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Emi Ogawa · Masaki Kanazawa · Shigenori Yamamoto
Satoko Ohtsuka · Atsushi Ogawa · Akira Ohtake
Masaki Takayanagi · Yoichi Kohno

Expression analysis of two mutations in carnitine palmitoyltransferase IA deficiency

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Abstract Carnitine palmitoyltransferase I (CPT I) is one of the carnitine cycle enzymes that plays a role in the transportation of long-fatty acids into the mitochondria for β-oxidation. Hepatic carnitine palmitoyltransferase I (CPT IA) is one of the isozymes of CPT I, and its deficiency results in an autosomal recessive mitochondrial fatty acid oxidation disorder. To date, 19 patients with CPT IA deficiency and 9 CPT IA mutations have been reported. Recently, six novel mutations in the *CPT IA* gene were reported in Japanese patients with CPT I deficiencies who were clinically diagnosed as having a Reye-like syndrome. One of these mutations was a missense mutation, 1079A>G (E360G). The other was a splicing mutation, 2027-2028+2delAAGT, which caused aberrant splicing transcripts, whereas 1876-2028del, 2027-2028insGTCTCTTCC ACTTCTTCC, and 2026-2028del were three aberrant transcripts that kept reading in-frame. In this report, an expression assay using SV40 transformed fibroblasts was performed to investigate the consequences of these two mutations on enzyme activity and protein levels. Molecular analysis in this study revealed that the two mutations 1079A>G and 2028+2delAAGT were the disease-causing mutations.

Key words Carnitine palmitoyltransferase · Carnitine cycle · Mitochondrial β-Oxidation · Hypoketotic hypoglycemia · Reye-like syndrome · Expression study

E. Ogawa (✉) · M. Kanazawa · S. Yamamoto · S. Ohtsuka
A. Ogawa · Y. Kohno
Department of Pediatrics, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
Tel. +81-43-226-2144; Fax +81-43-226-2145
e-mail: eogawa@pediat3.m.chiba-u.ac.jp

A. Ohtake
Department of Pediatrics, Saitama Medical University, Saitama, Japan

M. Takayanagi
Division of Metabolism, Chiba Children's Hospital, Chiba, Japan

S. Yamamoto
Division of Pediatrics, National Shimoshizu Hospital, Chiba, Japan

Introduction

Long-chain fatty acids serve as an important source of energy, and the mitochondrial β-oxidation of long-chain fatty acids is a major source of energy production, especially during conditions of fasting or metabolic stress. Mitochondrial β-oxidation is a complex process that requires the concerted action of several enzymes. This enzymatic complex is made up of three distinct proteins named carnitine palmitoyltransferase I (CPT I) (MIM600528), carnitine-acylcarnitine translocase (CACT) (MIM 212138), and carnitine palmitoyltransferase II (CPT II) (MIM 600650) (Bieber 1988; McGarry and Brown 1997). The first component of the process, CPT I, is tightly associated with the outer mitochondrial membrane and transfers long-chain acyl residues from acyl-coenzyme A (CoA) esters to carnitine.

Two different isoforms of CPT I have been described: a liver type (CPT IA) and a muscle type (CPT IB) (Britton et al. 1995; Yamazaki et al. 1996). CPT IA deficiency is a rare autosomal recessive disorder, usually presenting in infancy and characterized by recurrent episodes of hypoketotic hypoglycemia, hepatomegaly, hepatic dysfunction, seizure, and coma. These symptoms are similar to Reye syndrome and triggered by fasting or a viral illness.

Recently, *CPT IA* cDNA has been cloned in rat (Esser et al. 1993) and human (Britton et al. 1995). Nineteen unrelated patients with CPT IA deficiency (Bergman et al. 1994; Bonnefont et al. 1989; Bougneres et al. 1981; Brown et al. 2001; Demaugre et al. 1988; Falik-Borenstein et al. 1992; Gray et al. 1991; Haworth et al. 1992; Innes et al. 1997, 2000; IJlst et al. 1998; Schaefer et al. 1997; Stanley et al. 1992; Tein et al. 1989; Vianey-Saban et al. 1993) and nine mutations in the *CPT IA* cDNA (Brown et al. 2001; IJlst et al. 1998; Prip-Buus et al. 2001) have been reported to date. Detection of the molecular and biological findings of affected patients has an important impact on the elucidation of the structure–function relationships concerning CPT IA.

