Brain & Development 41 (2019) 862–869

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

Original article

Thermolabile polymorphism of carnitine palmitoyltransferase 2: A genetic risk factor of overall acute encephalopathy

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Received 28 March 2019; received in revised form 10 June 2019; accepted 10 July 2019

Abstract

Objectives: Acute encephalopathy is an acute brain dysfunction after preceding infection, consisting of multiple syndromes. Some syndromes, such as acute encephalopathy with biphasic seizures and late reduced diffusion (AESD), are severe with poor outcome, whereas others, such as clinically mild encephalitis/encephalopathy with reversible splenial lesion (MERS), are mild with favorable outcome. Previous study reported the association of the thermolabile polymorphism in Carnitine Palmitoyltransferase 2 (*CPT2*) gene and severe syndromes of acute encephalopathy. To further explore the pathogenetic role of CPT2 in acute encephalopathy, we conducted a case-control association study of a typical thermolabile *CPT2* polymorphism, rs2229291, in 416 patients of acute encephalopathy, including both severe and mild syndromes.

Methods: The case cohort consisted of 416 patients, including AESD, MERS, and other syndromes. The control subjects were 100 healthy Japanese. rs2229291 was genotyped by Sanger sequencing. Genetic distribution was compared between the patients and controls using Cochran-Armitage trend test.

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https://doi.org/10.1016/j.braindev.2019.07.008

Results: Minor allele frequency of rs2229291 was significantly higher in AESD ($p = 0.044$), MERS ($p = 0.015$) and entire acute encephalopathy ($p = 0.044$) compared to the controls. The polymorphism showed no significant association with influenza virus, or with outcome.

Conclusions: This study provided evidence that *CPT2* is a susceptibility gene for overall acute encephalopathy, including both severe and mild syndromes, and suggested that impairment of mitochondrial metabolism is common to various syndromes of acute encephalopathy.

Keywords: Carnitine palmitoyltransferase 2; Thermolability; Energy failure; Acute encephalopathy; Susceptibility gene; Predisposing factor; Genetic risk factor; Clinically mild encephalitis/encephalopathy with reversible splenial lesion; Acute encephalopathy with biphasic seizures and late reduced diffusion; Acute necrotizing encephalopathy; Acute encephalitis with refractory; Repetitive partial seizures/febrile infection-related epileptic syndrome

1. Introduction

Acute encephalopathy is an acute brain dysfunction which mostly occurs in the early, febrile period of infectious diseases. The pathogenic mechanism remains to be elucidated, however, numerous previous studies have highlighted three major pathologic processes: energy failure, excitotoxicity, and excessive inflammatory response, so-called cytokine storm. Acute encephalopathy is classified in two ways: virological classification based on the pathogens of precedent infection, and syndromic classification based on the clinicopathological features of encephalopathy [1]. With regard to pathogens of preceding infection, influenza virus is the most frequent, followed by human herpesvirus-6 (HHV-6) and rotavirus [1]. Despite worldwide distribution of these viruses, the incidence of acute encephalopathy is much higher in East Asians than in Caucasians, suggesting the involvement of genetic, predisposing factors. With regard to syndromes, acute encephalopathy with biphasic seizures and late reduced diffusion (AESD) is the most common, followed by clinically mild encephalitis/encephalopathy with reversible splenial lesion (MERS) and acute necrotizing encephalopathy (ANE). The syndromes are quite variable as to the severity and outcome [2]. In the severest syndrome, ANE, both deaths and severe neurologic sequelae are common. Other severe syndromes, such as AESD and acute encephalitis with refractory, repetitive partial seizures (or febrile infection-related epileptic syndrome, AERRPS/FIRES), are characterized by poor neurologic outcome, despite low mortality. On the other hand, MERS, a typical mild syndrome, causes neither death nor severe neurologic sequelae [1].

