ADORA2A polymorphism predisposes children to encephalopathy with febrile status epilepticus

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ADORA2A polymorphism predisposes children to encephalopathy with febrile status epilepticus

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ABSTRACT

Objective: Acute encephalopathy with biphasic seizures and late reduced diffusion (AESD) is a childhood encephalopathy following severe febrile seizures, leaving neurologic sequelae in many patients. However, its pathogenesis remains unclear. In this study, we clarified that genetic variation in the adenosine A2A receptor (*ADORA2A*), whose activation is involved in excitotoxicity, may be a predisposing factor of AESD.

Methods: We analyzed 4 *ADORA2A* single nucleotide polymorphisms in 85 patients with AESD. The mRNA expression in brain samples, mRNA and protein expression in lymphoblasts, as well as the production of cyclic adenosine monophosphate (cAMP) by lymphoblasts in response to adenosine were compared among *ADORA2A* diplotypes.

Results: Four single nucleotide polymorphisms were completely linked, which resulted in 2 haplotypes, A and B. Haplotype A (C at rs2298383, T at rs5751876, deletion at rs35320474, and C at rs4822492) frequency in patients was significantly higher than in controls ($p = 0.005$). Homozygous haplotype A (AA diplotype) had a higher risk of developing AESD (odds ratio 2.32, 95% confidence interval 1.32–4.08; $p = 0.003$) via a recessive model. mRNA expression was significantly higher in AA than AB and BB diplotypes, both in the brain ($p = 0.003$ and 0.002, respectively) and lymphoblasts ($p = 0.035$ and 0.003, respectively). In lymphoblasts, ADORA2A protein expression ($p = 0.024$), as well as cellular cAMP production ($p = 0.0006$), was significantly higher in AA than BB diplotype.

Conclusions: AA diplotype of *ADORA2A* is associated with AESD and may alter the intracellular adenosine/cAMP cascade, thereby promoting seizures and excitotoxic brain damage in patients.

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GLOSSARY

ADORA1 = adenosine A1 receptor; **ADORA2A** = adenosine A2A receptor; **AEIMSE** = acute encephalopathy with inflammation-mediated status epilepticus; **AESD** = acute encephalopathy with biphasic seizures and late reduced diffusion; **cAMP** = cyclic adenosine monophosphate; **CI** = confidence interval; **CPT2** = carnitine palmitoyltransferase II; **G6PDH** = glucose-6-phosphate dehydrogenase; **OR** = odds ratio; **SMRI** = Stanley Medical Research Institute; **SNP** = single nucleotide polymorphism.

During the course of acute febrile diseases, such as influenza and exanthema subitum, some children develop repetitive or prolonged seizures, followed by sustained impairment of consciousness. These conditions are collectively termed acute encephalopathy with inflammation-mediated status epilepticus (AEIMSE).[1] Among AEIMSE, acute encephalopathy with biphasic seizures and late reduced diffusion (AESD)[2] is the most common in Japan, affecting hundreds of children every year.[3] Hemiconvulsion-hemiplegia syndrome, a condition encountered worldwide, often occurs during an infectious disease, and is regarded as a subgroup of AESD.[4] AESD typically shows a biphasic clinical course, consisting of a prolonged febrile seizure

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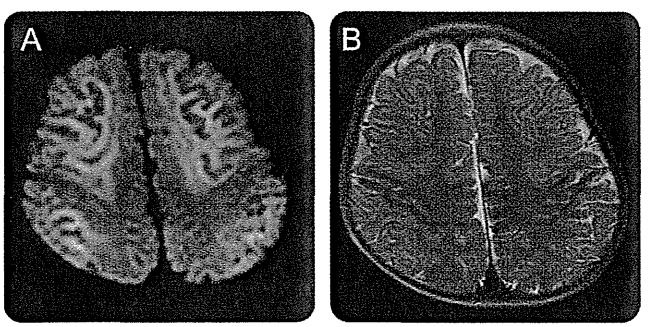
on the first day and a cluster of complex partial seizures several days later (late seizure), each followed by postictal coma. Cranial MRI reveals high signal intensity lesions in the cerebral subcortical white matter on diffusion-weighted images, which appear around the occurrence of late seizure (figure 1).[5,6] Excitotoxicity is considered to be the main pathologic mechanism of AESD.[2,4] The genetic background of AESD remains to be elucidated. Recently, polymorphism of a gene encoding a mitochondrial enzyme, carnitine palmitoyltransferase II (*CPT2*), was identified as a genetic predisposition for AESD[7]; however, some patients with AESD have no such polymorphism, suggesting the involvement of genes other than *CPT2*.

