Table 1 Clinical features of 6 individuals (probands) with *SLC20A2* mutations

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Mutation	c.1909A>C	c.1909A>C	c.344C>T	c.212G>A	c.1399C>T	c.152C>T	c.260_261delTC
	S637R	S637R	T115M	R71H	R467X	A51V	L87Hfs*6
Zygosity	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero
Exon	11	11	3	2	8	2	2
Proband information							
Age at detection of calcification, y	60	51	60	73	23	71	74
Age at onset, y	58	50	60	71	15	71	57
Onset symptom	Dysarthria	Dysarthria	Dementia	Parkinsonism	PKC	Dementia	Athetosis
Neurologic findings							
Cognitive impairment (MMSE)	27	24	20	16	30	22	22
Pyramidal sign	+	+	–	–	–	–	–
Extrapyramidal sign	+	+	–	+	–	–	+
Family information (except the proband)							
No. of other individuals with calcification	1	2	5	1	1	0[a]	0[a]
No. of other individuals with confirmed mutations	1	NE	5	NE	1	NA	NA
No. of other symptomatic individuals	0	0	2	0	0	NA	NA
Other symptoms (no.) in the family	—	—	Mental disorder (1), alcoholism (1)	—	—	NA	NA

Abbreviations: MMSE = Mini-Mental State Examination; NA = not applicable; NE = not examined; PKC = paroxysmal kinesigenic choreoathetosis.
[a] Because there was no other family member who had any neurologic symptoms, brain CT screening of other family members was not performed.

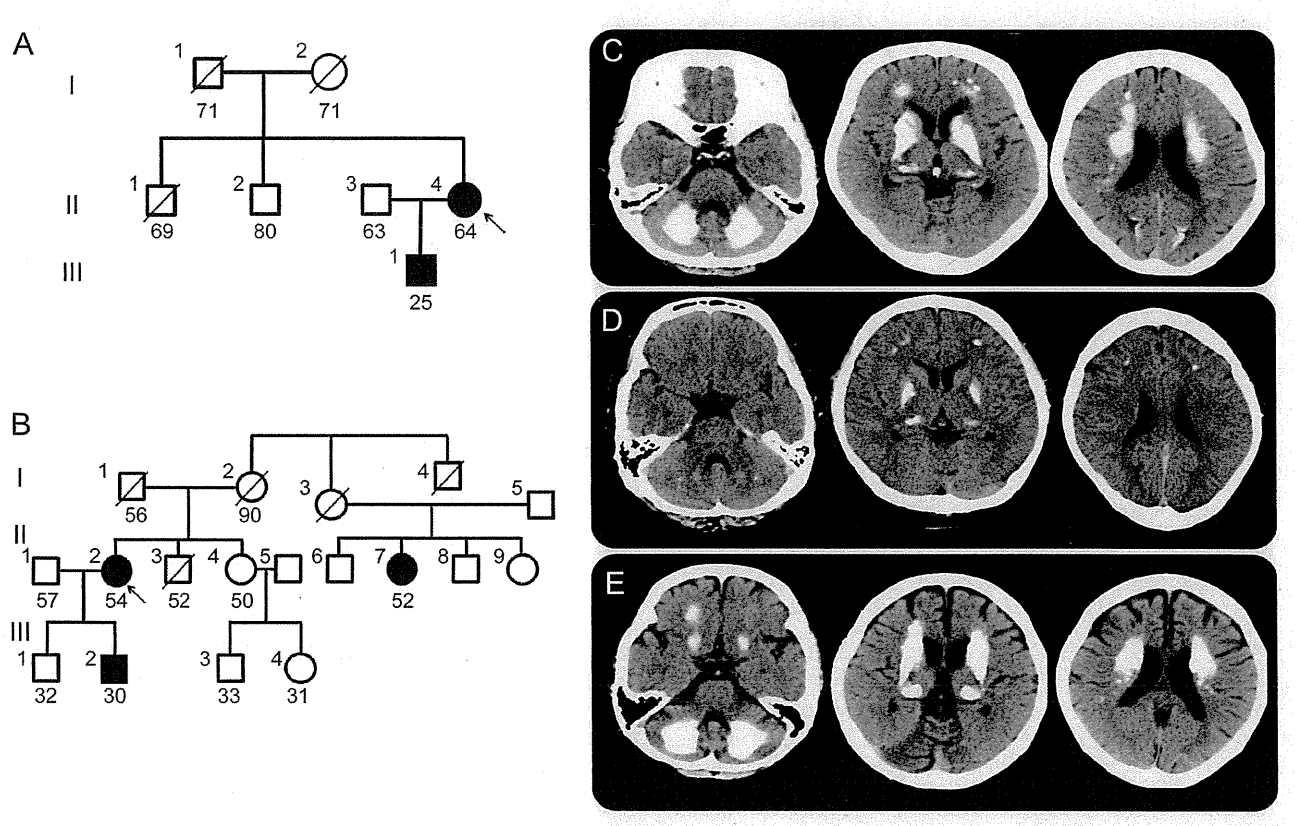
same mutation in exon 11 that had been found in his mother.

