest a possible effect of this mutation on the kinetic properties of the enzyme, with a marginal contribution, if any, to protein instability.

Family Studies of the E174K and F383Y Mutations

The restriction-site-generating PCR analysis was performed to demonstrate transmission of F383Y and E174K within the families of patients 1 and 2.

The F383Y mutant allele was detected by digesting 100-bp PCR products with *Apo*I. The normal sequence can be cleaved by *Apo*I to yield 83- and 17-bp fragments, whereas the PCR product from the mutant sequence would remain undigested. Analysis of case 1 family members showed that the patient was homozygous and both of his parents were heterozygous for F383Y (Fig. 2a).

In the family of case 2, F383Y was transmitted from the father to the patient and her brother (Fig. 2b, panel b). The other mutation (E174K) found in this patient was also examined by restriction-site-generating PCR. Genomic DNA was amplified with primers CPT-5-M13R and CPT-E174K-MboII and the resulting 230-bp fragment was digested with *Mbo*II. Digestion of PCR fragments amplified from normal sequence should produce three fragments of 179, 31, and 20 bp because of the presence of a naturally occurring *Mbo*II site along with the newly generated one. By contrast, the mutant E174K allele should produce only two fragments of 179 and 51 bp. Case 2 was thus found to be heterozygous for the E174K mutation that she had received from her mother (Fig. 2b, panel a). The analysis indicated that the brother was also a compound heterozygote for E174K and F383Y.

Haplotype Analysis of 50 Normal Japanese Individuals With F352C, V368I, and M647V Polymorphisms

For detecting the F352C substitution, 192-bp PCR products amplified with primers CPT-F352C-SgrAI and CPT-6 were digested by *Sgr*AI. The PCR products containing the mutant sequence were cleaved into 178 and 15 bp fragments, whereas those containing the normal sequence remained undigested (Fig. 2c). The F352C polymorphism as well as the previously reported V368I and M647V (Taroni et al., 1992; Verderio et al., 1993) were examined in 50 unrelated Japanese individuals. Allelic frequencies of each polymorphism and heterozygosity are shown in Table 3a. Interestingly, the F352C polymorphic variant was not detected in 35 Caucasian individuals of European origin.

Genotyping with the three polymorphisms enabled us to classify the Japanese population into five haplotypes (Table 3b). Three haplotypes, F1V1M1, F1V2M1, and F2V2M1, were common in the Japa-