Recent studies have revealed a positive association of thermolabile polymorphisms of Carnitine Palmitoyltransferase 2 (*CPT2*) gene and acute encephalopathy. CPT2 is an enzyme related to β-oxidation, which localizes in the mitochondrial inner membrane and catalyzes formation of acyl-CoA [3]. Chen et al. found that compound heterozygotes for rs2229291 (c. 1055 T > G, p. Phe352Cys) and rs1799821 (c. 1102G > A, p. Val368Ile) show reduced CPT2 activity at a very high body temperature, and showed a high frequency of the thermolabile polymorphisms in patients of influenza-associated encephalopathy with fatal or severe outcome [4]. The same group subsequently observed that heterozygous rs2229291 alone reduces enzymatic activity [5]. Kubota et al. reported that the outcome of acute encephalopathy is poorer in cases with rs2229291 than those without [6]. We previously reported that minor allele frequency (MAF) of rs2229291 was significantly higher in 29 patients with severe acute encephalopathy, consisting of AESD and ANE [7]. Taken together, previous studies have shown the association of *CPT2* thermolabile polymorphism with severe syndromes of acute encephalopathy. To date, no study has ever attempted to explore the association of *CPT2* polymorphism and mild syndromes of acute encephalopathy.

The purpose of this study is to investigate the association between the *CPT2* polymorphism, rs2229291, and overall acute encephalopathy including mild syndromes such as MERS. We performed a case-control association study of rs2229291 in acute encephalopathy in 416 patients, the largest cohort ever. We also assessed the effect of influenza virus as a confounding factor, and the association between polymorphism and outcome.

2. Materials

2.1. Patients

We recruited Japanese patients with acute encephalopathy from all over Japan. We classified the patients into 5 groups: AESD, MERS, ANE, AERRPS/FIRES and Others, according to diagnostic criteria [2,8]. A total of 416 patients, including 239 with AESD, 40 with MERS, 47 with ANE, 18 with AERRPS/FIRES and 72 with Others, were enrolled. Of the 416, 15 with AESD and 10 with ANE had previously been genotyped and published [5]. All patients

were Japanese and mutually unrelated. The clinical characteristics of the patients are shown in Table 1.

We obtained written informed consent from the parents of the patients. This study was approved by the Ethics Committee of the Graduate School of Medicine, the University of Tokyo (No. G-3504).

2.2. Controls

As the control subjects, we used the rs2229291 genotype information obtained by our previous study [7]. In this study, we analyzed the *CPT2* genotype of control subjects, consisting of 100 healthy Japanese adults, 50 males and 50 females, at 20–69 years of age. Purified DNA from the controls was extracted from PSC (Pharma SNP Consortium) B cell lines and was supplied by the Human Science Research Resources Bank.

3. Methods

3.1. CPT2 genotyping

Peripheral blood samples were collected from the patients. Genomic DNA was extracted from the blood using standard protocols. Polymerase chain reaction (PCR) amplification of *CPT2* exons 4 was performed using AmpliTaq PCR kits (Applied Biosystems). The reaction mixture contained 2 μl buffer, 2 μl of 2 mM dNTP, 1 μl forward and reverse primers (10 pmol), 0.12 μl AmpliTaq and 1 μl genomic DNA (30 ng). Primer sequences for exon 4 was constructed based on the GenBank database in the National Center for Biotechnology Information (NCBI). Forward and reverse primers were 5′-GGAAATCCAGGCACATC TGA-3′ and 5′-TAGCAGCTGTGATGCCAGTC-3′, respectively. The PCR amplification protocol was as follows: denaturation at 95 °C for 9 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 7 min. The sequences of the PCR products of *CPT2* exons 4 were analyzed with an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit using 310 Genetic Analyzer (Applied Biosystems).

3.2. A case-control association study of rs2229291

We conducted case-control association studies of rs2229291 and the syndromes. We also made comparisons between influenza-associated and non-influenza-

Table 1
Clinical characteristics of the patients.

