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ORIGINAL ARTICLE

Carnitine palmitoyl transferase 2 polymorphism may be associated with enterovirus 71 severe infection in a Chinese population

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Abstract Genetic polymorphism in the carnitine palmitoyl transferase 2 (CPT2) gene has been reported to be a susceptibility factor in a number of syndromes of acute encephalopathy with various infectious diseases, but evidence of its effect on enterovirus 71 (EV71) infection is lacking. The goal of this study was to examine the relationship between genetic polymorphism of CPT2 and severity of EV71 infection in a Chinese population. PCR of five exons of the CPT2 gene was carried out to identify single-nucleotide polymorphisms (SNPs) in EV71-infected subjects (n = 333), including mild cases (n = 271) and severe cases (n = 62) as well as healthy controls (n = 328). Blood ATP levels were measured within 24 h of admission. The frequency of the A allele of rs1799821 ($P = 0.023$) and the G allele of rs2229291 ($P = 0.009$) in the CPT2 gene was higher in patients with severe EV71 infection. The A-G haplotype of rs1799821and rs2229291 was directly linked to EV71 severe infection risk when compared to all other haplotypes (OR = 2.005, 95 % CI = 1.087-3.700, $P = 0.024$). The blood ATP levels of severe cases were significantly lower than in mild cases ($P < 0.01$) and controls ($P < 0.01$). A significant negative correlation was observed in haplotype A-G between ATP levels and physical findings in severe cases ($P < 0.05$). These findings suggest that CPT2 polymorphism may be associated with severity of EV71 infection and that the A-G haplotype of the CPT2 gene is involved in the inflammatory process of EV71 infection.

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Introduction

Enterovirus 71 (EV71) belongs to the species *Enterovirus A* of the genus *Enterovirus* within the family *Picornaviridae* [1]. Since the initial description of EV71 in 1974, outbreaks of infection with this virus have occurred periodically throughout the world [1, 2]. EV71 infection causes hand, foot and mouth disease (HFMD) in children, which usually resolves spontaneously [3, 4]. Compared to other enterovirus family members, EV71 is highly neurotropic, with severe cases having a high mortality and disability rate. It can even involve the central nervous system (CNS) to cause meningitis, brainstem encephalitis, poliomyelitis-like paralysis and convulsions associated with brain edema [5–9]. It has long been recognized that the ability of the blood-brain barrier (BBB) to exclude substances from the CNS is often compromised during infection and inflammation of the CNS [10]. Furthermore, the accumulation of mini-plasmin in the cerebral capillaries of mice with a congenital or acquired abnormality of mitochondrial fatty acid β-oxidation and the resulting prototypical destruction of the BBB after influenza virus infection also suggests a role of a disorder of mitochondrial β-oxidation and the destruction of the BBB in the molecular mechanisms of encephalopathy and acute brain edema [11].

Carnitine palmitoyl transferase 2 (CPT2) (EC 2.3.1.21) is an enzyme localized on the mitochondrial inner membrane. It removes the acyl group from acylcarnitine, transfers it to coenzyme A (CoA) to form acyl-CoA in the mitochondrial matrix and produces adenosine triphosphate

(ATP) through mitochondrial fatty acid β-oxidation [12–14]. The human CPT2 protein is a homotetramer encoded by a single gene, CPT2, which spans ~20 kilobases on human chromosome 1p32 and contains five exons [15–17]. Mutations of the CPT2 gene cause CPT2 deficiency (OMIM: 600650). CPT2 deficiency can induce a disorder of β-oxidation of long-chain fatty acids and impaired energy production, the effects of which are most pronounced during fasting or infection, when fatty acid breakdown is an essential energy source [12, 13, 18, 19]. When patients with CPT2 deficiency are infected with viruses, some develop energy failure and show a clinical course resembling that of acute encephalopathy [20].

In 2005, Chen et al. [21] first reported an association between genotypic variants of CPT2 and influenza-associated encephalopathy (IAE) with a markedly poor prognosis in Japanese patients. Yao et al. [19] also demonstrated that the thermolabile CPT2 variants might cause mitochondrial fuel utilization failure in various organs and endothelial cells during periods of high fever and thus might play an important role in the pathogenesis of brain edema in IAE. A similar study of 29 syndromes of acute encephalopathy patients with biphasic seizures and late reduced diffusion (AESD) or acute necrotizing encephalopathy (ANE) by Shinohara et al. confirmed these findings [22]. However, it is unclear whether the CPT2 gene variants are associated with EV71 encephalitis or not. Furthermore, the relationship between blood ATP levels and the susceptibility of children to severe EV71 infection is still unknown. Studies of the relationship between the genotypes of CPT2 on β-oxidation and ATP levels might clarify the molecular mechanisms of the neurological complications in patients suffering from EV71.

In the present study, we conducted a single-nucleotide polymorphism (SNP) analysis of CPT2 in EV71-associated HFMD patients and normal subjects. We also determined blood ATP levels in these samples to study the impact of these SNPs on the severity of EV71 infection. All cases were from the Chinese Han population.

Materials and methods

Study design and patient selection

This investigation was approved by the Ethics Review Committee of the Affiliated Hospital of Qingdao University, China. All participants' caregivers gave written informed consent.