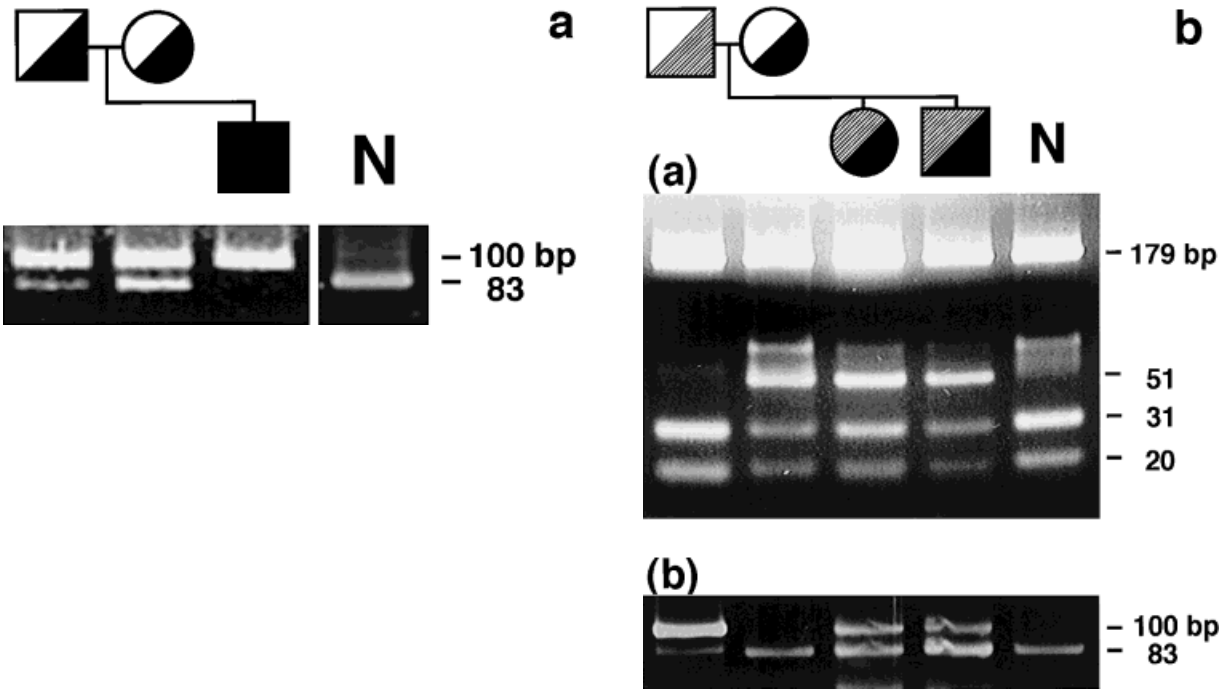

FIGURE 2. **(a)** Detection of the F383Y mutation in family members of case 1. PCR fragments were digested with *Apo*I. Case 1 was homozygous and both of his parents were heterozygous for F383Y. Lane N, normal control. **(b)** Detection of the E174K and F383Y mutations in the family of case 2. Carriers are indicated by black (E174K) and hatched (F383Y) half boxes. (a) E174K was detected by digesting PCR fragments with *Mbo*II. (b) F383Y was detected by digesting PCR fragments with *Apo*I. Case 2 and her younger brother were both compound heterozygotes of E174K and F383Y. E174K was transmitted from their mother as indicated by the presence of the 51 bp-fragment (a), whereas F383Y was transmitted from their father (b). Lane N, normal control. **(c)** Detection of the F352C polymorphism by restriction-site-generating PCR. Amplified genomic DNA was digested with *Sgr*AI and analyzed by agarose gel electrophoresis. Lane M, DNA molecular weight markers; lane 1, individual homozygous for the wild-type allele; lane 2, individual heterozygous for the F352C polymorphism; lane 3, individual homozygous for the F352C polymorphism.

nese population, accounting for 90% of alleles. In all the three families with CPT II deficiency, the E174K mutation resided only on F1V1M1 alleles, whereas the F383Y mutation was observed on F2V2M1 alleles.

DISCUSSION

We have identified two mutations in the *CPT2* gene that are responsible for CPT II deficiency in three infants presenting with two different clinical phenotypes. These two mutations have been independently reported also by Yamamoto et al. (1996) in a Japanese patient. This patient turned out to be the same individual as patient 2 in our report (S. Yamamoto, pers. comm.). However, expression analysis of each mutation was not performed in their study, and the pathogenicity of these two mutations was not demonstrated. In addition to the E174K and F383Y mutations, the patient also carried the F352C substitution. It must be noted that this mutation has been originally described by Hamajima et al. (1994) as a possible disease-causing mutation. Therefore, the effects of each amino acid substitution on the catalytic activity of CPT II had remained unsolved. Our study provides the first experimental evidence that E174K and F383Y are indeed responsible for CPT II deficiency and that F352C is a benign polymorphism. Moreover, Mendelian inheritance of each mutation was documented in families of cases 1 and 2.

Both the E174K and the F383Y mutations are nonconservative substitutions that might be predicted

TABLE 3. Haplotype Analysis of 50 Healthy Japanese Individuals

(a) Allelic frequency and heterozygosity

Polymorphisms	Frequency	Heterozygosity	
		Expected	Observed
F352C	F1=0.79, F2=0.21	0.33	0.18
V368I	V1=0.30, V2=0.70	0.42	0.36
M647V	M1=0.96, M2=0.04	0.08	0.08

(b) Haplotypes and their frequencies (expected/observed)

