Thermolabile phenotype of carnitine palmitoyltransferase II variations as a predisposing factor for influenza-associated encephalopathy

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Abstract To assess the etiology of influenza-associated encephalopathy (IAE), a surveillance effort was conducted during 2000–2003 in South-West Japan. All fatal and handicapped patients except one (4/34 patients) exhibited a disorder of mitochondrial β-oxidation evoked by the inactivated carnitine palmitoyltransferase II (CPT II) with transiently elevated serum acylcarnitine ratios ($C_{16:0} + C_{18:1})/C_2 > 0.09$ during high-grade fever. Analyses of genotypes and allele compositions of CPT II revealed a thermolabile phenotype of compound heterozygotes for [1055T > G/F352C] and [1102G > A/V368I], which shows a higher frequency in IAE patients than healthy volunteers ($P < 0.025$). The thermolabile phenotype of CPT II variations may be a principal genetic background of IAE in Japanese.

Keywords: Carnitine palmitoyltransferase II; DNA sequence variation; Influenza-associated encephalopathy; Thermal inactivation

1. Introduction

Influenza-associated encephalopathy (IAE) is a severe neurologic complication of influenza infection which is known to be distinct from Reye's syndrome [1,2]. The number of cases of IAE has increased in recent years, with more than 100 children aged below 5–6 years dying annually from IAE in Japan [3,4]. IAE results in high morbidity and mortality and is characterized by a high-grade fever accompanied within 12–48 h by febrile convulsions, often leading to coma and multiple-organ failure. Because the frequency of IAE is higher in Japanese than it is in Caucasians, it is possible that genetic factors play an important role in the aetiology of IAE.

During the last four influenza seasons of 2000–2003, we conducted a survey to investigate the etiology and constitutional predispositions in patients susceptible IAE in South-West Japan. We found that almost all fatal or handicapped IAE patients exhibited transiently elevated the serum levels of long-chain acylcarnitines during high-grade fever >40 °C. The result suggests that the high-risk patients have thermolabile genetic backgrounds of enzymes in the long-chain fatty acid metabolism. In the present study, we report thermolabile carnitine palmitoyltransferase II (CPT II) variation, predominantly found in Japanese IAE children, and discuss the etiology of IAE as a 'thermolabile phenotype of polymorphic variation'.

2. Materials and methods

2.1. Patients

This investigation was approved by the ethics review committee for human genome analysis at our institution. All participants granted their written informed consent. Surveillance for IAE was conducted during the influenza seasons of 2000 through 2003 in South-West Japan, and a total of 34 patients were diagnosed as having IAE. The diagnosis of IAE was made according to the clinical signs of the disease [3]. All 34 patients had viral antigen, the abrupt onset of seizure and coma that occur within 12–48 h after beginning of a high-grade fever, although these patients did not have previous episodes. One patient (patient #21) had ingested dichophenac sodium at the onset of fever and later died, but fatty degeneration in the liver, a typical pathological finding of Reye's syndrome [5,6], was not observed. Thirteen IAE patients, four familial relations of patient #21 and 79 healthy volunteers agreed to undergo the genome analyses.

2.2. Clinical data analyses

EDTA-treated peripheral blood, urine and specimens from throat swabs were obtained from the patients. Profiles of organic acids in urine and acylcarnitines in serum were analyzed by gas chromatography–mass spectrometry [7] (Shimazu Qp5000 Model, Shimazu, Kyoto, Japan) and electrospray tandem mass spectrometry [8] (TSQ7000 Model, Thermo-Quest, Tokyo, Japan), respectively. Influenza virus antigen was detected by enzyme-linked immunosorbent assay (Becton–Dickinson) in specimens from throat swabs.

2.3. Assay of CPT II activity

CPT II activities were measured in the homogenates of liver biopsies and COS-7 cells transfected with wild-type (WT) and polymorphic variant CPT II cDNAs in the presence of 1% Tween 20 in the reaction mixture, by detecting of the palmitoyl-L-[methyl-^{3}H]carnitine formed from L-[methyl-^{3}H] carnitine and palmitoyl-CoA [9]. For the analysis of the heat stability of WT and variants of CPT II, the activities of liver and cellular homogenates were measured after incubating the samples at 37 and 41 °C.

2.4. Analysis of genomic CPT II

Genomic DNA from whole blood was purified as previously described [10]. PCR of five exons of the *CPT II* gene was carried out with intron-based primers (Table 1) in genomic DNA. For haplotype analysis the CPT II exon 4 region was cloned into pCR®2.1

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Table 1
Exon primers used for PCR amplification of the *CPT2* gene; MA or MB primer used for variant induction

Region	Forward primer	Reverse primer	Product size (bp)
Exon 1	cttgtgtttagactccagaactcc	gtcatgagtgactgcagtcaggttg	292
Exon 2	ctgtcagccttacactgaccc	aactctcggggcttggtc	305
Exon 3	tttagggctatgctgttggg	aggaagggaggatgagacgt	358
Exon 4-1	ctctggaggttgatgccatt	acccaagcactgaggacaag	1472
Exon 4-2	tagagttcagtgggtagctggct	atccaggcacatctgaagtac	401
Exon 5	tttcctgaggtccttttccatcctg	atgaggaagtgatggtagcttttca	425
MA-V352I	ggcacaaaccgctggtgtgataaat	atttatcacaccagcggtttgtgc	
MB-V368I	ctactgccgtccactttagcactcttg	caagagtgctcaaagtggacggcagtag	

vector (Invitrogen). The sequences of the PCR products and cloned *CPT II* gene were analyzed with the ABI DyeDeoxy Terminator Cycle Sequencing Kit on an ABI-PRISM 3100 Genetic Analyzer (PE-Applied Biosystems). Each PCR product was sequenced in both strands, and the analysis was performed at least twice independently.