Yamamoto et al. (2000) reported three nonsense mutations, one missense mutation, and two splicing mutations in

four Japanese patients with CPT IA deficiency. In the report, they described one missense mutation, 1079A>G, and a splicing mutation, 2028+2delAAGT. The missense mutation, 1079A>G, causes just one amino acid change, E360G. The splicing mutation, 2028+2delAAGT, causes the aberrant splicing transcripts, 1876-2028del, 2027-2028insGTCTCTTCCACTTCTTCC (abbreviated to 2027-2028ins18nt), and 2026-2028del; however, these aberrant transcripts were deleted or inserted, maintaining their reading frame. Therefore, it is necessary to confirm that these two mutations decrease the activity of CPT I and are the disease-causing mutations. In this report, an expression assay using transformed fibroblast was performed to investigate the effect of these mutations on the enzyme activity and protein level.

Patients and methods

Case reports

Table 1 shows the clinical and laboratory data, and the results of the molecular and enzymatic characterization of the two patients, which were reported by Yamamoto et al. (2000). The patients were a boy and a girl from unrelated families. The age of onset was 21 months and 9 months, respectively. They were the children of nonconsanguineous parents. Both presented with almost normal growth and development. Patient 1 had one sibling who died from a Reye-like syndrome. Patient 1 and patient 2 had episodes of Reye-like syndrome associated with hypoketotic hypoglycemia. The blood glucose of each patient was 22 mg/dl and 28 mg/dl, respectively. No ketones were detected in the urine at the time of collapse. Free carnitine levels were elevated in the serum. They were given intravenous glucose. From the clinical and laboratory data, these patients were suspected as having fatty acid oxidation disorders. Skin fibroblasts from these patients were examined for CPT activity, sequence analysis, immunoblot analysis, and Northern blot analysis.

The CPT I activity of each patient was markedly reduced. Patient 1 was compound heterozygous for 96T>G (Y32X) and 1079A>G (E360G); patient 2 carried a 2027-2028+2delAAGT in the paternal allele. The other mutation was not found in the maternal allele in the analysis of reverse transcriptase-polymerase chain reaction (RT-PCR) products. In immunoblot experiments using an antibody raised against CPT IA, the signal from the CPT IA protein was very weak in the patients' fibroblasts.

Informed consent for this study was obtained from each parent of the patients.

Northern blot analysis

Samples of 1 μg of mRNA isolated from control fibroblasts or fibroblasts of patient 2 were analyzed. Northern blot analysis was conducted essentially as described previously (Thomas 1980). The blot was hybridized with an RNA probe corresponding to nt875-1961 of the cDNA and a β-actin cDNA probe as a control for RNA loading. The probes were labeled with digoxigenin-uridinetriphosphate using a DIG RNA Labeling kit (SP6/T7) (Boehringer Mannheim, Mannheim, Germany).

Expression studies

Plasmid construction. The expression plasmids were constructed for expression of mutations: 1079A>G, 1876-

Table 1. Clinical and laboratory data, and the results of molecular analysis of two patients with carnitine palmitoyltransferase IA deficiency (Yamamoto et al. 2000)

	Patient 1	Patient 2
Age of onset	21 Months	9 Months
Sex	Male	Female
Family history		
Consanguineous marriage	None	None
Sibling's death	1 Sibling	None
Blood glucose	22 mg/dl	28 mg/dl
Ketone body	No ketonuria	No ketonuria
Clinical diagnosis	Reye-like illness	Reye-like illness
Carnitine (μM)		
Free/acyl	104.1/66.0	88.6/137.2
cDNA	96T>G	1876-2028del
	1079A>G	2027-2028ins18nt
		2026-2028del
Genomic DNA	96T>G (Maternal)	2027-2028+2delAAGT(Paternal)
	1079A>G (Paternal)	
Protein	Y32X	626-676 del
	E360G	676 ins 6aa
		E676 del
CPT I activity	None	None
Immunoblot analysis	Remarkably decreased	Remarkably decreased

CPT I, Carnitine palmitoyltransferase I; del, deletion; ins, insertion; nt, nucleotide; aa, amino acid

2028del, 2027-2028ins18nt, and 2026-2028del. The complete open reading frame of the gene coding for CPT IA from the control and the patients was amplified. The amplified wild-type and mutant *CPT IA* cDNAs were sequenced to assess the integrity of the RT and PCR processes. Both cDNAs were then retrieved from pGEM-T (Promega, Madison, WI, USA) as *Eco*RI fragments and subcloned into the mammalian expression vector pCAGGS (Niwa et al. 1991) previously cut by *Eco*RI to construct the pCAGGS-wild type, pCAGGS-1079A>G, pCAGGS-1876-2028del, pCAGGS-2027-2028ins18nt, and pCAGGS-2026-2028del.