Haplotypes	Number of observed alleles	Frequency	
		Expected	Observed
Type 1 (F1V1M1)	29	0.228	0.290
Type 2 (F1V1M2)	0	0.009	0.000
Type 3 (F1V2M1)	46	0.531	0.460
Type 4 (F1V2M2)	4	0.022	0.040
Type 5 (F2V1M1)	1	0.060	0.010
Type 6 (F2V1M2)	0	0.003	0.000
Type 7 (F2V2M1)	20	0.141	0.200
Type 8 (F2V2M2)	0	0.006	0.000
Total	100	1.000	1.000

to induce substantial changes in the secondary structure of the protein. Neither mutation, however, lies in any of the six clusters of homology that have been identified following sequence alignment of different acyltransferases from several species (Corti et al., 1994). Furthermore, glutamic acid 174 is conserved in some, but not all, acyltransferases, whereas phenylalanine 383 is found only in rat and human CPT II. A deleterious effect of the mutations was therefore not obvious. Direct evidence of their pathogenic role was ultimately provided by transient expression in transfected COS-1 cells. Both mutant CPT II were normally synthesized but exhibited significant reduction of activity (see below). Furthermore, the drastically reduced steady-state levels of F383Y mutant CPT II demonstrated by Western blot analysis strongly suggest that, similarly to the other CPT II mutations (Taroni et al., 1992, 1993; Verderio et al., 1995; Bonnefont et al., 1996), the F383Y mutation alters the stability of the mutant enzyme, perhaps interfering with folding and/or assembly. Notably, such effect was not observed in the case of the E174K substitution. In this case, the normal steady-state levels of the mutant CPT II would rather suggest that the E174K might affect the kinetic properties of the enzyme, perhaps altering the enzyme's substrate-binding affinity.

It has been reported that an apparently benign polymorphism can modify both qualitatively and quantitatively the phenotypic expression of a pathogenic mutation in the same gene (Romeo and McKusick, 1994). Such polymorphisms, so-called modifiers, were previously described in the *CPT2* gene: V368I and M647V substitutions exacerbated the effects of R631C disease-causing mutation, but they did not affect enzymatic activity alone (Taroni et al., 1992). The frequency of modifiers in various genetic disorders may be underestimated, because few of in vitro expression studies analyze interaction between common polymorphisms and mutations. Our expression analysis using single, double, and triple mutants indicated that F352C did not have modifying effect on E174K or F383Y.

Case 1 was homozygous for F383Y and showed infantile onset and hepatic symptoms. However, case 3 was homozygous for E174K and exhibited the clinical features of the classical muscular form of CPT II deficiency. Interestingly, case 2, who was a compound heterozygote for E174K and F383Y, showed a hepatic phenotype. Despite the clinical heterogeneity, the residual activities in lymphoblasts from the three patients were uniformly low (6.3% in case 1, 2.2% in case 2 and 5.8% in case 3). It has been reported that the residual CPT II activity in different tissues from a patient may vary significantly. In a patient with neonatal lethal phenotype, CPT II activity ranged from 1–2% of normal control in muscle and heart to 17% in liver (Hug et al., 1991). Another patient, who was homozygous for S113L and showed a muscular phenotype, had 7.0%, 28.1%, and 20.0% of residual CPT II activities in skeletal muscle, fibroblasts and lymphoblasts, respectively (Taroni et al., 1993), indicating that CPT II activity in patients' cultured cells may not reflect the actual residual activity in functionally more important organs, such as muscle and liver. Modeling mutations in a cellular system is therefore necessary in order to elucidate the functional consequences of amino acid changes on enzyme activity and to delineate the correlation between genotype and phenotype. When F383Y and E174K were expressed in COS-1 cells, different residual activities were observed: the F383Y mutation decreased CPT II activity to 4.29% of wild-type activity, whereas the E174K mutant enzyme retained 10.80% of activity (Table 2). It thus appears that there was an inverse correlation between the levels of residual CPT II activity and the severity of the disease, the mutation (E174K) with less impaired CPT II activity being associated with milder clinical symptoms as those observed in patient 3.