We hypothesized that the adenosine-mediated signal pathway is altered in AESD because theophylline, a nonselective adenosine receptor antagonist, aggravates AESD.[4] To test this hypothesis, we studied the haplotype frequency of 4 single nucleotide polymorphisms (SNPs) located in the linkage disequilibrium block of the adenosine A2A receptor (*ADORA2A*) gene, and then examined the effects of *ADORA2A* diplotypes on their mRNA and protein expression, and those on cyclic adenosine monophosphate (cAMP) production in response to adenosine.

METHODS

Subjects. We recruited patients with AESD from hospitals in Japan during 2008–2011 based on the diagnostic criteria.[3] Eighty-five Japanese patients, 39 male and 46 female aged from 6 months to 10 years and 3 months (median, 1 year and 10 months), participated in this study. Detailed clinical data are shown in table e-1 on the *Neurology*® Web site at www.neurology.org. All patients had their first convulsion, mostly status epilepticus, within 24 hours from the onset of fever, followed by impairment of consciousness that improved on the second day in most cases. On the fourth to sixth day of illness, there was a recurrence of convulsions or a cluster of partial seizures, followed again by impairment of consciousness. Cranial MRI was normal on the first to second day of illness, but showed high signal intensity lesions in the cerebral subcortical white matter on the third to ninth day (figure 1). Pathogens of antecedent infections included human herpesvirus 6 (28 cases), influenza virus (5 cases), respiratory syncytial virus, rotavirus, adenovirus, mumps virus, and *Mycoplasma pneumoniae*.

Figure 1 Typical MRI findings of a patient with acute encephalopathy with biphasic seizures and late reduced diffusion



Magnetic resonance study of a 1-year-old boy on day 8 demonstrated lesions in the subcortical white matter that showed high signal intensity on diffusion-weighted (A) and T2-weighted (B) images. The lesions were prominent along the U-fibers with sparing of the peri-Rolandic region.

Standard protocol approvals, registrations, and patient consents. The procedures in this study were approved by the University of Tokyo Ethics Committee. Written informed consent was obtained from all guardians of participants in the study.

Controls. We analyzed the *ADORA2A* genotypes of control subjects, consisting of 100 healthy Japanese adults, 50 men and 50 women, 20 to 69 years of age, using DNA extracted from Pharma SNP Consortium B cell lines obtained from the Human Science Research Resources Bank (Osaka, Japan). We searched the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) in the National Center for Biotechnology Information for the variation frequencies of *ADORA2A* SNPs and combined the data of 100 controls from the Pharma SNP Consortium and those of 84 Japanese in the National Center for Biotechnology Information dbSNP database.

Brain samples. To examine *ADORA2A* gene expression levels in the brain, 100 human brain DNA and RNA samples were obtained from Stanley Medical Research Institute (SMRI) (Bethesda, MD). DNA and RNA were extracted from the occipital and anterior cingulate cortex, respectively. In this experiment, the ethnic background was Caucasian in the vast majority (at least 98 samples).

Lymphoblasts. For expression studies and functional assays, we used 15 lymphoblast cell lines from control Japanese adults, obtained from control subjects at the University of Tokyo Hospital.

Procedures. Peripheral blood samples were collected from the patients. Genomic DNA was extracted from the blood using standard protocols. All 5 exons of *ADORA2A* were PCR amplified with flanking intronic primers and standard PCR conditions (primer sequences are described in table e-2). PCR products of *ADORA2A* were sequenced on a 310 Genetic Analyzer, 3100 Genetic Analyzer, or 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA). To identify rs5751876 and rs2298383 SNPs, the PCR–restriction fragment length polymorphism method was adopted.[8] For quantitative PCR, total RNA was isolated from control lymphoblasts using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. Total RNA was reversely transcribed to cDNA by a Ready-To-Go You-Prime First-Stand Beads cDNA synthesis kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. Random Primer (Takara Bio, Otsu, Japan) was used. Gene expression was evaluated by the relative Quantification ABI PRISM 7000 Sequence Detection System (Life Technologies) with FastStart Universal SYBR Green Master [ROX] (Roche, Basel, Switzerland) reagent. The relative *ADORA2A* mRNA expression level was calculated using glucose-6-phosphate dehydrogenase (G6PDH) as the internal standard. Primer sequences of real-time PCR for *ADORA2A* and *G6PDH* are described in table e-3. Each value is shown as the mean value of 2 independent experiments in triplicate. For SMRI brain samples, genotyping and the gene expression study of *ADORA2A* were performed by the same methods as for AESD patient samples. Western blotting

