1. Introduction

Mitochondrial fatty acid oxidation (FAO) disorders are among the causes of neuromuscular symptoms as well as acute encephalopathy or even sudden death. In particular, the carnitine cycle is important in energy-producing pathway for cardiac and skeletal muscle and for preventing from hypoglycemia especially during prolonged fasting or increased muscular exercise. Carnitine–acylcarnitine translocase (CACT, EC 2.3.1.21) is one of the enzymes in the carnitine cycle, which catalyzes the transfer of the long-chain fatty acylcarnitines across the inner mitochondrial membrane in exchange of free carnitine. CACT deficiency (OMIM 212138) was first described in 1992 [1]. It is an autosomal-recessive disease caused by mutations of the SLC25A20 gene located in chromosome 3p21.31 [2]. The gene consists of 9 exons and encodes protein comprising 301 amino acids [3]. CACT deficiency is a very rare disorder with so far as approximately 30 patients have been described, and accounted for 10% of patients with FAO disorders in French population [4]. However, it might be a common FAO disorder in some East Asian countries such as Hong Kong with the estimated incidence of 1 in 60,000 live births, and accounted for 33% of patients with FAO disorders [5]. Most patients develop neonatal-onset encephalopathy with nonketotic hypoglycemia, hyperammonemia, and hypothermia, or sudden death from cardiac arrhythmias. Cardiomyopathy and hepatic dysfunction may be the associated complications. CACT deficiency could be detected by elevations of C16 and C18 acylcarnitines, and low free carnitine in acylcarnitine profiles. However, the same profile could be found in neonatal carnitine palmitoyltransferase-2 (CPT2) deficiency. Therefore, confirmation of diagnosis requires CACT enzyme assay or molecular analysis of the SLC25A20 gene [6]. Treatment includes intravenous glucose for acute decompensation, and avoidance of long fasting with frequent meals. Long-chain fatty acids may be restricted in diet, but medium-chain triglyceride (MCT) oil is supplemented instead. Carnitine therapy is still controversial. Despite aggressive treatment, most patients still died in infancy [7]. However, there have been some patients who received early treatment with good outcomes [8,9]. Novel therapy for FAOD using bezafibrate, which is a hypolipedimic drug acting as a peroxisome proliferator-activated receptor (PPAR) agonist has been reported. The clinical trials of bezafibrate showed clinical improvement in adult patients with CPT2 deficiency [10], and a child with glutaric acidemia type 2 (GA2) [11]. In vitro probe acylcarnitine (IVP) assay can be used to evaluate FAO disorders [12], and determine the effect of bezafibrate [13]. We herein report the clinical findings of two unrelated cases with neonatal-onset CACT deficiency, and in vitro bezafibrate response using the IVP assay.

2. Patients and methods

2.1. Patients

2.1.1. Case 1

This patient was the first child of possibly consanguineous parents from the southern province of Thailand. He was born at 37 weeks of gestation with birth weight of 2460 g (25th percentile), length 48 cm (3rd percentile), and head circumference 30 cm (<3rd percentile). He developed hypothermia at 10 h of age. Sepsis was suspected, but the patient rapidly responded to rewarming treatment. However, after rooming-in with the mother, he developed hypothermia again. At 60 h after birth, he had cardiac arrest. On physical examination, no abnormalities were found. Serum glucose was 1.2 mmol/L and acetoacetate was 0 mmol/L. Venous blood pH was 7.24 and serum bicarbonate was 13 mmol/L with an anion gap of 20. Plasma ammonia was 471 µmol/L (normal, <110 µmol/L). There were mildly elevated liver enzymes aspartate aminotransferase (AST) (97 U/L; normal, 0–32) and alanine aminotransferase (ALT) (78 U/L; normal, 0–33). Serum creatine kinase was 4439 U/L (normal, <190). He had a good response to treatment with intravenous glucose administration. Urine organic acids were unremarkable. A dried blood spot acylcarnitine profile by tandem mass spectrometry (MS/MS) showed free carnitine (C0), 5.26 µM (10–60); C16-acylcarnitine, 14.14 µM (0.6–7); C18-acylcarnitine, 2.71 µM (0.15–2.1); C18:1-acylcarnitine, 4.3 µM (0.3–3.2); and a (C16 + C18)/C0 ratio, 3.21 (0.007–0.5). The profile was consistent with CPT2 or CACT deficiency. The patient has been treated with a modular medical formula, which has been composed of modified fats (long-chain fatty acid restriction along with supplementation of 83% of fat as medium-chain triglyceride oil), protein, maltodextrins, minerals, and fat-, and water-soluble vitamins. L-Carnitine at a daily dosage of 100–150 mg/kg has been supplemented. Thereafter, he has had several episodes of hypoglycemia, hyperammonemia, and metabolic acidosis following infections. At 8 months of age, he developed cholestasis and hepatomegaly. At 9 months of age, an echocardiogram revealed hypertrophic cardiomyopathy. At the age of 15 months, he had mild developmental delay and generalized hypotonia. He could stand with support, put block in cup, and say one word. Then he had a metabolic crisis, and developed generalized weakness. After he recovered from encephalopathy, neurologic examination revealed normal cranial nerves, muscle weakness (grade 3/5), and decreased muscle tone and deep tendon reflexes (1+) in all extremities. A brain computed tomography scan was normal. Serum creatine kinase was elevated (1419 U/L). A nerve conduction study showed no evidence of demyelination. He had been ventilator-dependent since then. At 2½ years of

age, he had several complications including chronic liver disease, upper gastrointestinal bleeding, and osteoporosis. He died at the age of 2 years and 8 months from upper gastrointestinal bleeding and metabolic decompensation.

