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IV. 研究成果の刊行物・別刷

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.
***Neurology*® 2013;80:1–6**

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

Supplemental data at www.neurology.org

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Go to Neurology.org for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

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*.

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.

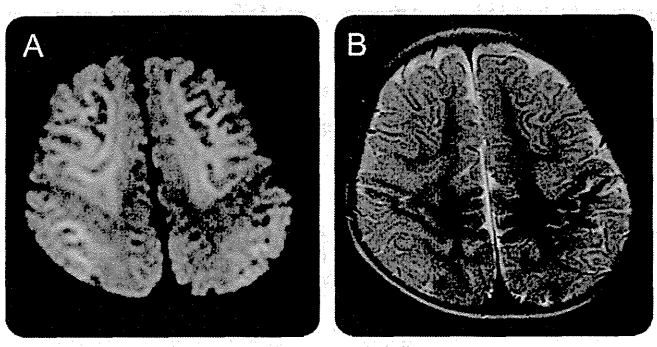
Controls. We analyzed the *ADORA2A* genotypes of control subjects, consisting of 100 healthy Japanese adults, 50 men and 50 women, 20 to 69 years of age, using DNA extracted from Pharma SNP Consortium B cell lines obtained from the Human Science Research Resources Bank (Osaka, Japan). We searched the 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

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.

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

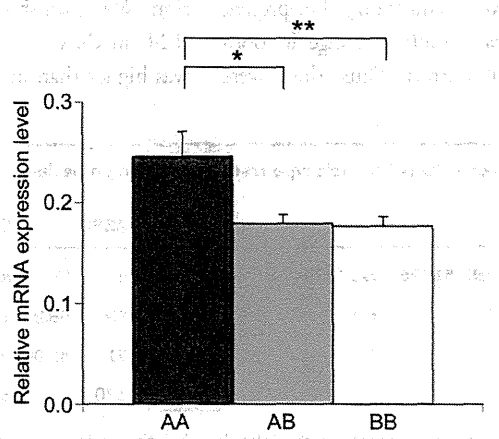
Diplotype	AESD (n = 85), n (%)	Control (n = 184),[a] n (%)	OR (95% CI), *p* value	
AA	32 (37.6)	38 (20.6)	2.62 (1.29-5.32), 0.007	
AB	35 (41.2)	90 (49.0)	1.21 (0.62-2.34), 0.612	
BB	18 (21.2)	56 (30.4)	1.00 (reference)	
Genetic model				***p* Value for trend test**
Recessive	AA vs AB + BB		2.32 (1.32-4.08),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

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

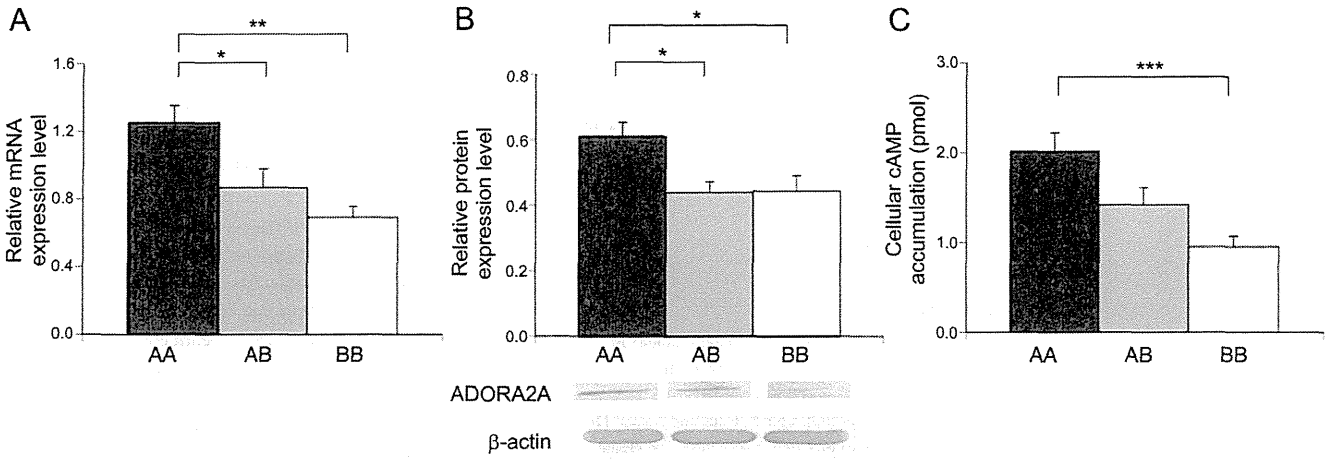


Relative ADORA2A mRNA expression level (ADORA2A/G6PDH) in the brain was higher in the AA diplotype (n = 19) than in AB (n = 38, *p = 0.003) and BB (n = 38, **p = 0.002).