	AESD	ANE	MERS	AERRPS/FIRES	Others	Entire
Cases	239	47	40	18	72	416
Sex (F/M)	132/107	27/20	20/19	3/15	33/37	215/98
Age (months), mean (*SD*)	26.6(25.9)	32.1(28.4)	99.7(129.2)	81.4(35.4)	68.6(100.7)	43.9(67.4)
Pathogens (%)						
	HHV-6 (35)	HHV-6 (23.4)	influenza virus (30)	influenza virus (11)	influenza virus (13.8)	HHV-6 (24.8)
	influenza virus (10)	influenza virus (19.1)	*Salmonella* (5.0)	RSV (5.6)	HHV-6 (8.3)	influenza virus (15.1)
	RSV (4)	others and NI (57.5)	rotavirus (5.0)	others and NI (83.4))	rotavirus (4.2)	RSV (2.9)
	others and NI (51)		HHV-6 (5.0)		others and NI (73.7)	others and NI (57.2)
			others and NI (5 5)			
Outcome (%)						
Death	0 (0)	0 (0)	0 (0)	0 (0)	7(9.7)	7(1.7)
Profound sequelae	25(10.5)	9(19.1)	0 (0)	4(22.2)	14(19.4)	52(12.5)
Severe sequelae	45(18.8)	8(17.0)	0 (0)	1(5.6)	7(9.7)	61(14.7)
Moderate sequelae	27(11.3)	3(6.4)	0 (0)	6(33.3)	2(2.8)	38(9.1)
Mild sequelae	49(20.5)	8(17.0)	2(5.0)	5(27.8)	9(12.5)	73(17.5)
Full recovery	50(20.9)	3(6.4)	36(90)	0 (0)	22(30.6)	111(26.7)
NA	43(18.0)	16(34.1)	2(5.0)	2(11.1)	11(15.3)	74(17.8)

Abbreviations: AESD, acute encephalopathy with biphasic seizures and late reduced diffusion; ANE, acute necrotizing encephalopathy; MERS, clinically mild encephalitis/encephalopathy with reversible splenial lesion; AERRPS/FIRES, acute encephalitis with refractory, repetitive partial seizures/febrile infection-related epileptic syndrome; F, female; M, male; *SD*, standard deviation; HHV-6, human herpesvirus 6; RSV, respiratory syncytial virus; NI, not identified; NA, not available.

[*] Pathogens are listed in descending order of frequency.

[**] The frequency of influenza virus was significantly lower in AESD than in MERS and the remaining syndromes including ANE, AERRPS/FIRES and Others ($p = 0.00843$, significance threshold $\alpha = 0.017$).

associated cases to explore the influence of pathogens, and between poor and favorable outcome to assess the impact of rs2229291 on the disease severity.

3.3. Statistical analysis

Comparison of categorical information of each syndrome was assessed by chi-square or Fisher's exact test. Hardy-Weinberg Equilibrium (HWE) of rs2229291 in the case and control group was tested by chi-square or Fisher's exact test. Genetic association studies were conducted by Cochran-Armitage trend test. Bonferroni correction or Benjamini-Hochberg method (BH) was applied for multiple testing correction. False discovery rate q of BH methods was set to be $q < 0.05$, and adjusted p value for BH was described as pBH. The threshold for significance was set to $p < 0.05$.

Statistical analyses were performed using R software [9] and its package "epitools" [10].

4. Results

4.1. Clinical characteristics of the patients

This study noted differences among multiple syndromes as to precedent infection and outcome, in agreement with the findings of previous studies [1,2]. With regard to precedent infection, the most common pathogen was HHV-6 in AESD, and influenza virus in MERS and ANE. Influenza virus was significantly less frequent in AESD than in MERS and the remaining syndromes after Bonferroni correction ($p = 0.0084$, significance threshold $\alpha = 0.017$) (Table 1). With regard to outcome, the ratios of patients with moderate to profound sequelae in AESD, ANE, and AERRPS/FIRES were 40.6, 42.4 and 61.1%, respectively. MERS showed a favorable prognosis, with 90% of patients having no sequelae.

4.2. HWE of rs2229291 in the cases and in the controls

Based on the HWE tests, the genotypes distribution of the cases and the controls was in accordance with the universal law ($p > 0.05$). The results are shown in the Table 2.

