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.

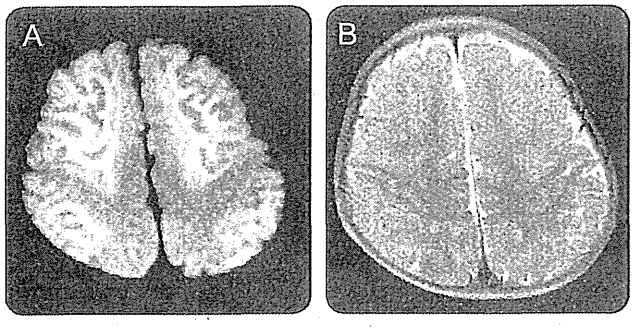
Controls. We analyzed the *ADORA2A* genotypes of control subjects, consisting of 100 healthy Japanese adults, 50 men and 50 women, 20 to 69 years of age, using DNA extracted from Pharma SNP Consortium B cell lines obtained from the Human Science Research Resources Bank (Osaka, Japan). We searched the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

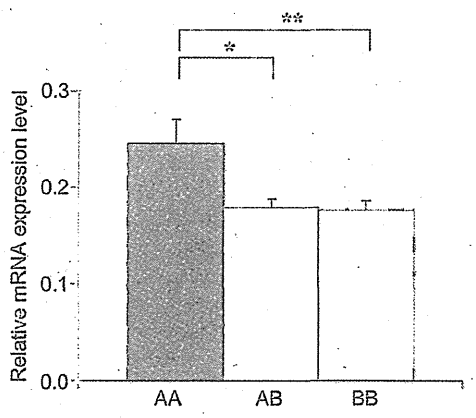
Diplotype	AESD (n = 85), n (%)	Control (n = 184),[a] n (%)	OR (95% CI), *p* value	
AA	32 (37.6)	38 (20.6)	2.62 (1.29-5.32), 0.007	
AB	35 (41.2)	90 (49.0)	1.21 (0.62-2.34), 0.612	
BB	18 (21.2)	56 (30.4)	1.00 (reference)	
Genetic model				*p* Value for trend test
Recessive	AA vs AB + BB		2.32 (1.32-4.08),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

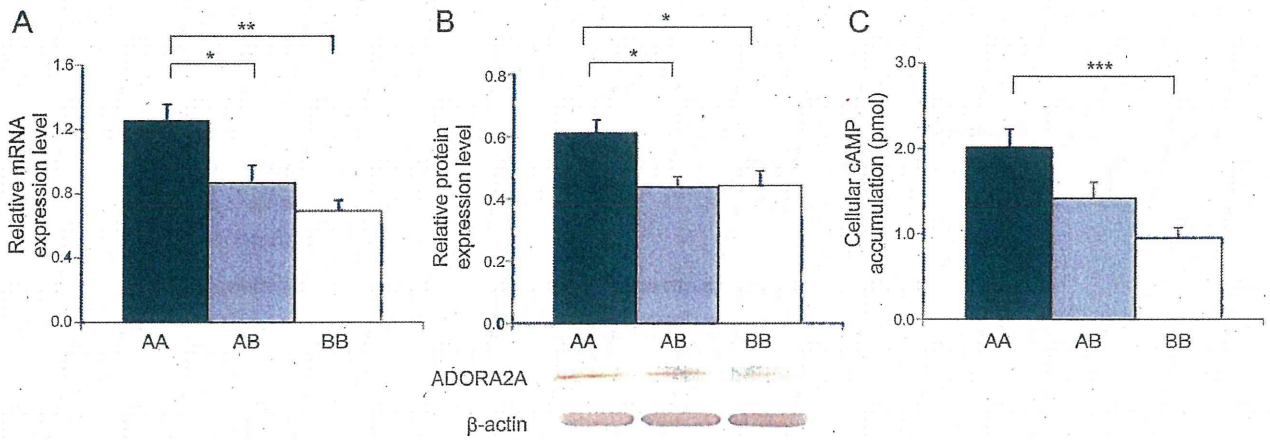


Relative *ADORA2A* mRNA expression level (*ADORA2A*/*G6PDH*) in the brain was higher in the AA diplotype (n = 19) than in AB (n = 38, *$p = 0.003$) and BB (n = 38, **$p = 0.002$).