DNA transfection and enzyme assay. Fibroblasts from a Japanese patient with CPT IA deficiency reported by Yamamoto et al. (2000) were immortalized by SV40, and were used for expression experiments. This patient was a compound heterozygote with nonsense mutations W475X and Y498X, and exhibited remarkably reduced CPT I activity and undetectable CPT IA protein by immunoblot analysis. Because CPT IA protein is a monomer enzyme, we suggest that the CPT IA protein of the patient does not interfere with the results of the expression study. Transfected cells were cultured in 10-cm culture dishes in RPMI-1640 medium, supplemented with 20% fetal bovine serum, and incubated at 37°C in 5% CO_2. Ten micrograms of each expression plasmid was cotransfected with 3 μg of a plasmid bearing the bacterial β-galactosidase gene (pAc-lacZ [Miyazaki et al. 1989]) into the cells using the transfection kit, FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany). After 18 h the cells were harvested by trypsinization and washed twice with phosphate-buffered saline. The cells were sonicated and the extracts were used for the CPT I assays, β-galactosidase assay, and immunoblot analysis.

CPT activity. CPT activity was measured essentially as described by Demaugre and co-workers (1991). Briefly, it was assayed as palmitoyl-L-(methyl-^{14}C) carnitine formed from L-(methyl-^{14}C) carnitine and palmitoyl-CoA. The palmitoyl-L- (methyl-^{14}C) carnitine was extracted with isopropanol, and measured for CPT I activity. CPT I activity was expressed by the difference of the CPT I activity with and without malonyl-CoA. Results of the assay were expressed as nanomoles of palmitoyl-L-(methyl-^{14}C) carnitine/min/mg protein.

Immunoblot analysis. Polyacrylamide gel (7.5%) electrophoresis was performed by the methods of Laemmli (1970). The cell lysate was prepared as described above. The dose of protein applied was varied in inverse proportion to β-galactosidase activity. Samples containing protein that had 13.6×10^{-5} units of β-gal actosidase activity (2.57–10.0 μg of protein) were applied to each lane. Proteins were transferred to a polyvinylidine difluoride (PVDF) membrane, and incubated with anti-human CPT IA antiserum. Antibodies against CPT IA were produced in a rabbit by injecting the chemically synthesized 16-residue peptide (14V-29R: the cytoplasmic domain). Immunization protocols were as described (Kanazawa et al. 1997). CPT IA proteins were then detected with the ABC-PO kit (Vector, Burlingame, CA, USA) and the ECL kit (Amersham Pharmacia Biotech, Tokyo, Japan).

Results

Northern blot analysis

Using mRNA obtained from fibroblasts from patient 2, we performed Northern blot analysis (Fig. 1). β-Actin mRNA used as the control was equal both in this patient and in the control. Northern blot analysis of patient 2 revealed two mRNA species. One was a 4.7-kb mRNA, which was the same size as the control mRNA band. The other mRNA species was 6.7 kb in length. Because the cell viability of the fibroblasts from patient 1 was not good, we could not obtain mRNA from the fibroblasts and could not perform a Northern blot analysis for patient 1.

Expression studies

CPT I activity of each mutation. Table 2 shows the results of the enzyme activities observed in the expression analysis of each mutation. Homogenates of both transfected and untransfected cells were prepared and were measured for CPT I activity. Both the untransfected control cells and the cells transfected with the expression plasmid pCAGGS did not display any CPT activity. Transfection with plasmids encoding mutations 1079A>G, 1876-2028del, 2027-2028ins18nt, and 2026-2028del resulted in 0%, 7.80%, 22.4%, and 101% of control, respectively.

Immunoblot analysis. Figure 2 shows the results of an immunoblot from the expression analysis of each mutation.