4.3. Association between rs2229291 and each syndrome

MAF of rs2229291 was 20.7%, 28.8%, and 20.8% in AESD, MERS and entire acute encephalopathy, respectively. The G allele of rs2229291 had a significant association with MERS, AESD, and entire acute encephalopathy (Cochran-Armitage trend test, additive model, $pBH = 0.015$, 0.044, and 0.044, respectively) (Table 2). The remaining syndromes, including ANE and AERRPS/FIRES, showed no significant difference from the controls.

4.4. Comparison between influenza-associated and non-influenza-associated encephalopathy

The ratios of the pathogenic viruses were quite different among syndromes (Table 1). In order to evaluate the confounding effect of the virus, we compared the genotype distribution between influenza-associated and non-influenza-associated cases in AESD, MERS and entire acute encephalopathy using Cochran-Armitage trend test, and observed no significant differences in any of them after Bonferroni correction (AESD,

Table 2
Association of rs2229291 with acute encephalopathy syndromes.

	AESD (n = 239) n (%)	MERS (n = 40) n (%)	ANE (n = 47) n (%)	AERRPS (n = 18) n (%)	Entire (n = 416) n (%)	control (n = 100) n (%)
Genotype						
TT	150(62.8)	21(52.5)	32(68.1)	12(66.7)	261(62.7)	74(74)
TG	79(33.1)	15(37.5)	14(29.8)	6(33.3)	137(32.9)	25(25)
GG	10(4.1)	4(10.0)	1(2.1)	0(0.0)	18(4.4)	1(1)
p (HWE)	0.92	0.69	1.00	1.00	1.00	0.87
Allele						
T	379(79.3)	57(71.2)	78(83.0)	30(83.3)	659(79.2)	173(86.5)
G	99(20.7)	23(28.8)	16(17.0)	6(16.7)	173(20.8)	27(13.5)
Cochran-Armitage trend test additive model						
p	0.027	0.0029	0.41	0.6	0.019	
p_{BH}	0.044*	0.015*	0.51	0.6	0.044*	

Abbreviations: AESD, acute encephalopathy with biphasic seizures and late reduced diffusion; ANE, acute necrotizing encephalopathy; MERS, clinically mild encephalitis/encephalopathy with reversible splenial lesion; AERRPS/FIRES, acute encephalitis with refractory, repetitive partial seizures/febrile infection-related epileptic syndrome; HWE, Hardy-Weinberg Equilibrium.
The genetic association studies were conducted between 5 cohorts (AESD, MERS, ANE, AERRPS/FIRES and Entire) and the controls by Cochran-Armitage trend test (additive model). The Benjamini-Hochberg (BH) method was applied to regulate the false discovery rate.

Table 3
Comparison between influenza-associated and non-influenza-associated encephalopathy.

	AESD		MERS		All	
	Flu	non-Flu	Flu	non-Flu	Flu	non-Flu
Genotype						
TT	0	10	1	3	2	16
TG	10	69	2	13	18	119
GG	14	136	9	12	43	218
Allele						
T	10	89	4	19	22	151
G	38	341	20	37	104	555
Cochran-Armitage trend test Additive model						
p	0.98		0.13		0.32	

Abbreviations: AESD, acute encephalopathy with biphasic seizures and late reduced diffusion; MERS, clinically mild encephalitis/encephalopathy with reversible splenial lesion; Flu, influenza-associated; non-Flu, non-influenza-associated.
Statistical analysis was conducted by Cochran-Armitage trend test.
There was no significant difference related to influenza virus. (Cochran-Armitage trend test, additive model, AESD, $p = 0.98$; MERS, $p = 0.13$; Entire, $p = 0.32$; significance threshold $\alpha = 0.017$).

$p = 0.98$; MERS, $p = 0.13$; Entire, $p = 0.32$; significance threshold $\alpha = 0.017$) (Table 3).

4.5. Association between outcome and rs2229291

We obtained the outcome information of 342 patients. Based on Cochran-Armitage trend test, there was no significant association between outcome and rs2229291 genetic distribution, in any of AESD, ANE and entire acute encephalopathy after Bonferroni correction (AESD, $p = 0.81$; ANE, $p = 0.89$; Entire, $p = 0.31$; significance threshold $\alpha = 0.017$) (Table 4).