of the cell lysate from control lymphoblasts was performed by the standard protocol using a rabbit polyclonal antibody to human ADORA2A (Abcam, Cambridge, UK) at a dilution of 1:500. The relative ADORA2A protein expression level was calculated using β-actin as the internal standard. Each value is shown as the mean value of 3 independent experiments in duplicate. The cAMP concentration in lymphoblasts was measured after stimulation by adenosine (10 nM) and 8-cyclopentyl-1,3-dipropylxanthine (10 nM), a selective adenosine A1 receptor (ADORA1) antagonist, using the cAMP-Screen Direct System (Life Technologies) according to the manufacturer's protocol. Cellular cAMP levels were determined using SpectraMax Pro 5.3 software (Molecular Devices, Sunnyvale, CA). Each value is shown as the mean value of 2 independent experiments in triplicate.

Statistical analysis. Differences in the demographic characteristics of the genotypes between patients (85 cases) and controls were assessed by Pearson χ^2 test and Fisher exact test for categorical data. Goodness-of-fit to the Hardy-Weinberg equilibrium and differences in genotype and allele frequencies between AESD and control groups were examined by χ^2 analysis. Significant differences were defined as $p < 0.05$ in conditional analysis. We estimated the odds ratio (OR) together with the 95% confidence interval (CI) for each allele haplotype frequency with AESD using Microsoft Office Excel 2010. Patients with AESD were compared with the controls under dominant, recessive, and additive models using a likelihood ratio χ^2 test. These genetic models were also assessed using the Cochran-Armitage test for trend. The differences in mRNA and protein expression levels and cellular cAMP accumulation, expressed as the mean ± SEM, were calculated using analysis of variance followed by the Tukey-Kramer test in the case of multiple comparisons. $p < 0.05$ was considered a significant difference.

RESULTS

***ADORA2A* haplotype frequency.** First, we analyzed the entire coding region of *ADORA2A* in patients with AESD and found no mutations. Second, we analyzed genetic variations of *ADORA2A* in patients with AESD and control subjects. Distribution of the *ADORA2A* polymorphisms in both AESD and controls met the Hardy-Weinberg equilibrium ($p = 0.15$ and 0.86, respectively). Four SNPs (figure e-1) in this gene, rs2298383, rs5751876, rs35320474, and rs4822492, had previously been reported to show complete linkage disequilibrium in 84 Japanese (human HapMap project, http://Apr2011.archive.ensemble.org). The present study also supported their complete linkage in both 85 AESD cases and 100 controls. Thus, there were only 2 haplotypes, haplotype A (C at rs2298383, T at rs5751876, deletion at rs35320474, and C at rs4822492) and haplotype B (T at rs2298383, C at rs5751876, T at rs35320474, and G at rs4822492). Table 1 shows haplotype frequency for the *ADORA2A* SNPs in AESD and control groups. Haplotype A was significantly more frequent in AESD than in controls ($p = 0.005$). The frequency of homozygous haplotype A (AA diplotype) in AESD and controls was 37.6% and 20.6%, respectively. There was a significant association between AA diplotype and increased risk of developing AESD for recessive model comparison (OR 2.32, 95% CI 1.32–4.08; $p = 0.003$) and additive model comparison (OR 2.62, 95% CI 1.29–5.32; $p = 0.007$), but not for the dominant model comparison (OR 1.63, 95% CI 0.89–2.99; $p = 0.142$) (table 2). The most significant p value was obtained under the recessive model using χ^2 test, as well as Cochran-Armitage test for trend.

