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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

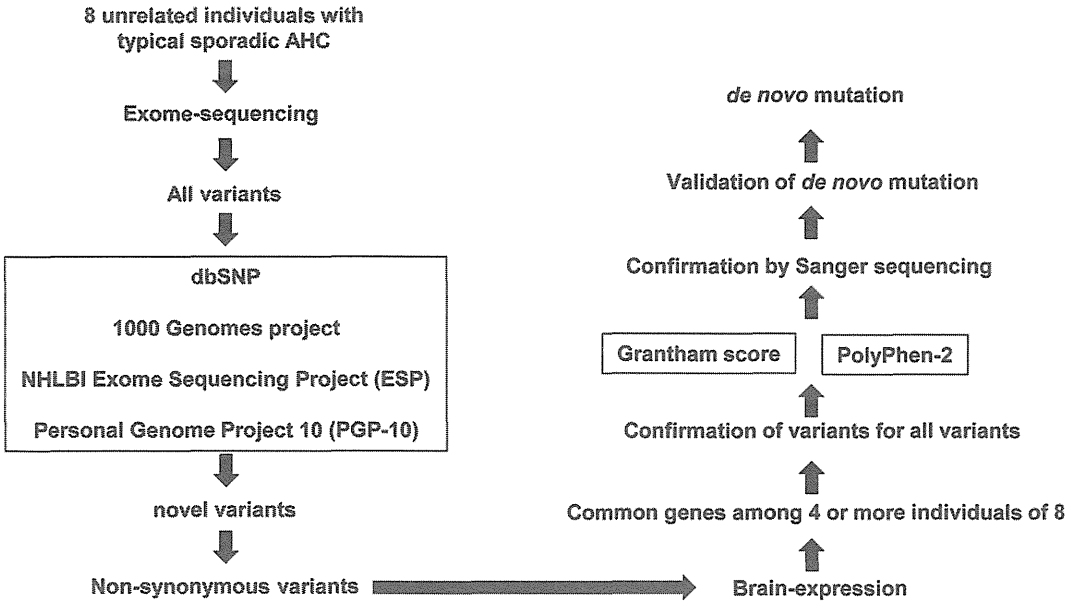

Figure 1. Pipeline for detection of novel *de novo* mutations. The pipeline was used to identify pathogenic mutations of alternating hemiplegia of childhood (AHC). All genetic variants detected by exome sequencing are sequentially filtered through the pipeline. First, variations are screened according 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.
doi:10.1371/journal.pone.0056120.t002

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

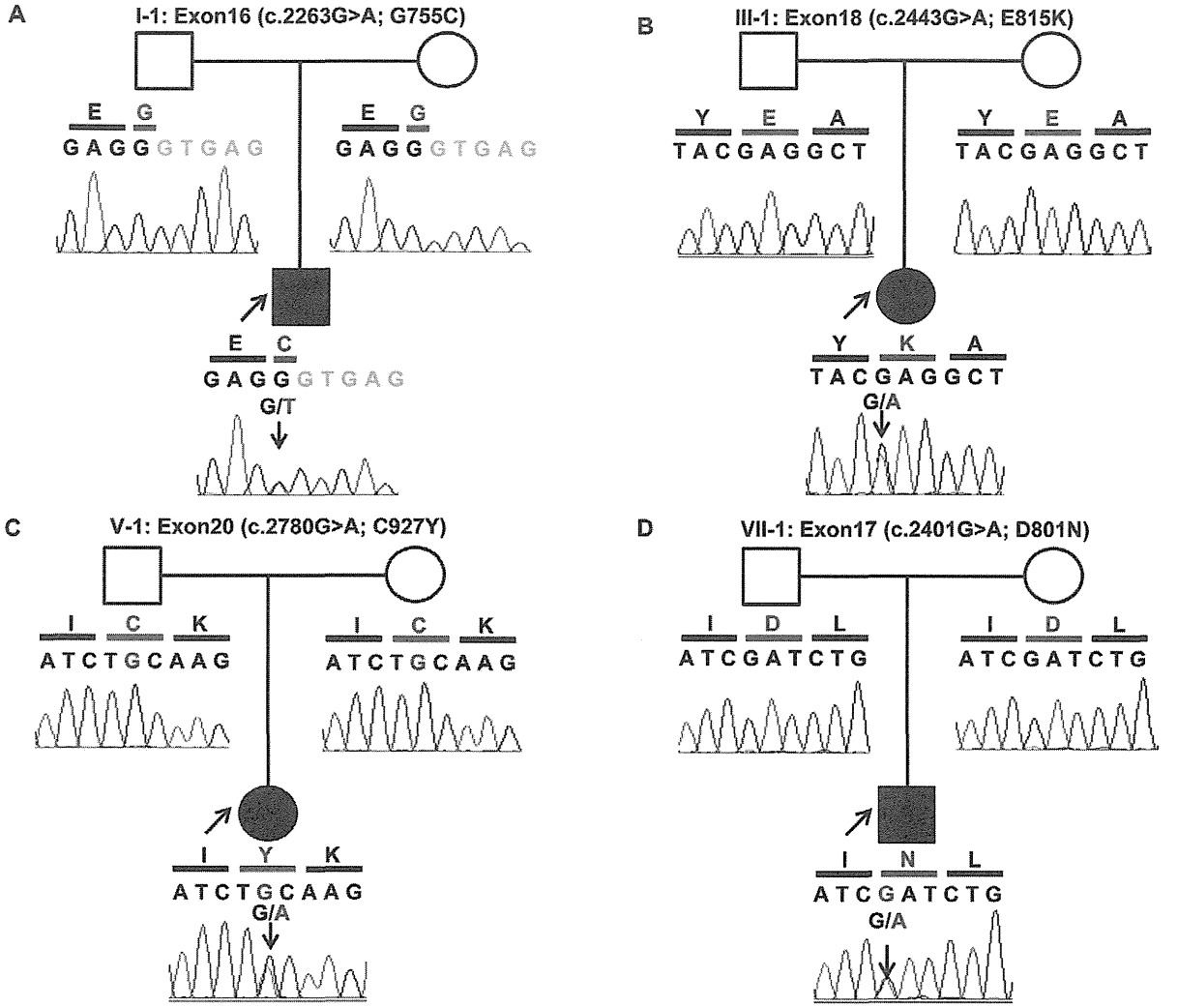

Figure 2. Chromatograms of four *de novo* mutations identified in *ATP1A3*. Data were obtained by Sanger sequencing during the confirmation process. In trio of each pedigree, black shadow represents the proband. In the chromatograms, *Black letters* show exonic nucleotide sequences, *gray letters* show intronic nucleotide sequences. Amino acids are shown in a single letter notation. Nucleotides and amino acids in red indicate mutations. (A) G755C was identified only in Patient I-1. (B) E815K was identified in Patients II-1, III-1, IV-1, IX-1 and X-1. (C) C927Y was identified in Patient V-1 only. (D) D801N was identified in Patients VI-1, VII-1 and VIII-1. None of the mutations was detected in the father or mother except for Patient IX-1, whose parents refused to undergo genetic analysis.
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function. In fact, *in silico* analysis of the mutations identified in the present study suggests a profound damage of the ATPase molecule and hence accord well with functional deficits of the ATPase encountered with the recently described mutations [10].