2.1.2. Case 2

The patient was the first child of nonconsanguineous parents. She was born at 35 weeks of gestation with a birth weight of 2.3 kg (50th percentile), length 44 cm (25th percentile), and head circumference 30 cm (10th percentile). At 2 days after birth, she developed lethargy, poor feeding, and cardiac arrest. Blood glucose was 0.56 mmol/L. She responded to cardiac resuscitation and intravenous glucose infusion. Serum acetoacetate was 0 mmol/L. Venous blood pH was 7.39 and serum bicarbonate was 13 mmol/L with an anion gap of 20. Plasma ammonia was 157 µmol/L (normal, <110 µmol/L). There were elevated liver enzymes AST (638 U/L; normal, 0–32) and ALT (83 U/L; normal, 0–33). Plasma lactate dehydrogenase (LDH) was 522 U/L (normal, 240–480). An echocardiogram revealed no cardiomyopathy. A dried blood spot acylcarnitine profile by MS/MS analysis showed C0, 13.8 µM (10–60); C16-acylcanitine, 15 µM (0.6–7); C18-acylcarnitine, 4.3 µM (0.15–2.1); C18:1-acylcarnitine, 5.9 µM (0.3–3.2); and a (C16 + C18)/C0 ratio, 1.4 (0.007–0.5). The profile was consistent with either CPT2 or CACT deficiency. The patient had been treated with a high-MCT formula (Portagen®, Mead Johnson Nutritionals), and 100 mg/kg/day of L-carnitine. At 1 month of age, she developed anemia from Hb AE Bart's disease – a thalassemia intermedia resulting from the interaction between α-thalassemia and heterozygous Hb E, which required monthly blood transfusion. At the age of 4 months, she had poor feeding and cardiac arrest. Blood glucose was 0.5 mmol/L. The patient died without any response to resuscitation. An autopsy revealed left ventricular hypertrophy, micro/macrovesicular steatosis of the liver with focal areas of bridging fibrosis, and abnormal lipid accumulation in skeletal muscles and the proximal renal tubules.

2.2. Materials and methods

This study was approved by the Siriraj Institutional Review Board. The written informed consents for the mutation analysis, IVP assay, and bezafibrate trial were obtained from the parents. Genomic DNA was extracted from leukocytes. Mutation analyses of the *CPT2* and *SLC25A20* genes were performed in case 1, and only *SLC25A20* gene in case 2. All coding exons and their flanking intron sequences (up to 20 bases for both sides) of the *CPT2* and *SLC25A20* genes were PCR-amplified and directly sequenced according to the previously described method [14]. The IVP assay was performed using the skin fibroblasts in the absence and presence of bezafibrate according to the previously described method [11].

3. Results

3.1. Mutation analysis and IVP assay

Mutation analysis of the *SLC25A20* gene identified homozygous c.199-10T>G (IVS2-10T>G) mutation in both patients, and heterozygous mutation in their parents (Fig. 1). Mutation analysis of the *CPT2* gene revealed no pathogenic mutation in Case 1. The IVP assay profiles revealed increased C16, C16:1 acylcarnitines, and decreased C2 (acetylcarnitine) indicating a typical pattern of CPT2 or CACT deficiency, with substantial reduction of long-chain acylcarnitines by the presence of bezafibrate in the cultured fibroblasts from both patients (Fig. 2). However, C2 acylcarnitine did not increase as expected.

3.2. Clinical trial of bezafibrate

We started a clinical trial of bezafibrate in case 1 at age of 2 years and 2 months, after the IVP assay which
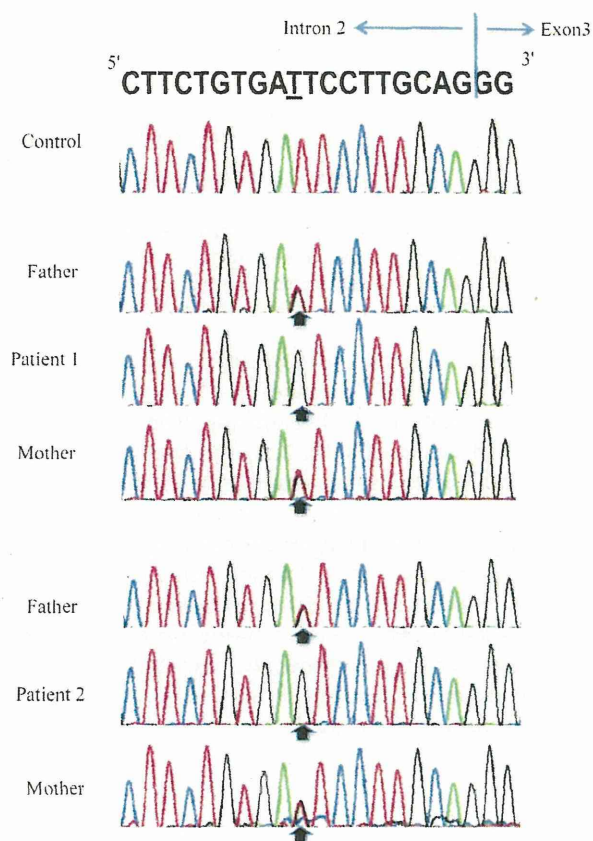