2.5. Expression of WT and variant CPT IIs in COS-7 cells

A full-length WT CPT II cDNA clone (pCMV6-WT) containing the entire coding region of human CPT II was a gift of V. Esser, the University of Texas. Plasmid pCNV6-WT was used as a parental vector to generate three full-length variant CPT II cDNA clones, pCMV6-MA [1055T > G/F352C], pCMV6-MB [1102G > A/V368I] and pCMV6-MA + B [1055T > G/F352C] + [1102G > A/V368I] by means of a QuickChange® site-directed mutagenesis Kit (Stratagene). The primers used for variant induction are listed in Table 1. The substitutions and integrity of the CPT II cDNAs were confirmed by sequence analysis. pSVβ-Galactosidase control vector (Promega) was co-transfected with various pCMV-6-CPT II plasmids as an internal standard for the monitoring of transfection efficiency. Mock transfection was also carried out as control. After transfection for 72 h, COS-7 cells were washed twice with saline and CPT II activities of WT and variants were analyzed.

3. Results

3.1. Patients and acylcarnitine ratios

Thirty-four patients (22 male and 12 female) with no underlying disease, ranging in age from 0 to 16 years, with a mean age of 4.7 ± 2.8 years, had IAE. Influenza A, B and A + B virus antigen were detected in nasopharyngeal swabs at 91.2%, 5.9% and 2.9%, respectively. A single patient (patient #21, which was one of the fatal cases) used dichrophenac during the influenza episode.

Laboratory tests of patient serum revealed that 41.2% of the IAE patients exhibited characteristic elevations of serum acylcarnitine ratios [11] $(C_{16:0} + C_{18:1})/C_2 > 0.048$ an upper cutoff value [8,11] (Fig. 1). In particular, over half the patients in the severe IAE group (seven patients), i.e., those with the ratios >0.09, turned to fatal (three female) and handicapped (one male) outcomes. These data indicate that patients in the high-risk group have a marked disorder(s) of mitochondrial long-chain fatty acid metabolism. The most common inborn errors of mitochondrial fatty acid β-oxidation and related metabolism in Japanese are due to CPT II deficiency, with an accumulation of long-chain acylcarnitine in serum and glutaric aciduria type 2 (GA2) at 26.6% and 21.9%, respectively [12]; however, the frequency of these are relatively low at 11% and 5.5%, respectively, in Caucasians, who have the most common deficiency of medium-chain acyl-CoA dehydrogenase (MCAD), which occurs at about 36.6% [13]. The $(C_{16:0} + C_{18:1})/C_2$ ratios of all IAE patients tested, however, normalized or decreased to the borderline ratios between

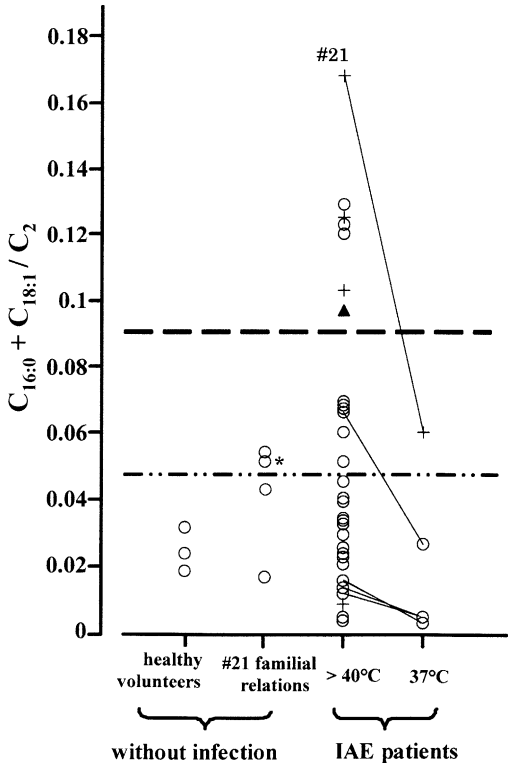

Fig. 1. Distribution of $(C_{16:0} + C_{18:1})/C_2$ ratios of the patients with IAE at the time of high-grade fever/convulsion and normal temperature conditions. The acylcarnitine ratios of patients suffering from IAE, and the family members of patient #21 and one volunteer without infection were analyzed. Upper cut-off range = 0.048 and high-risk patient range = 0.09 are indicated by the thin dashed and bold dashed lines, respectively. (+) Fatal; (▲) handicapped; (*) brother, who had the same genotype as in patient #21.

0.048 and 0.06 at normal temperature after febrile convulsion. These results suggest that the ratios are transiently elevated during febrile convulsions at >40 °C. Familial relations except the mother of patient #21, who carried thermolabile phenotype of CPT II, as described below, exhibited borderline ratios between 0.042 and 0.054 under normal temperature, and a brother who had identical alleles with patient #21, exhibited the ratio of 0.051. Two patients in the high-risk group with ratios >0.09 recovered after infection for 3–4 weeks without any sequelae.

There was one fatal IAE patient (patient #16) with the ratio of 0.004 (Fig. 1), who was diagnosed GA2 based upon an abnormal urinary organic acid profile (data not shown), a disorder of electron transfer in mitochondria. All the other IAE patients with the ratios <0.09 recovered without any severe sequelae. Octanoylcarnitine, a diagnostic marker of MCAD