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 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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Review Article

Influenza encephalopathy and related neuropsychiatric syndromes

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Influenza is occasionally complicated by CNS disorders, in particular impairment of consciousness. Severe disorders encompass multiple, distinct syndromes manifesting acute encephalopathy, whereas mild disorders represent multiple, ill-defined neuropsychiatric syndromes. Acute encephalopathy is manifested with seizures and coma, with or without multi-organ involvement. The outcome varies from death or neurologic sequelae to recovery and differs among syndromes. Transient neuropsychiatric disorders are manifested with delirium and/or abnormal behavior. There also are multiple syndromes. The outcome is usually favorable, although occasional fatal accidents warrant caution.

Keywords Acute encephalopathy, coma, delirium, influenza, oseltamivir.

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Neurologic complication of influenza

Except for classical Reye syndrome, central nervous system (CNS) complications of influenza attracted little attention of physicians until around 1985, when Japanese pediatricians became aware of the fact that cases of acute encephalopathy cluster during an influenza epidemic.[1] In 1996–2000, influenza encephalopathy was widely recognized in Japan (population, 130 million), where its incidence was estimated to be 100–500 cases per year. The majority of patients were young children under 5 years of age, presenting with seizures and severe impairment of consciousness, namely coma. The fatality was about 30 percent.[2–4] A research committee was organized by the Japanese Government, and a society of patients' parents was established. In 2005–2007, other types of CNS complication drew attention, in both medical and social contexts in Japan. Patients with these conditions had mild impairment of consciousness, typically delirium and/or hallucination. Despite their benign and self-limited nature, these neuropsychiatric syndromes caused tragic deaths in some of the patients who either rushed onto a busy highway or jumped off from a high-rise apartment. Because many of these patients had taken oseltamivir, causal relationship between the drug use and delirious behavior was raised.[5]

Influenza-associated acute encephalopathy and other CNS syndromes have also been described in countries other than Japan. During the 2009 pandemic, there were multiple reports of such disorders.[6–9] In addition, several CNS disorders, such as autoimmune encephalitis, narcolepsy, and parkinsonism, are suspected as late complications of influenza.[10–12]

This article reviews CNS complications of influenza, characterized by impairment of consciousness, based primarily on the experience in Japan. The first half describes several syndromes of acute encephalopathy, and the second half discusses other neuropsychiatric syndromes.

Acute encephalopathy associated with influenza

Acute encephalopathy refers to syndromes of acute CNS dysfunction due to diffuse or widespread, non-inflammatory brain edema. Its onset is usually during the febrile period of an antecedent infection, which is viral in the majority of cases. The incidence is highest in infancy and early childhood. Its main symptoms include impaired consciousness, seizures, and signs of increased intracranial pressure.[13] In acute encephalopathy, impairment of consciousness is severe and sustained, with the level of consciousness equal to or below 13 on the Glasgow Coma Scale, and duration of impairment longer than 24 hours, according to the diagnostic criteria of the Japanese research committee on influenza encephalopathy.[14,15]

Acute encephalopathy is commonly classified in two ways: one based on the pathogen of infection and the other on the clinicopathological features of encephalopathy. Diagnoses by the virologic classification include influenza, human herpesvirus-6 (HHV-6), and rotavirus encephalopathies, whereas those by the syndrome classification include Reye syndrome, acute necrotizing encephalopathy (ANE), acute encephalopathy with biphasic seizures and late reduced diffusion (AESD), and clinically mild encephalitis/encephalopathy with a reversible splenial lesion (MERS). Any virus can cause any syndrome, but there is clear tendency that one virus is more likely to cause certain syndromes than other viruses.[13] For example, influenza is strongly associated with ANE and MERS, and HHV-6 with AESD. Among the encephalopathy syndromes following influenza, MERS is the most common, followed by AESD (10%) and ANE (6%).[16,17]

Epidemiology of influenza encephalopathy

Recent epidemiologic studies in Japan have shown that encephalopathy and conditions that often accompany ANE, such as multiple organ failure (MOF) and disseminated intravascular coagulation (DIC), are the leading cause of death from influenza in children <15 years of age.[18] Influenza is the commonest pathogen of acute encephalopathy, accounting for 27% of the cases. The incidence of influenza encephalopathy is estimated to be 200–300 cases per annum. Influenza encephalopathy affects all age groups, but is most common in children <10 years of age. In the pediatric cases, the median and mean age is 6 and 6·3 ± 3·4 years, respectively. Boys and girls are equally affected. The outcome is variable; 7% of the patients die, 17% survive with neurologic sequelae, and 76% show a full recovery. Notably, fatality has markedly declined from 30% to 7% during the last two decades. Although pediatric mortality was very low in Japan during the 2009 pandemic, acute encephalopathy was still a leading cause of influenza-associated childhood deaths.[18]