Fig. 1. The reference DNA sequence of an intron 2/exon 3 boundary of the *SLC25A20* gene, and the IVS2-10T>G mutation identified in both patients and their parents denoted by black arrows and the underlined letter.
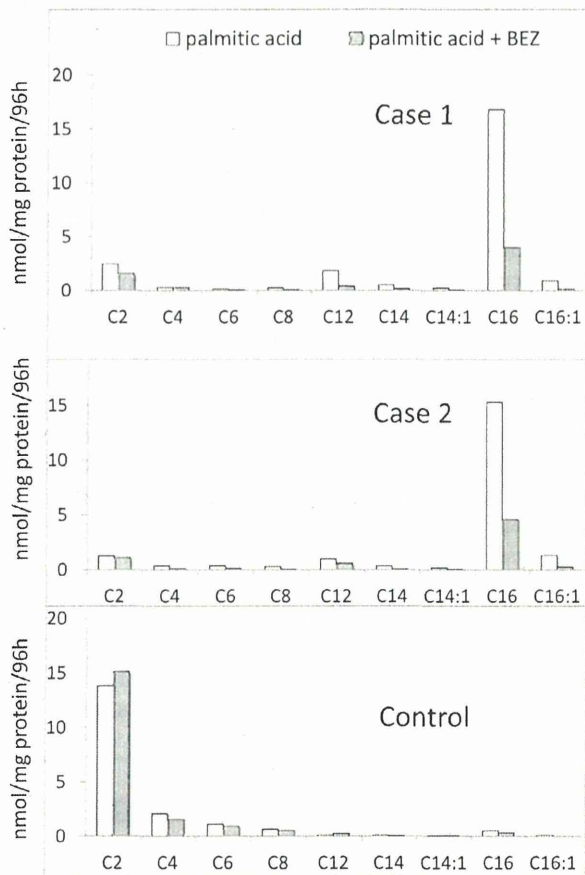
Fig. 2. Acylcarnitine profiles of IVP assay in the presence and absence of bezafibrate (BEZ) of cases 1, 2, and normal control respectively. Unit of vertical lines, nmol/mg protein of acylcarnitines (ACs); the horizontal lines represent acylcarnitines from C2, C4, C6, C8, C12, C14, C14:1, C16, and C16:1. The experiments for each were performed in triplicate, and the mean values of ACs are illustrated with bars.

showed some improvement in acylcarnitine profiles with bezafibrate. We used a dosage of 17–25 mg/kg/day as previously described [11]. Monitoring of liver functions, lactate dehydrogenase (LDH), creatine kinase (CK), and lipid profiles showed no adverse effects of bezafibrate. A short-term evaluation, after 6 months of the trial, did not show clinical improvement except for slightly increased back muscle strength noted by the mother. An echocardiography showed stable but no improvement in a left ventricular mass index. Acylcarnitine profiles in dried blood spots and other biochemical parameters did not show improvement (data not shown). Case 2 died before a clinical trial was considered.

4. Discussion

We report 2 unrelated cases of CACT deficiency with molecular confirmation first identified in Thailand. The c.199-10T>G (IVS2-10T>G) nucleotide change was the most prevalent mutation and identified in 14 out of 76 mutant alleles [15]. This mutation was homozygously identified in three Vietnamese and three Chinese patients. In the present study, in spite that two families had no consanguineous history, both patients were also a homozygotes of the c.199-10T>G mutation. In Japan, three CACT deficient patients have been described. Among them the same mutation was identified heterozygously in only one patient [14]. We propose that this mutation is a founder mutation in Asian populations. Clinical history of the three Chinese patients with homozygous c.199-10T>G mutation were reported [16]. All of them developed cardiac arrest within two days of age, as well as our two patients. Hence the phenotype of homozygotes of c.199-10T>G mutation is severe. This mutation was suggested to reside at a consensus lariat branch point sequence resulting in skipping of exons 3 and 4 or exon 3 alone, which leads to truncation of the protein [17].

Although our cases 1 and 2 were homozygotes of the same mutation, Case 1 survived until 2 years and 8 months and Case 2 died at 4 months of age. Several factors might attribute to their different clinical outcomes: (1) Thalassemia disease in case 2 which required repeated blood transfusions might affect cardiac functions by chronic hypoxia, iron overload, or decreased carnitine [18]; (2) differences in possible modifier genes such as *SLC25A29* gene (CACT-like, CACL) which has palmitoyl-carnitine transporting activity [19]; and (3) different formulas using in our cases, one is a synthetic modular formula and the other is a commercial formula. However, the rationale of both special formulas for diet therapy is a reduction in long-chain fatty acids together with supplementation of medium-chain triglyceride oil to be a caloric source shunting an obstruction of long-chain fatty acid β-oxidation.

Although increased FAO flux induced by bezafibrate was clearly shown in fibroblasts only from patients with mild phenotypes of FAO disorders, increased mRNA expression after bezafibrate exposure also occurred in cell lines from patients with severe phenotypes [20]. This could explain *in vitro* response to bezafibrate observed in fibroblasts of patient 1 and 2. Despite the severe genotype leading to barely detectable enzyme activity [21], we believe that there should be some FAO flux which could be enhanced by bezafibrate in these patients. Our hypothesis is if there is entirely absent FAO flux in these patients, they should have anomalies like those found in a lethal neonatal form of CPT2 deficiency or GA2 [22], even though there has been no report of such findings in CACT deficiency. To our knowledge, patient 1 is the first case of neonatal-onset CACT deficiency who underwent a clinical trial of bezafibrate after showing an *in vitro* response by IVP assay. However, no beneficial short-term effect was shown. This might indicate the irreversible damage of the affected organs esp. the cardiac and skeletal muscles, and liver. Moreover, the difference between the *in vitro* and *in vivo* responses is