We investigated 579 Chinese Han children with HFMD (346 males and 233 females) treated at the Pediatric Department of the Affiliated Hospital of Qingdao University and Qingdao Women & Children's Hospital, China, between May 2009 and November 2013. Of these patients, 214 were excluded for diagnosis as non-EV71associated HFMD, and another 32 refused to participate in the study. After that, a total of 333 EV71-associated HFMD patients were enrolled in this study. HFMD was diagnosed by clinical manifestations with characteristic oral ulcers and skin eruptions on the hands and feet, according to the hand, foot and mouth disease diagnosis and treatment guidelines (2010) published by the Chinese Ministry of Health [23]. In accordance with the guidelines, HFMD patients without any complications (mild cases, n = 271) were characterized as herpetic stomatitis and a rash on the hands and feet, with or without fever. Patients with CNS involvement (severe cases, n=62) were classified into four clinical groups, which were diagnosed by pediatric neurologists, with or without additional neuroimaging evidence (magnetic resonance imaging or computed tomography) [23–25]: (1) aseptic meningitis (n = 16) characterized by fever, headache, vomiting, and neck stiffness, compatible with CSF pleocytosis (white blood cell count, 5/mm^3), without the other neurologic manifestations; (2) brainstem encephalitis (n = 7) characterized by myoclonus, tremor, ataxia, nystagmus, oculomotor palsies, bulbar palsy, and autonomic dysregulation such as pulmonary edema, in various combinations, with or without neuroimaging evidence; (3) acute flaccid paralysis (n = 2) characterized by acute-onset motor weakness of the extremities; (4) encephalitis (n = 37) characterized by decreased consciousness, seizure, and fever without myoclonus, tremor, ataxia, or autonomic dysregulation, with or without neuroimaging evidence (Fig. 1).

Reverse transcription polymerase chain reaction (RT-PCR) was used to detect EV71 nucleic acid in stool specimens, throat swabs, and/or cerebrospinal fluids (CSF) from each patient on the day of admission. The strain was phylogenetically linked to clade C4a, the same strain identified in Shandong in 2007 [5]. The normal control population consisted of 328 unrelated Han Chinese (184 males and 144 females, mean age, 3.0 ± 1.2 years) who underwent a health examination during the same period. The control subjects had not been in contact with EV71-infected individuals, as confirmed by testing RNA extracted from throat swabs and stool specimens, and those with other metabolism disorders, cardiopathy, hypoplasia or cerebral palsy caused by innateness, trauma or other non-viral infections were excluded.

Data collection

We collected results of laboratory assays such as white blood cell count (WBC), C-reactive protein (CRP), blood glucose (BG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase-myocardial isozyme (CK-MB), cells and proteins in cerebrospinal fluid (CSF), as well as

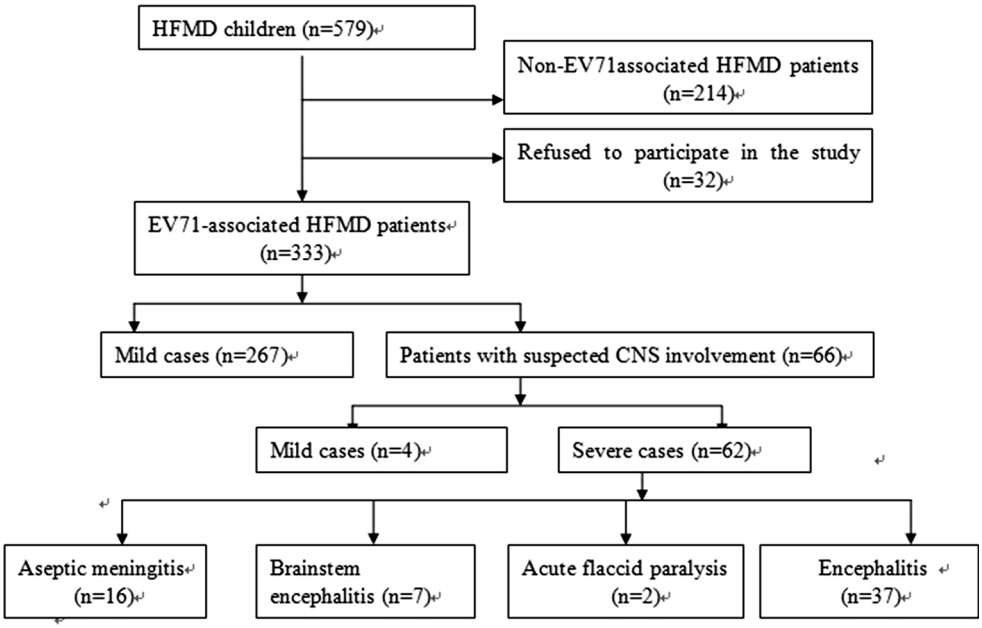

Fig. 1 Flowchart showing the process of selection of patients for this study. We recruited 579 HFMD patients, 214 of which were excluded because of a diagnosis of non-EV71-associated HFM and 32 of which were excluded because they refused to participate in the study. Finally, 333 patients were enrolled and divided into mild cases and severe cases based on whether they had CNS complications. Of the 66 patients with suspected CNS involvement, four had only a temporary headache, without any neurologic manifestations or neuroimaging evidence

electroencephalogram (EEG), and brain computed tomography (CT)/magnetic resonance (MRI) data.