The genotype/phenotype correlation in CPT II deficiency has been well documented among Caucasian patients. Two mutations, S113L and P50H, were reported to be associated with the muscular type of CPT II deficiency (Taroni et al., 1993; Verderio et al., 1995), whereas R631C and Y628S were described in the early-onset hepatocardiomyopathic form (Taroni et al., 1992; Bonnefont et al., 1996). Another mutation, L302fs, which resulted in a premature translation termination codon, caused a lethal neonatal phenotype, the most severe form of the disease (Gellera et al., 1992). These

mutations have been expressed in COS cells using the same expression vector as in the current report (Taroni et al., 1992, 1993; Verderio et al., 1995; Bonnefont et al., 1996). The residual activity of each CPT II mutation, including the two identified in this study, is presented in Table 4 along with the clinical features of the patients homozygous for the mutation. S113L and P50H, which showed a relatively high residual activity of 33.9% and 16.7%, respectively, were associated with muscular manifestations. By contrast, the F383Y substitution showed the lowest activity and was associated with a hepatic phenotype. The L302fs mutation, which would produce a significantly truncated polypeptide that would be unlikely to retain any residual activity, was linked to a severe neonatal lethal form. Although the D553N showed significantly low residual activity (6.7%), it was identified only in heterozygous form in a patient who also carried the S113L on the other allele and showed a muscular phenotype (Verderio et al., 1995). The other three mutations exhibited intermediate residual activities, ranging from 14.7% to 10.2%, and the associated clinical phenotypes were variable. These observations suggest that, although the magnitude of residual CPT II activity highly influences the metabolic and clinical consequences of CPT II deficiency as previously proposed (Demaugre et al., 1991; Bonnefont et al., 1996), there is a significant overlap of residual activity between the two clinical phenotypes. This would be consistent with previous reports describing clinical heterogeneity in patients with identical *CPT2* genotypes. For example, a boy homozygous for the S113L showed muscular symptoms of CPT II deficiency including recurrent rhabdomyolysis and myoglobinuria (Handig et al., 1996). One of his cousins died at the age of 16 years during a severe attack of muscle injury. By contrast, another cousin, also homozygous for the S113L, was almost asymptomatic and never had episodes of acute muscle injury. Clinical heterogeneity was also observed in the family of our patient 3 who had a muscular phenotype. An elder brother of this patient died of Reye-like syndrome at 3 years of age. Although no specimen was available from the deceased brother for further investigation, it seems likely that the brother had the same E174K mutation that caused a different phenotype in his sister. This observation suggests that the two clinical phenotypes associated with CPT II deficiency, the muscular form and the hepatic form, may not be genetically distinct and can be caused by the same mutation.

Clinical symptoms in CPT II-deficient patients are usually provoked by various types of stress, such as prolonged exercise, fasting, and infection, implying that environmental factors may have significant influence on the onset as well as the clinical picture of the disease. The fact that various clinical pictures in CPT II deficiency can be caused by the same genotype is probably due to some unique metabolic features of the β-oxidation pathway, an essential energy-producing pathway that is fully activated by fasting (Taroni and Uziel, 1996). In fact, medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, one of the most frequent fatty-acid oxidation disorders, is known to present with variable clinical pictures, from sudden infant death to absence of symptoms in adults (Matsubara et al., 1992), caused by the same genotype. The weak genotype/phenotype correlation in MCAD deficiency has been explained by interindividual variation of alternative metabolic pathways as well as the degree of metabolic stress (fasting, infection, etc.) to which each patient is exposed (Matsubara et al., 1992).

We detected a polymorphism, F352C, in the *CPT2* gene that had no significant effect on CPT II activity when expressed in COS cells. The experimental results were consistent with our observation that six homozygotes for F352C were identified among 50 Japanese healthy individuals. The polymorphism was not observed among Caucasian populations (data not shown), appearing to be uniquely present in the Japanese population. The allelic frequencies of V368I (V1=0.49, V2=0.51) and M647V (M1=0.75, M2=0.25) in Southern European populations have been reported previously (Verderio et al., 1995). Taking

TABLE 4. **Correlation of Mutations and Clinical Phenotypes**

Mutation	Residual activity[a]	Clinical phenotype of a patient homozygous for the mutation	References
S113L (+V368I+M647V)[b]	33.9 ±5.8%	muscular form	Taroni et al., 1993
P50H (+V368I)[b]	16.7 ±1.8%	muscular form	Verderio et al., 1995
R631C (+V368I+M647V)[b]	14.7 ±4.0%	hepatic form	Taroni et al., 1992
E174K	10.8 ±2.1%	muscular form	this study
Y628S	10.2 ±1.9%	hepatic form	Bonnefont et al., 1996
D553N	6.7 ±2.8%	unknown	Verderio et al., 1995
F383Y (+V368I+F352C)[b]	4.3 ±2.0%	hepatic form	this study
L302fs	not determined	neonatal lethal form	Gellera et al., 1992