It is noteworthy that several mutations of *ATP1A3* have been reported to cause RDP [9]. RDP is an autosomal dominant disease characterized by abrupt onset of dystonia and Parkinsonism, developing within minutes to days of onset [13–16]. Recently reported were two infantile RDP patients with *ATP1A3* mutations (R756H and D923N); onset began for one of them at 11 months and for the other at 4 years of age. Major symptoms included motor delay, hypotonia, and ataxia [17,18]. Involuntary movements such as dystonia overlap with AHC, however, their clinical features and age of onset are different than those of AHC, which mainly shows repeated attacks of alternating hemiplegia and which begins with abnormal ocular movements by 3 months of age. Both typical and infantile RDP show different clinical features and processes than AHC, although *ATP1A3* seems to be pathologically involved in both disorders. In particular, D801N, one of the *ATP1A3* mutations identified in the present study, affected D801, where D801Y had been found in RDP. Thus, it seems that two substitutions in the same amino acid result in two distinguished phenotypes. Initially, we could not identify D801N in *ATP1A3* from novel variant. The reason for the erroneous results was the extraction of novel variants from all the variants using chromosome position only during the collation of databases. The position 42474557 of chromosome 19, where the G to A transversion resulted in D801N identified by our exome sequencing, had been registered as the nucleotide where the G to T transition is identified in rapid-onset dystonia-parkinsonism. Based on the backup plans involving reconfirmation of the gene identified with novel variants, using all variants, and to re-sequence the gene in our pipeline with the Sanger sequencer, D801N was not overlooked in the present study. These results suggest that confirmation by Sanger sequencer is useful in avoiding any oversight in the field of gene identification.

Table 3. Clinical data of 10 unrelated individuals with AHC.

Patient ID	I-1	II-1	III-1	IV-1	V-1	VI-1	VII-1	VIII-1	IX-1	X-1
Mutations	**G755C**	**E815K**	**E815K**	**E815K**	**C927Y**	**D801N**	**D801N**	**D801N**	**E815K**	**E815K**
Age (year)/sex	18/male	13/male	32/female	6/male	16/female	17/male	9/male	12/male	9/male	1/male
Age at onset (day)	60	17	2	1	60	1	120	0	Infant	Neonatal
Age at onset of paralysis (month)	6	10	12	4	12	4	9	9	Infant	9
Initial symptoms/signs	L versive movement of neck, monocular deviation of L eye to the left	Tonic fits	Tonic fits	Upward gaze, tonic fits	Nystagmus, ocular deviation to right	Nystagmus, focal clonic seizure	Clonic seizure	Nystagmus	Apnea	Nystagmus, downward gaze, tonic fits
Paralytic type	Flaccid	Flaccid	Flaccid	Flaccid	Rigid	Flaccid	Flaccid	Flaccid	Flaccid	Flaccid
Paralytic symptoms	Paralysis of unilateral arm or leg on R or L, or hemiparesis, sometimes continues with shift to opposite side. Rarely quadriplegia.	Paralysis of unilateral arm or leg on R or L, or hemiparesis, sometimes shifts to opposite side. Rarely quadriplegia.	Hemiparesis. Sometimes quadriplegia. No episodic paralysis since stabilizing of quadriplegia at 14 years.	Paralysis or hemiparesis of R arm.	Rigidity of R arm. Alternating flaccid hemiplegia since 1 year of age.	Alternating hemiparesis every 2–3 months	Alternating hemiplegia (R>L), only a few days every month.	R or L unilateral arm or leg paralysis, sometimes systemic paralysis. Tendency to occur following tonic fits.	Quadriplegia with/without bulbar palsy, for a few min to several hrs every day. Sometimes hemiplegia. Sometimes paralysis shifts to other parts.	Exterior ocular deviation on R side. Systemic cataplexy. Alternating paraparesis
Other neurological abnormalities	Choreoathetosis, aphonia	Choreoathetosis, facial dyskinesia	Dystonia, oral or facial dyskinesia	Aphonia	Spastic diplegia	None	Left hemidystonia	Dystonia	Dystonia	Head lag, nystagmus, ocular deviation
Motor development	walks alone	stands with support	walks with support	sits alone	walks alone	walks alone	walks alone	walks with support	Unable to sit	rolling over
Intellectual development	two words	only words	only words	no words	Normal	three phrases	three word phrases	only words	No words	delay
Regression	No	Yes	Yes	No	Yes	No	No	Yes	Yes	No
Epilepsy	4 years	2 years	4 years	None	None	None	4months	8 years	Yes	9months
Epileptic status	No	Yes	Yes	No	No	No	No	Yes	Yes	Yes
Headache	Yes	Yes	No	No	No	No	No	No	unknown	unknown
Head MRI	Normal	Cerebellar atrophy	Cerebellar atrophy	Normal	Mild enlargement of inferior horns bilaterally	Normal	Normal	High intensity in hippocampus	N/A	Normal
Respiratory status	Apnea	Normal	Use of ventilator	Apnea	Normal	Normal	Normal	Apnea	Apnea	Apnea
Effective drugs for paralysis	flunarizine	CZP	CZP, flunarizine	flunarizine	CZP	flunarizine	flunarizine	flunarizine	none (flunarizine not tried)	MDL
Family history	None	None	None	Headache, epilepsy	None	None	Migraine	Headache, epilepsy	Headache	None
Gestational age	40 weeks	34 weeks 3days	42 weeks	40 weeks	unknown	41 weeks 4 days	39 weeks 3 days	41 weeks	40 weeks	37 weeks 3 days
Birth weight (g)	3148	2218	3260	3392	unknown	3526	3200	3008	3550	2962
Asphyxia	None	No crying unless stimulated	Unknown	None	unknown	None	unknown	None	None	None

MDL: midazolam, CZP: clonazepam, L: left, R: right.