Case 2 (in family 2). The proband in family 2 was a 54-year-old woman who had dysarthria and gait disturbance for 4 years. She showed mild mental deterioration in Mini-Mental State Examination (MMSE) score of 24 points, frontal signs, dysarthria, mild parkinsonism (rigidity of bilateral wrist joints and bradykinesia), adiadochokinesis, spasticity, and small steppage gait. Her CT images revealed severe calcification at the bilateral globus pallidus, caudate nuclei, thalamus, subcortical white matter, and dentate nuclei (figure 2E). Although her son and cousin also showed calcification in CT images, they were asymptomatic. Her DNA analysis revealed the same mutation as that in family 1.

Case 3 and other symptomatic individuals (in family 3). The proband was a 69-year-old woman (II-1 in the pedigree in figure 3). She was admitted to a hospital at the age of 65 because of forgetfulness since the age of 60 years. Her MMSE score was 20, which indicated a possibility of dementia (MMSE score below 22). Decreased blood flow was detected in the bilateral basal ganglia and thalamus and the right frontal lobe in particular by SPECT. She had a positive family history of brain calcification, as shown in figure 3A. The initial clinical diagnosis had been diffuse neurofibrillary tangles with calcification (DNTC),[17] although to our knowledge familial cases of DNTC have not been reported. Her son had psychological disorders including violent behavior; unfortunately, no brain CT had yet been performed on him. In the patients in family 3, the degree of calcification was mild compared with that observed in the other families (figure 3, B–G). Her brother with calcification in the brain (II-7) had a mental disorder and another (II-8) presented with alcoholism. The 3 other relatives with calcification were asymptomatic (II-5, II-9, and III-3). The symptomatic patients (II-1, II-7, and II-8) showed more apparent brain atrophy than the others (figure 3, B, D, and E, respectively). The individuals with calcification on the CT images (II-1, II-5, II-7, II-8, II-9, and III-3) had the same mutation in exon 3 in *SLC20A2*. However, the individuals with no calcification (III-2, III-5, and IV-1) revealed no mutation in *SLC20A2*. In summary, 6 patients had calcification among the 10 individuals examined by CT scan in family 3 and all of them carrying the *SLC20A2* mutation exhibited similar calcification on CT images. However, persons without the mutation did not show calcification.

Case 4 (in family 4). Family 4 had a mutation in exon 2. The proband developed clumsiness of her hands and gait unsteadiness at the age of 71 years, and she was diagnosed as having Parkinson disease. Visual

Figure 2 **CT images and family trees of families 1 and 2**



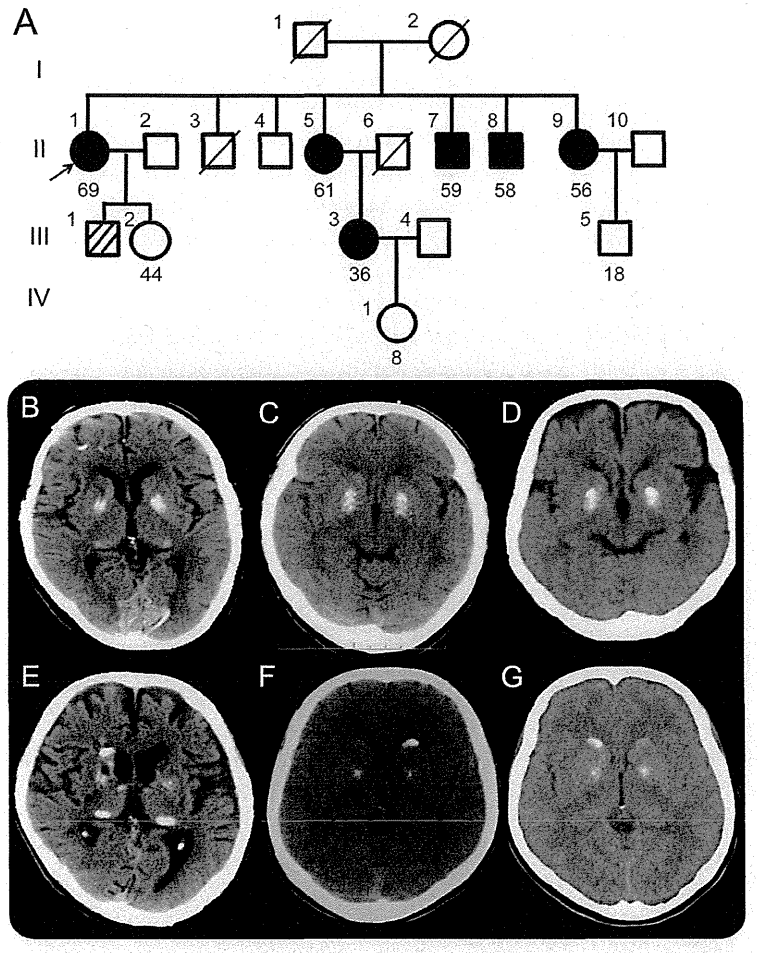
(A) Family tree of family 1. (B) Family tree of family 2. The arrow indicates the index subject. Filled symbols represent patients affected by brain calcification. We show the ages of persons under symbols in the family tree for those we could obtain. (C) CT images of proband (II-4 in pedigree of family 1, part A). (D) CT images of the proband's son (III-1 in pedigree of family 1, part A). (E) CT images of the proband (II-2 in pedigree of family 2, part B). All have mutation of S637R.