Major syndromes of influenza encephalopathy

Acute necrotizing encephalopathy (ANE)

Acute encephalopathy is the most common cause of death from childhood influenza in Japan. Among encephalopathy syndromes, ANE is characterized by the highest rate of fatal outcome.[16] First described in the 1990s, ANE is a fulminant type of encephalopathy characterized by the multiple and symmetric brain lesions, affecting the bilateral thalami, and by the involvement of systemic organs leading to DIC or MOF in the severest cases.[19] The main pathogenesis of ANE is cytokine-driven, systemic inflammatory responses.[13] Viral infections, such as influenza[20] and exanthem subitum (HHV-6), when combined with certain genetic predisposition, such as mutation of a nuclear membrane protein, Ran-binding protein 2 (RANBP2), and polymorphism of a mitochondrial enzyme, carnitine palmytoyltransferase II (CPT2), cause encephalopathy.[21–26] Excessive production and action of proinflammatory cytokines, such as tumor necrosis factor-α and interleukin-6, cause vascular endothelial injury and apoptosis of parenchymal cells in the brain and other organs, resulting in brain edema and systemic organ damage.[13] Due to the breakdown of blood–brain barrier, cerebrospinal fluid (CSF) often contains an increased amount of protein, despite the absence of inflammatory cells and influenza viruses.[16] The use of non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac sodium and mefenamic acid, increases the fatality of influenza-associated encephalopathy, including ANE.[13]

Acute necrotizing encephalopathy is most prevalent in young children between 1 and 5 years of age. The outcome of ANE is poor; many patients die (28%) or are left with sequelae (61%). In Japan, the incidence of influenza-associated ANE is estimated to be about 10 cases per annum.[16,17]

Acute encephalopathy with biphasic seizures and late reduced diffusion (AESD)

The term AESD has several eponyms, including acute encephalopathy with febrile convulsive status epilepticus (AEFCSE).[13] First described as AEFCSE in a Japanese journal (2000),[27] and as AESD in 2006 in an international journal (2006),[28] this syndrome is characterized by the biphasic clinical course and by a typical magnetic resonance imaging (MRI) finding of subcortical white matter lesions in the cerebral cortex. The main pathogenesis of AESD is considered to be excitotoxicity; febrile infections, when coupled with genetic predisposition such as polymorphism of CPT2 and adenosine receptor A2A (ADORA2A),[24,29] cause status epilepticus. Excessive release of glutamate, an excitotoxin, may provoke selective and delayed death of the cerebral cortical neurons.[13] AESD is most prevalent in infants <2 years of age. Its outcome is characterized by the low fatality (1%) and high probability of neurologic sequelae (66%), including intellectual and motor deficits and epilepsy. In Japan, the incidence of influenza-associated AESD is about 20 cases per annum.[16,17]

Clinically mild encephalitis/encephalopathy with a reversible splenial lesion (MERS)

In contrast to ANE and AESD, MERS is a mild and benign disorder. First described in 2004, this syndrome is characterized by neurologic symptoms, such as delirium, stupor, and seizures, and by the typical MRI finding of a lesion in the splenium of the corpus callosum.[30] Pathogenesis remains unclear. MERS is most prevalent in children between 3 and 8 years of age and is the commonest type of encephalopathy

among schoolchildren. The outcome is good; most patients show full recovery within 10 days. The rate of death and sequelae was 0 and 7%, respectively. The incidence of influenza-associated MERS is about 40 cases per annum.[16,17]

Other syndromes

Although less common compared with the above disorders, other encephalopathy syndromes occasionally occur in association with influenza. Congenital disorders of metabolism of organic and fatty acids may be rapidly exacerbated by influenza, mimicking acute encephalopathy.[31] There also are case reports of acute encephalitis with refractory, repetitive partial seizures (AERRPS), and posterior reversible encephalopathy syndrome (PRES).[16,17,32,33]

Guideline for influenza encephalopathy

A research committee on influenza encephalopathy, supported by the Ministry of Health, Labour, and Welfare of Japan, compiled a guideline for influenza encephalopathy. The first edition was published in 2005,[14] and the second edition in 2009.[15] This guideline has been widely read and used by pediatricians in Japan, and translation into English is now under way.

Neuropsychiatric syndromes associated with influenza

Influenza is associated with a wide spectrum of neuropsychiatric complications. This review focuses on delirium and abnormal behavior that are commonly seen in children and adolescents. Essentially, they are mild, transient, and reversible impairment of consciousness. Many patients have delirium with varying degrees of mental confusion with excitement and/or anxiety. Some also have hallucination, which is visual in most cases.