Virus load determination

Fluorescent quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to determine the number of copies of viral RNA present in samples. Total RNA was extracted from 150 μL of various swab samples using an RNAiso Plus Kit (Takara, Dalian, China) according to the manufacturer's instructions. Next, total RNA was reverse transcribed with random hexamers using a Reverse Transcription Kit (Thermo Scientific). The cDNA was subjected to quantitative PCR in a 50-μL reaction mixture (Thermo Scientific DyNAmo SYBR Green qRT-PCR Kit) with the primers EV71-S (5′-GTT CTTAACTCACATAGCA-3′, nucleotides 2643–2661) and EV71-A (5′-TTGA CAAAAACTGAGGGTT-3′, nucleotides 2983–2965) for EV71 VP1, and the conditions consisted of a denaturation step at 95 °C for 15 min and 40 cycles of thermal cycling at 95 °C for 10 s and 60 °C for 60 s. A series of dilutions containing 1×10^7, 1×10^6, 1×10^5, and 1×10^4 copies/μL of a DNA fragment derived from EV71 was used to make a standard curve for calculating the copy numbers of virus RNA in various samples. Quantitative real-time RT-PCR was performed using the MxproMx3000P system.

CPT2 genotyping

Genomic DNA specimens were prepared from 200 μL of peripheral blood using a commercial kit following the manufacturer's instructions (Omega, USA). Genomic DNA was evaluated by electrophoresis in a 1 % agarose gel and diluted to a working concentration of 5-10 μg/ml.

We obtained the sequences of *Homo sapiens* CPT2 (accession no. NM_000098.2) genes from the GenBank database, and primers were designed for the coding regions of these genes using Primer Primier version 3.0 software. PCR of five exons of the CPT2 gene in the genomic DNA was carried out using intron-based primers (Table 1).

PCR reaction

P2F1/R1 fragment

A 10-μl mixture was prepared for each reaction and included 1x GC buffer, 0.2 mM dNTP, 0.2 μM each primer (Table 1), 0.25 U of Ex Taq polymerase (Takara) and 1 μl of template DNA. The cycling program was 95 °C for 5 min; 35 cycles of 96 °C for 10 s, 68 °C for 1 min per cycle; 72 °C for 2 min.

Other fragments

A 10-μl mixture was prepared for each reaction and included 1x Ex Taq buffer, 2.0 mM Mg^{2+}, 0.2 mM dNTP, 0.2 μM each primer, 0.25 U of Ex Taq polymerase (Takara) and 1 μl of template DNA. The cycling program was 95 °C for 5 min; 11 cycles of 94 °C for 15 s, 62 °C-0.5 °C per cycle for 40 s, 72 °C for 1 min; 24 cycles of 94 °C for 15 s, 56 °C for 30 s, 72 °C for 1 min; 72 °C for 2 min.

Table 1 PCR primers for the coding regions of the CPT2 gene

Primer name	Sequence (5′ to 3′)	Length (nt)	Region
CPT II P2F1	CGCTCGGTTTCCCGCCTCCT A	21	2218-2771
CPT II P2R1	GGGGGCGGAAACGGGTTCACT	21	
CPT II P3F1	TGGTTTTGGGGGAGGAAATTA	21	6181-6445
CPT II P3R1	GCTTGGTCCACCACTACTTGC	21	
CPT II P4F1	TGTTGGGGACCCAAAACTCTA	21	7995-8278
CPT II P4R1	CCTGCATCCTTTTCAGACAGG	21	
CPT II P5F1	GGAGGTTGATGCCATTTCCTT	21	15,476-16,144
CPT II P5R1	CCCAGATGTCTCGGTTCTCAC	21	
CPT II P6F1	CCCAGTATTTTCGGCTTTTCA	21	15,890-16,525
CPT II P6R1	CCCAGTATTTTCGGCTTTTCA	21	
CPT II P7F1	TTTGAGCACTCTTGGGGTGAT	21	16,354-16,970
CPT II P7R1	TTGGAGTAGAATGATTTAGGCTTGC	25	
CPT II P8F2	CTTGAGCTGCTCTGAAGGTT	20	18,668-19,215
CPT II P8R2	CAGTTTTCATGATGAGGAAGTGAT	24	

Homo sapiens chromosome 1, GRCh37.p10 primary assembly
Coordinates 53660101 to 53681869=21768 GPC_000000025

Sequencing reaction

The reaction mixture included 2 μl of BigDye3.1 mix, 2 μl of sequencing primer (0.4 μM) and 1-2 μl of purified PCR product. The cycling program was 96 °C for 1 min; 28 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min.

The samples were analyzed using an ABI3130XL DNA sequencer. Data were analyzed using Polyphred software.

Measurement of Blood ATP levels

ATP concentrations in whole blood lysate were measured using an ENLITEN ATP assay system bioluminescence detection kit (Promega) according to the instructions provided by the manufacturer, and the values were expressed as ATP levels in whole blood. The ATP standard curve was generated using the ATP standard (10^{-7} mol/L) included in the kit. Briefly, 0.5 % trichloroacetic acid (TCA) was added to the blood for efficient ATP release, and a pH 7.75 buffer (25 mM Tris-acetate) was used for neutralization. After addition of recombinant rL/L reagent, light output from the rL/L reaction was measured in a luminometer. A 2-second delay time after L/L Reagent injection and a 10-second RLU signal integration time were used. Blood ATP levels were measured within 24 h of admission and during the convalescent phase.

Statistical analysis

The observed genotype frequencies in controls were tested for Hardy-Weinberg equilibrium using the chi-square test. Normally distributed parametric data were expressed as mean ± SD, whereas non-parametric data were described using median and range and analysed using the Kruskal–Wallis or Mann–Whitney test, with values expressed as median (25th–75th percentile value). The frequencies of genotypes and alleles were compared between different groups using the chi-square test. Differences in ATP levels were tested by independent samples *t*-test and paired samples *t*-test. A *P*-value less than 0.05 was considered statistically significant. The association of polymorphism and the risk of EV71 infection were evaluated by calculating odds ratios (OR) and 95 % confidence intervals (95 % CI) using logistic regression. The ATP levels, duration of fever, and WBC, CRP and BG levels were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test or Student's *t*-test. Pearson's correlation was used to analyze the relationship between ATP levels and physical findings and EV71 load. All analyses were performed using Statistical Package for Social Science (IBM SPSS software, USA) version 17.0.