hallucinations started with the initiation of medication. She showed parkinsonism (rigidity, bradykinesia, and postural instability), which responded to levodopa. Her MMSE score was 16. Her brain CT images revealed calcification at the globus pallidus, caudate nuclei, and dentate nuclei, and her daughter, who was asymptomatic, also had intracranial calcification (figure e-2C). Brain CT was not performed in her other children. Her SPECT images showed decreased perfusion in the bilateral frontal, temporal, and parietal regions of the brain. She died of pneumonia at the age of 79. Neuropathologic examination revealed neuronal loss and Lewy bodies in the substantia nigra, locus ceruleus, amygdala, and parahippocampal gyrus indicative of Parkinson disease, and prominent deposition of calcium in the parenchyma and the wall of arteries in the globus pallidum and dentate nuclei compatible with the pathologic findings of IBGC.

Case 5 (in family 5). The proband was a 24-year-old man who had paroxysmal kinesigenic choreoathetosis (PKC). His laboratory data were normal except for CT findings. He presented with an attack of PKC after exercise and his symptom responded well to carbamazepine. His CT images revealed calcification at the globus pallidus, thalamus, subcortical white matter, and dentate nuclei (figure e-2B [A]). We had an opportunity to examine his parents, who had no symptoms or signs. Mutational analysis of *SLC20A2* of his parents with their informed consent revealed the same mutation in exon 8 in his mother as he had. Brain CT scan of his mother confirmed calcification at the globus pallidus, subcortical white matter, and dentate nuclei.

Sporadic cases. ***Case 6.*** The patient had a mutation in exon 2. She was a 72-year-old woman who noticed forgetfulness at the age of 71. She had no motor deficits. Her MMSE score was 22, and her score on the revised Hasegawa Dementia Scale was 24. Her Frontal Assessment Battery score at bedside was 5, indicating a frontal lobe deficit (cutoff score, 11/12). The index scores of the revised Wechsler Memory Scale were as follows: attention and concentration, 86; verbal memory, 89; general memory, 85; attention/concentration, 71; and delayed recall, 75. Her brain CT images revealed calcification at the globus pallidus, caudate nuclei, thalamus, subcortical and periventricular white matter, and dentate nuclei (figure e-2B [B]). Her SPECT images showed decreased perfusion in the left frontal,

Figure 3 **Pedigree and CT images of family 3**



(A) Pedigree of family 3. The arrow indicates the index subject. Filled symbols represent patients affected by brain calcification. We show the ages of persons under symbols in the family tree for those we could obtain. The striped symbol represents a symptomatic patient, although his CT image and DNA sample were not available for the study. (B) CT image of the proband (II-1 in pedigree of family 3). (C) CT image of asymptomatic II-5. (D) CT image of symptomatic II-7. (E) CT image of symptomatic II-8. (F) CT image of asymptomatic II-9. (G) CT image of asymptomatic III-3. All have mutation of T115M.

temporal, and parietal regions of the cerebrum and bilateral cerebellum. [^{11}C] Pittsburgh compound B (PiB) retention was not observed by [^{11}C]PiB PET. There were no other family members presenting with similar neurologic symptoms. CT scan was not performed for other individuals in the family.

Case 7. The patient was a 78-year-old man who had a frameshift in exon 2. Involuntary movement of the left thumb and index finger like "pill-rolling" began in his sixth decade. His family first noticed memory impairment at the age of 75. Gait disturbance appeared at the age of 77 and oral dyskinesia and left shoulder shrugging appeared at the age of 78. His scores on the MMSE and Frontal Assessment Battery were 22 and 10, respectively. His brain CT images showed calcification at the globus pallidus, thalamus, subcortical and periventricular white matter, and dentate nuclei (figure e-2B [C]). His SPECT images showed decreased perfusion in the bilateral (predominantly in the left) frontal and temporal regions of the cerebrum and bilateral cerebellum. [^{11}C]PiB retention was not observed by [^{11}C]PiB PET, which was performed at the age of 81. There were no other family members presenting with similar neurologic symptoms. CT scan was not performed for other individuals in the family.