Syndromes of influenza-associated neuropsychiatric disorders

There are at least four syndromes that vary in their association with age, sex, and oseltamivir use.

1. *MERS:* This is the mildest form of acute encephalopathy described in the previous section. In some patients with MERS, impairment of consciousness is too mild and transient to strictly meet the diagnostic criteria of acute encephalopathy. Thus, MERS constitutes a part of the spectrum of mild neuropsychiatric disorders and accounts for a sizable subpopulation of children with delirious behavior associated with influenza.[34–36] There is no evidence for the association of this condition with oseltamivir.
2. *Febrile delirium:* Febrile delirium is a common but ill-defined syndrome prevalent among young children with acute febrile diseases.[35–38] Clinically, this condition is a mild impairment of consciousness, manifested with fear, anxiety, disorientation, and hallucination, lasting for several minutes or hours. Hundreds of Japanese children with influenza reportedly have this condition every year. Although some Japanese investigators suspected a decrease in the risk of febrile delirium by oseltamivir use, there are no published data to support this notion.
3. *Delirium with rushing/jumping behavior:* According to the national surveillance in Japan, about 100 cases of rushing onto a busy street and jumping off from a high-rise apartment occur every year. Children between 5 and 15 years of age are most frequently affected, with a marked male preponderance (male: female = 2–4:1).[39] There is no clear temporal relationship between oseltamivir use and delirious behavior. The same kind of abnormal behavior is observed also in some patients with influenza but without oseltamivir.[40,41] Thus, it is evident that oseltamivir is not the main cause for this condition. Whether oseltamivir increases its risk attracted much attention, but still remains obscure.[42]
4. *Reproducible neuropsychiatric adverse effects of oseltamivir:* This condition is extremely rare. To the author's knowledge, only four cases have ever been reported to the Japanese government. All the patients were children under 10 years of age and had multiple episodes of delirium, each consisting of oseltamivir intake, latency for 1–2 hours, abnormal behavior and/or hallucination up to 2 hours, sleep for several hours, and full recovery. Multiple episodes with a clear temporal relationship suggest causality. Although this condition may represent "true" adverse effects of oseltamivir, the number of cases is tiny, and the information is only anecdotal.

Controversy over oseltamivir's relationship to neuropsychiatric symptoms

Influenza-associated delirium and hallucination are transient and benign, but occasionally cause abnormal behavior leading to accidental trauma and sometimes death, particularly in Japanese teenagers. In the 2005–2006 season, controversy arose over alleged adverse effects of oseltamivir, which had been taken by many of these patients.[43] Warning was issued in 2007 against prescription of oseltamivir for teenagers.

The Japanese government organized multiple research committees to conduct epidemiologic and pharmacologic studies, which led to the following conclusions:

1. Regardless of oseltamivir use, influenza was occasionally complicated by mild neuropsychiatric symptoms.
2. Children under 10 years of age were most commonly affected, followed by teenagers. With regard to the rushing/jumping behavior, teenagers were the most common.
3. Some of the patients had family history or history of parasomnia, a sleep disorder.

In the 2006–2007 season, a nation-wide epidemiologic study was conducted in Japan to reveal the relationship between drugs and neuropsychiatric symptoms. About 10 000 patients with influenza under 18 years of age were recruited. Based on the data obtained, one study group concluded that they found no positive association between oseltamivir and abnormal behavior.[44] Using the same data, however, another group suggested that oseltamivir may slightly increase the risk of this condition, with a hazard ratio of 1·51 (95% confidence interval, 0·95–2·40; $P = 0·061$) adjusted for risk factors, including body temperature, sex, age, and history of impaired consciousness or abnormal behavior, by multivariate analysis of the proportional hazard model. This study also reported an increase in the incidence of unconsciousness with oseltamivir use, with a hazard ratio of 1·79 ($P = 0·0389$).[45] This discrepancy may have resulted partly from the different hypothesis and methodology and partly from the heterogeneity of the conditions studied, as described above. As a consequence, controversy remained unsettled over the adverse effects of oseltamivir in Japan.[5] It was impossible to exclude the possibility that oseltamivir slightly increased the risk of impairment of consciousness in Japanese children and adolescents infected with influenza.

Conclusions

Recent studies of acute encephalopathy clearly defined multiple syndromes, such as ANE, AESD, and MERS, and enabled classification of many of the influenza-associated cases into either of these conditions. Improved options for diagnosis and treatment were described in the Japanese guideline for influenza encephalopathy, contributing to the decline of fatality in Japan. Genetic predispositions and pathogenetic mechanisms have also been elucidated. By contrast, much remains obscure with regard to other neuropsychiatric syndromes, many of which are poorly defined. Their etiology and pathogenesis, as well as association with antiviral drugs, require further investigation.