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.

ACKNOWLEDGMENT

The authors thank the Stanley Medical Research Institute Brain Collection for providing the SMRI specimens, the contributors (listed on the *Neurology*® Web site at www.neurology.org) for providing patient samples and clinical records, and Ms. Kiyomi Noyama and Ms. Aya Shoda for technical assistance.

STUDY FUNDING

No targeted funding reported.

DISCLOSURE

M. Shinohara reports no disclosures. M. Saitoh is funded by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science No. 22591176. D. Nishizawa and K. Ikeda report no disclosures. S. Hirose is funded by a by a Grant-in-Aid for Nervous and Mental Disorder Grant 24-7 and H24-Nanji-Ippan-29 from the Ministry of Health, Labour and Welfare, Japan. J. Takanashi is funded by a Grant-in-Aid for research on Measures for Intractable Diseases H23-Nanji-Ippan-78 from the Ministry of Health, Labour and Welfare, Japan. J. Takita and K. Kikuchi report no disclosures. M. Kubota is funded by a Grant-in-Aid for research on Measures for Intractable Diseases H23-Nanji-Ippan-78 from the Ministry of Health, Labour and Welfare, Japan. G. Yamanaka, T. Shiihara, A. Kumakura, M. Kikuchi, M. Toyoshima, T. Goto, and H. Yamanouchi report no disclosures. M. Mizuguchi is funded by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science No. 20390293 and by a Grant-in-Aid for research on Measures for Intractable Diseases H23-Nanji-Ippan-78 from the Ministry of Health, Labour and Welfare, Japan. Go to Neurology.org for full disclosures.

Received September 13, 2012. Accepted in final form January 10, 2013.