5. Discussion

The present study demonstrated that the *CPT2* thermolabile polymorphism rs2229291 is a predisposing factor for overall acute encephalopathy, including not only AESD, but also MERS. Two major syndromes of acute encephalopathy, AESD and MERS, were quite different

Table 4
Association of outcome and rs2229291.

		AESD		ANE		All	
		Poor	Not Poor	Poor	Not Poor	Poor	Not Poor
Genotype							
	TT	44	80	13	11	78	139
	TG	24	39	4	3	40	70
	GG	2	7	0	0	2	13
Allele							
	T	112	199	30	25	196	348
	G	28	53	4	3	44	96
Cochran-Armitage trend test additive model							
p		0.81		0.89		0.31	

Abbreviations: AESD, acute encephalopathy with biphasic seizures and late reduced diffusion; ANE, acute necrotizing encephalopathy; DQ, Developmental Quotient.
Poor: severe to profound neurological deficit.
Favorable: full recovery and mild to moderate neurological deficit.
Intellectual deficit was graded into none, mild (DQ 50–70), moderate (DQ 35–50), severe (DQ 20–35) and profound (DQ < 20).
Motor deficit was graded into none, mild (able to walk without support), moderate (unable to walk without support), severe (unable to sit), and profound (bedridden). The motor deficit criteria can be adjusted according to patients' age and patients' motor development before the onset of acute encephalopathy.
Outcome was judged by pediatricians who treated the patients.
Patients' outcome was defined according to intellectual and motor deficits. If intellectual and motor deficits were different in degree, the severer one was taken as the outcome.
There was no significant association between outcome and genotype distribution in AESD, ANE, or entire acute encephalopathy. (Cochran-Armitage trend test, additive model, AESD, $p = 0.81$; ANE, $p = 0.89$; Entire, $p = 0.31$; significance threshold $\alpha = 0.017$).

as to precedent infection and outcome. However, this study found no significant difference in the frequency of rs2229291, either between influenza-associated and non-influenza-associated cases, or between poor and favorable outcome.

This study showed for the first time the association of *CPT2* rs2229291 with MERS, a mild syndrome, whereas several previous studies had reported the association with severe syndromes [4,6] including AESD and ANE [6]. Taken together, the thermolabile CPT predisposes children to multiple syndromes of acute encephalopathy, ranging from mild to severe. Among the syndromes, there exist both similarities and differences. The syndromes are similar in clinical presentation: onset in the acute febrile period of common infectious diseases, and with convulsion and coma. Many of them are similar also from an epidemiological viewpoint: high incidence in young children in East Asia. On the other hand, they show highly different outcomes, as was noted also in this study. Blood laboratory findings are distinct, as are the topography and nature (vasogenic versus cytotoxic) of brain edema. This diversity is ascribed to the predominant pathogenetic mechanism specific to each syndrome [1].

During the last decade, we and other investigators in Japan have vigorously studied the genetic background of these syndromes. Many of the studies adopted a candidate gene approach, by selecting target genes based on the predominant pathologic mechanisms: systemic inflammatory response in ANE, and neuronal excitation in AESD. Several studies, on the other hand, used hypothesis-free approaches, such as linkage analysis and next generation sequencing. Both types of studies have successfully identified many risk gene variants. For ANE, an encephalopathy syndrome with cytokine storm, human leukocyte antigen (HLA) *DRB1*09:01* and *DQB1*03:03* are predisposing factors in Japanese patients [11], whereas in Caucasians, missense mutations of *RANBP2*, encoding for a nuclear membrane protein, cause a familial, recurrent variant of ANE [12]. For AESD, polymorphisms of *ADORA2A,* upregulating the expression of an excitatory neuromodulator, adenosine receptor A2A, renders Japanese children susceptible to excitotoxic neuronal damage [13]. Mutations of *CACNA1A*, *RHOBTB2* and *HNRNPU* have been identified in hemiconvulsion-hemiplegia-epilepsy syndrome, a condition highly overlapping with AESD [14–16]. For MERS, a reversible encephalopathy with suspected intramyelinic edema, a missense mutation of *MYRF*, encoding a transcriptional factor necessary for oligodendroglial differentiation and myelin maintenance, has recently been demonstrated to be causative in two pedigrees of familial cases [17]. Mutations of *GJB1*, another gene encoding for a myelin protein, connexin 32, also cause a recurrent, MERS-like encephalopathy [18]. All these gene variants, either polymorphisms or mutations, are specifically linked to a single syndrome.