Conflict of interests

The author has no competing interests to declare.

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Clinical and radiologic features of encephalopathy during 2011 *E coli* O111 outbreak in Japan

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ABSTRACT

Objective: To elucidate the clinical and radiologic features and analyze factors associated with neurologic outcomes of encephalopathy secondary to Shiga toxin-producing *Escherichia coli* (STEC) O111.

Methods: We reviewed medical records and neuroimaging in 22 patients with neurologic symptoms among 86 with STEC O111 infection.

Results: Twenty-one (6 males and 15 females, 10 children and 11 adults) of the 22 patients were diagnosed with encephalopathy. All patients with encephalopathy also presented with hemolytic-uremic syndrome. Five patients died, from day 1 to 6 months (days 1–5 in 4 patients), due to progressive encephalopathy with severe cerebral edema observed in neuroimaging (4 patients). Fifteen of the 16 surviving patients clinically recovered completely. Statistical analysis revealed differences between patients with poor (n = 6) and good (n = 15) outcomes in the interval from hemolytic-uremic syndrome presentation to encephalopathy, creatinine levels, and the methylprednisolone administration ratio.

Conclusion: We note a high incidence of encephalopathy in the Toyama STEC O111 outbreak. All fatal cases resulted from progressive encephalopathy. Methylprednisolone pulse therapy represents a possible therapeutic choice.

Classification of evidence: This study provides Class III evidence that methylprednisolone pulse therapy increases the probability of a good outcome for patients with encephalopathy associated with STEC O111. *Neurology*® 2014;82:564–572

GLOSSARY

ADC = apparent diffusion coefficient; **Gb3** = globotriaosylceramide; **HUS** = hemolytic-uremic syndrome; **IL-1β** = interleukin-1β; **IVIg** = IV immunoglobulin; **mPSL** = methylprednisolone; **STEC** = Shiga toxin-producing *Escherichia coli*; **Stx** = Shiga toxin; **TNF-α** = tumor necrosis factor-α.

Diarrhea-associated hemolytic-uremic syndrome (HUS) is a complication in 6% to 9% of patients infected with Shiga toxin (Stx)-producing *Escherichia coli* (STEC), and approximately 15% of those are younger than 10 years.[1] CNS involvement occurs in some patients with STEC-HUS and may be predictive of poor outcomes.[2,3] Renal lesions in HUS are characterized by thrombotic microangiopathy; however, the pathogenesis of the CNS involvement is not well understood.[4]

Although the STEC O157 serotype is the most prevalent, other strains also cause outbreaks, as in the case of the STEC O104 in northern Germany and O111 in Oklahoma.[5–7] From April to May 2011, a STEC O111 outbreak occurred predominantly in Toyama Prefecture, Japan, among persons who had eaten raw meat at several branches of a barbeque restaurant chain; the outbreak included a high frequency of severe complications. Thirty-four of the 86 patients with STEC O111 infection (40%) in this particular outbreak had STEC-HUS; in those 34 patients, we encountered patients with severe encephalopathy, which sometimes resulted in brain

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herniation and death. Herein, we report the clinical and radiologic features of STEC O111-encephalopathy and suggest the efficacy of methylprednisolone (mPSL) pulse therapy.

METHODS **Primary research question and classification of evidence.** We conducted a retrospective review of 21 patients with STEC O111-encephalopathy, and analyzed factors associated with their neurologic outcomes. We sought Class III evidence for increasing the probability of a good outcome.

Definitions. For our purposes, we defined STEC O111 infection as illness in a person who visited one of the branches of the implicated barbecue restaurant chain, had at least one gastrointestinal symptom, and positive bacterial cultures of stool samples or sera for anti-*E coli* O111 antibody by microagglutination assay. Gastrointestinal symptoms included diarrhea, bloody stools, abdominal pain, and vomiting. Diagnosis of HUS required findings of thrombocytopenia, hemolytic anemia, and acute renal dysfunction (hematuria, proteinuria, or an elevated creatinine level).[8] Diagnosis of encephalopathy was defined as acute onset of impaired consciousness lasting more than 12 hours, often associated with seizures or delirious behavior, according to the guidelines for influenza encephalopathy.[9] We defined the day of encephalopathy onset as day 0.

Patients. In Toyama and other prefectures in Japan between late April and early May 2011, 86 patients (21 children [15 years or younger], 65 adults [older than 15 years]) with STEC O111 infection were identified, of whom 34 (40%, 11 children and 23 adults) had HUS. We retrospectively collected clinical and radiologic information on 22 patients with neurologic manifestations associated with the STEC O111 infection, and reviewed neuroimaging results (CT and MRI) and reports for these patients, including information on symptoms, clinical diagnosis, treatments, laboratory data, and outcome (monitored until at least 1 year after the encephalopathy). We classified patients with encephalopathy (n = 21) into the poor outcome group (patients who died or had neurologic sequelae) (n = 6) and those with complete recovery (n = 15) into the good outcome group.