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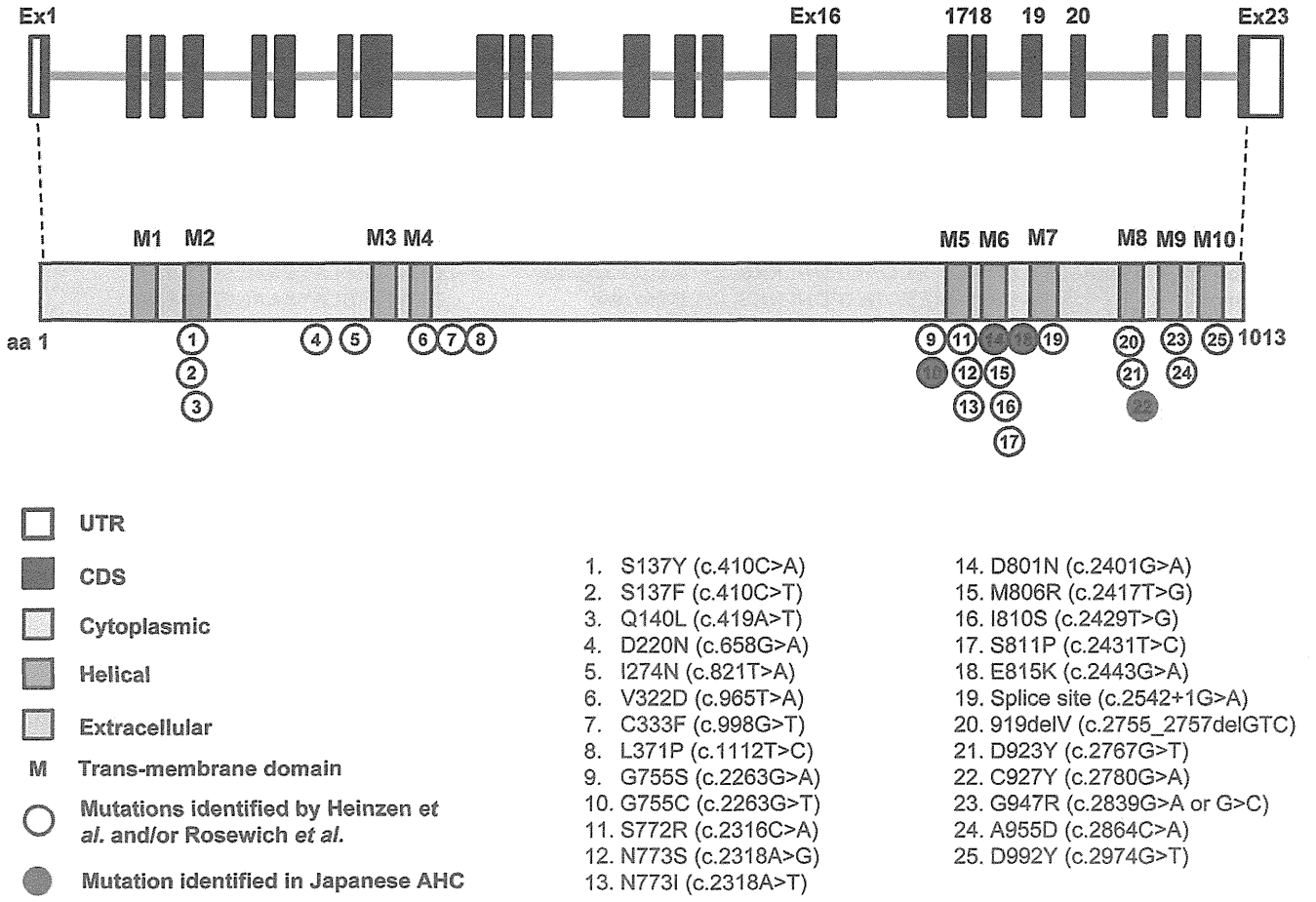

Figure 3. *ATP1A3* mutations and their protein domain structures. *Black lined circle:* Mutations reported recently [10,11]. *Red colored circle:* Mutations identified in the present study in a Japanese cohort with AHC. The *ATP1A3* gene consists of 23 exons that encode several domains in the ATP1A3 protein molecule, including 6 cytoplasmic, 10 helical and 5 extracellular domains. G755C and E815K were located in the cytoplasmic domains. Notably, E815K was resident of the transmembrane domain rather than the cytoplasmic domain. D801N and C927Y were located in the helical domains. C927Y was identified in this study only and hence considered novel.
doi:10.1371/journal.pone.0056120.g003

Functional analysis of *ATP1A3* mutations in RDP by haploinsufficiency demonstrated low protein levels of the corresponding ATPase [9]. In addition, Heinzen *et al.* demonstrated that none of the mutations causes AHC reduced protein levels, whereas both mutations of AHC and those of RDP reduced ATPase activity [10]. These studies suggested that mutations identified in AHC affect the Na^+/K^+ ATPase pump function due to inhibition of ion binding. This implies that D801N substitutions can cause pump dysfunction more than D801Y. Heterozygous knock-out mice and knock-in mice deficient in *ATP1A3* have been generated. The *ATP1A3* knock-out mice were found to have reduced NMDA receptors and exhibited neurological abnormalities such as hyperactivity, spatial learning and memory deficit [19]. The mice harboring mutation I810N of *ATP1A3*, which were neither RPD nor AHC, developed seizures [20]. While these phenotypes do not necessarily correspond with the typical clinical manifestations observed in either RDP or AHC, some similarities do exist.

In total, we identified four *ATP1A3* mutations in 10 Japanese AHC patients. All were heterozygous and *de novo*. Although the number of patients was small (10 individuals), E815K and D801N were observed in 5 (50%) and 3 (30%) of the 10 patients, respectively.

The exact mechanism of *de novo* mutation identified in this study is not clear at present. The nucleotides of both E815K and D801N are located in the GC-rich sequences of *ATP1A3*, and within 6-bp palindrome. These features may be related to the development of these *de novo* mutations.

Intriguingly, E815K mutation of *ATP1A3* found in half of our patients was associated with the presence of severe neurological symptoms, respiratory failure, status epilepticus and resistance to medications. The attending physicians consider, with hindsight clinical experience that flunarizine seems to be less effective in individuals with E815K mutation, compared to those with other mutations. However, the association between genotype and phenotype remains undefined due to the small number of the cohort. The present findings and those of other groups on AHC associated with *ATP1A3* mutations warrant further studies to understand the relation between genotype and phenotype in AHC and to develop new tools for the diagnosis and treatment of AHC.

Patients and Methods

Ethics statement

The present study was approved by the Ethics Review Committees of Fukuoka University and the University of Tokyo.

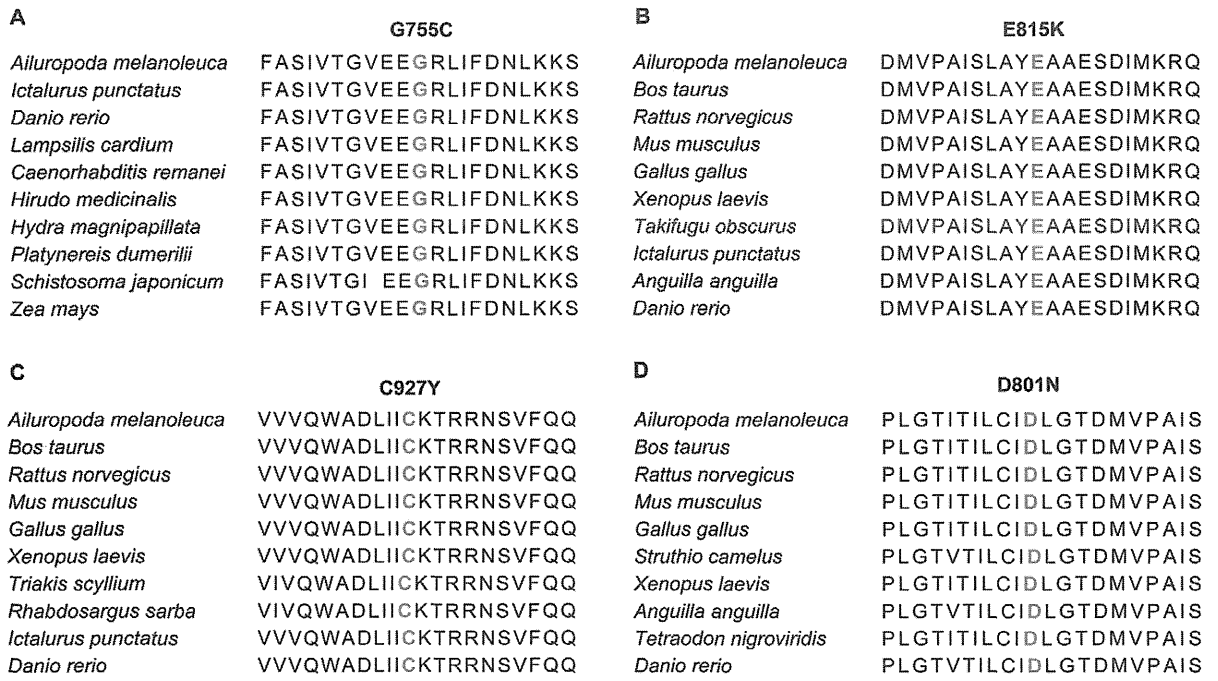

Figure 4. Homologous comparison of altering-protein. *Blue letters:* altering-protein by mutation, *red letters:* differential protein with human. (A) G755C changed by novel SNVs (c.2263G>T) of *ATP1A3* in Patient I-1. (B) E815K changed by novel SNVs (c.2443 G>A) of *ATP1A3* in Patients II-1, III-1, IV-1, IX-1 and X-1. (C) C927Y changed by novel SNVs (c.2780 G>A) of *ATP1A3* in Patient V-1. (D) D801N changed by novel SNVs (c.2401 G>A) of *ATP1A3* in Patient VI-1, VII-1 and VIII-1.
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Parents of each patient and the parents themselves provided signed informed consent before the study.