***ADORA2A* mRNA expression in the brain.** Second, to evaluate the association of *ADORA2A* diplotypes with gene expression in the CNS tissue, we measured the amount of *ADORA2A* mRNA in SMRI samples after genotyping. Because the 4 SNPs were completely linked in 95 of 100 subjects (diplotype AA, 19 subjects; AB, 38 subjects; and BB, 38 subjects), we used these 95 samples. The relative expression level of *ADORA2A* mRNA (mean ± SEM) in AA, AB, and BB diplotypes was 0.246 ± 0.025, 0.179 ± 0.009, and 0.177 ± 0.009, respectively (figure 2). The expression level was 1.4-fold higher in the AA diplotype than in AB and BB, showing a significant difference ($p = 0.003$ and 0.002, respectively).

***ADORA2A* mRNA and protein expression and production of cAMP in lymphoblasts.** ADORA2A is highly expressed in brain, heart, kidney, and lymphocytes.[9,10] Because protein samples from the brain were unavailable, we used lymphoblast cell lines to determine the effect of *ADORA2A* diplotypes on ADORA2A protein expression. We again showed that the expression of *ADORA2A* mRNA in lymphoblasts with AA diplotype was higher than in those with AB and BB (figure 3A,

Table 1 Comparison of *ADORA2A* haplotype frequency between patients with AESD and controls[a]

	Genotype				AESD		Control[b]		Test for allele haplotype frequency
Haplotype	rs2298383	rs5751876	rs35320474	rs4822492	n	%	n	%	OR (95% CI)
A	C	T	del	C	99	58.2	166	45.1	1.70 (1.17–2.45)
B	T	C	T	G	71	41.8	202	54.9	
Total					170		368		

Abbreviations: AESD = acute encephalopathy with biphasic seizures and late reduced diffusion; CI = confidence interval; OR = odds ratio.
[a] Difference in haplotype frequency between patients and controls was statistically significant ($p = 0.005$).
[b] Data of Pharma SNP Consortium B cell samples and those of HapMap (JPT) were combined.

Table 2 Comparison of *ADORA2A* diplotype distribution between patients with AESD and controls

Diplotype	AESD (n = 85), n (%)	Control (n = 184),[a] n (%)	OR (95% CI), p value	
AA	32 (37.6)	38 (20.6)	2.62 (1.29-5.32), 0.007	
AB	35 (41.2)	90 (49.0)	1.21 (0.62-2.34), 0.612	
BB	18 (21.2)	56 (30.4)	1.00 (reference)	
Genetic model				p Value for trend test
Recessive	AA vs AB + BB		2.32 (1.32-4.08), 0.003	0.003
Dominant	AA + AB vs BB		1.63 (0.89-2.99), 0.142	0.114
Additive	AA vs BB		2.62 (1.29-5.32), 0.007	0.006

Abbreviations: AESD = acute encephalopathy with biphasic seizures and late reduced diffusion; CI = confidence interval; OR = odds ratio.
[a] Data of Pharma SNP Consortium B cell samples and those of HapMap (JPT) were combined.

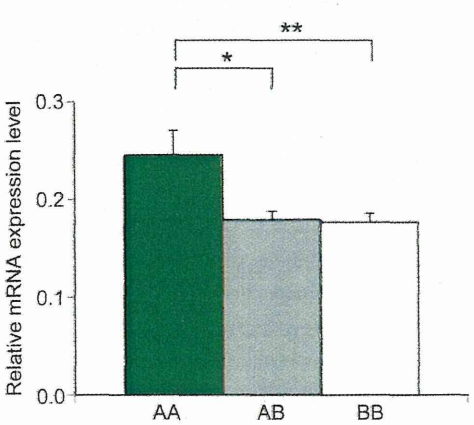
p = 0.035 and 0.003, respectively). By Western blotting, the relative ADORA2A protein level (mean ± SEM) was evaluated as 0.611 ± 0.045, 0.439 ± 0.022, and 0.443 ± 0.044 for AA, AB, and BB, respectively (figure 3B). The protein expression was significantly higher in AA diplotype than in AB and BB (p = 0.021 and 0.024, respectively). Next, to elucidate the difference of intracellular signal transduction among 3 *ADORA2A* diplotypes, cAMP assay was performed. The cellular cAMP accumulation level (mean ± SEM) for AA, AB, and BB diplotypes was 2.016 ± 0.207, 1.421 ± 0.186, and 0.953 ± 0.118 pmol, respectively (figure 3C). As the number of haplotype A alleles increased, so did the adenosine-stimulated cAMP production. The cAMP level was significantly higher in the AA diplotype than in BB (p = 0.0006).