probably due to the difference of bezafibrate concentration used in the IVP assay (400 μmol/L) and typical concentrations obtained in patients on bezafibrate therapy (50–200 μmol/L) [23]. Another possible reason is inadequate acetyl-CoA production despite bezafibrate treatment. This hypothesis is supported by persistently low C2 acylcarnitines in IVP assays of our cases and a previous case with CACT deficiency [11]. Moreover, C16 acylcarnitine did not decrease to the control level after bezafibrate treatment. Overall, although some improvement of acylcarnitine profile was shown in the patient 1 and 2's fibroblasts in IVP assay with bezafibrate, the effect of bezafibrate was less than those in fibroblasts from patients with mild forms of FAO disorders [11,24]. Hence clinical improvement in this patient was thought to be limited. Since CACT-deficient patients who developed metabolic decompensation in early neonatal period had poor prognosis with routine management [7], we decided to use bezafibrate treatment in patient 1. He survived until two years of age with bezafibrate treatment. However, it is uncertain whether this longer survival owed to the effect of bezafibrate treatment or not, since no apparent improvement of clinical laboratory data was obtained.

In conclusion, CACT deficiency may be a common FAO disorder in East Asian populations probably from a founder effect. IVP assay of fibroblasts could determine a response to bezafibrate treatment. A long-term clinical trial and more enrolled patients are required for evaluation of this therapy.

Acknowledgements

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Research Article

Changes in Cerebrospinal Fluid Biomarkers in Human Herpesvirus-6-Associated Acute Encephalopathy/Febrile Seizures

Naoyuki Tanuma,[1,2] Rie Miyata,[2] Keisuke Nakajima,[2] Akihisa Okumura,[3] Masaya Kubota,[4] Shin-ichiro Hamano,[5] and Masaharu Hayashi[2]

[1] *Department of Pediatrics, Tokyo Metropolitan Fuchu Medical Center for the Disabled, 2-9-2 Musashidai, Fuchu, Tokyo 183-8553, Japan*
[2] *Department of Brain Development and Neural Regeneration, Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8506, Japan*
[3] *Department of Pediatrics, School of Medicine, Juntendo University, Tokyo 113-8421, Japan*
[4] *Department of Neurology, National Center for Child Health and Development, Tokyo 157-8535, Japan*
[5] *Division of Neurology, Saitama Children's Medical Center, Saitama 339-8551, Japan*

Correspondence should be addressed to Naoyuki Tanuma; naoyuki_tanuma@member.metro.tokyo.jp

To determine the involvement of oxidative stress in the pathogenesis of acute encephalopathy associated with human herpesvirus-6 (HHV-6) infection, we measured the levels of oxidative stress markers 8-hydroxy-2′-deoxyguanosine (8-OHdG) and hexanoyl-lysine adduct (HEL), tau protein, and cytokines in cerebrospinal fluid (CSF) obtained from patients with HHV-6-associated acute encephalopathy (HHV-6 encephalopathy) ($n = 16$) and complex febrile seizures associated with HHV-6 (HHV-6 complex FS) ($n = 10$). We also examined changes in CSF-8OHdG and CSF-HEL levels in patients with HHV-6 encephalopathy before and after treatment with edaravone, a free radical scavenger. CSF-8-OHdG levels in HHV-6 encephalopathy and HHV-6 complex FS were significantly higher than in control subjects. In contrast, CSF-HEL levels showed no significant difference between groups. The levels of total tau protein in HHV-6 encephalopathy were significantly higher than in control subjects. In six patients with HHV-6 infection (5 encephalopathy and 1 febrile seizure), the CSF-8-OHdG levels of five patients decreased after edaravone treatment. Our results suggest that oxidative DNA damage is involved in acute encephalopathy associated with HHV-6 infection.

1. Introduction

Viral infection-associated acute encephalopathy/encephalitis is a serious complication with neurological sequelae. The main symptoms of the acute phase are impaired consciousness and convulsive status epilepticus with hyperpyrexia. Several subtypes of acute encephalopathy have been established based on clinical, radiologic, and laboratory findings. Acute encephalopathy with biphasic seizures and late reduced diffusion (AESD) is a new subtype characterized by a prolonged febrile seizure (FS) on day 1, which usually lasts longer than 30 min, as the initial neurological symptom [1, 2]. The initial seizures are followed by secondary seizures, most often a cluster of complex partial seizures on days 4–6. Magnetic resonance imaging (MRI) shows no acute abnormalities

until day 1 or 2 but reveals reduced subcortical diffusion from day 3 onwards. Hoshino et al. reported that AESD was the most frequent syndrome in a nationwide survey on the epidemiology of acute encephalopathy in Japan and that human herpesvirus-6 (HHV-6) was the most common preceding pathogenic infection in AESD [3]. Recent studies demonstrated three potential major pathomechanisms of viral associated encephalopathy: metabolic error, cytokine storm, and excitotoxicity [4]. However, the exact pathogenesis remains unknown.

Oxidative stress originates from an imbalance between the production of reactive oxygen species (ROS) and, to a lesser extent, reactive nitrogen species (RNS), and the antioxidant capacities of cells and organs [5]. Recently,

oxidative stress was confirmed to play a role in adult-onset neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [6, 7]. We confirmed the involvement of oxidative neuronal damage in child-onset neurodegenerative diseases, such as subacute sclerosing panencephalitis [8], xeroderma pigmentosum [9], Cockayne syndrome [10], and spinal muscular atrophy [11].

In the present study, we measured the levels of oxidative stress markers (8-hydroxy-2′-deoxyguanosine: 8-OHdG and hexanoyl-lysine adduct: HEL), tau protein, and cytokines in cerebrospinal fluid (CSF) obtained from patients with HHV-6-associated encephalopathy and complex FS associated with HHV-6 infection.