Results

Baseline demographics and characteristics

As shown in Table 2, we examined a group of 333 EV71-infected HFMD patients. In severe cases, 41 of 62 (77 %) patients were male, and 21 were female. Forty-three of 62 (69 %) patients with severe disease had abnormal EEG, 24 of 62 (39 %) had abnormal brain CT/MRI, and 45 of 62 (73 %) had pleocytosis. Five patients died, and two were left with severe mental retardation.

Table 2 Physical findings at the time of admission of EV71-infected patients to the hospital

Variable	Severe cases (n = 62)	Mild cases (n = 271)	controls (n = 328)	*P*-value
Average age (years),	2.4 ± 0.7	3.1 ± 1.1	3.0 ± 1.2	0.042[b]
Male, n (%)	41 (66)	139 (51)	184 (56)	
Female, n (%)	21 (34)	132 (49)	144 (44)	0.092[a]
Duration of fever days	3.0 (2.0–3.5)	2.0 (1.5–3.5)	-	<0.01[c]
WBC ($\times 10^9$/L)	12.8 (7.7-14.6)	9.7 (7.0–13.7)	-	<0.01[c]
BG (mmol/L)	12.14 ± 1.93	6.24 ± 1.20	-	<0.01[b]
CRP (mg/L)	8.7 (6.4-9.9)	7.0 (4.9-9.6)	-	<0.01[c]
ALT (U/L)[a]	20.0 (15.0–25.0)	21.0 (17.0–26.0)	-	0.209[c]
AST (U/L)[a]	27.0 (21.0–37.0)	27.0 (21.0–34.0)	-	0.566[c]
CK-MB (U/L)[a]	15.2 (12.0–23.7)	15.0 (10.0–20.3)	-	0.195[c]
EV71 load (log10 copies/ul)	4.1 (3.6–4.5)	4.0 (3.5–4.3)		0.862
CSF(WBC>5/mm^3), n (%)	45(73)	-	-	-
EEG abnormal, n (%)	43(69)	-	-	-
Brain MRI or CT abnormal, n (%)	24(39)	-	-	-

[a] Groups were compared using the chi-square test

[b] Values expressed as mean ± SD

[c] Values expressed as median (25th–75th percentile values)

Significant differences were observed in patients with EV71 infection between severe cases and mild cases with respect to average age and duration of fever ($P = 0.042$, $P < 0.01$). There were significant increases in CRP levels, WBC counts, and BG concentrations in severe cases when compared to mild cases. The median WBC counts in severe cases and mild cases were 12.8 $\times 10^9$/L and 9.7 $\times 10^9$/L, respectively ($P < 0.01$). BG concentrations were 12.14 ± 1.93 mmol/L and 6.24 ± 1.20 mmol/L, respectively ($P < 0.01$). The median CRP levels were 8.7 mg/L and 7.0 mg/L, respectively ($P < 0.01$). However, no significant differences were found with regard to gender, ALT, AST, CK-MB levels, or viral load ($P = 0.092$, P =0.209 P =0.566, P =0.195, $P = 0.862$).

The genotype distributions and allele frequencies of CPT2

Sequence analysis of the CPT2 gene revealed three significant SNP sites with missense mutations: rs1799821 (NM_000098.2:c.1102G>A), rs1799822 (NM_000098.2: c.1939A>G) and rs2229291(NM_000098.2:c.1055T>G). The genotype distributions of each group conformed to Hardy-Weinberg equilibrium ($p > 0.05$). The genotype distributions, allele frequencies and carriage frequencies are shown in Tables 3 and 4.

As shown in Table 3, there were no statistical differences in the genotype distribution, allele frequency and carriage frequency in rs1799821 ($P = 0.213$, $P = 0.088$ and $P = 0.152$). Concerning the rs2229291 polymorphism, the frequencies of the TT, TG, and GG genotypes in patients and controls were significantly different ($\chi^2 = 7.309$, $P = 0.026$). The frequency of G allele among the patients was significantly higher than that of controls (26.9 % vs. 20.4 %, OR = 1.432, 95 %CI = 1.109-1.713, $p = 0.006$). No statistical differences were found in the genotype distributions, allele frequencies and carriage frequencies among different groups in rs1799822.

As shown in Table 4, in EV71-infected patients, the frequencies of the AA, GA, GG genotypes of rs1799821 in mild cases were 52.0, 40.6, 7.4 %, and they were 64.5, 35.5, 0 % in severe cases. The severe cases had a significantly lower frequency of the G allele compared with mild cases (17.7 % vs. 27.7 %, OR = 0.564, 95 %CI = 0.343-0.927, $P = 0.023$). Concerning the rs2229291 polymorphism, the severe cases had a significantly higher frequency of the G allele compared to mild cases (36.3 % vs. 24.7 %, OR = 1.734, 95 %CI = 1.145-2.626, $P = 0.009$).