REFERENCES

1. Nabbout R, Vezzani A, Dulac O, Chiron C. Acute encephalopathy with inflammation-mediated status epilepticus. Lancet Neurol 2011;10:99–108.
2. Takanashi J, Oba H, Barkovich AJ, et al. Diffusion MRI abnormalities after prolonged febrile seizures with encephalopathy. Neurology 2006;66:1304–1309.
3. Hoshino A, Saitoh M, Oka A, et al. Epidemiology of acute encephalopathy in Japan, with emphasis on the association of viruses and syndromes. Brain Dev 2012;34:337–343.
4. Mizuguchi M, Yannanouchi H, Ichiyama T, Shiomi M. Acute encephalopathy associated with influenza and other viral infections. Acta Neurol Scand 2007;115:45–56.
5. Takanashi J, Tada H, Terada H, Barkovich AJ. Excitotoxicity in acute encephalopathy with biphasic seizures and late reduced diffusion. AJNR Am J Neuroradiol 2009;30:132–135.
6. Takanashi J. Two newly proposed infectious encephalitis/encephalopathy syndromes. Brain Dev 2009;31:521–528.
7. Shinohara M, Saitoh M, Takanashi J, et al. Carnitine palmitoyl transferase II polymorphism is associated with multiple syndromes of acute encephalopathy with various infectious diseases. Brain Dev 2011;33:512–517.
8. Yu LQ, Frith MC, Suzuki Y, et al. Characterization of genomic organization of the adenosine A2A receptor gene by molecular and bioinformatics analyses. Brain Res 2004;1000:156–173.
9. Olah ME, Stiles GL. Adenosine receptor subtypes: characterization and therapeutic regulation. Annu Rev Pharmacol Toxicol 1995;35:581–606.
10. Huang S, Apasov S, Koshiba M, Sitkovsky M. Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. Blood 1997;90:1600–1610.
11. Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM. Adenosine and brain function. Int Rev Neurobiol 2005;63:191–270.
12. Dragunow M. Adenosine: the brain's natural anticonvulsant. Trends Pharmacol Sci 1986;7:128–130.
13. Avsar E, Empson RM. Adenosine acting via A1 receptors, controls the transition to status epilepticus-like behaviour in an in vitro model of epilepsy. Neuropharmacology 2004;47:427–437.
14. Zeraati M, Mirnajafi-Zadeh J, Fathollahi Y, Namvar S, Rezvani ME. Adenosine A1 and A2A receptors of hippocampal CA1 region have opposite effects on piriform cortex kindled seizures in rats. Seizure 2006;15:41–48.
15. El Yacoubi M, Ledent C, Parmentier M, et al. Absence of the adenosine A2A receptor or its chronic blockade decrease ethanol withdrawal-induced seizures in mice. Neuropharmacology 2001;40:424–432.
16. Fukuda M, Suzuki Y, Hino H, Morimoto T, Ishii E. Activation of central adenosine A2A receptors lowers the seizure threshold of hyperthermia-induced seizure in childhood rats. Seizure 2011;20:156–159.
17. Rogers PJ, Hohoff C, Heatherley SV, et al. Association of the anxiogenic and alerting effects of caffeine with ADORA2A and ADORA1 polymorphisms and habitual level of caffeine consumption. Neuropsychopharmacology 2010;35:1973–1983.
18. Childs E, Hohoff C, Deckert J, Xu K, Badner J, de Wit H. Association between ADORA2A and DRD2 polymorphisms and caffeine-induced anxiety. Neuropsychopharmacology 2008;33:2791–2800.
19. Alsene K, Deckert J, Sand P, de Wit H. Association between A2a receptor gene polymorphisms and caffeine-induced anxiety. Neuropsychopharmacology 2003;28:1694–1702.
20. King DC, Taylor J, Elnitski L, Chiaromonte F, Miller W, Hardison RC. Evaluation of regulatory potential and conservation scores for detecting cis-regulatory modules in aligned mammalian genome sequences. Genome Res 2005;15:1051–1060.
21. Conde L, Vaquerizas JM, Dopazo H, et al. PupaSuite: finding functional single nucleotide polymorphisms for large-scale genotyping purposes. Nucleic Acids Res 2006;34:W621–W625.
22. Higley MJ, Sabatini BL. Competitive regulation of synaptic Ca2+ influx by D2 dopamine and A2A adenosine receptors. Nat Neurosci 2010;13:958–977.
23. Skeberdis VA, Chevaleyre V, Lau CG, et al. Protein kinase A regulates calcium permeability of NMDA receptors. Nat Neurosci 2006;9:501–510.
24. Cunha RA. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem Int 2001;38:107–125.
25. Fredholm BB, Chern Y, Franco R, Sitkovsky M. Aspects of the general biology of adenosine A2A signaling. Prog Neurobiol 2007;83:263–276.
26. O'Regan MH, Simpson RE, Perkins LM, Phillis JW. The selective adenosine A2 receptor agonist CGS 21680 enhances excitatory transmitter amino acid release from the ischemic rat cerebral cortex. Neurosci Lett 1992;138:169–172.
27. Jones PA, Smith RA, Stone TW. Protection against hippocampal kainate excitotoxicity by intracerebral administration of an adenosine A2A receptor antagonist. Brain Res 1998;800:328–335.

CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Epidemiology of Hereditary Sensory and Autonomic Neuropathy Type IV and V in Japan

Nobuhiko Haga,[1]* Masaya Kubota,[2] and Zenzo Miwa[3]

[1]Department of Rehabilitation Medicine, The University of Tokyo, Tokyo, Japan

[2]Division of Neurology, National Center for Child Health and Development, Tokyo, Japan

[3]Department of Pediatric Dentistry, Tokyo Medical and Dental University Graduate School, Tokyo, Japan