DISCUSSION We have obtained clinical information of 161 patients with brain calcification in a nationwide study. We discovered that 3 patients had hypoparathyroidism, Aicardi-Goutières syndrome, and Cockayne syndrome during the survey. CT images revealed varying degrees of calcification, from marked calcification in the basal ganglia to patchy calcification in various regions, suggesting diversity in the etiologies. Some patients were incidentally found to have calcification by CT performed for head injury caused by accidents. Because our previous survey revealed a considerable frequency (1%–2%) of patchy calcification in the CT images of all patients in 2 university hospitals in Japan,[18] more asymptomatic IBGC patients with patchy calcification may exist than the number that we had previously assumed to be present in the population in Japan. After the examination by neurologists, we collected 69 DNA samples from patients who met the criteria for IBGC.[2,3] Symptoms and neurologic findings varied widely from asymptomatic to variable symptoms including headaches, psychosis, and dementia.

In this study, we investigated mutations in *SLC20A2* in 69 patients with IBGC in Japan and identified 4 new mutations in 10 familial cases (the same mutation in 2 families) and 2 other new mutations in 46 sporadic cases. The frequency of families with mutations in *SLC20A2* was 50% (5 of the 10 families), and that of sporadic patients was 4.3% (2 of the 46 patients). The frequency of the mutations in *SLC20A2* in FIBGC in Japan was as high as in other countries in a previous report.[10] Case 5 indicates that it is difficult to reliably determine sporadic cases without brain CT scans and genetic studies of all members in the family.

The mutations in our study existed in exons 2, 3, 8, and 11. One of these mutations (R467X) in exon 8 resulted in a substitution to a TGA stop codon, and the other (c.260_261delTC) in exon 2 was a frameshift. None of the mutations were reported previously, indicating heterogeneities of the mutations in *SLC20A2*. Taken together with other reports, causative mutations identified in *SLC20A2* include 6 mutations in exon 2, 1 in exon 3, 3 in exon 4, 1 in exon 5, 1 in exon 7, 10 in exon 8, 2 in exon 9, 4 in exon 10, and 4 in exon 11.[9–12] It does not seem that there

are mutation hot spots in *SLC20A2*. The in silico analysis using PolyPhen-2 for the missense mutations predicted all to be likely damaging, as determined from the residue changes. We drew the structure model of the PiT-2 protein using the TOPO2 software (http://www.sacs.ucsf.edu/TOPO/top.html). The schematic structure of the PiT-2 protein with the mutations is shown in figure 4.

Although the clinical features varied widely among the families with IBGC with *SLC20A2* mutations, the patients in families 1 and 2 with the same *SLC20A2* mutation exhibited similar clinical manifestations including dysarthria, mild cognitive decline, pyramidal signs, and extrapyramidal signs as well as similar ages at detection of calcification and onset of symptoms. Of note, the CT images among the affected individuals in the 2 families are similar (figure 2). In family 3, in contrast, 3 symptomatic patients presented with dementia, psychological disorder, and alcoholism, accompanied with brain atrophy in CT images. None of them showed movement disorders such as those in families 1 and 2.

Although mutational analysis and CT scan were not performed in other familial members of cases 6 and 7, concordance of the presence of mutations of *SLC20A2* and brain calcification were confirmed in 15 individuals, and we did not observe any individuals who carried the mutation and did not show brain calcification. These observations strongly support a high penetrance of the *SLC20A2* mutations regarding brain calcification.

Correlations of genotypes and neurologic phenotypes, however, have been controversial. *SLC20A2* mutations in patients with FIBGC have been described to show variability in clinical manifestations among the families. In the present study, the 2 affected individuals in families 1 and 2, who carried the same mutation, exhibited quite similar neurologic manifestations and clinical courses, suggesting a genotype-phenotype correlation of the S637R mutation. Of note, 2 individuals aged 56 and 61 years in family 3 did not exhibit any neurologic manifestations despite carrying the mutation and having brain calcification, indicating that penetrance regarding the neurologic manifestations is incomplete.