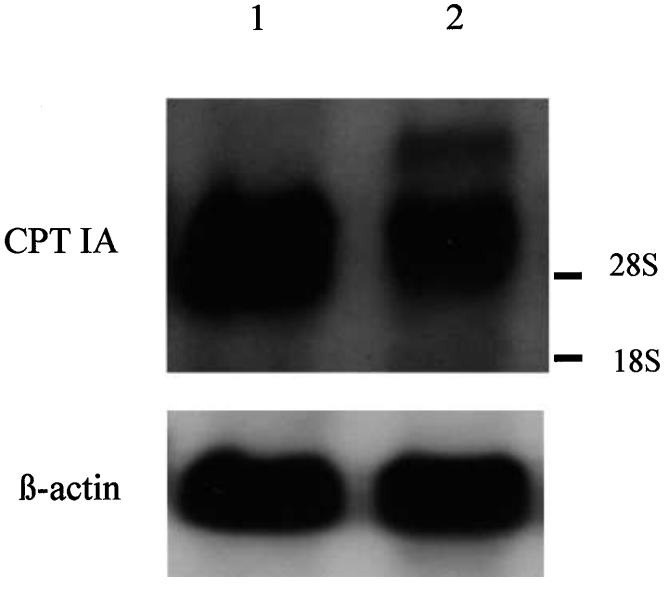

Fig. 1. The results of Northern blot analysis. *Lane 1*, control subjects; *lane 2*, patient 2. One microgram of mRNA was applied to each lane

Table 2. Activity of carnitine palmitoyltransferase I in the expression study

Plasmid	CPT I			β-galac tosidase	CPT I/β-galac tosidase	
	Malonyl-CoA–	Malonyl-CoA+	activity			
Untransfected	0.29	0.27	0.02	13.6	1.47	3.38%
pCAGGS	0.31	0.28	0.03	52.7	0.569	1.31%
pCAGGS-wild type	2.29	1.19	1.10	25.3	43.6	100%
pCAGGS-1079A>G	0.25	0.25	0.00	23.4	0	0%
pCAGGS-1876-2028del	0.24	0.17	0.07	21.2	3.40	7.80%
pCAGGS-2027-2028ins18nt	0.52	0.33	0.19	19.44	9.80	22.4%
pCAGGS-2026-2028del	1.77	0.91	0.86	19.5	44.3	101%

CPT I activity was assayed in fibroblast homogenates as described in Methods
CPT I activity, nmol/min/mg protein
β-galactosidase activity, U/g protein
CPT I, Carnitine palmitoyltransferase I; del, deletion; ins, insertion; nt, nucleotide

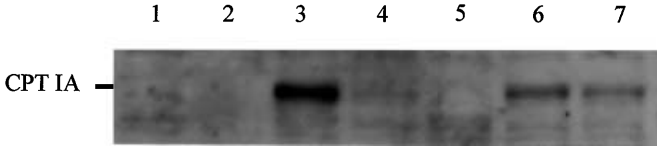

Fig. 2. Immunoblot showing expression of wild-type, deletion, and point mutant carnitine palmyltransferase IA (*CPT IA*). *Lane 1*, untransfected; *lane 2*, pCAGGS; *lane 3*, pCAGGS-wild type; *lane 4*, pCAGGS-1079A>G; *lane 5*, pCAGGS-1876-2028del; *lane 6*, pCAGGS-2027-2028ins18nt; *lane 7*, pCAGGS-2026-2028del

In fibroblasts transfected with the pCAGGS-wild type, pCAGGS-2027-2028ins18nt, and pCAGGS-2026-2028del, the CPT IA protein was found to be clearly present, whereas the signals were weak in fibroblasts transfected with the pCAGGS-1079A>G and pCAGGS-1876-2028del, which showed relatively low CPT I activity.

Discussion

We performed an expression analysis of CPT IA mutations, including 1079A>G, 1876-2028del, 2027-2028ins18nt, and 2026-2028del, to investigate the effect of these mutations on enzyme activity.