2. Patients and Methods

2.1. Patients. We analyzed CSF obtained in the acute phase of inpatients with HHV-6-associated encephalopathy (HHV-6 encephalopathy) ($n = 16$) and complex FS associated with HHV-6 (HHV-6 complex FS) ($n = 10$) during the period from 2008 to 2010. Laboratory diagnoses of HHV-6 infection were based on a virus-specific polymerase chain reaction (PCR) assay or detection of virus-specific antibodies. Diagnosis of acute encephalopathy or complex FS was performed by the attending physician and later confirmed by examination of available clinicoradiological information. All cases of HHV-6-associated encephalopathy were diagnosed based on the clinical course and MRI findings. The complex FS group consisted of children who presented with fever and seizure but were later found to be free from acute neurological damage based on the clinical course, laboratory data, and brain imaging. Another 16 children (15 with fever but not central nervous system infection and 1 with hypoglycemia) were also enrolled as control subjects. Parent consent was obtained in all subjects in accordance with the Helsinki Declaration and all protocols were approved by the institutional ethics committee of the Tokyo Metropolitan Fuchu Medical Center for the Disabled.

2.2. Sample Collection and Measurement of CSF Biomarkers. CSF samples were obtained from each patient at any point during the disease and immediately stored at −80°C until they were analyzed. The amount of DNA oxidative stress marker, 8-hydroxy-2′-deoxyguanosine (8-OHdG), and the early stage lipid peroxidation marker, hexanoyl-lysine adduct (HEL), was examined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Japan Institute for the Aging, Shizuoka, Japan). Total tau protein was determined using sandwich ELISA (Invitrogen Corporation, Camarillo, CA). The levels of cytokines were evaluated by multiplex bead-based immunoassay (BioPlex 200 system) (Bio-Rad Laboratories, Inc., Hercules, CA). All assays were carried out according to the manufacturer's protocols. The detection limit for each ELISA kit was 0.06 ng/mL (8-OHdG), 2.6 ng/mL (HEL), and 15 pg/mL (total tau protein).

2.3. Edaravone Treatment. Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a free radical scavenging drug that

is clinically used in Japan for treatment of acute ischemic stroke [12, 13]. Several studies have shown that edaravone has preventive effects on brain injury following ischemia and reperfusion in patients with brain attack [14, 15]. Based on these observations, six patients with HHV-6 infection (5 patients with encephalopathy and 1 patient with complex FS) received free radical scavenger edaravone treatment in addition to conventional therapy for acute encephalopathy. A standard treatment protocol is edaravone 0.5 mg/kg every 12 hours (1 mg/kg daily) intravenously for 7–12 days. Parent consent was obtained in all patients before the treatment.

2.4. Statistical Analysis. Data were analyzed by GraphPad Prism version 5.0. Differences in oxidative stress markers, tau protein, and cytokine levels among each group were analyzed by one-way analysis of variance (ANOVA) and Dunn's multiple comparison test. Correlations between CSF-8OHdG and other biomarkers were evaluated using Spearman's rank correlation coefficient. We used Fisher's exact test to examine the relationship between increased levels of each biomarker and the presence or absence of neurological sequelae in HHV-6 encephalopathy. Comparisons of levels of CSF biomarkers before and after edaravone treatment were performed by paired t-test. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Study Population and Clinical Features. The characteristics of the patients included in the study are summarized in Table 1. There were no significant differences of age among each group. Thirteen of 16 patients (81.3%) with HHV-6 encephalopathy were AESD, and only five patients (31.3%) recovered without sequelae from HHV-6 encephalopathy. In contrast, all patients with complex FS associated with HHV-6 infection were without neurological sequelae.

3.2. Oxidative DNA Damage and Lipid Peroxidation in HHV-6 Encephalopathy and Complex FS. The CSF-8-OHdG levels in HHV-6 encephalopathy (0.129 ± 0.07 ng/mL, mean ± SD, $P < 0.01$) and HHV-6 complex FS (0.116 ± 0.061 ng/mL, mean ± SD, $P < 0.05$) patients were significantly higher than in control subjects (0.063 ± 0.01 ng/mL, mean ± SD) (Figure 1(a)). CSF-HEL levels (mean ± SD) in HHV-6 encephalopathy, HHV-6 complex FS, and control subjects were 3.59 ± 1.87 nmol/L, 5.24 ± 3.63 nmol/L, and 3.62 ± 1.08 nmol/L, respectively. There were no significant differences in CSF-HEL levels between all groups (Figure 1(b)). These data are summarized in Table 2.

3.3. Total Tau Protein Levels in HHV-6 Encephalopathy and Complex FS. Total tau protein levels in HHV-6 encephalopathy patients ($n = 16$) ($13,905.6 \pm 14,201.1$ pg/mL, mean ± SD) were significantly higher than in control subjects (609.0 ± 342.0 pg/mL, mean ± SD) ($P < 0.05$, Figure 2). However, there were no significant differences in CSF tau protein levels between the HHV-6 encephalopathy group and HHV-6 FS group (654.7 ± 213.7 pg/mL, mean ± SD). We then divided

TABLE 1: Clinical characteristics of HHV-6 encephalopathy and febrile seizure patients.