Patients

We initially recruited 10 unrelated Japanese individuals with clinical features of typical sporadic AHC. The diagnosis of AHC was based on the criteria of AHC [1,2]. The clinical presentations of these patients were typical but the neurological symptoms showed some variations, including aphonia, choreoathetosis, dyskinesia, epilepsy, and episodic apnea. Furthermore, variability in the response to different medications, such as flunarizine, was also noted among the patients (Table 3). Flunarizine was used for the treatment of 9 patients to control paralysis. The frequency of the paretic symptom decreased somewhat following the treatment, compared to that with other medications. However, the response to treatment, as evaluated subjectively by the attending physician, was not remarkable. Two patients (II-1 and V-1) showed a better response to clonazepam than to flunarizine.

The patients studied were 8 males and 2 females with similar clinical presentation, including infantile onset and psychomotor retardation. MRI images showed high-intensity hippocampal region in patient VIII-1 (Table 3), which was considered secondary to repeated episodes of epileptic convulsions. MRI images in patients II-1 and III-1 showed cerebellar atrophy, which was considered a primary lesion similar to FHM. The MRI findings in patient V-1 were considered non-specific.

Based on the availability of samples from the parents of the 9 patients, we selected 8 probands (subjects I-1 to VIII-1, Table 3) for exome sequencing analysis. After the identification of *de novo* heterozygous mutations in 8 patients, we also collected samples from the parents of patient IX-1 and also samples from patient X-1 and his parents. Parents of the patients with available genomic DNAs were also enrolled in this study. We also recruited 96 unrelated healthy Japanese volunteers as the control group who were free of seizures or history of epilepsy.

Genomic DNA was prepared from EDTA-Na_2-containing blood samples using the QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany), using the protocol provided by the manufacturer.

Exome sequencing

The exonic sequences were enriched using the Agilent SureSelect technology for targeted exon capture (213,383 exons, covering approximately 50 Mb of the CCDS database) (Agilent Technologies, Santa Clara, CA) from 3 μg of genomic DNA, using the protocol provided by the manufacturer. The captured DNAs were subjected to massively parallel sequencing (100 bp paired-end reads) on the Illumina Hiseq2000 (Illumina, San Diego, CA). The average of 1.3 billion bases of the sequence data was obtained for each individual. On average, 99.08% of the total bases were mapped to the reference genome with a mean coverage of 182.8x, which encompassed 92.99% of the targeted regions with coverage >10x. Burrows Wheeler Aligner [21] and Samtools [22] were used as default settings for alignment of raw reads and detection of variations. The variants were filtered against dbSNP (build 135). The aligned short reads were viewed using the University of Tokyo Genome Browser (UTGB) [23].

Sanger sequencing

Sanger sequencing was performed to validate the presence of each variant detected by exome sequencing in patients with AHC and the absence of each in the parental genomes. The entire exons and the intron-exon boundaries of *ATP1A3*, *CNTN4* (NM_175607) and *SYNE1* (NM_033071) were amplified by PCR using the

designed PCR primers (Table S1 lists the primer sequences and the PCR conditions). The PCR products were purified in ExoSAP-IT for PCR Product Clean-Up (Affymetrix, Santa Clara, CA) set at one cycle of 15 min at 37°C and 15 min at 80°C. The purified PCR products were sequenced using the ABI PRISM BigDye 3.1 terminator method (Applied Biosystems, Foster City, CA) and the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

URLs

BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD = Web&PAGE_TYPE = BlastHome Japanese Society of Alternating hemiplegia of childhood: http://www008.upp.so-net.ne.jp/ahc/

Accession numbers

Reference sequences are available from NCBI under the following accession codes: *CACNA1A*:NM_000068
ATP1A2:MN_000702
CNTN4: NM_175607
ATP1A3: NM_152296
SYNE1: NM_033071

Supporting Information

Figure S1 Rations of single nucleotide variations (SNVs) overlapping with known polymorphisms in various ethnic backgrounds.
(DOC)

Note S1 Brain-expressed genes.
(DOC)

Table S1 PCR primers and conditions designed for *ATP1A3*.
(DOC)

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Author Contributions

Confirmed the diagnosis in each patients participating in this study: MS YS. Conceived and designed the experiments: AI YS SM MS ST SH. Performed the experiments: AI JM HI. Analyzed the data: AI JY. Contributed reagents/materials/analysis tools: MS YS HA SY SK HO. Wrote the paper: AI ST SH.

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ONLINE FIRST

The Neurogenomics View of Neurological Diseases

Shoji Tsuji, MD, PhD

The availability of high-throughput genome sequencing technologies is expected to revolutionize our understanding of not only hereditary neurological diseases but also sporadic neurological diseases. The molecular bases of sporadic diseases, particularly those of sporadic neurodegenerative diseases, largely remain unknown. As potential molecular bases, various mechanisms can be considered, which include those underlying apparently sporadic neurological diseases with low-penetrant mutations in the gene for hereditary diseases, sporadic diseases with de novo mutations, and sporadic diseases with variations in disease-susceptible genes. With unprecedentedly robust power, high-throughput genome sequencing technologies will enable us to explore all of these possibilities. These new technologies will soon be applied in clinical practice. It will be a new era of datacentric clinical practice.

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The elucidation of the molecular bases of neurological diseases is fundamental to the development of disease-modifying and preventive therapies.[1] Over the past 3 decades, we have witnessed remarkable progress in the identification of the genes that cause hereditary neurological diseases (**Figure 1**).[2-4] This has been accomplished mainly on the basis of the research paradigm known as "positional cloning,"[5,6] which uses linkage studies to pinpoint the position of genes on chromosomes followed by the identification of the causative gene. The identification of causative genes has further made it possible to develop disease models for hereditary neurological diseases[7-10] and to develop therapeutic strategies.[11]

The majority of neurological diseases, however, are sporadic without any obvious familial occurrence. We are thus faced with the challenge of elucidating the molecular bases of sporadic diseases. Intriguingly, the clinical presentations and neuropathological findings of hereditary forms of neurodegenerative diseases are often indistinguishable from those of sporadic diseases, raising the possibility that common pathophysiologic pathways underlie both hereditary and sporadic neurodegenerative diseases.

In contrast to the molecular bases of hereditary neurological diseases, the molecular bases of sporadic neurological diseases, particularly those of sporadic neurodegenerative diseases, largely remain unknown. A potential clue to the molecular bases of sporadic neurological diseases may be the clinical observation that siblings and relatives of a patient with a neurological disease are at an increased risk of developing the same disease; this phenomenon has been observed with regard to Parkinson disease (PD)[12] and amyotrophic lateral sclerosis.[13] These clinical observations suggest the involvement of genetic factors in these diseases (Figure 1). Until recently, it has been difficult to elucidate the genetic factors underlying sporadic neurological diseases. Rapid advancements in genome science, particularly the availability of massively parallel sequencing technologies that use next-generation sequencers (NGSs), are revolutionizing the neurogenomics view of sporadic neurological diseases. The elucidation of the genomic variants underlying sporadic diseases is expected to provide some answers that will help us to develop disease-modifying and preventive therapies.