DISCUSSION Previous studies have shown the complex roles of adenosine in the brain, deriving from the diversity of receptor subtypes. In the CNS, ADORA2A competes with ADORA1 in various neural functions. For synaptic transmission, ADORA2A enhances excitatory neurotransmitter release, whereas ADORA1 exerts an inhibitory effect.[11] The role of adenosine as an endogenous anticonvulsant is mediated via ADORA1.[12] Inhibition of ADORA1 function has been shown to cause status epilepticus.[13] In a rat model of seizure kindling, ADORA1 in the hippocampal CA1 region reduces seizures, whereas ADORA2A promotes them.[14] *Adora2a* knockout mice show a reduction of ethanol-induced seizures,[15] whereas activation of ADORA2A renders rat pups susceptible to hyperthermia-induced seizures.[16] Despite these findings, the association of *ADORA2A* variations with a seizure disorder has never been reported. They are known to be associated with anxiety induced by caffeine, an antagonist of ADORA1 and ADORA2A.[17–19]

The present study showed for the first time the association between an *ADORA2A* genetic variant and AESD, a typical syndrome of AEIMSE during early childhood. The results suggest that *ADORA2A* AA diplotype predisposes children to AESD by altering the intracellular adenosine/cAMP signal cascade.

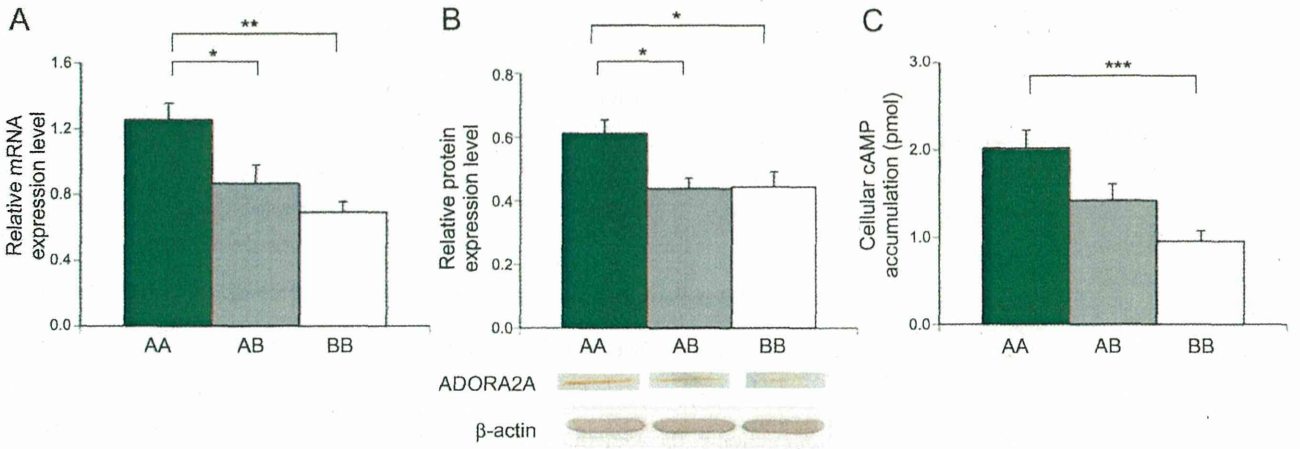
We demonstrated that the frequency of *ADORA2A* AA diplotype was significantly higher in patients with AESD than in controls (table 2). These data show an apparent association between AA diplotype and AESD, although whether the recessive or additive model most accurately describes this association is unclear at this time. Haplotype A consists of 4 SNPs, rs2298383, rs5751876, rs35320474, and rs4822492, which show complete linkage disequilibrium with one another in Japanese. The rs2298383 SNP is located in a potential promoter region upstream of the recently identified exon variant,[8] with a regulatory element predicted from alignment of human and other mammalian genes.[20] Further evidence of its importance in gene expression regulation is provided by *in silico* analyses,[21] which indicated the position

Figure 2 *ADORA2A* mRNA expression in the brain with different *ADORA2A* diplotypes



Relative *ADORA2A* mRNA expression level (*ADORA2A*/*G6PDH*) in the brain was higher in the AA diplotype (n = 19) than in AB (n = 38, *p = 0.003) and BB (n = 38, **p = 0.002).