Association between haplotypes of CPT2 and susceptibility to EV71 infection

We next utilized Haploview software to identify the haplotype blocks and then performed Pearson's correlation analysis to associate haplotype blocks with EV71 infection risk. The haplotype analysis showed that the A-G haplotype (AG-AG, AG-GG) was directly linked to the risk of severe EV71 infection when compared to all other haplotypes (OR = 2.005, 95 % CI = 1.087-3.700, $P = 0.024$)

Table 3 Genotype and allele frequencies of rs1799821, rs1799822, rs2229291and rs7554022 in CPT2 gene between EV71-infected patients and controls

SNP	EV71-infected patients n = 333 (%)	Controls n = 328 (%)	*P*-value	OR (95 % CI)
rs1799821				
Genotype				
AA	181 (54.4)	160 (48.8)		
AG	132 (39.6)	139 (42.4)	χ^2 = 3.089	
GG	20 (6.0)	29 (8.8)	0.213*	
Allele				
G	172 (25.8)	197 (30.0)	χ^2 = 2.904	0.811
A	494 (74.2)	459 (70.0)	0.088†	(0.638-1.032)
Carriage frequency				
AG+GG	152 (45.6)	168 (51.2)	χ^2 = 2.056	0.800
AA	181 (54.4)	160 (48.8)	0.152†	(0.589-1.086)
rs1799822				
Genotype				
AA	282 (84.7)	259 (79.0)		
AG	48 (14.4)	65 (19.8)	χ^2 = 3.641	
GG	3 (0.9)	4 (1.2)	0.162*	
Allele				
G	54 (8.1)	73 (11.1)	χ^2 = 3.471	0.075
A	612 (91.9)	583 (88.9)	0.062†	(0.487-1.020)
Carriage frequency				
AG+GG	51 (15.3)	69 (21.0)	χ^2 = 3.640	0.679
AA	282 (84.7)	259 (79.0)	0.056†	(0.455-1.012)
rs2229291				
Genotype				
TT 181 (54.4)		211 (64.3)		
TG	125 (37.5)	100 (30.5)	χ^2 = 7.309	
GG	27 (8.1)	17 (5.2)	0.026*	
Allele				
G	179 (26.9)	134 (20.4)	χ^2 = 7.609	1.432
T	487 (73.1)	522 (79.6)	0.006†	(1.109-1.713)
Carriage frequency				
TG+GG	152 (45.6)	117 (35.7)	χ^2 = 6.812	1.514
TT	181 (54.4)	211 (64.3)	0.009†	(1.108-2.070)

* Control subjects vs. EV71-infected patients using the χ^2 test with a 3 × 2 contingency table

† Control subjects vs. EV71-infected patients using the χ^2 test with a 2 × 2 contingency table

in block 1 (including rs1799821and rs2229291) (Fig. 2,Table 5).

Blood ATP levels in patients with HFMD

ATP levels in the extracts of whole blood of patients differed significantly among controls, mild cases and severe cases (Fig. 3). The blood ATP levels in severe cases in the acute phase (0.67 ± 0.10 mmol/L) were significantly lower than those in mild cases (0.95 ± 0.11 mmol/L, $P < 0.01$) and controls (1.24 ± 0.14 mmol/L, $P < 0.01$) (Fig. 3A).

ATP levels were measured for each genotype of rs1799821, rs1799822 and rs2229291 in EV71-infected patients. In the AA (GA+GG) genotype of rs1799821, the ATP levels of the AA genotype were statistically lower than that of the GA+GG genotype (0.64 ± 0.07 mmol/L vs. 0.69 ± 0.08 mmol/L, $P < 0.01$), and the ATP levels of the AA genotype showed a similar trend in mild cases (0.93 ± 0.08 mmol/L vs. 0.97 ± 0.05mmol/L, $P = 0.036$) (Fig. 3B). In TT (TG+GG) genotypes of rs2229291, the ATP levels of the TG+GG genotype were statistically lower than those of the TT genotype in severe cases (0.63 ± 0.06 mmol/L vs. 0.69 ± 0.08 mmol/L, $P < 0.01$)

Table 4 Genotype and allele frequencies of rs1799821, rs1799822, rs2229291and rs7554022 in the CPT2 gene in severe and mild cases

SNP	Severe cases n = 62 (%)	Mild cases n = 271 (%)	*P*-value	OR (95 % CI)
rs1799821				
Genotype				
AA	40 (64.5)	141 (52.0)		
AG	22 (35.5)	110 (40.6)	χ^2 = 6.355	
GG	0 (0)	20 (7.4)	0.042*	
Allele				
G	22 (17.7)	150 (27.7)	χ^2 = 5.198	0.564
A	102 (82.3)	392 (72.3)	0.023†	(0.343-0.927)
Carriage frequency				
AG+GG	22 (35.5)	130 (48.0)	χ^2 = 3.171	0.597
AA	40 (64.5)	141 (52.0)	0.075†	(0.337-1.057)
rs1799822				
Genotype				
AA	52 (83.9)	230 (84.9)		
AG	10 (16.1)	38 (14.0)	χ^2 = 0.848	
GG	0 (0)	3 (1.1)	0.655*	
Allele				
G	10 (8.1)	44 (8.1)	χ^2 = 0.000	0.993
A	114 (91.9)	498 (91.9)	0.984†	(0.485-2.032)
Carriage frequency				
AG+GG	10 (16.1)	41 (15.1)	χ^2 = 0.039	1.079
AA	52 (83.9)	230 (84.9)	0.844†	(0.508-2.293)
rs2229291				
Genotype				
TT	27 (43.6)	154 (56.8)		
TG	25 (40.3)	100 (36.9)	χ^2 = 7.839	
GG	10 (16.1)	17 (6.3)	0.020*	
Allele				
G	45 (36.3)	134 (24.7)	χ^2 = 6.870	1.734
T	79 (63.7)	408 (75.3)	0.009†	(1.145-2.626)
Carriage frequency				
TG+GG	35 (56.4)	117 (43.2)	χ^2 = 3.586	1.706
TT	27 (43.6)	154 (56.8)	0.058†	(0.978-2.977)