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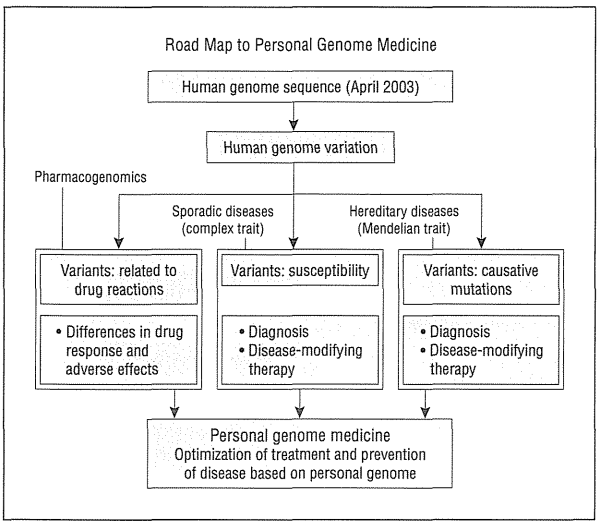

Figure 1. Diagram showing the road map to personal genome medicine. Since the completion of the human genome sequence in 2003, the research focus in human genetics has moved to how human genome variations affect human health. Human genome variations are considered to be associated not only with hereditary diseases but also with sporadic diseases. In addition, human genome variations are also associated with differences in drug responses and adverse effects. Optimization of treatment and prevention based on personal genome information will soon be a realistic paradigm in clinical practice.

Another important field is pharmacogenomics, in which genomic variations underlie differences in drug responses and adverse drug effects (Figure 1). This field is currently being introduced into clinical practice.

Thus, it will be essential to better understand how human genome variations affect our health with regard to diseases with Mendelian or complex traits, as well as with regard to pharmacogenomics. Herein, the neurogenomics view of neurological diseases and the future directions of clinical practice are discussed.

HIGH-THROUGHPUT GENOME SEQUENCING TECHNOLOGIES

Emerging new technologies for nucleotide sequencing have brought about a remarkable revolution in analyses of the human genome sequence. Compared with a conventional technology (namely, the Sanger method),[14,15] the throughput of massively parallel sequencing that uses NGSs[16] is increasing dramatically, with the current throughput at 600 GB per run, which means that a sufficient amount of sequence data can be obtained for whole-genome sequencing of at least 4 individuals.[17] In typical experiments, billions of short reads (100-150 base pairs [bp]) are obtained. These short reads are aligned to human genome reference sequences, and sequence variations are called through computational analyses.

Currently, 2 types of sequencing strategy (namely, whole-exome and whole-genome sequence analyses) are used. Because the cost of whole-genome sequencing is still considerably high, it is not easy to conduct whole-genome sequencing for a large number of individuals. In whole-exome sequence analysis, the enrichment of exonic sequences using oligonucleotide "baits," which is followed by sequencing, has been preferentially used. With this strategy, all exonic sequences in the human genome can be efficiently enriched.[18-20] With this approach, more than 90% of target regions can be enriched, and these enriched genomic regions are then subjected to massively parallel sequencing using NGSs. This approach is currently being used a lot for the identification of disease-relevant variants[21-31] and even for diagnostic purposes.[32-35]

Given the ever-increasing throughput of NGSs and the dramatically decreasing costs, it will soon be a realistic approach to conduct whole-genome sequencing for various research applications (**Figure 2**).[36-40] Studies have shown that there are more than 3 million variations in the human genome of each individual. In one study,[40] among the 3.3 million single-nucleotide polymorphisms (SNPs), 8996 known nonsynonymous SNPs and 1573 novel nonsynonymous SNPs were identified. Interestingly, 32 alleles exactly matched mutations previously registered in the Human Gene Mutation Database. In addition, 345 insertions/deletions were observed to overlap in a coding sequence and may alter protein function.[40] These findings indicate that, among the numerous candidate variations, it will be a challenge to determine which variations are relevant to diseases.

Given the enormous number of short read sequences (~100 bp), informatics analyses, including mapping to reference sequences and indentifying variations, require a huge computational power.[41-45] Furthermore, mutations can be variable, including single base substitutions, insertions/deletions, and structural variations. It is difficult to efficiently identify all the variations using currently available NGSs and software. For example, expansions of repeat motifs identified in frontotemporal dementia and amyotrophic lateral sclerosis[46] are difficult to identify using NGSs.

As already stated, most of the currently available NGSs produce billions of short reads of 100 to 150 bp. This is the limitation in analyzing various structural variations, some of which may be relevant to neurological diseases. Very recently, single-molecule sequencing technology has become available from Pacific Biosciences; this type of technology enables the acquisition of nucleotide sequences as large as 10 kilobases.[47,48] Another single-molecule sequencing technology using nanopores, which allows for the acquisition of much longer sequences,[49] will soon become available.

EFFECT OF HIGH-THROUGHPUT GENOME SEQUENCING ON UNDERSTANDING THE MOLECULAR BASES OF HEREDITARY NEUROLOGICAL DISEASES

The strategies for identifying causative genes for hereditary diseases have been well established.[5,6] The chromosomal localization of the disease-causing genes is pinpointed by linkage analysis using polymorphic DNA markers.[50-52] Although a number of genes have been identified by applying these technologies, more than 50% of the genes causing familial amyotrophic lateral sclerosis remain to be identified.[53] In families with hereditary diseases, the availability of affected and unaffected individuals is often limited owing to small family sizes and the small number of family members with a confirmed clinical and/or a pathological diagnosis. These circumstances pose a challenge to positional cloning because the candidate regions cannot be narrowed down to small regions that are sufficient for identifying the