[a]Each mutation was expressed in COS cells in order to determine the residual CPT II activity.
[b]The V368I, M647V, and F352C polymorphisms associated with the mutations on the patients' alleles were also incorporated in CPT II cDNAs used for the expression study.

into account the absence of F2 allele in Caucasians, three haplotypes appear to occur in the Caucasian population F1V1M1, F1V2M1, and F1V2M2, with a relative frequency of 0.49, 0.26, and 0.25, respectively. This haplotype distribution is significantly different from that observed in the Japanese population (Table 3).

Both E174K and F383Y were associated with a single polymorphic haplotype in the patients: E174K was present on F1V1M1 alleles in patients 2 and 3, whereas F383Y was identified on F2V2M1 alleles in patients 1 and 2. The association in apparently unrelated families suggests that each mutation has a single origin. The molecular characterization of additional patients will allow us to examine the prevalence of these two mutations as well as to elucidate genotype/phenotype correlation among Japanese individuals with CPT II deficiency.

ACKNOWLEDGMENTS

We thank Dr. M. Takayanagi for his helpful advice on CPT II assay.

REFERENCES

Bieber LL (1988) Carnitine. Ann Rev Biochem 57:261–283.

Bonnefont J-P, Taroni F, Cavadini P, Cepanec C, Brivet M, Saudubray J-M, Leroux J-P, Demaugre F (1996) Molecular analysis of carnitine palmitoyltransferase II deficiency with hepatocardiomuscular expression. Am J Hum Genet 58:971–978.

Corti O, Finocchiaro G, Rossi E, Zuffardi O, DiDonato S (1994) Molecular cloning of cDNAs encoding human carnitine acetyltransferase and mapping of the corresponding gene to chromosome 9q34.1. Genomics 23:94–99.

Demaugre F, Bonnefont J-P, Mitchell G, Nguyen-Hoang N, Pellet A, Rimoldi M, DiDonato S, Saudubray J-M (1988) Hepatic muscular presentation of carnitine palmitoyltransferase deficiency: Two distinct entities. Pediatr Res 24:308–311.

Demaugre F, Bonnefont J-P, Colonna M, Cepanec C, Leroux J-P, Saudubray J-M (1991) Infantile form of carnitine palmitoyltransferase II deficiency with hepatomuscular symptoms and sudden death. J Clin Invest 87:859–864.

Finocchiaro G, Taroni F, Rocchi M, Martin AL, Colombo I, Tarelli GT, DiDonato S (1991) cDNA cloning, sequence analysis and chromosomal localization of the gene for human carnitine palmitoyltransferase. Proc Natl Acad Sci USA 88:661–665.

Gellera C, Witt DR, Verderio E, Cavadini P, DiDonato S, Taroni F (1992) Molecular study of lethal neonatal carnitine palmitoyltransferase II (CPTII) deficiency. Am J Hum Genet 51 (supplement): A168.

Gellera C, Verderio E, Floridia G, Finocchiaro G, Montermini L, Cavadini P, Zuffardi O, Taroni F (1994) Assignment of the human carnitine palmitoyltransferase II gene to chromosome 1p32. Genomics 24:195–197.

Guzman M, Geelen MJH (1993) Regulation of fatty acid oxidation in mammalian liver. Biochim Biophys Acta 1167:227–241.

Hamajima N, Sugiyama N, Nonaka M, Sasaki M, Wada Y (1994) Gene analysis in a case of carnitine palmitoyltransferase (CPT) II deficiency. J Jpn Soc Study Inborn Errors Metab 10:154 (in Japanese).

Handig I, Dams E, Taroni F, Van Laere S, de Barsy T, Willems PJ (1996) Inheritance of the S113L mutation within an inbred family with carnitine palmitoyltransferase enzyme deficiency. Hum Genet 97:291–293.

Holton TA, Graham MW (1991) A simple and efficient method for direct cloning of PCR products using ddT-tailed vectors. Nucleic Acids Res 19:1156.