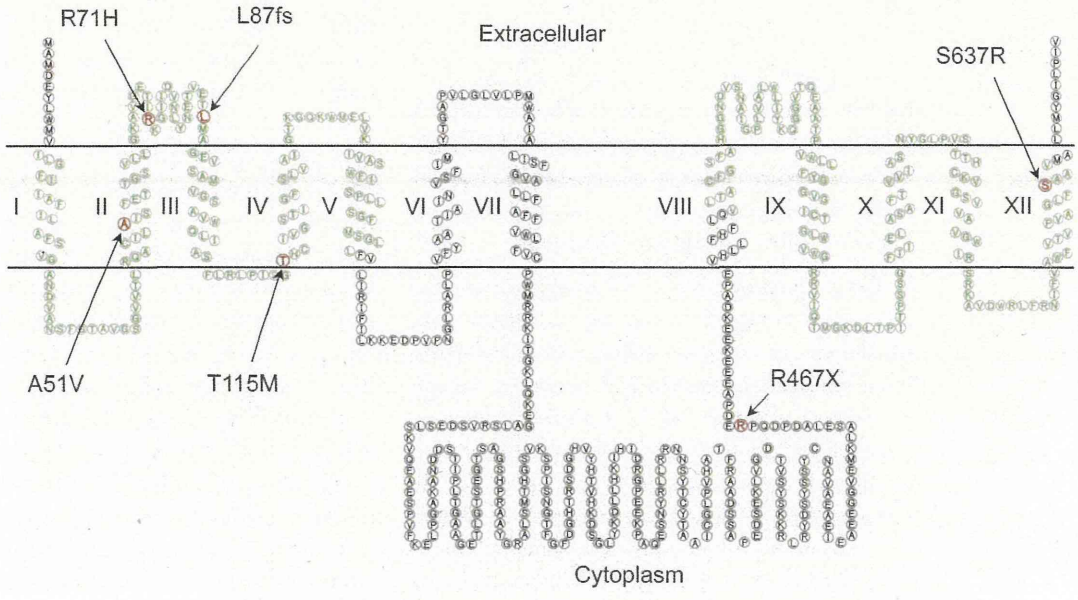
In case 4, interestingly, the proband showed pathologic findings of both IBGC and Parkinson disease. Because Parkinson disease is a common disorder in aged people, there remains a possibility that the presence of IBGC and Parkinson disease is coincidental.

Case 5 had a mutation that leads to a premature stop codon, making an incomplete structure of PiT-2. His neurologic symptom was PKC controllable by carbamazepine. Intriguingly, several patients with IBGC have been reported to present with PKC or paroxysmal nonkinesigenic dyskinesia.[19,20] For these cases of PKC or paroxysmal nonkinesigenic dyskinesia, mutational analyses of not only *SLC20A2* but also *PRRT2* and MRI will be indispensable.[21,22]

Herein, we have reported 5 cases of FIBGC and 2 cases of IBGC with *SLC20A2* mutations in Japan. We could not find any characteristic features of Japanese patients, although we had discovered that each case has a new mutation in *SLC20A2*, respectively.

The mechanisms of calcification and cell damage remain to be elucidated. Despite that the expression of PiT-2 encoded by *SLC20A2* is distributed widely in the human body,[23] mutations in *SLC20A2* cause

Figure 4 **Schematic structure of PiT-2 (type III sodium-dependent phosphate transporter) with the mutations**



calcification only in the brain. Mutations in *SLC34A2* have been reported to cause pulmonary alveolar microlithiasis.[24] Because Npt2b encoded by *SLC34A2* is the only phosphate transporter that is highly expressed in the lungs,[25] the mutations in *SLC34A2* are compatible with the lesion of the alveolar type II cells in the lungs.[24] Because the limitation of calcification to the brain cannot be explained by only the mutation in *SLC20A2* followed by abnormalities of inorganic phosphate (Pi) transport via PiT-2, there might be some other genes responsible for calcification in the brain, or the mutations in *SLC20A2* may take some toxic gain of function. The dysfunction of Pi transport can explain the accumulation of various metals in regions of the brain and the abnormal distribution of metals, which we observed in CSF[26] and hair in the patients with IBGC.[27] We have recently shown that PiT-2 immunopositivity was expressed predominantly in neurons, astrocytes, and vascular endothelial cells in the mouse brain.[28] PDGF-B is expressed in endothelial cells and neurons.[29] PDGF-B homodimer (PDGF-BB) enhanced the expression of PiT-1 mRNA encoded by *SLC20A1* in human aortic smooth muscle cells.[30] The hypomorph of PDGF-B in mice has recently been revealed to cause brain calcification through pericyte and blood-brain barrier impairment.[15] Recently, simple knockout of *SLC20A2* has also been shown to lead to calcification in the mouse brain.[31] PiT-2, PDGF, and as yet undetermined other molecules are considered to have pivotal roles in blood vessel–associated calcification and neuronal death in patients with IBGC. Elucidation of the molecular basis underlying IBGC will contribute to the development of therapeutic measures for patients with calcification in the brain.

AUTHOR CONTRIBUTIONS

Principal investigator: Isao Hozumi. Study supervision: Shoji Tsuji, Gen Sobue, Takashi Inuzuka, and Kortaro Tanaka. Manuscript draft preparation: Megumi Yamada and Masaki Tanaka. Acquisition and collection of data: Seiju Kobayashi, Yoshiharu Taguchi, Shutaro Takashima, Tetsuo Touge, Hiroyuki Hatsuta, and Shigeo Murayama. Analysis and interpretation: Megumi Yamada, Masaki Tanaka, Mari Takagi, Yuichi Hayashi, Masayuki Kaneko, Naoki Atsuta, Nobuyuki Shimozawa, Hiroyuki Ishiura, and Jun Mitsui.