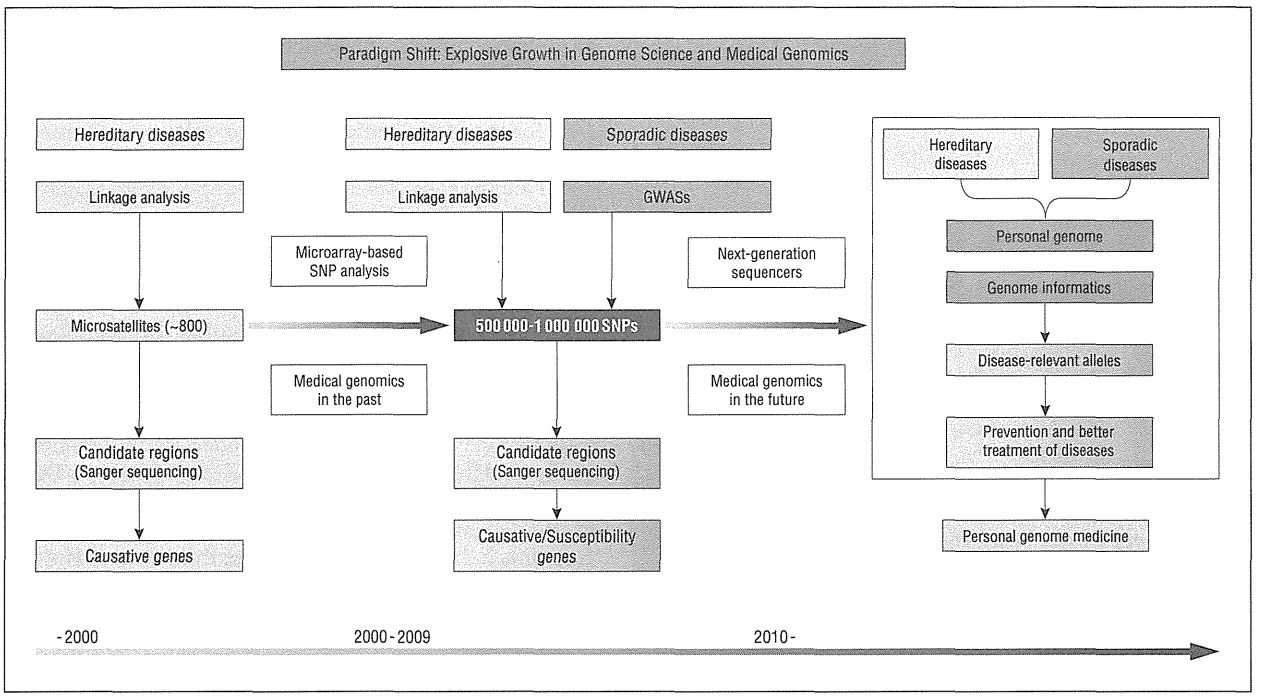

Figure 2. Diagram showing the paradigm shift (ie, the explosive growth in genome science and medical genomics). Over the past decade, genome-wide association studies (GWASs) using common single-nucleotide polymorphisms (SNPs) have been conducted to identify genomic variations in sporadic neurological diseases. The theoretical framework of GWASs is the common disease–common variants hypothesis. Although GWASs have successfully revealed numerous susceptibility genes for common diseases such as diabetes mellitus, as well as neurodegenerative diseases, the odds ratios associated with these risk alleles are generally low and account for only a small proportion of estimated heritability. The availability of high-throughput genome sequencing technologies will enable us to identify all the genomic variants, and eventually those of disease-relevant alleles based on the common disease–multiple rare variants hypothesis.

causative genes by sequencing individual genes in the candidate regions. Despite these difficult circumstances, the availability of NGSs with unbelievably high throughput has made the identification of causative genes possible.[31,54,55] Given the large capacity of NGSs, the most essential step (and the bottleneck) is now the collection of as many samples from patients and their families as possible based on well-characterized clinical information, including the correct diagnosis, regardless of family size or number.

EFFECT OF HIGH-THROUGHPUT GENOME SEQUENCING ON UNDERSTANDING THE MOLECULAR BASES OF SPORADIC NEUROLOGICAL DISEASES

The elucidation of the molecular bases of sporadic neurological diseases is now a big challenge. We need to take various mechanisms into account as the molecular bases of sporadic neurological diseases, which include (1) apparently sporadic diseases with low-penetrant mutations in the gene for hereditary diseases, (2) sporadic diseases with de novo mutations, (3) sporadic diseases with variations in disease-susceptible genes, and (4) sporadic diseases with other mechanisms. These different molecular bases are reviewed.

APPARENTLY SPORADIC NEUROLOGICAL DISEASES WITH LOW-PENETRANT MUTATIONS IN THE GENE FOR HEREDITARY DISEASES

There are numerous examples of low-penetrant mutations in apparently sporadic cases of neurological diseases. Sporadic cases of amyotrophic lateral sclerosis due to low-penetrant *SOD1* mutations have been well characterized.[56-61] In prion diseases, patients with V180I or M232R mutations in the prion protein (*PRNP*) gene rarely have a family history of prion diseases, indicating that these patients are usually diagnosed as having sporadic Creutzfeldt-Jakob disease.[62]

SPORADIC NEUROLOGICAL DISEASES WITH DE NOVO MUTATIONS

Alternating hemiplegia of childhood is a rare neurological disorder characterized by early-onset episodes of hemiplegia, dystonia, various paroxysmal symptoms, and developmental impairments. Almost all cases are sporadic, but the concordance of alternating hemiplegia of childhood in monozygotic twins and the dominant transmission in a family with a milder phenotype have been reported. With this background information, Rosenwich et al[63] conducted whole-exome sequencing of 3 proband-parent trios to identify a disease-associated gene and then examined whether mutations in the gene were also present in the remaining patients and their healthy parents. Whole-exome sequencing indeed showed 3 heterozygous de novo missense mutations.[63] Similar approaches have been used for a number of diseases, including severe epileptic encephalopathy,[64] autism, and schizophrenia.[65] The rationale for these approaches is based on the hypothesis that patients with severe phenotypes associated with reduced reproductive fitness may harbor de novo mutations.[65,66]

Twin studies in which differences in the phenotypes of monozygotic and dizygotic twins were compared have

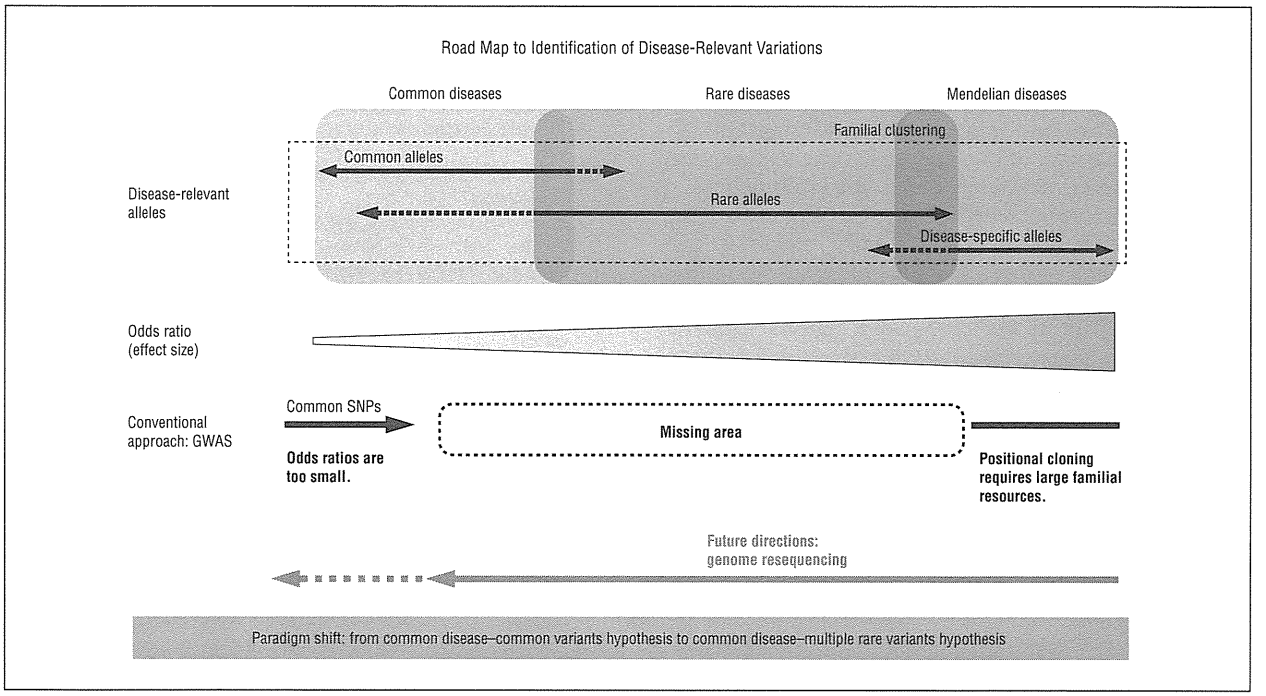

Figure 3. Diagram showing the road map to the identification of disease-relevant variations. Shifting the paradigm from the common disease–common variants hypothesis to the common disease–multiple rare variants hypothesis will lead to the elucidation of the molecular bases of sporadic neurological diseases. Relatively rare sporadic neurological diseases will be good candidates for identifying disease-relevant alleles with large effect sizes because, depending on the effect sizes, the sample sizes can be small.