Manuscript Received: 19 September 2012; Manuscript Accepted: 9 November 2012

Hereditary sensory and autonomic neuropathy (HASN) refers to a group of rare congenital disorders characterized by loss of pain sensation and other sensory or autonomic abnormalities. Among them, a relatively large proportion of patients with HSAN type IV, which is accompanied by anhidrosis and intellectual disability, are reported from Israel and Japan. HSAN type V, with normal sweating and mental development, is rarely reported in Japan. In 2009, we founded a research group for congenital insensitivity to pain and performed the first epidemiological survey of HSAN types IV and V in Japan. Questionnaires were sent to a total of 3,488 certified training institutions of five nationwide medical societies comprising pediatricians, neurologists, orthopedic surgeons, and dentists. Answers were obtained from 1,610 institutions, and 192 HSAN patients (152 with type IV and 28 with type V) were reported from 105 institutions. After excluding duplicated patients, we identified a total of 62 current, 36 past, and five deceased patients for HSAN-IV, and a total of 14 current, 13 past, and 0 deceased patients for HSAN-V. Using these figures, we estimated that the number of Japanese patients with HSAN types IV and V as 130–210 and 30–60 patients, respectively. We identified no gender differences, and patients with a family history of the disorder were limited to affected siblings in both conditions. Most patients with HSAN-IV were 5–40 years of age, whereas half of the patients with HSAN-V were 40 years or older. © 2013 Wiley Periodicals, Inc.

Key words: congenital insensitivity to pain; hereditary sensory and autonomic neuropathy; epidemiology; prevalence

How to Cite this Article:
Haga N, Kubota M, Miwa Z. 2013. Epidemiology of hereditary sensory and autonomic neuropathy type IV and V in Japan.
Am J Med Genet Part A 161A:871–874.

INTRODUCTION

Congenital insensitivity to pain (CIP) comprises a group of disorders manifesting in a congenital loss of pain sensation that stems from peripheral neuropathy. Dyck et al. [1983] named these disorders as hereditary sensory and autonomic neuropathies (HASN), and classified them into types I–V [Nagasako et al., 2003]. Recently, HSAN type VI was added to the list [Edvardson et al., 2012]. Among them, type III is most common, with a relatively high incidence of 27 among 100,000 live births reported for Israel [Maayan et al., 1987]. Several hundred patients with HSAN type IV (HSAN-IV) have been reported [Feldman et al., 2009], but its prevalence has not been estimated. Other types are considered less common.

HSAN-IV, also called congenital insensitivity to pain with anhidrosis (CIPA), is an autosomal recessive disorder manifesting in a generalized loss of pain and thermal sensation, a lack of sweating, and is associated with variable degrees of intellectual disability and/or learning deficits. CIPA results from loss-of-function mutations in the *NTRK1* gene encoding TrkA (tropomyosin-related kinase A), a receptor tyrosine kinase for nerve growth factor (NGF) [Indo et al., 1996]. Articles written in English on more than 10 patients are only from Israel [Shatzky et al., 2000] and Japan [Amano et al., 1998, 2006; Iijima and Haga, 2010]. Although additional case reports indicate a relatively high prevalence of CIPA among the Japanese population [Ishii et al., 1988; Hasegawa et al., 1990; Okuno et al., 1990; Iwanaga et al., 1996; Yotsumoto et al., 1999; Uehara et al., 2001; Hiura et al., 2002; Ohto et al., 2004], no epidemiological study of CIPA in Japan has been conducted to date.

Grant sponsor: Health and Labor Sciences Research Grants for Research on Intractable Diseases, the Ministry of Health, Labor, and Welfare.
The authors declare that they have no conflict of interest with regard to this study.
*Correspondence to:
Dr. Nobuhiko Haga, MD, PhD, Department of Rehabilitation Medicine, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: hagan-reh@h.u-tokyo.ac.jp
Article first published online in Wiley Online Library (wileyonlinelibrary.com): 12 March 2013
DOI 10.1002/ajmg.a.35803

Similarly, the prevalence of HSAN-V amongst the Japanese population is unknown, with only one English article reporting a Japanese patient with HSAN-V found [Miura et al., 2008]. HSAN-V is also an autosomal recessive disorder characterized by a loss of pain and thermal sensation. Contrastingly, sweating and mental development are usually normal in these patients. In northern Sweden, a large family with six members affected by HSAN-V has been reported, with mutations in the *NGFB* (nerve growth factor, beta subunit) gene detected [Minde et al., 2004].