* Severe cases vs. mild cases using the χ^2 test with a 3×2contingency table

† Severe cases vs. mild cases using the χ^2 test with a 2×2contingency table

and mild cases (0.93 ± 0.08 mmol/L vs. 0.96 ± 0.05 mmol/L, $P = 0.025$) (Fig. 3C). No significant differences were found with ATP levels in AA (GA+GG) genotypes of rs1799822 in severe cases (0.67 ± 0.10 mmol/L vs. 0.64 ± 0.08 mmol/L, $P = 0.288$) or mild cases (0.95 ± 0.05 mmol/L vs, 0.92 ± 0.08 mmol/L, P =0.06) (Fig. 3D). In the haplotype analyses, the ATP levels of patients carrying haplotype A-G were statistically lower than that of haplotype A-T (0.65 ± 0.09 mmol/L vs. 0.69 ± 0.07 mmol/L, $P < 0.01$) and haplotype G-T (0.65 ± 0.09 mmol/L vs. 0.69 ± 0.05 mmol/L, $P < 0.01$) in severe cases (Fig. 3E). A significant negative correlation was observed in haplotype A-G between ATP levels and physical findings (duration of fever, CRP levels, WBC counts and BG concentrations) in severe cases ($P < 0.05$), which were not seen in mild cases (Table 6).

Discussion

EV71 can cause large epidemics with severe CNS and fatal pulmonary complications [26]. Regular epidemics have since been seen in countries across the Asia-Pacific region, including an epidemic in Taiwan in 1998 [27] that was

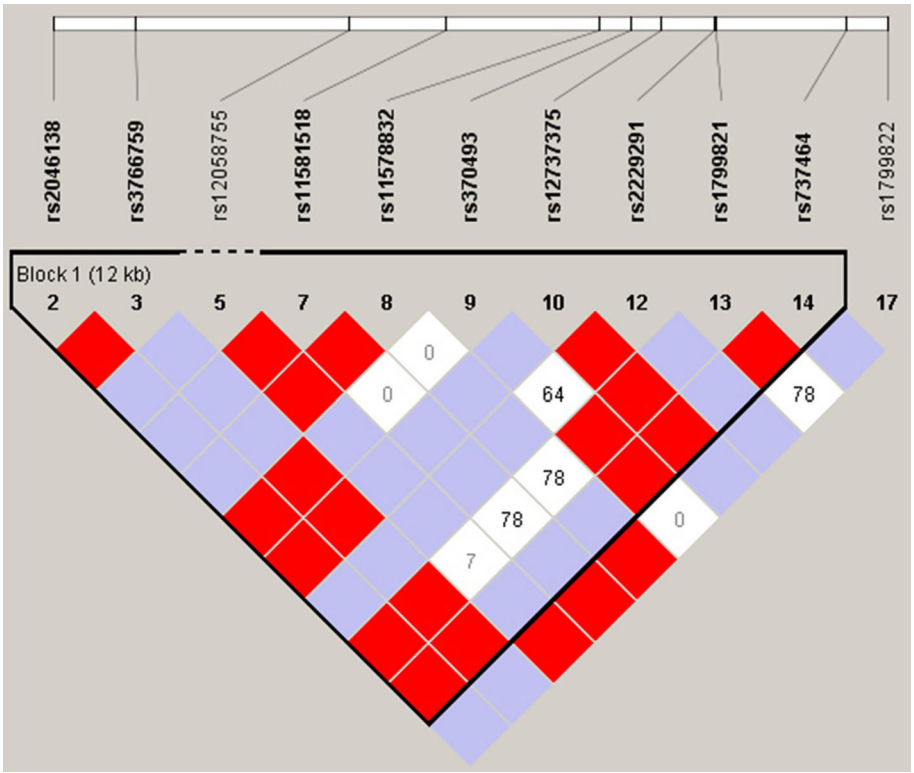

Fig. 2 Structure of haplotype block 1, including rs1799821 and rs2229291, in EV71 infection patients and healthy controls. The darker color indicates a higher level of linkage disequilibrium (LD), while the lighter color indicates less LD

Table 5 Haplotype structure and frequency analysis of *CPT2* genetic polymorphisms in EV71-infection patients and controls

Block	Loci	Case/controls	Haplotype	Frequency (%)		OR (95 % CI)	*P*-value
				Case	Control		
1	rs1799821and rs2229291	EV71-infected	A-T	42.0	44.2	0.915 (0.673-1.246)	0.574
		patients/controls	A-G	27.9	22.0	1.378 (0.967-1.964)	0.076
			G-T	30.7	33.8	0.839 (0.605-1.164)	0.293
		Severe cases/mild case	A-T	43.5	47.6	0.849 (0.487-1.480)	0.564
			A-G	32.3	19.2	2.005 (1.087-3.700)	0.024
			G-T	24.2	33.2	0.642 (0.341-1.210)	0.168

thought to involve millions of people and an outbreak of HFMD in China [4–10], during which nearly 500,000 cases were reported. Chang et al. [28] reported that HLA-A33, which is a common phenotype in Asian populations but is rare in white populations, was most significantly associated with EV71 infection, which explains why EV71 outbreaks occur much more frequently in Asian countries than in Western countries. This observation indicated that genetic factors might be involved in the differences in physical responses to the same infectious agent, in addition to the virulence of the pathogen.