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DISCLOSURE

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Evaluation of *SLC20A2* mutations that cause idiopathic basal ganglia calcification in Japan

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Identification of *ATP1A3* Mutations by Exome Sequencing as the Cause of Alternating Hemiplegia of Childhood in Japanese Patients

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Abstract

Background: Alternating hemiplegia of childhood (AHC) is a rare disorder characterized by transient repeated attacks of paresis and cognitive impairment. Recent studies from the U.S. and Europe have described *ATP1A3* mutations in AHC. However, the genotype-phenotype relationship remains unclear. The purpose of this study was to identify the genetic abnormality in a Japanese cohort of AHC using exome analysis.

Principal Findings: A total of 712,558 genetic single nucleotide variations in 8 patients with sporadic AHC were found. After a series of exclusions, mutations of three genes were regarded as candidate causes of AHC. Each patient harbored a heterozygous missense mutation of *ATP1A3*, which included G755C, E815K, C927Y and D801N. All mutations were at highly conserved amino acid residues and deduced to affect ATPase activity of the corresponding ATP pump, the product of *ATP1A3*. They were *de novo* mutations and not identified in 96 healthy volunteers. Using Sanger sequencing, E815K was found in two other sporadic cases of AHC. In this study, E815K was found in 5 of 10 patients (50%), a prevalence higher than that reported in two recent studies [19 of 82 (23%) and 7 of 24 (29%)]. Furthermore, the clinical data of the affected individuals indicated that E815K resulted in a severer phenotype compared with other *ATP1A3* mutations.

Interpretation: Heterozygous *de novo* mutations of *ATP1A3* were identified in all Japanese patients with AHC examined in this study, confirming that *ATP1A3* mutation is the cause of AHC.

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Introduction

Alternating hemiplegia of childhood (AHC) (MIM 104290) is a rare disorder characterized by transient repeated attacks of paresis on either one or both sides of the body, occulomotor and autonomic abnormalities, movement disorders, and cognitive impairment [1,2]. AHC is predominantly observed in sporadic cases without familial history, although familial AHC with autosomal dominant inheritance has also been reported [3]. Only about 50 patients with sporadic AHC have been reported in Japan and the estimated prevalence of AHC is one in a million births [4].

Since the clinical features of AHC share similarity with those of familial hemiplegic migraine (FHM), previous studies applied mutational analyses of *CACNA1A* (NM_000068) and *ATP1A2* (MN_000702), which are responsible for two types of FHM, FHM1 (MIM 601011) [5] and FHM2 (MIM 182340) [6,7], respectively, to explore the genetic cause of AHC. Although T378N, a mutation of *ATP1A2*, was identified in four affected

members of a Greek family with familial AHC [3], mutations of *ATP1A2* have neither been observed in other familial cases nor in sporadic cases of AHC. Thus, candidate gene approaches have been unsuccessful in identifying the molecular pathogenic mechanism of AHC.

To elucidate the molecular basis of AHC, we hypothesized that sporadic AHC is caused by *de novo* mutations among novel non-synonymous coding variants, which are shared in patients with AHC. To test this hypothesis, we built a *de novo* mutation detection pipeline using the exome sequencing method (Figure 1). Using this technique, we found that *de novo* mutations of *ATP1A3* (NM_152296) cause sporadic AHC.

Results

A total of 712,558 genetic single nucleotide variations (SNVs) and 141,933 small indels were found, including previously known and synonymous genomic variations (Table 1). The ratios of non-overlapping variations in these patients are comparable to those of Asian or Japanese populations (Figure S1). The candidate variants were selected in the following processes based on the pipeline designed in the present study (Figure 1).

To select variants as candidate mutations for AHC, variations that are registered in the genomic variation databases were excluded, which resulted in a total of 39,414 single nucleotide variants and 48,056 indels. The next step was designed to select non-synonymous coding variations and those affecting splice sites, which resulted in the identification of 2,449 variations in 2,131 genes and 246 indels in 232 genes.

We then selected variations in genes expressed in the central nervous system (CNS) (Note S1) [8]. Using this filter, we further narrowed the list to 718 non-synonymous SNVs and 76 indels (Table 1). We then identified variations that were frequently shared among the 8 patients with sporadic AHC. We found that six patients (II-1, III-1, IV-1, VI-1, VII-1, and VIII-1) carried a common variant (c.2813T>G: V938G) of *CNTN4*, four patients carried heterozygous variants of *SYNE1* (c.3955G>A: E1319K in VII-1, c.7196T>G: V2399A in III-1, c.10126A>G: M3376V in V-1, and c.24665G>A: R8222Q in I-1) and five patients carried heterozygous variants (c.2263G>T: G755C, c.2443G>A: E815K, and c.2780G>A: C927Y) of *ATP1A3* (Table 2). These variations were then subjected to validation by Sanger sequencing. The SNV of c.2813T>G of *CNTN4* was not confirmed by Sanger sequencing, indicating that it is an error of exome sequencing.