	HHV-6 encephalopathy	HHV-6 complex febrile seizures	Controls
Number of patients	16	10	16
Age (months)	15.1 ± 5.4	12.6 ± 3.9	11.1 ± 10.8
Sex ratio (M : F)	8 : 8	5 : 5	11 : 5
Sampling time (day of illness)	1–8	1	—
MRI abnormality	14/16	ND	ND
Outcome (without sequelae)	5/16	10/10	—

HHV: human herpesvirus; No.: number; ND: not done; M: male; F: female; MRI: magnetic resonance imaging.
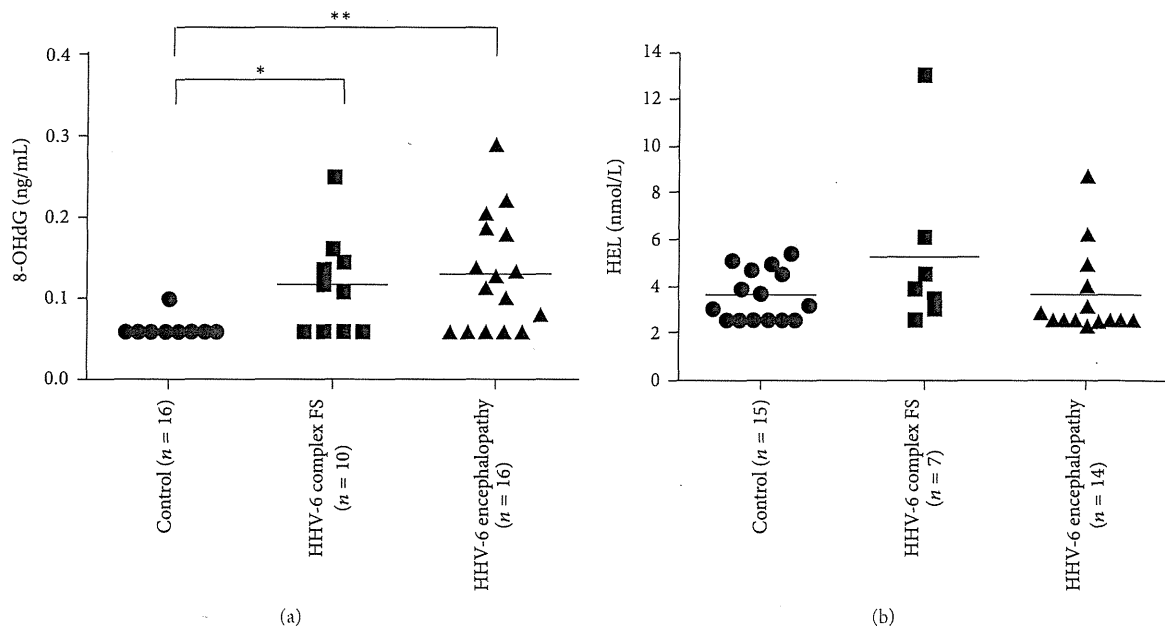

(a)   (b)

FIGURE 1: Cerebrospinal fluid (CSF) levels of oxidative stress markers in HHV-6 encephalopathy, HHV-6 complex febrile seizures (FS), and controls. (a) 8-hydroxy-2′-deoxyguanosine: 8-OHdG, (b) hexanoyl-lysine adduct: HEL. $^*P < 0.05$, $^{**}P < 0.01$. The horizontal bar indicates the mean value of each group. CSF-8OHdG levels (mean ± SD) in HHV-6 encephalopathy, HHV-6 complex FS, and controls are 0.129 ± 0.07 ng/mL, 0.116 ± 0.061 ng/mL, and 0.063 ± 0.01 ng/mL, respectively. CSF-HEL levels (mean ± SD) in HHV-6 encephalopathy, HHV-6 complex FS, and control subjects are 3.59 ± 1.87 nmol/L, 5.24 ± 3.63 nmol/L, and 3.62 ± 1.08 nmol/L, respectively.

TABLE 2: Descriptive statistics for the biomarkers examined[a].

Biomarkers	Controls	HHV-6 complex FS	HHV-6 encephalopathy	Global	P Controls versus HHV-6 FS	P Controls versus HHV-6 encephalopathy	P HHV-6 FS versus HHV-6 encephalopathy
8-OHdG, ng/mL	0.063 (0.01)	0.116 (0.061)	0.129 (0.07)	0.0025	<0.05	<0.01	ns
HEL, nmol/L	3.62 (1.08)	5.24 (3.63)	3.59 (1.87)	0.1863	ns	ns	ns
Tau, pg/mL	609.0 (342.0)	654.7 (213.7)	13,905.6 (14,201.1)	0.0028	ns	<0.05	ns
IL-6, pg/mL	3.2 (3.0)	5.8 (5.3)	74.6 (116.9)	0.0349	ns	<0.01	ns
IL-10, pg/mL	0.4 (0.3)	0.6 (0.8)	1.4 (2.1)	0.1663	ns	ns	ns
TNF-α, pg/mL	0.1 (0.1)	0.3 (0.5)	3.4 (4.0)	0.0036	ns	<0.01	<0.05