In the present study, we investigated CPT2 polymorphism in Chinese Han children with EV71 infection. We found that the variant allele G of rs2229291 in the CPT2 gene was markedly overrepresented in patients with severe EV71 infection compared to mild cases and controls, which suggested that polymorphism in rs2229291 might be associated with EV71 infection in the Chinese Han population. It has been reported that variations in the CPT2 gene (rs2229291) may be associated with multiple syndromes of acute encephalopathy with various infectious diseases [22], and the rs2229291 and rs1799821 variants of the CPT2 gene might be associated with the development of acute encephalitis [29]. These observations are in line with ours. Although no statistical differences were found in the genotype distributions, allele frequencies and carriage frequencies in rs1799821 between EV71-infected patients and controls, we did find significantly lower frequencies of the G allele of rs1799821 in patients with CNS involvement compared to patients with EV71-related HFMD without any complications. This suggests that the rs1799821 polymorphism confers susceptibility to severe

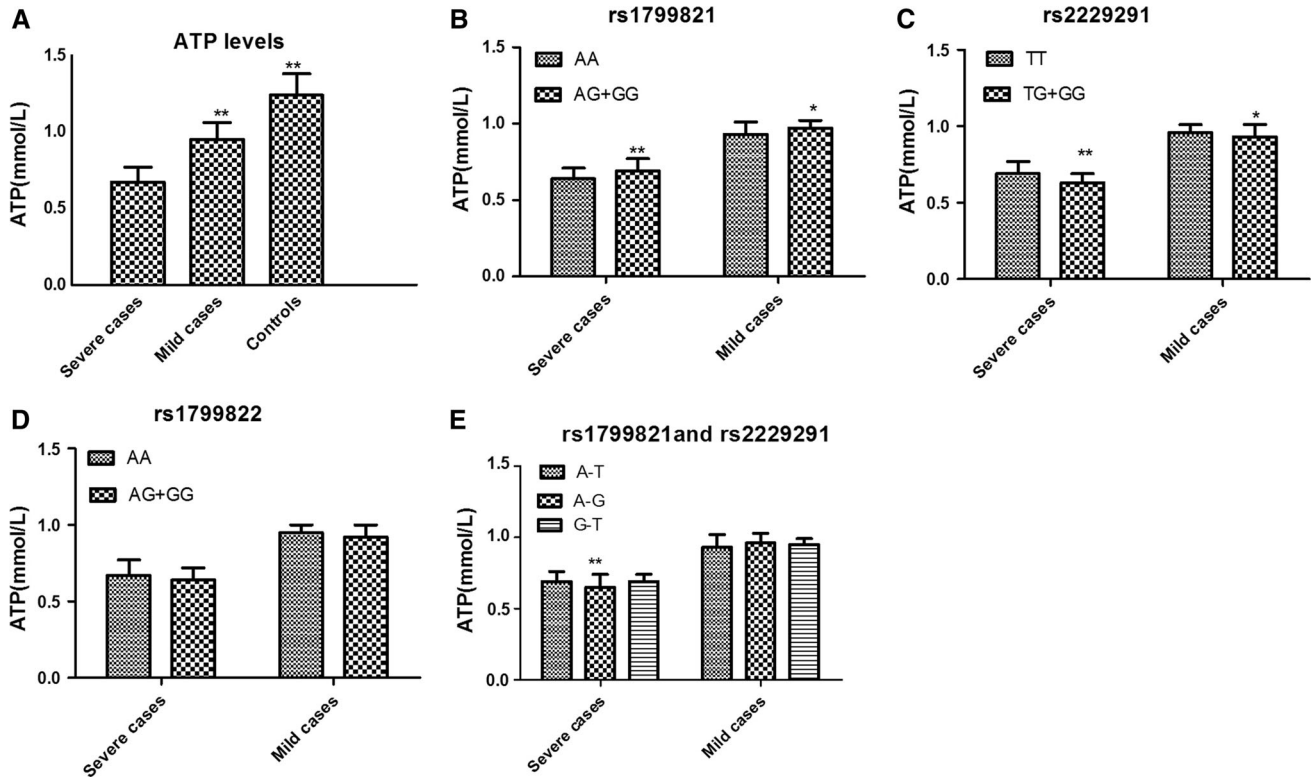

Fig. 3 Blood ATP levels in EV71-infected patients and controls. Values are expressed as mean ± SD. ATP levels of severe cases in the acute phase were significantly lower than in mild cases ($P < 0.01$) and controls ($P < 0.01$) (**, $p < 0.01$) (A). In rs1799821, ATP levels of the AA genotype were statistically lower than those of the GA+GG genotype ($P < 0.01$), and the AA genotype showed a similar trend in mild cases ($P = 0.036$) (**, $p < 0.01$, *, $p < 0.05$) (B). In rs2229291, the ATP levels of the TG+GG genotype were statistically lower than those of the TT genotype in severe cases ($P < 0.01$) and mild cases ($P = 0.025$) (**, $p < 0.01$, *, $p < 0.05$) (C). No significant differences were found between the ATP levels in AA, (GA+GG) genotypes of rs1799822 in severe cases ($P = 0.288$) and mild cases ($P = 0.06$) (D). The ATP levels of patients carrying haplotype A-G were statistically lower than those of haplotype A-T (0.65 ± 0.09 mmol/L vs. 0.69 ± 0.07 mmol/L, $P < 0.01$) and haplotype G-T (0.65 ± 0.09 mmol/L vs. 0.69 ± 0.05 mmol/L, $P < 0.01$) in severe cases (**, $p < 0.01$, *, $p < 0.05$) (E)

EV71 infection in the Han Chinese population. Haplotype analysis integrating rs1799821 and rs2229291 also implied that A-G carriers were more inclined to suffer from severe EV71 infection than carriers of other haplotypes. We did not find any statistical difference in rs1799822 between different groups, which could be due to the smaller number of samples available for this study.