In 2009, we founded a research group for CIP in Japan to establish treatment and support guidelines for HSAN-IV and V. We chose these two conditions because members of this group had experienced diagnosing and treating many patients with these two types and only a few with other types of HSAN. The purpose of this study is to reveal the epidemiological data on the Japanese patients with HSAN-IV and V.

MATERIALS AND METHODS

The present research included a questionnaire that was sent to pediatricians, neurologists, orthopedic surgeons, and dentists who were believed to deal with CIP patients. Questionnaires were sent to a total of 3,488 certified training institutions of the Japan Pediatric Society (520 institutions), the Japanese Society of Neurology (734 institutions), the Japanese Orthopaedic Association (1,994 institutions), the Japanese Society of Pediatric Dentistry (47 institutions), and the Japanese Society for Disability and Oral Health (193 institutions, excluding duplication with those of Japanese Society of Pediatric Dentistry). These questionnaires were sent in November and December 2009, and the answers were gathered and analyzed in 2010.

The questionnaire included the experiences of examining or treating patients with HSAN-IV and V. The diagnosis was based on the clinical findings, physiological tests, and sometimes nerve biopsy and/or gene analyses. Because we did not ask which of these the diagnosis of each patient was based on, we could not find out how many patients had molecular confirmation of the diagnosis. If the institution had experiences with these patients, the questionnaire asked the initials of the patient's name, gender, date of birth, the prefecture where the patient lives, and the presence or absence of a family history of the disorder. Physicians were also asked to detail the status of the patients with respect to the clinic: current patients, past patients (those no longer attending the clinic), and deceased patients.

This study was approved by the Ethics Committee, Faculty of Medicine, The University of Tokyo, Japan (approval number 2769).

RESULTS

Of the institutions targeted for our study, 1,610 institutions answered the questionnaire, providing an answer rate of 46.2%. Of these, 105 institutions reported the identification of 192 patients with HSAN-IV or V, among which 152 patients were characterized as having HSAN-IV, and 28 patients with HSAN-V (Table I). Twelve patients with unknown diagnoses were excluded.

To avoid patient duplication, we determined patients with overlapping demographic details: the same initials, gender, date of birth, and the place of residence. After exclusion of suspected duplications, a total of 62 current, 36 past, and five deceased patients were identified for HSAN-IV. For the five deceased patients, the ages at death were 1, 2, 7, 18, and 22 years. For HSAN-V, a total of 14 current, 13 past, and 0 deceased patients were identified.

To estimate the number of patients in Japan, we set the minimum number of patients by dividing the number of current patients by the answer rate of the questionnaire and the maximum number by dividing the sum of the current and past patients by the answer rate of the questionnaire. These results led to estimations of 130–210 patients with HSAN-IV, and 30–60 patients with HSAN-V. Using the Japanese population statistics from 2010 of approximately 127,522,000 people, we calculated the prevalence of HSAN-IV and V as 1 in 600,000–950,000 and 1 in 2,200,000–4,200,000, respectively.

For the current patients, we analyzed patient gender, heredity, and age distribution at the time of the questionnaire. Patients with HSAN-IV included 27 males and 35 females; patients with HSAN-V included eight males and five females (the gender of one patient was not described). No apparent gender difference existed in both HSAN-IV and V. Fifteen out of 46 patients with HSAN-IV, and seven out of 14 patients with HSAN-V had a positive family history for the disorder, all of whom were siblings, including several sets of twins. As

TABLE I. Raw Results of the Questionnaire According to the Medical Department

	Pediatrics	Neurology	Orthopedics	Dentistry	Total
Questionnaires sent	520	734	1,994	240	3,488
Questionnaires answered	257	312	919	122	1,610
Answer rate (%)	49.4	42.5	46.1	50.8	46.2
Institutions with patients	28	7	51	19	105
Total no. of patients	44	7	86	55	192
No. of HSAN-IV patients	31	1	70	50	152 (62/36/5)[a]
No. of HSAN-V patients	8	6	12	2	28 (14/13/0)[a]
No. of unknown diagnosis	5	0	4	3	12

[a]Figures in the parentheses indicate the actual numbers of the current/past/deceased patients after excluding suspected duplication of patients.