Patient 1 is a compound heterozygote for the nonsense mutation Y32X and the missense mutation E360G. This nonsense mutation causes premature termination, resulting in immature CPT IA proteins that are unlikely to function normally. The consequence of the E360G substitution on enzyme activity and protein level was investigated by expressing normal and mutated *CPT IA* cDNA using transformed fibroblasts. This mutation decreased CPT I activity, as demonstrated by expression studies with the 1079A>G mutant *CPT IA* cDNA, which displayed 0.0% of the activity of the expressed wild-type *CPT IA* cDNA. Moreover, the level of expressed protein of the 1079A>G mutant CPT IA was remarkably decreased compared with that of the wild type. These data show that the 1079A>G (E360G) mutation is the disease-causing mutation and that the marked reduction of CPT I activity observed in the patient was caused by instability of the enzyme. The glutaric acid at position 360 is located in a well-conserved region (McGarry and Brown 1997) in both human and rat; therefore, this amino acid change is thought to destroy an enzyme activity of CPT I. Indeed, the role of the C-terminal domain as part of a catalytic domain has been reported by Zammit and coworkers (Zammit et al. 2001). As described above, these two mutations found in patient 1 do not produce a normal CPT IA protein, and are proposed to be disease-causing mutations in the gene.

In patient 2, the splicing mutation 2028+2delAAGT was detected. The 4-bp deletion changes the consensus sequence of the splicing donor site and is likely to be the source of all the mutations that were found in this patient by an analysis of RT-PCR products. The splicing mutation led to three truncated transcripts, 1876-2028del, 2027-2028ins18nt, and 2026-2028del. One of these abnormally spliced transcripts, 1876-2028del, which is a 153-bp deletion within frame, that is deleted of exon 15, causes a deletion of 51 amino acids from amino acid positions 626 to 676. In this expression analysis, this mutation resulted in the absence of detectable CPT I activity when compared with expressed wild-type CPT IA. The expressed protein derived from this transcript was hardly detectable in immunoblot analysis. These findings suggest that this mutation makes the CPT IA protein immature and unstable, explaining the deficient CPT I activity.

The second transcript, 2027-2028ins18nt, which was revealed to have inserted 18 nucleotides adjunct to the 3' end of exon 15, resulted in the insertion of six amino acids from amino acid positions 676 to 677. In this expression analysis, this mutation resulted in 22.4% of CPT I activity when compared with the expressed wild-type CPT IA. The expression level of the mutated protein was slightly lower than that of the wild type. This mutation was suspected to cause a slight impairment in the stability of the enzyme. A CPT IA protein derived from this mutated transcript is supposed to have inserted six amino acids; however, we suggest from these results that this insertion does not remarkably influence secondary and tertiary structures of the CPT IA protein, and does not severely influence the enzyme activity of CPT I.

The third transcript, 2026-2028del, which was revealed to result in the deletion of three nucleotides adjunct to the 5′ end of exon 15, causes one amino acid deletion within frame, E676del. Although the expression level of the mutated protein was slightly lower than that of the wild type, this mutation resulted in 101% of CPT I activity when compared with the expressed wild-type CPT IA in this expression analysis. These data showed that this mutation was not the disease-causing mutation.

The result of the Northern blot analysis from patient 2, who had the splicing mutation 2027-2028+2delAAGT, revealed that the transcript showing almost the same size as the control was derived from these three truncated mRNAs, 1876-2028del, 2027-2028ins18nt, and 2026-2028del. Yamamoto et al. (2000) could identify only one mutation in this patient after analyzing RT-PCR products and supposed that this patient had another mutation because the CPT IA deficiency is an autosomal recessive disorder. A 6.7-kb mRNA was also detected in our Northern blot analysis. The other mutation could not be identified in the previous report (Yamamoto et al. 2000), and it is possible that it was because this long truncated 6.7-kb mRNA, which is thought to carry the other mutation, could not be amplified by RT-PCR.

Both the activity and the protein level of the CPT IA of the fibroblasts from patient 2 were remarkably reduced, while the expression analysis showed the CPT I activity from the transcripts of the 2027-2028ins18nt and 2026-2028del did not reveal a dramatic decrease. We hypothesize that the amount of the two truncated mRNAs, 2027-2028ins18nt and 2026-2028del, were remarkably smaller than that of the 1876-2028del.

To date, reported expression studies of CPT IA mutations were performed using *Saccharomyces cerevisiae* (IJlst et al. 1998; Prip-Buus et al. 2001), but handling and culturing *S. cerevisiae* is complicated. We performed expression studies using SV40 transformed fibroblasts obtained from the patient who was a compound heterozygote with the nonsense mutations W475X and Y498X. We think it was very useful to use this fibroblast line in the expression study instead of *S. cerevisiae* because of the ease of handling and culturing.

Molecular biological analysis revealed that these two mutations, 1079A>G (E360G) and 2028+2delAAGT, decrease the enzyme activity of CPT I and were the disease-causing mutation in the *CPT IA* gene.

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