CPT2 plays a pivotal role in the transport of long-chain fatty acids (LCFA) into mitochondria [18]. CPT2 facilitates the β-oxidation of LCFA in mitochondria and is ubiquitously expressed in all tissues that require fatty acid oxidation as an energy-producing pathway [13, 14, 30]. Kubota et al. [31] reported reduced blood ATP levels and systemic mitochondrial dysfunction in patients with thermolabile CPT2 with acute encephalopathy and febrile seizures. In that study, blood ATP levels in the acute phase of encephalopathy during high fever were significantly lower than those in the convalescent phase and also with those of patients with febrile seizure status. However, there are few reports about the associations between CPT2 gene variants and blood ATP levels [13, 29, 31]. In this work, we observed a significant decrease in blood ATP levels in patients with CNS involvement compared to mild cases and normal controls. Furthermore, in patients with severe EV71 infection, ATP levels were obviously lower in subjects with the A-G haplotype than in those with haplotype A-T and haplotype G-T, which indicates a possible association between haplotype A-G and ATP production, and mitochondrial energetic failure may be more severe in patients with this haplotype.

Considering the clinical manifestations and the laboratory data, we found significantly higher fever duration, WBC counts, and BG concentrations in EV71 encephalitis patients than in EV71-related HFMD patients without any complications, and these have often been considered the key factors for severe HFMD [32]. Furthermore, we also found that patients with severe EV71 infection had an elevated CRP level, which was a common indicator of an inflammatory response. We also observed a significant negative correlation between ATP levels and these physical

Table 6 Correlation of ATP level and physical findings in EV71-infected patients

Genotype	Parameter	Mild cases		Severe cases	
		ATP level Pearson's correlation	Significance (two-tailed)	ATP level Pearson's correlation	Significance (two-tailed)
rs1799821and rs2229291					
A-T	Duration of fever	−0.815	0.062	−0.799	0.066
	WBC ($\times 10^9$/L)	−0.807	0.091	−0.815	0.058
	BG (mmol/L)	0.358	0.282	0.477	0.216
	CRP (mg/L)	−0.802	0.065	−0.824	<0.05
	EV71 load(log10 copies/ul)	0.331	0.422	0.273	0.472
A-G	Duration of fever	0.622	0.183	−0.825	<0.01
	WBC ($\times 10^9$/L)	−0.814	0.071	−0.901	<0.01
	BG (mmol/L)	0.502	0.277	−0.822	<0.05
	CRP (mg/L)	−0.782	0.099	−0.901	<0.01
	EV71 load(log10 copies/ul)	−0.709	0.071	−0.561	0.201
G-T	Duration of fever	0.601	0.203	0.572	0.302
	WBC ($\times 10^9$/L)	0.263	0.328	0.800	0.093
	BG (mmol/L)	−0.375	0.293	0.622	0.217
	CRP (mg/L)	−0.762	0.103	−0.815	0.082
	EV71 load(log10 copies/ul)	−0.811	0.062	−0.799	0.061

findings in severe forms of EV71 infection in patients carrying the A-G haplotype of rs1799821and rs2229291. This suggests that CPT2 polymorphisms are involved in the inflammatory process in EV71 infection. However, the underlying mechanisms of these associations have not yet been determined. We did not find a significant correlation between the virus load and ATP level in the different groups, which may due to the small sample size or to the limited number of clinical specimens.

The BBB is composed of highly specialized endothelial cells in the CNS vasculature [33]. It is particularly susceptible to acute hypoxic insult when there is an inadequate supply of ATP [34]. BBB breakdown may occur at an initial stage of encephalopathy under conditions of ATP reduction, thus leading to subsequent brain edema due to a complex cascade of hypercytokinemia, excitotoxicity, and oxidative stress [35]. Moreover, recent studies have suggested that direct lesions and/or a systemic inflammatory response syndrome produced by the release of cytokines and chemokines appears to play an important role in the elicitation of the immune response to severe complications in EV71 [8–10]. In a review of previous studies, we found that endothelial cell damage and BBB breakdown might in turn induce cytokine production, resulting in neuronal damage in patients with severe EV71 infection carrying the A-G haplotype of CPT2.

However, our current study does have some limitations. The sample size of this study was relatively small, and only one ethnic group was included. Further studies with a larger sample size and multiple ethnic groups are required to determine the effects of genetic variants of the CPT2 gene. Studying the enzymatic properties of CPT2 variants would help in elucidating the pathophysiology of EV71 infection.

In conclusion, we report three variants of the CPT2 gene in a Chinese Han population. Our present results demonstrate a statistically significant correlation of the A-G haplotype of rs1799821and rs2229291 in the CPT2 gene with the severity of EV71 infection. Our study suggests that the A-G haplotype of CPT2 gene is involved in the inflammatory process of EV71 infection. However, the mechanism by which variations in CPT2 affect the expression and function of the CPT2 protein need to be investigated further.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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