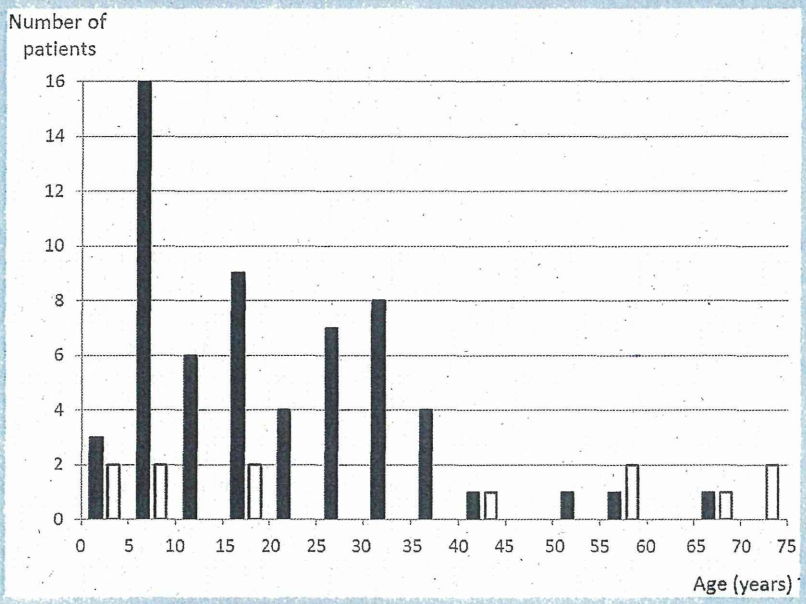


FIG. 1. Age distribution of patients. Black bar indicates patients with HSAN-IV and white bar indicates patients with HSAN-V. One patient with HSAN-IV and two with HSAN-V with unknown ages are not included in this graph.

for the age distribution (Fig. 1), most patients with HSAN-IV were under 40 years, with those under 5 years of a relatively smaller number. Comparatively, the age distribution of patients with HSAN-V was relatively equal, with half of them aged 40 years or older.

DISCUSSION

This study describes the prevalence of Japanese patients with HSAN-IV and V as 1 in 600,000–950,000 and 1 in 2,200,000–4,200,000, respectively. While these figures are small, the estimated number of 130–210 HSAN-IV patients from institutions across Japan seems large considering the number of reported patients from non-Israeli and non-Japanese populations.

Mutation in the *NTRK1* and *NGFB* genes are linked with HSAN patients who had consanguineous parents and in families with a history of consanguineous marriages [Shatzky et al., 2000; Minde et al., 2004]. The present results reported that the family history only consisted of affected siblings, which is consistent with the autosomal recessive inheritance of HSAN-IV and V; however, the consanguinity in the family was not asked in our questionnaire. Though Japanese may have higher frequency of *NTRK1* mutation, more precise investigation of pedigrees in Japanese patients is mandatory. An article from Sweden reports a pedigree in which patients with heterozygous mutation of *NGFB* shows a milder phenotype of HSAN-V [Minde et al., 2004].

The age distribution of patients may be affected by the age at diagnosis, follow-up conditions, and the life expectancy. In HSAN-IV, the relatively small number of patients under the 5 years of age indicates the difficulty of diagnosis in young patients. It is unknown if the decline in patient numbers after adulthood is caused by decreased visits to a medical facility or shorter life expectancy, although the reported ages of death in this study indicates a shorter life expectancy for these patients with HSAN-IV. In HSAN-V, the lack of such a decline in adult patient numbers suggests a longer life expectancy for these patients.