By contrast, a small number of genes are known to be involved in multiple syndromes. *SCN1A* and *SCN2A* are genes encoding for subunits of voltage-gated, sodium channels of neurons. Dravet syndrome, a severe genetic epilepsy typically caused by *SCN1A* mutations, is occasionally complicated by acute encephalopathy [19,20]. *SCN1A* mutations have indeed been detected in patients with Dravet syndrome complicated by acute encephalopathy [21–23]. Furthermore, *SCN1A* mutations are also found in patients with acute encephalopathy, but without Dravet syndrome [24–26]. Acute encephalopathy associated with *SCN1A* mutations included AESD, AERRPS/FIRES, ANE and others. Likewise, missense mutations of another sodium channel subunit, *SCN2A*, have occasionally been detected in cases of AESD, AERRPS/FIRES and others [26,27]. Thus, the pathogenetic roles of neuronal sodium channels in multiple, severe syndromes of acute encephalopathy have been highlighted. The present study, together with several previous studies [4,6,7], identified *CPT2*, encoding an enzyme essential for cellular energy metabolism, as another gene underlying pathogenic mechanisms of acute encephalopathy consisting of multiple syndromes. The spectrum of *CPT2*-associated acute encephalopathy ranges from mild to severe syndromes, being apparently wider compared to *SCN1A* and *SCN2A*.

This study provides genetic evidence that impairment of mitochondrial metabolism during fever is a common pathologic event in acute encephalopathy. CPT2 is an enzyme that catalyzes formation of acyl-CoA utilized for long-chain fatty acid oxidation. During exposure to fever, production of extra energy demand is met by oxidation of fatty acids, so the deficit of CPT2 causes energy failure [3]. Pathogenic mutations in *CPT2* cause CPT2 deficiency which is the most common inherited disorder of long-chain fatty acids oxidation affecting skeletal muscles [3]. The most common mutation is rs74315254 (c. 338C > T, p. S113L) found in about 70% of mutant alleles [28]. Other than these pathogenic mutations, thermolabile polymorphisms significantly reduce CPT2 activity at high temperature via a dominant negative effect [4,5]. In particular, rs2229291 reduces the specific activity of CPT2 by increasing Km values for L-carnitine [5]. It is worth noting that the polymorphism is more frequent in East Asians than in other populations in the world, which could partially account for the higher incidence of acute encephalopathy in East Asia. From the perspective of therapy, this study may provide a piece of rationale recommending the introduction of metabolic rescue, including L-carnitine, vitamin B1, and others [29].

Limitation of the present study is sampling bias varying among syndromes. The sample size of MERS was

unduly small compared to AESD, considering the incidence of the two syndromes published previously [2]. This study could have failed to recruit many of the rapidly fatal cases of ANE, which may underestimate the association between genotype and outcome.

In conclusion, we demonstrated that the *CPT2* thermolabile polymorphism rs2229291 is a genetic risk factor of overall acute encephalopathy, ranging from AESD, a severe syndrome, to MERS, a mild syndrome. The findings suggest that mitochondrial dysfunction during high fever is an underlying pathogenic mechanism shared by various syndromes of acute encephalopathy.

Acknowledgement

The authors are grateful to Ms. Aya Shoda for technical assistance, and to the Collaborative Research Supporting Committee of the Japanese Society of Child Neurology for promoting this study. We wish to thank all the pediatricians for providing us their patients' samples, and the patients and their parents for participating this study. This research was supported by Grant-in-Aid for Scientific Research, No. 15H04872, from the Japan Society for the Promotion of Science, and by Grant-in-aid for Policy Research for Intractable Diseases, No. H30-Nanjitou-Ippan-007, from the National Institute of Public Health, Japan.

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