Hug G, Bove KE, Soukup S (1991) Lethal neonatal multiorgan deficiency of carnitine palmitoyltransferase II. N Engl J Med 325:1862–1864.

Matsubara Y, Narisawa K, Tada K (1992) Medium-chain acyl-CoA dehydrogenase deficiency: molecular aspects. Eur J Pediatr 151:154–159.

Morita J, Yano S, Nisiyori J, Nakao M, Yoshida I, Yoshino M, Yamasita H, Inoguchi T (1991) Infantile form of carnitine palmitoyltransferase II deficiency with dicarboxylic aciduria at acute phase. J Jpn Soc Study Inborn Errors Metab 7:110 (in Japanese).

Ogasawara M, Matsubara Y, Mikami H, Narisawa K (1994) Identification of two novel mutations in the methylmalonyl-CoA gene with decreased levels of mutant mRNA in methylmalonic acidemia. Hum Mol Genet 3:867–872.

Romeo G, McKusick VA (1994) Phenotypic diversity, allelic series and modifier genes. Nature Genet 7:451–453.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Taniwaki T, Kira J, Kitaguchi T, Goto I, Sugiyama N (1989) A female case of carnitine palmitoyltransferase deficiency. Clin Neurol 29:382–384.

Taroni F, Rosenberg LE (1991) The precursor of the biotin-binding subunit of mammalian propionyl-CoA carboxylase can be translocated into mitochondria as apo- or holoprotein. J Biol Chem 266:13267–13271.

Taroni F, Verderio E, Fiorucci S, Cavadini P, Finocchiaro G, Uziel G, Lamantea E, Gellera C, DiDonato S (1992) Molecular characterization of inherited carnitine palmitoyltransferase II deficiency. Proc Natl Acad Sci USA 89:8429–8433.

Taroni F, Verderio E, Dworzak F, Willems PJ, Cavadini P, DiDonato S (1993) Identification of a common mutation in the carnitine palmitoyltransferase II gene in familial recurrent myoglobinuria patients. Nature Genet 4:314–320.

Taroni F, Gellera C, Cavadini P, Baratta S, Lamantea E, Dethlefs S, DiDonato S, Reik RA, Benke PJ (1994) Lethal carnitine palmitoyltransferase (CPT) II deficiency in newborns: A molecular genetic study. Am J Hum Genet 55 (supplement): A245.

Taroni F, Uziel G (1996) Fatty acid mitochondrial β-oxidation and hypoglycemia in children. Curr Op Neurol 9:477–485.

Verderio E, Cavadini P, Pandolfo M, DiDonato S, Taroni F (1993) Two novel polymorphisms of the human carnitine palmitoyltransferase II (CPTII) gene. Hum Mol Genet 2:334.

Verderio E, Cavadini P, Montermini L, Wang H, Lamantea E, Finocchiaro G, DiDonato S, Gellela C, Taroni F (1995) Carnitine palmitoyltransferase II deficiency: Structure of the gene and characterization of two novel disease-causing mutations. Hum Mol Genet 4:19–29.

Woeltje KF, Kuwajima M, Foster DW, McGarry JD (1987) Characterization of the mitochondrial carnitine palmitoyltransferase enzyme system. J Biol Chem 262:9822–9827.

Woeltje KF, Esser V, Weis BC, Cox WF, Schroeder JG, Liao ST, Foster DW, McGarry JD (1990) Inter-tissue and inter-species characteristics of the mitochondrial carnitine palmitoyltransferase enzyme system. J Biol Chem 265:10714–10719.

Yamamoto S, Abe H, Kohgo T, Ogawa A, Ohtake A, Hayashibe H, Sakuraba H, Suzuki Y, Aramaki S, Takayanagi M, Hasegawa S, Niimi H (1996) Two novel gene mutations (Glu174Lys, Phe383Tyr) causing the "hepatic" form of carnitine palmitoyltransferase II deficiency. Hum Genet 98:116–118.

Zierz S (1994) Carnitine palmitoyltransferase deficiency. In Engel AG, Franzini-Armstrong C (eds): Myology, 2nd ed. New York: McGraw-Hill, pp 1577–1586.