long been conducted to delineate the involvement of genetic factors. Therefore, the comparison of whole-genome sequences of discordant monozygotic twins is expected to accelerate the discovery of genomic variations responsible for the disease phenotypes.[67,68]

SPORADIC NEUROLOGICAL DISEASES WITH VARIATIONS IN DISEASE-SUSCEPTIBILITY GENES

Over the past decade, genome-wide association studies (GWASs) using common SNPs have been conducted to identify genomic variations associated with sporadic neurological diseases. The theoretical framework of GWASs is the "common disease–common variants" hypothesis, in which common diseases are attributable in part to allelic variants present in more than 5% of the population.[69-71] Although GWASs have successfully revealed numerous susceptibility genes for common diseases such as diabetes mellitus, as well as neurodegenerative diseases, the odds ratios associated with these risk alleles are generally low and account for only a small proportion of estimated heritability.[72-75]

In GWASs, the general finding that the odds ratios associated with risk alleles identified for disease susceptibility are low indicates that GWASs based on the common disease–common variants hypothesis are not effective in identifying genetic risks with large effect sizes. The current experience with GWASs strongly suggests that rarer variants that are difficult to detect by GWASs may account for the "missing" heritability.[17,74] Such rare variants may have large effect sizes as genetic risk factors for diseases. Thus, the paradigm should be shifted from the "common disease–common variants" hypothesis to the "common disease–multiple rare variants" hypothesis to identify disease-relevant alleles with large effect sizes (**Figure 3**).

An excellent example of rare variants with substantially large effect sizes is the recent discovery of the glucocerebrosidase (*GBA*) gene as a robust genetic risk factor for PD.[76,77] A population-based study[78] coupled with genealogy information demonstrated that the estimated risk ratio for PD for siblings of patients with PD was significantly high, indicating that genetic factors substantially contribute to the development of sporadic PD. Recent clinical observations[79] have suggested the association of sporadic PD with heterozygous mutations in the *GBA* gene encoding the enzyme that is deficient in patients with Gaucher disease, an autosomal recessive lysosomal storage disease. Furthermore, the comorbidity of PD and Gaucher disease was previously described.[80] We conducted an extensive resequencing analysis of *GBA* in patients with PD and controls, and we found that *GBA* variants that are pathogenic for Gaucher disease confer a robust susceptibility to sporadic PD and even account for the familial clustering of PD.[77] The combined carrier frequency of the "pathogenic variants" was as high as 9.4% in patients with PD and significantly higher than that in controls (0.37%), with a markedly high odds ratio of 28.0 (95% CI, 7.3-238.3) for patients with PD compared with controls.

We can draw the following conclusions from the discovery of the major disease-susceptibility gene (*GBA*) with a large effect size: (1) a genetic factor with a large effect size has been discovered in sporadic PD; (2) in accordance with the large effect size, there is a tendency of familial clustering (multiplex families such as affected siblings); and (3) the disease-relevant allele could not be

identified by GWASs using common SNPs and was identified only by nucleotide sequence analysis. These conclusions strongly encourage us to search for disease-susceptibility genes with large effect sizes based on the common disease–multiple rare variants hypothesis. Although the majority of rare missense variants have been suggested to be functionally deleterious in humans,[81] it remains controversial whether a comparison of allele frequencies of rare variants (in particular, missense variants) is a sufficient method for identifying variants associated with diseases. Functional annotation of all the variants obtained by comprehensive genome sequencing will no doubt increase the robust power for detecting significant associations of variants with diseases.

SPORADIC NEUROLOGICAL DISEASES WITH OTHER MECHANISMS

Besides the mechanisms already mentioned, there may be others underlying sporadic neurological diseases. The involvement of somatic mutations occurring in certain cell lineages in sporadic neurological diseases is a potentially interesting mechanism. Such a mechanism in certain types of cancer is well established.[82] The involvement of epigenetics in the development of sporadic neurodegenerative diseases is also a potentially attractive mechanism.[83,84] Recently, there have been an increasing number of studies suggesting that "prion-like" processes (ie, the propagation of misfolded proteins leading to abnormal aggregation) may be involved in the pathogenesis of sporadic neurodegenerative diseases.[85,86] In the field of autoimmune diseases such as multiple sclerosis, the involvement of genetic factors is well characterized. The application of massively parallel sequencing to extensively characterize T-cell receptor repertoires[87,88] and immunoglobulin heavy chain genes,[89] along with sequence-based typing of HLAs,[90,91] will provide new insights into the molecular bases of autoimmune diseases.

As discussed in this review, the availability of robust technologies using NGSs will revolutionize our research paradigms for exploring the molecular bases of hereditary and sporadic neurological diseases. Furthermore, these technologies will soon be applied in clinical practice. It will be a new era of datacentric clinical practice. Are we prepared for this new era?

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ORIGINAL CONTRIBUTION

C9ORF72 Repeat Expansion in Amyotrophic Lateral Sclerosis in the Kii Peninsula of Japan

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Background: In the Kii peninsula of Japan, high prevalences of amyotrophic lateral sclerosis (ALS) and parkinsonism-dementia complex have been reported. There are 2 major foci with a high prevalence, which include the southernmost region neighboring the Koza River (Kozagawa and Kushimoto towns in Wakayama prefecture) and the Hohara district (Mie prefecture).

Objective: To delineate the molecular basis of ALS in the Kii peninsula of Japan, we analyzed hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 (*C9ORF72*) gene, which has recently been identified as a frequent cause of ALS and frontotemporal dementia in the white population.

Design: Case series.

Setting: University hospitals.

Patients: Twenty-one patients (1 familial patient and 20 sporadic patients) with ALS from Wakayama prefecture, and 16 patients with ALS and 16 patients with parkinsonism-dementia complex originating from Mie prefecture surveyed in 1994 through 2011 were enrolled in the study. In addition, 40 probands with familial ALS and 217 sporadic patients with ALS recruited from other areas of Japan were also enrolled in this study.

Main Outcome Measures: After screening by repeat-primed polymerase chain reaction, Southern blot hybridization analysis was performed to confirm the expanded alleles.

Results: We identified 3 patients with ALS (20%) with the repeat expansion in 1 of the 2 disease foci. The proportion is significantly higher than those in other regions in Japan. Detailed haplotype analyses revealed an extended shared haplotype in the 3 patients with ALS, suggesting a founder effect.

Conclusions: Our findings indicate that the repeat expansion partly accounts for the high prevalence of ALS in the Kii peninsula.

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AMYOTROPHIC LATERAL SCLEROSIS (ALS) is a devastating neurodegenerative disorder primarily affecting motor neurons. Although the prevalence of ALS is basically similar around the world, an extraordinarily high prevalence rate has been reported in the southern coast areas of the Kii peninsula of Japan as well as in the island of Guam and in West New Guinea.[1-5] In the Kii peninsula, there are 2 major foci with a high prevalence, which include the southernmost region neighboring the Koza River (Kozagawa and Kushimoto towns) and the Hohara district (**Figure 1**).