Clinical features of 4 patients (including 3 patients who died) with STEC O111-encephalopathy (none of whom had mPSL therapy), and the serum cytokine profiles of 14 patients with STEC O111-HUS (8 patients with encephalopathy) were previously reported.[8,10]

Statistics. We compared the good and poor outcome groups using the Welch test with the following variables: age of patient; the interval from ingestion of contaminated food to enteritis, from enteritis to HUS, and from HUS to encephalopathy; as well as laboratory data (the highest or lowest values during acute HUS and encephalopathy), including white blood cell, hemoglobin, platelet, aspartate aminotransferase, creatinine, and C-reactive protein data. We performed Fisher exact test for the following variables: sex; brain lesions in the basal ganglia and thalamus; and therapies, including hemodialysis, plasma exchange, polymyxin-B immobilized column direct hemoperfusion, thrombomodulin, mPSL, and IV immunoglobulin (IVIg). We used SPSS software (version 20; IBM Corp., Armonk, NY) for statistical analyses and set $p < 0.05$ as statistically significant. In addition, we calculated 95% confidence intervals of the data for patients with poor and good outcomes. Because of the small sample size, we did not use adjustments by multivariate analysis.

Standard protocol approvals, registrations, and patient consents. Institutional Review Boards of the Kameda Medical Center and the National Institute of Infectious Diseases approved this study.

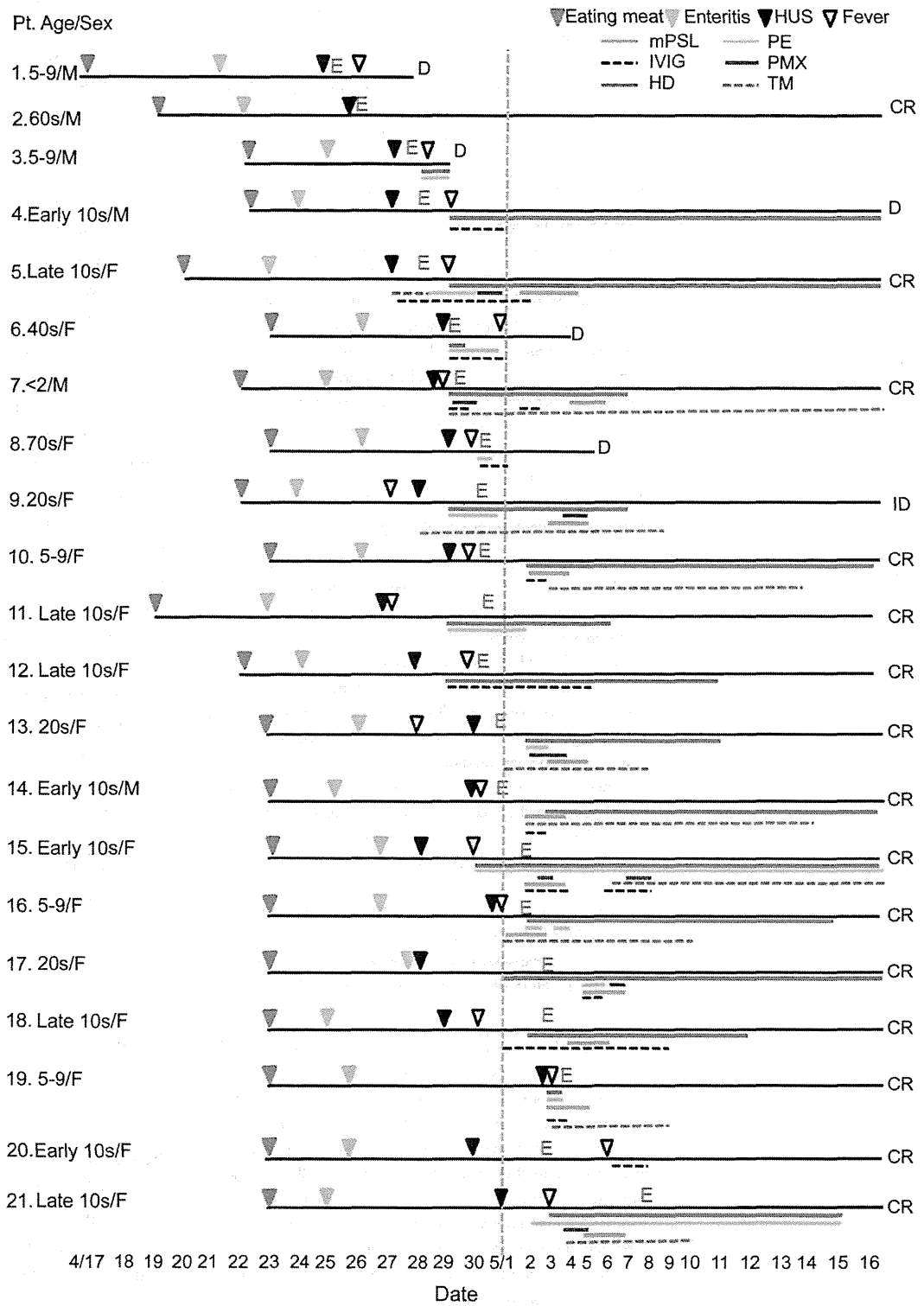
RESULTS Twenty-one of the 22 patients with neurologic symptoms showed encephalopathy, and the remaining patient had idiopathic intracranial hypertension. Figure 1 and table 1 show clinical symptoms and courses. All of the 21 patients with STEC O111-encephalopathy also presented with HUS. Ten of 11 children and 11 of 23 adults with STEC O111-HUS had encephalopathy. All 21 patients had eaten raw meat on days −7 to −15 (April 17–23, 2011) and developed gastrointestinal symptoms on days −3 to −13. The diagnosis of HUS was made on days 0 to −7.