We then sought all non-synonymous coding variants of *SYNE1* in all variants identified by exome sequencing regardless of whether they were novel or had been reported previously. A total of 19 non-synonymous coding SNVs (10 in I-1, 10 in II-1, 8 in III-1, 10 in IV-1, 9 in V-1, 8 in VI-1, 10 in VII-1, and 9 in VIII-1) were found in 8 patients. Sanger sequencing was performed to search for the 4 novel variants, which were found in the 4 patients, in 96 controls and parents of the 4 patients. Among the novel variants, E1319K, V2399A and M3376V of *SYNE1* were found in 2, 2 and 2 individuals of the 96 controls, respectively. R8222Q was not found in the control. However, each of the 4 variants including R8222Q was inherited from one of the healthy parents of the probands. Taken together, these results suggest that *SYNE1* is unlikely to be the gene responsible for AHC.

Three heterozygous variants (c.2263G>T: G755C, c.2443G>A: E815K, and c.2780G>A: C927Y) of *ATP1A3* were found in 5 of the 8 patients (Table 2). We then reviewed the data of exome analysis, with a special focus on *ATP1A3*, and found another variant (c.2401G>A: D801N) in the other 3 patients. The D801N was not initially classified as a novel variant through our pipeline, since a variant involving D801 had already been registered (though the mutation was D801Y). The D801Y

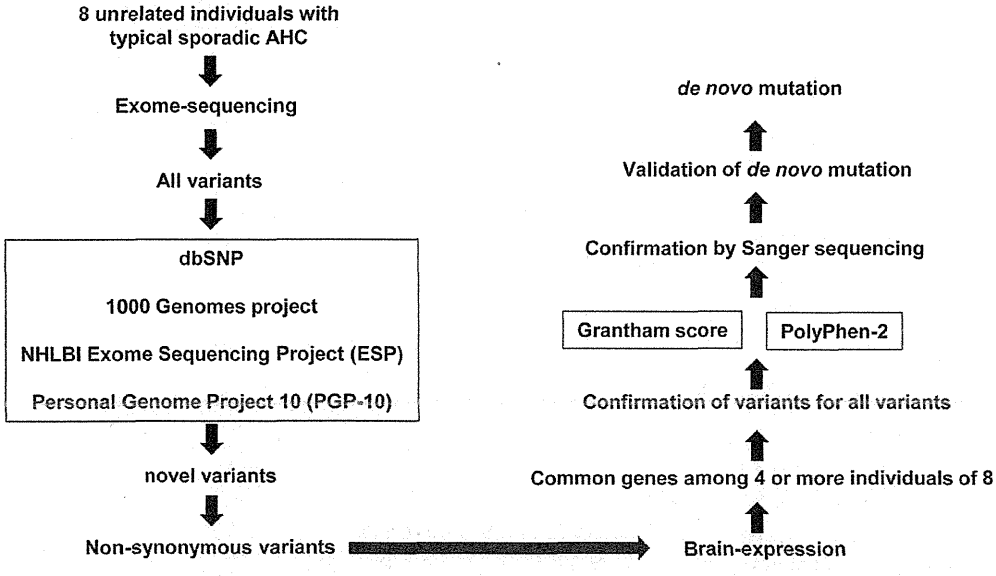

Figure 1. Pipeline for detection of novel *de novo* mutations. The pipeline was used to identify pathogenic mutations of alternating hemiplegia of childhood (AHC). All genetic variants detected by exome sequencing are sequentially filtered through the pipeline. First, variations are screened according to databases of registered single nucleotide polymorphisms (SNP) and only non-registered SNP undergo the next selection as "Novel variants". In the next step, non-synonymous novel variants of genes expressed in the central nervous system are selected. When variations of the same gene are found in the patient, the impact of such variation is evaluated *in silico* using Grantham score and PolyPhen-2. Mutations identified at this stage are reconfirmed by Sanger sequence. *De novo* mutation is validated by analyzing samples from parents. Mutations considered pathogenic are sought in other patients with AHC if necessary.
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Table 1. Distribution of novel non-synonymous single nucleotide polymorphisms including brain-expressed genes in eight patients with AHC.