Figure 3 *ADORA2A* mRNA expression, ADORA2A protein expression, and cAMP production in lymphoblasts with different *ADORA2A* diplotypes



(A) Relative *ADORA2A* mRNA expression level (*ADORA2A*/*G6PDH*) is higher in AA diplotype than in AB (*$p = 0.035$) and BB (**$p = 0.003$) (n = 5 for each diplotype). (B) Relative ADORA2A protein expression level (ADORA2A/β-actin) was higher in AA than in AB (*$p = 0.021$) and BB (*$p = 0.024$) (n = 5 for each diplotype). Lower panel shows results of a representative Western blot, showing increasing band intensity with the number of haplotype A. (C) Cyclic adenosine monophosphate (cAMP) production in response to adenosine was higher in diplotype AA than in BB (***$p = 0.0006$) (n = 5 for each diplotype).

of rs2298383 SNP within a triplex-forming oligonucleotide target sequence. The 35320474 SNP is located in the 3′ untranslated region including U-rich motifs. U-rich motifs are conserved across species and provide active sites for interaction with RNA-binding proteins. Thus, any of these SNPs may possibly alter the expression level of mRNA.

In fact, we found that the *ADORA2A* AA diplotype causes a high expression of *ADORA2A* mRNA in the brain and lymphoblasts, and a high expression of ADORA2A protein in lymphoblasts. Given its excitatory function, increased expression of ADORA2A may cause a functional imbalance between ADORA1 and ADORA2A, resulting in hyperexcitation of cerebral neurons.

In the present study, cellular cAMP accumulation in response to adenosine was high in lymphoblasts with *ADORA2A* AA diplotype. ADORA2A, together with coupled Gs proteins, activates adenylate cyclase and increases the cellular cAMP level. In this study, we observed high cellular cAMP in the AA diplotype, which supports our hypothesis that the signal cascade downstream of ADORA2A is excessively activated in AESD. cAMP promotes protein kinase A, which in turn enhances Ca^{2+} influx through the L-type Ca^{2+} channel in the basal ganglia, hippocampus, and striatum. Ca^{2+} then enhances glutamate efflux from the endoplasmic reticulum to the extracellular space, leading to excitotoxicity.[22–25] An increase of extracellular glutamate in the brain lesion of AESD has recently been demonstrated by magnetic resonance spectrometry.[5]

Involvement of ADORA2A in the pathogenesis of AESD may have therapeutic implications. Experimental studies have previously shown that an ADORA2A antagonist, but not an ADORA1 agonist, can terminate or suppress seizures.[26,27] Pharmacologic blockade or genetic disruption of ADORA2A may protect neurons from seizures by reducing glutamate release and excitotoxicity.[27] Thus, ADORA2A antagonists are promising candidate drugs to ameliorate seizure-induced brain damage. Because this study showed alteration of the ADORA2A signal cascade in AESD, these drugs may also be particularly useful in the treatment of AESD. However, our data showed that 20% of patients with AESD have the BB diplotype, suggesting the involvement of factors other than *ADORA2A* in the etiology of AESD.

In conclusion, the present study demonstrated that polymorphisms of the *ADORA2A*, or AA diplotype, are risk factors of AESD, an acute encephalopathy with febrile status epilepticus. This diplotype showed a high ADORA2A expression level and high cAMP accumulation in response to adenosine, suggesting the involvement of the adenosine/cAMP signal cascade in the pathogenesis of AESD. Pharmacologic intervention in this pathway may improve the treatment of children with this devastating encephalopathy.

AUTHOR CONTRIBUTIONS

M. Shinohara contributed to analysis and interpretation of the data. M. Saitoh contributed to design and conceptualization of the study, interpretation of the data, draft and revision of the manuscript for intellectual content. D. Nishizawa contributed to analysis and interpretation of the data. K. Ikeda contributed to design and conceptualization of the study, interpretation of the data, draft and revision of the manuscript for intellectual content. S. Hirose contributed to analysis and interpretation of the data. J. Takanashi, J. Takita, K. Kikuchi, M. Kubota, G. Yamanaka, T. Shiihara, A. Kumakura, M. Kikuchi, M. Toyoshima, T. Goto, and H. Yamanouchi contributed to interpretation of the data. M. Mizuguchi

contributed to the design and conceptualization of the study, interpretation of the data, draft and revision of the manuscript for intellectual content.

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DISCLOSURE

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Research Article

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

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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 ± 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