Therapy included blood purification, such as hemodialysis (n = 17), plasma exchange (n = 12), polymyxin-B immobilized column direct hemoperfusion (n = 7), and thrombomodulin treatment (n = 10), in addition to fluid and electrolyte management, red blood cell or platelet transfusion, and antihypertensive agents in most patients. Treatments for encephalopathy, which were introduced to improve possible hypercytokinemia after May 1, 2011, included mPSL (n = 12, 11 receiving pulse therapy [30 mg/kg × 3 days]), IVIg (n = 13), and brain hypothermia in one. We did not observe any obvious side effects from mPSL therapy. Six patients had a poor outcome, 5 died on day 1 to 6 months (days 1–5 in 4 patients) due to progressive encephalopathy, and another previously healthy patient had persistent intellectual disability. Fifteen patients recovered completely. None exhibited hypoglycemia during the course of encephalopathy. Renal function recovered to almost normal in the 16 surviving patients.

Both MRI and CT were performed in 11 patients, only MRI in 5, and only CT in 5 (figure 2; table e-1 on the *Neurology*® Web site at www.neurology.org). In 4 of the 5 patients who died, CNS lesions rapidly progressed within 2 days, leading to severe cerebral edema (figure 2, A–C) and finally death. Neuroimaging studies revealed no parenchymal lesions in 6 patients, and those in the other 15 patients revealed the following: 1) symmetrical lesions in the thalamus (most often in the lateral thalamus, figure 2, A–E and H) on days 0 to 7 in 12, including 4 patients who died; 2) the basal ganglia (putamen or globus pallidus) (figure 2, C–E and G) on days 0 to 7 in 10 (including 5 patients who died); 3) external capsule (figure 2, D, E, G, and H) on days 0 to 7 in 9 (including 5 patients who died); and 4) dorsal brainstem or cerebellum (figure 2F) on days 0 to 10 in 6 (including 3 patients who died).

Follow-up MRI in 7 patients performed later than day 28 showed mild cerebral atrophy (figure 2, I and J) in 4. We found no residual lesions in the thalamus on follow-up. We noted decreased apparent diffusion coefficient (ADC) values in the lateral thalamus (figure 2, E and H) in 4 patients scanned before or on day 7,

Figure 1 Clinical courses of 21 patients with STEC O111-encephalopathy from April 17 to May 16, 2011



< 2 = younger than 2 years; 5-9 = 5-9 years; early 10s = 10-15 years; late 10s = 16-19 years; 20s = 20-29 years; 40s = 40-49 years; 60s = 60-69 years; 70s = 70-79 years. The blue line indicates May 1, 2011, when we started to use mPSL and IVIg therapy for the treatment of encephalopathy. CR = complete recovery; D = dead; E = encephalopathy; HD = hemodialysis; HUS = hemolytic-uremic syndrome; ID = intellectual disability; IVIg = IV immunoglobulin; mPSL = methylprednisolone; PE = plasma exchange; PMX = polymyxin-B immobilized column direct hemoperfusion; Pt. = patient; STEC = Shiga toxin-producing *Escherichia coli*; TM = thrombomodulin.