8-OHdG: 8-hydroxy-2′-deoxyguanosine; HEL: hexanoyl-lysine adduct; HHV-6: human herpesvirus-6; FS: febrile seizure; ns: not significant; IL: interleukin; TNF: tumor necrosis factor.
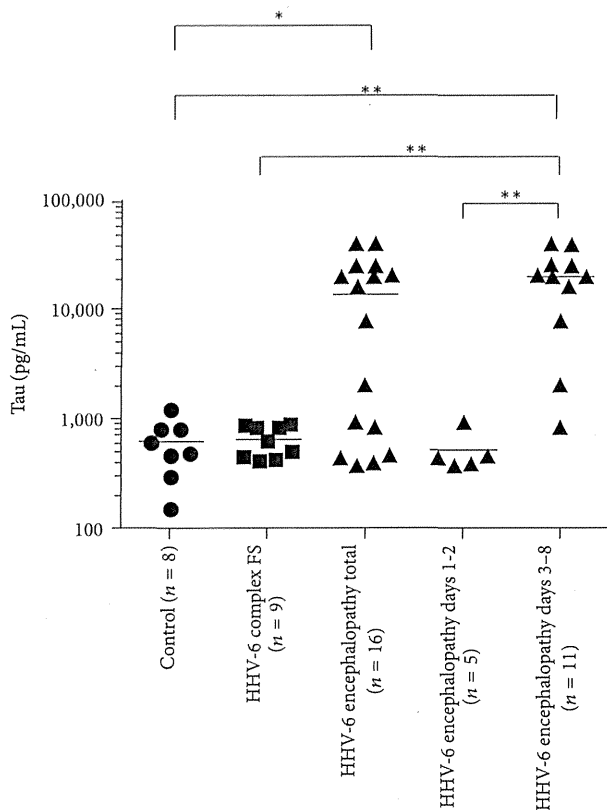[a]Values are expressed as the mean (standard deviation).

FIGURE 2: Cerebrospinal fluid (CSF) tau protein levels. The horizontal bar indicates the mean value of each group. CSF levels (mean ± SD) of tau protein in HHV-6 encephalopathy, HHV-6 complex febrile seizures (FS), and controls are 13,905.6 ± 14,201.1 pg/mL, 654.7±213.7 pg/mL, and 609.0±342.0 pg/mL, respectively. Total tau protein levels in HHV-6 encephalopathy patients are significantly higher than in control subjects ($^*P < 0.05$). The levels of tau protein in HHV-6 encephalopathy at days 3–8 (19,856.9 ± 13,121.9 pg/mL) are significantly higher than those of HHV-6 encephalopathy at days 1-2 (520.4 ± 229.6 pg/mL), HHV-6 complex FS, and controls ($^{**}P < 0.01$).

the HHV-6 encephalopathy group into two groups according to sampling time at days 1-2 ($n = 5$) and days 3–8 ($n = 11$), respectively. Consequently, we found that the levels of tau protein were significantly increased at days 3–8 in HHV-6 encephalopathy (19,856.9 ± 13,121.9 pg/mL, mean ± SD) compared with those of HHV-6 encephalopathy at days 1-2 (520.4 ± 229.6 pg/mL, mean ± SD) ($P < 0.01$), HHV-6 complex FS ($P < 0.01$), and controls ($P < 0.01$) (Figure 2).

3.4. CSF Cytokine Profile in Acute Encephalopathy and Complex FS.
We next confirmed the elevation of CSF IL-6 and TNF-α in patients with HHV-6 encephalopathy (Figure 3). The CSF IL-6 levels in patients with HHV-6 encephalopathy (74.6 ± 116.9 pg/mL, mean ± SD) were significantly higher than in controls (3.2 ± 3.0 pg/mL, mean ± SD) ($P < 0.01$) (Figure 3(a)). The CSF TNF-α levels in patients with HHV-6 encephalopathy (3.4 ± 4.0 pg/mL, mean ± SD) were also

significantly higher than those with complex FS (0.3 ± 0.5 pg/mL, mean ± SD) and in controls (0.10 ± 0.1 pg/mL, mean ± SD) ($P < 0.05$ and $P < 0.01$, resp.) (Figure 3(c)). In contrast, there were no significant differences of CSF IL-10 levels among patients with HHV-6 encephalopathy or HHV-6 complex FS and controls (Figure 3(b)).

3.5. Correlation Analysis of CSF Biomarkers in HHV-6 Encephalopathy.
We next examined correlations between CSF-8OHdG and other biomarkers in the HHV-6 encephalopathy group (Table 3). There was a significant positive correlation between IL-6 and TNF-α (Spearman $r = 0.783$, $P = 0.0006$). However, there were no significant correlations among other biomarkers. In addition, there was no correlation between the increased levels of each biomarker and the presence or absence of neurological sequelae in HHV-6 encephalopathy (data not shown).

3.6. Changes in CSF-8-OHdG and CSF-HEL Levels before and after Edaravone Treatment in HHV-6-Associated Acute Encephalopathy and Complex FS.
Finally, we compared the CSF levels of oxidative stress markers in six patients with HHV-6 infection (5 patients with encephalopathy and 1 patient with febrile seizures) before and after edaravone treatment. Clinical profile of patients with edaravone treatment is shown in Table 4. The mean initiation time of edaravone treatment was day 4.8 for the HHV-6 encephalopathy group. One patient with febrile seizures associated with HHV-6 infection who received edaravone treatment from day 1 did not develop encephalopathy and recovered without sequelae (patient 6). The CSF-8-OHdG levels decreased after edaravone treatment ($P = 0.0202$, paired t-test) (Figure 4(a)). Regarding the CSF-HEL levels, there were no significant differences between before and after edaravone treatment. We also compared the mean CSF levels of other biomarkers before and after treatment and observed no significant differences of mean values (data not shown).