Patient ID	Total		Novel				
	Variant	Gene	Variant	Variant (NS/SS)	Gene (NS/SS)	Brain expressed variant (NS/SS)	Brain expressed gene (NS/SS)
I-1	229,647	5,590	6,195	282	270	77	75
II-1	200,443	5,656	5,934	316	299	86	82
III-1	125,855	5,489	4,304	342	327	100	93
IV-1	251,550	5,701	7,568	405	376	129	118
V-1	174,045	5,503	6,251	323	302	95	91
VI-1	231,603	5,744	6,785	402	388	111	108
VII-1	177,446	5,613	5,344	330	313	101	96
VIII-1	178,175	5,608	4,767	295	282	78	77
Total	712,558	1,3517	39,414	2,449	2,131	718	630

NS: non-synonymous variants, SS: splice-site acceptor/donor variants.
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mutation was reported to cause rapid-onset dystonia-parkinsonism (RDP/DYT12) (MIM 128235) [9].

Sanger sequencing of *ATP1A3* confirmed four heterozygous mutations; D801N mutation in Patients I-1, VI-1 and VII-1, G755C mutation in Patient II-1, E815K in Patients III-1, IV-1 and V-1, and C927Y mutation in Patient VIII-1 (Figure 2). None of the variants were detected in the parents of each patient, indicating that these mutations were *de novo*. None of these variants was detected in any of the 96 healthy subjects.

Sanger sequence analysis for *ATP1A3* was further conducted in two other unrelated individuals with sporadic AHC (Patients IX-1 and X-1, Table 3). The analysis identified a heterozygous E815K in both patients while neither of the parents of these two patients had the mutation, confirming that the mutation was also *de novo*. These findings in the two patients provided compelling evidence for the pathogenic role of *ATP1A3* mutation in sporadic AHC. Taken together, we identified a total of four *ATP1A3* mutations in the 10 patients studied and these *de novo* mutations were considered pathogenic mutations involved in the etiology of AHC.

The clinical features of AHC patients with *de novo* mutations are summarized in Table 3. Four of the 5 patients with E815K and 1 of the 3 patients with D801N had respiratory abnormalities such as apnea, and one of the patients with E815K required mechanical ventilation. Furthermore, patients with E815K and D801N suffered from status epilepticus, and various involuntary movements were encountered in those harboring E815K mutation. Unfortunately, the small number of patients in our study precluded any firm conclusions backed by proper statistical analysis between genotype and phenotype. However, the results suggested the frequent presence of severe neurological complications, such as aphonia, choreoathetosis, dyskinesia and epilepsy, in individuals with E815K (Table 3). The attending physicians also provided answers to our survey on medications that were considered effective in the control of paralysis (Table 3).

Table 2. *ATP1A3* variants found in eight individuals with AHC.

Patient	Chromosome (position)	Exon	SNV	Amino acid change
I-1	19 (42479781)	16	c. 2263 G>T	G755C
II-1	19 (42474436)	18	c. 2443 G>A	E815K
III-1	19 (42474436)	18	c. 2443 G>A	E815K
IV-1	19 (42474436)	18	c. 2443 G>A	E815K
V-1	19 (42472976)	20	c. 2780 G>A	C927Y
VI-1	19 (42474557)	17	c. 2401 G>A	*D801N
VII-1	19 (42474557)	17	c. 2401 G>A	*D801N
VIII-1	19 (42474557)	17	c. 2401 G>A	*D801N

SNV: single nucleotide variation,
*D801N was initially not considered a novel mutation but confirmed later by re-analysis.
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Discussion

By applying the exome sequencing strategy, we have demonstrated in the present study that *de novo ATP1A3* mutations cause sporadic AHC. Our work provides evidence that *ATP1A3* is the responsible gene for sporadic AHC, a rare but devastating disease that lacks proper treatment so far. At the time of the writing of this communication, two independent research groups, one from the USA and the other from Germany [10,11], reported similar findings. Collectively, the three studies confirm that *ATP1A3* is the causative gene for AHC.

ATP1A3 is a member of the gene family that encodes the alpha subunits of Na^+/K^+ transporting ATPase, which regulates the electrochemical gradients of Na^+ and K^+ through active transport. These ions are essential for regulation of cellular osmolality and the action potentials of excitable membrane. *ATP1A1*, *ATP1A2* and *ATP1A3* encode alpha 1, 2 and 3 subunits, respectively, which are mainly expressed in interneurons and pyramidal cells[12], suggesting that they play important roles in the brain.

A total of 25 mutations identified to date reside in or near transmembrane domains (Figure 3). The G755C and E815K are at the cytoplasmic domain. However, E815K resides more in the transmembrane domain than in the cytoplasmic domain. The D801N and C927Y are at the transmembrane domains, M6 and M8, respectively, and form a helical structure. Also, C927Y identified in our study is a novel mutation.

The amino acids substituted in each mutation are highly conserved among Na^+/K^+ ATPase isoforms of various species (Figure 4), suggesting that the amino acids are crucial for ATPase