The diagnostic criteria for both HSAN-IV and V have not been established. As such, this study was limited by the diagnoses based on the clinical findings, physiological tests, and sometimes nerve biopsy and/or gene analyses from institutions across Japan. In addition, Carvalho et al. [2011] recently identified a homozygous loss-of-function mutation in the *NGFB* gene in a consanguineous Emirati Bedouin family with lack of pain sensation, mild intellectual disability and anhidrosis. This finding indicates the phenotypic overlap between HSAN-IV and V due to changes in the NGF/TRKA signaling pathway and may indicate further difficulty in accurately diagnosing patients with these disorders.

There are various methods to estimate a prevalence of a rare disorder. One of such methods is the protocol established by the Research Committee on the Epidemiology of Intractable Diseases funded by the Ministry of Health, Labor and Welfare of Japan [Kawamura et al., 2006]. This protocol recommends sampling hospitals to which questionnaires are sent, and asking the presence of patients during a limited term of years in the preliminary screening. In preparation for the present research on HSAN-IV and V, we estimated that the number of patients would be extremely small and each patient could be visiting multiple clinical departments in different large hospitals. So we did not follow the above-mentioned protocol, but sent questionnaires to certified training institutions of medical societies comprising pediatricians, neurologists, orthopedic surgeons, and dentists. We also tried to gather information on all current and past patients, and exclude duplicated reports.

In conclusion, the present study revealed estimations for the number of Japanese patients with HSAN-IV and V. We identified no gender differences and noted that patients with a family history of the disorder were limited to affected siblings for both conditions. Most patients with HSAN-IV were 5–40 years of age, whereas half of the patients with HSAN-V were 40 years or older.

ACKNOWLEDGMENTS

We appreciate the cooperation of five medical societies in Japan who provided us with the lists of certified training institutions and all doctors who answered the questionnaire. This study was supported by the Health and Labor Sciences Research Grants for Research on Intractable Diseases, the Ministry of Health, Labor and Welfare.

REFERENCES

Amano A, Akiyama S, Ikeda M, Morisaki I. 1998. Oral manifestations of hereditary sensory and autonomic neuropathy type IV. Congenital insensitivity to pain with anhidrosis. Oral Surg Oral Med Pathol Oral Radiol Endod 86:425–431.

Amano S, Fukuoka S, Usui T, Honda N, Ideta R, Ochiai M, Yamagami S, Araie M, Awaya Y. 2006. Ocular manifestations of congenital insensitivity to pain with anhidrosis. Am J Ophthalamol 141:472–477.

Carvalho OP, Thornton GK, Hertecant J, Houlden H, Nicholas AK, Cox JJ, Rielly M, Al-Gazali L, Woods CG. 2011. A novel NGF mutation clarifies the molecular mechanism and extends the phenotypic spectrum of the HSAN5 neuropathy. J Med Genet 48:131–135.

Dyck PJ, Mellinger JF, Reagan TJ, Horowitz SJ, McDonald JW, Litchy WJ, Daube JR, Fealey RD, Go VL, Kao PC, Brimijoin WS, Lambert EH. 1983. Not 'indifference to pain' but varieties of hereditary sensory and autonomic neuropathy. Brain 106:373–390.

Edvardson S, Cinnamon Y, Jalas C, Shaag A, Maayan C, Axelrod FB, Elpeleg O. 2012. Hereditary sensory autonomic neuropathy caused by a mutation in dystonin. Ann Neurol 71:569–572.

Feldman DS, Ruchelsman DE, Spencer DB, Straight JJ, Schweitzer ME, Axelrod FB. 2009. Peripheral arthropathy in hereditary sensory and autonomic neuropathy types III and IV. J Pediatr Orthop 29:91–97.

Hasegawa Y, Ninomiya M, Yamada Y, Hattori T. 1990. Osteoarthropathy in congenital sensory neuropathy with anhidrosis. Clin Orthop Relat Res 258:232–236.