4. Discussion

In the present study, we demonstrated that CSF-8-OHdG levels in HHV-6 encephalopathy and HHV-6 complex FS patients were significantly higher than in controls, suggesting increased oxidative stress is induced by HHV-6 infection. Recent studies revealed that oxidative damage is an emerging general mechanism of nervous system injury caused by viral infection. For example, oxidative injury is a component of acute encephalitis caused by herpes simplex virus type 1 (HSV-1) [16]. HSV-1 infection of nervous system tissues in mice was associated with the expression of inducible nitric oxide synthase (iNOS) and the release of cytokines including TNF-α from inflammatory cells. Thus, increased generation of ROS and RNS can be caused by the direct effects of virus on cells and the indirect effects of host inflammatory responses [17]. Regarding HHV-6 infection, Fukuda et al. reported that urinary 8-OHdG concentrations in a patient with HHV-6 encephalopathy on the first day of hospitalization were 1.5 times higher than the mean concentration
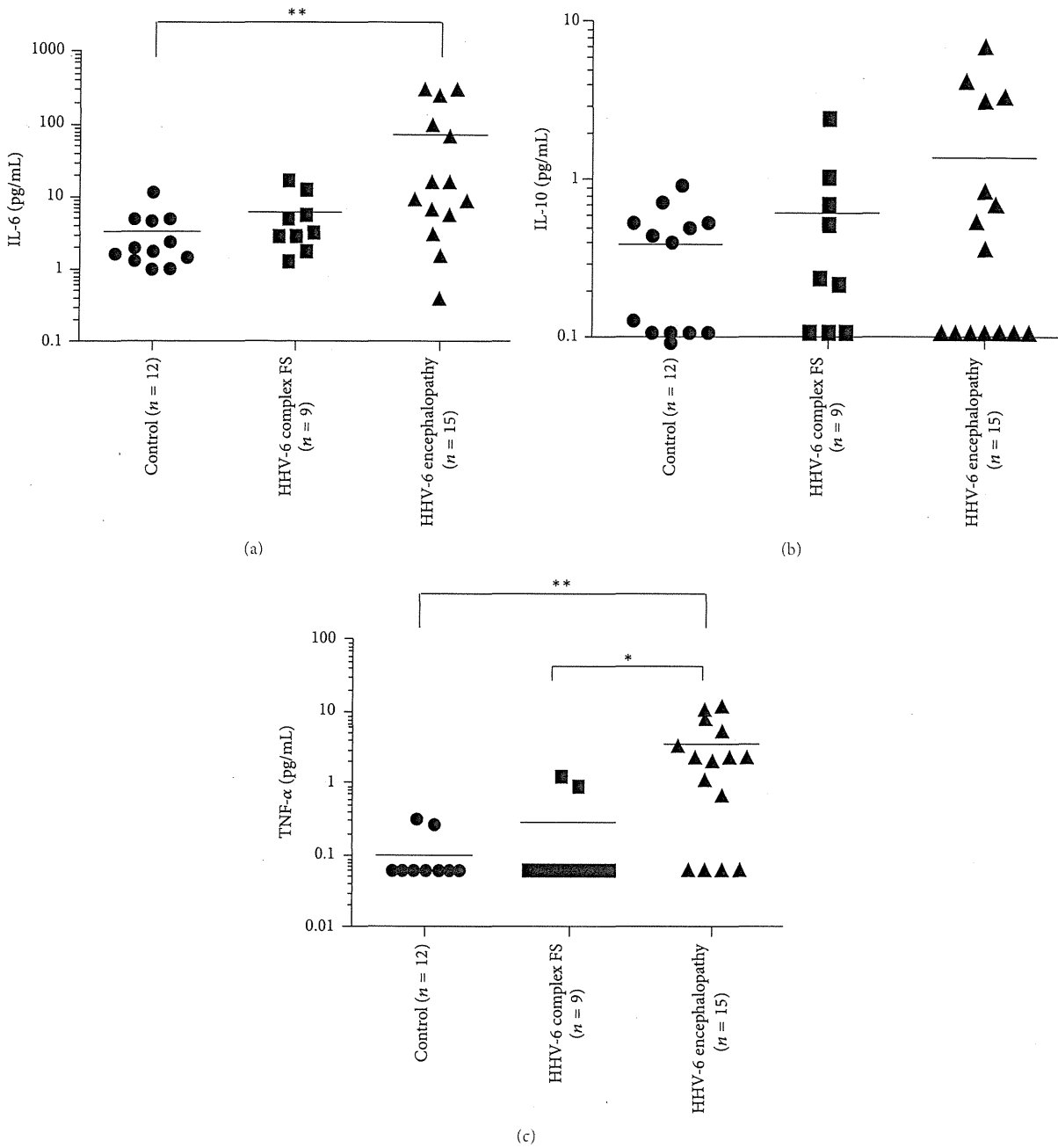
(a)

(b)

(c)

FIGURE 3: Cerebrospinal fluid (CSF) cytokine levels. The horizontal bar indicates the mean value of each group. (a) Levels of CSF IL-6 in patients with HHV-6 encephalopathy, HHV-6 febrile seizures (FS), and controls are 74.6 ± 116.9 pg/mL, 5.8 ± 5.3 pg/mL, and 3.2 ± 3.0 pg/mL, respectively. The CSF IL-6 levels in patients with HHV-6 encephalopathy are significantly higher than in controls ($^{**}P < 0.01$). (b) Levels of CSF IL-10 in patients with HHV-6 encephalopathy, HHV-6 febrile seizures (FS), and controls are 1.4 ± 2.1 pg/mL, 0.6 ± 0.8 pg/mL, and 0.4 ± 0.3 pg/mL, respectively. (c) Levels of CSF TNF-α in patients with HHV-6 encephalopathy, HHV-6 febrile seizures (FS), and controls are 3.4 ± 4.0 pg/mL, 0.3 ± 0.5 pg/mL, and 0.1 ± 0.1 pg/mL, respectively. The CSF TNF-α levels in patients with HHV-6 encephalopathy are significantly higher than those with complex FS and in controls ($^{*}P < 0.05$ and $^{**}P < 0.01$, resp.).