Table 1 **Clinical symptoms and outcomes in 21 patients with STEC O111-encephalopathy**

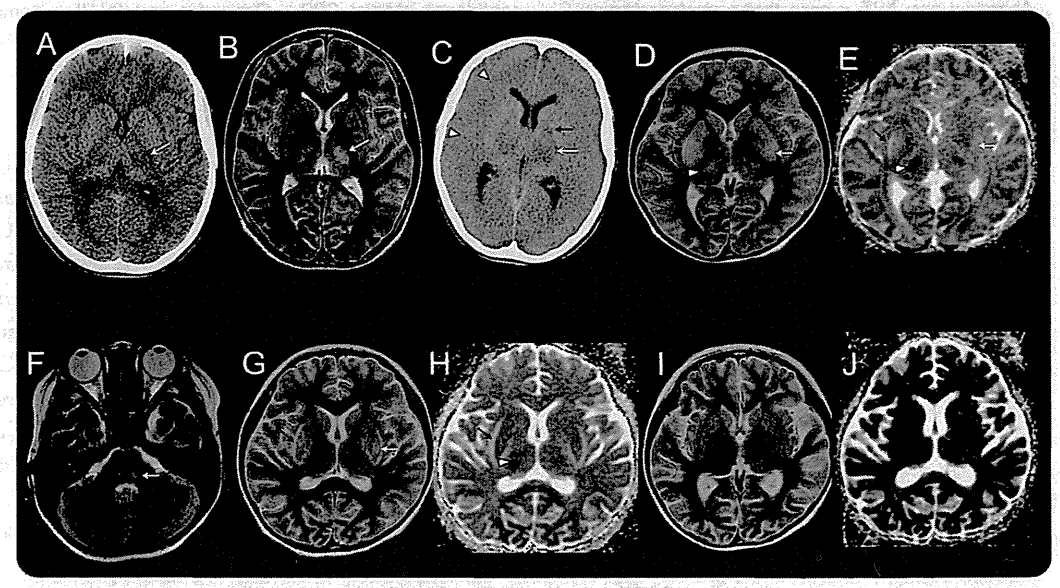
		Seizure	Consciousness disturbance	Delirium	Intubation	Others	Outcome		
							CR	ID	Died
Children, ≤15 y	n = 10	5	8	6	6		7	0	3
	Male (n = 5)	4	4	2	4	Myoclonus (n = 1)	2	0	3
	Female (n = 5)	1	4	4	2	Tremor (n = 1), aphasia (n = 1)	5	0	0
Adults, >15 y	n = 11	4	7	8	6		8	1	2
	Male (n = 1)	0	1	1	0		1	0	0
	Female (n = 10)	4	6	7	6	Tremor (n = 2), aphasia (n = 1)	7	1	2
All ages	n = 21	9	15	14	12		15	1	5
	Male (n = 6)	4	5	3	4		3	0	3
	Female (n = 15)	5	10	11	8		12	1	2

Abbreviations: CR = complete recovery; ID = intellectual disability; STEC = Shiga toxin-producing *Escherichia coli*.

and normal or increased values in 3 patients after day 7. However, ADCs in the putamen and external capsule (figure 2, E and H) were normal or increased in 3 patients examined on days 2, 6, 7, and 10. Lastly, we noted spotty lesions in the white matter or basal ganglia (figure 2G) suggesting lacunar infarctions in 5 patients on days 6 to 27.

Statistical analysis revealed differences between patients with poor and good outcomes in the interval from HUS to encephalopathy, the creatinine level, and the mPSL administration ratio (table 2). The characteristics of study patients showed no significant difference between patients with and without mPSL (table 3).

Figure 2 **Neuroimaging of patients 4 and 10**



CT images of patient 4 who died in 6 months show low density lesions in the lateral thalamus on day 1 (A, arrow). T2-weighted image on day 1 shows T2 prolongation in the thalamus (B, arrow). CT on day 2 (C) reveals cerebral edema with low density lesions in the cerebral white matter (arrowheads), thalamus (white arrow), and globus pallidus (black arrow). T2-weighted image of patient 10 (who subsequently recovered completely) on day 2 (D) shows bilateral T2 prolongation in the putamen (white arrow), external capsule (black arrow), and lateral thalamus (arrowhead). The apparent diffusion coefficient (ADC) map on day 2 (E) shows increased diffusion in the putamen (white arrow) and external capsule (black arrow) and reduced diffusion in the thalamus (arrowhead). At the level of the middle cerebellar peduncles, the T2-weighted image on day 2 (F) shows T2 prolongation in the dorsal midpons (arrow). The T2-weighted image on day 6 (G) shows high signal lesions in the bilateral external (black arrow) and internal capsules with lesions in the putamen (white arrow) and thalamus decreased in size and intensity and a new lesion in the left globus pallidus (black arrowhead). The ADC map on day 6 (H) shows increased diffusion in the external capsule (black arrow) and reduced diffusion in the thalamus (arrowhead). The T2-weighted image (I) and ADC map at 2 months (J) show mild atrophy with no abnormal signal lesion.