Hiura M, Torigoe K, Numata O, Yazaki S, Kuwabara A, Matsunaga M, Hasegawa S, Boku N, Ino H, Indo Y, Miura Y. 2002. Case of congenital indifference to pain with anhidrosis using sympathetic skin response as a useful supportive diagnostic method in the early stage of infancy. Pediatr Int 44:436–439.

Iijima M, Haga N. 2010. Evaluation of nonnociceptive sensation in patients with congenital insensitivity to pain with anhidrosis. Childs Nerv Syst 26:1085–1089.

Indo Y, Tsuruta M, Hayashida Y, Karim MA, Ohta K, Kawano T, Mitsubuchi H, Tonoki H, Awaya Y, Matsuda I. 1996. Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. Nat Genet 13:485–488.

Ishii N, Kawaguchi H, Miyakawa K, Nakajima H. 1988. Congenital sensory neuropathy with anhidrosis. Arch Dermatol 124:564–566.

Iwanaga R, Matsuishi T, Ohnishi A, Nakashima M, Abe T, Ohtaki E, Kojima K, Nagamitsu S, Ohbu K, Kato H. 1996. Serial magnetic resonance images in a patient with congenital sensory neuropathy with anhidrosis and complications resembling heat stroke. J Neurol Sci 142:79–84.

Kawamura T, Nagai M, Tamakoshi A, Hashimoto S. 2006. The nationwide epidemiological survey manual for investigating the number of patients and clinico-epidemiological features of intractable diseases, 2nd edition. Tokyo: Japanese Ministry of Health and Welfare (in Japanese).

Maayan C, Kaplan E, Shachar S, Peleg O, Godfrey S. 1987. Incidence of familial dysautonomia in Israel 1977–1981. Clin Genet 32:106–108.

Minde J, Toolanen G, Andersson T, Mennesmo I, Remahl IN, Svensson O, Solders G. 2004. Familial insensitivity to pain (HSANV) and the mutation in the NGFB gene. A neurophysiological and pathological study. Muscle Nerve 30:752–760.

Miura T, Amano S, Fukuoka S, Honda N, Arita R, Ochiai M, Yanagisawa M, Usui T, Ono K, Araki F, Yamagami S, Araie M, Awaya Y. 2008. In vivo confocal microscopy of hereditary sensory and autonomic neuropathy. Curr Eye Res 33:940–945.

Nagasako EM, Oaklander AL, Dworkin RH. 2003. Congenital insensitivity to pain: An update. Pain 101:213–219.

Ohto T, Iwasaki N, Fujiwara J, Ohkoshi N, Kimura K, Tanaka R, Matsui A. 2004. The evaluation of autonomic nervous function in a patient with hereditary sensory and autonomic neuropathy type IV with novel mutations of the TRKA gene. Neuropediatrics 35:274–278.

Okuno T, Inoue A, Izumo S. 1990. Congenital insensitivity to pain with anhidrosis. J Bone Joint Surg Am 72:279–282.

Shatzky S, Moses S, Levy J, Pinsk V, Hershkovitz E, Herzog L, Shorer Z, Luder A, Parvari R. 2000. Congenital insensitivity to pain with anhidrosis (CIPA) in Israel-Bedouins: Genetic heterogeneity, novel mutations in the TRKA/NGF receptor gene, clinical findings, and results of nerve conduction studies. Am J Med Genet 92:353–360.

Uehara K, Tsuchiya H, Kabata T, Sakurakichi K, Shimozaki E, Tomita K. 2001. Ankle arthrodesis and tibial lengthening for congenital sensory neuropathy with anhidrosis. J Orthop Sci 6:430–434.

Yotsumoto S, Setoyama M, Hozumi H, Mizoguchi S, Fukumaru S, Kobayashi K, Saheki T, Kanzaki T. 1999. A novel point mutation affecting the tyrosine kinase domain of the *TRKA* gene in a family with congenital insensitivity to pain with anhidrosis. J Invest Dermatol 112:810–814.