Detailed epidemiologic studies in these 2 areas started in the 1960s revealed that the prevalence rates of ALS were 100 to 150 times higher than those in other regions in Japan.[1] Follow-up studies revealed that the prevalence rates of ALS in these areas seemed to decrease in the 1980s, but they are still substantially higher in these regions than in other regions in Japan.[6-8]

Intensive clinical and neuropathologic studies have been conducted in the Hohara district and its vicinity (Minamiise town and Shima city), and the major pathologic findings have been described to consist of neurofibrillary tangles widely distributed in the brain and spinal cord, confirming the diagnosis of ALS/parkinsonism-dementia complex (ALS/PDC).[1,9] Although epidemiologic studies in the Hohara district have suggested the involvement of genetic components, the molecular basis of ALS or ALS/PDC in these 2 areas in the Kii peninsula remains to be elucidated.[10,11]

Recently, GGGGCC hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 (*C9ORF72*) gene has

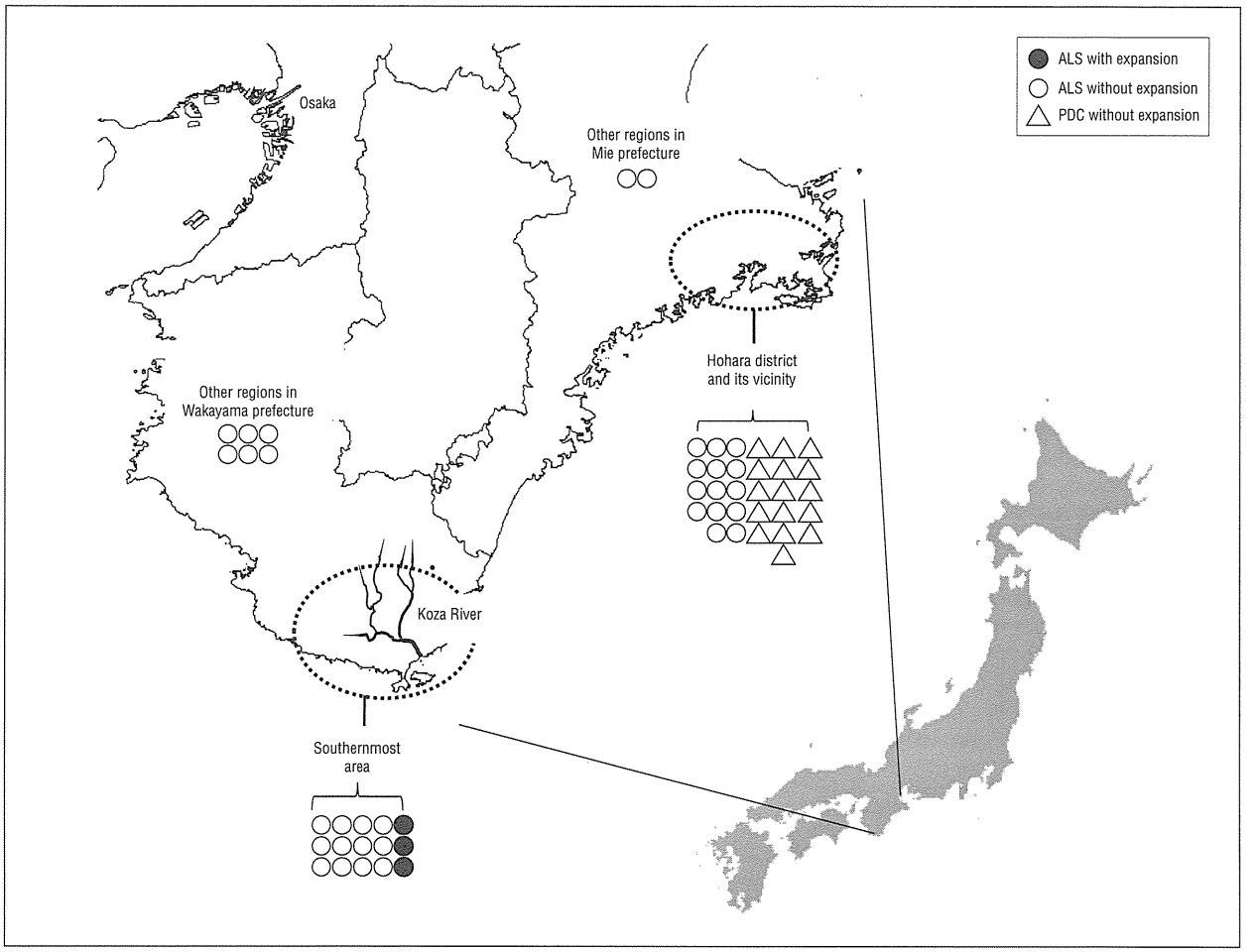

Figure 1. Map of Kii peninsula of Japan and distribution of patients with amyotrophic lateral sclerosis (ALS) and parkinsonism-dementia complex (PDC). The southernmost area neighboring the Koza River (Kozagawa and Kushimoto towns) and the Hohara district and its vicinity (Minamiise town and Shima city) shown in the figure are 2 disease foci. The circles represent examined patients with ALS. The filled-in circles designate patients with the repeat expansion in *C9ORF72*. The triangles represent patients with the PDC phenotype. Each symbol indicates the proband in the family when multiple affected family members were observed. Patients with hexanucleotide repeat expansion in *C9ORF72* are concentrated in the southernmost Kii peninsula.

been identified as the causative mutation in familial and sporadic ALS and frontotemporal dementia (OMIM 105550).[12,13] Given the potential clinical overlapping among ALS, frontotemporal dementia, and ALS/PDC, we investigated the GGGGCC hexanucleotide repeat expansion in *C9ORF72* in patients with ALS and PDC from the Kii peninsula.

METHODS

SUBJECTS AND DNA EXTRACTION

Sixteen patients with ALS and 16 patients with PDC originating from Mie prefecture and 21 patients (1 familial patient and 20 sporadic patients) with ALS from Wakayama prefecture surveyed in 1994 through 2011 were enrolled in the study. In addition, a total of 40 probands with familial ALS and 217 sporadic patients with ALS recruited from other areas of Japan were also enrolled in this study.[14] Genomic DNA was isolated from patients' blood leukocytes, lymphoblastoid cell lines, or autopsied brains using standard procedures. Written informed consent was obtained from all of the participants or the families of the deceased patients. The study was approved by the institutional review boards of the participating institutions.

REPEAT-PRIMED POLYMERASE CHAIN REACTION ANALYSIS

Because the expansion is too large to detect by a standard polymerase chain reaction, screening by repeat-primed polymerase chain reaction was performed, as reported previously.[12] Fragment analysis was performed using an ABI PRISM 3130xl sequencer and GeneScan software (Life Technologies).

SOUTHERN BLOT HYBRIDIZATION ANALYSIS

To independently confirm the repeat expansion in *C9ORF72*, Southern blot hybridization analysis was conducted, as described previously.[12]

HAPLOTYPE ANALYSIS

To investigate the possibility of a founder effect associated with the expanded alleles in *C9ORF72*, we genotyped the patients with expanded alleles using Genome-wide Human SNP array 6.0 (Affymetrix). Genotypes were called and extracted using Genotyping Console 4.0 (Affymetrix). In addition, we performed direct nucleotide sequence analysis of 42 single nucleotide polymorphisms to compare the haplotype with the Finnish haplotype.[14]

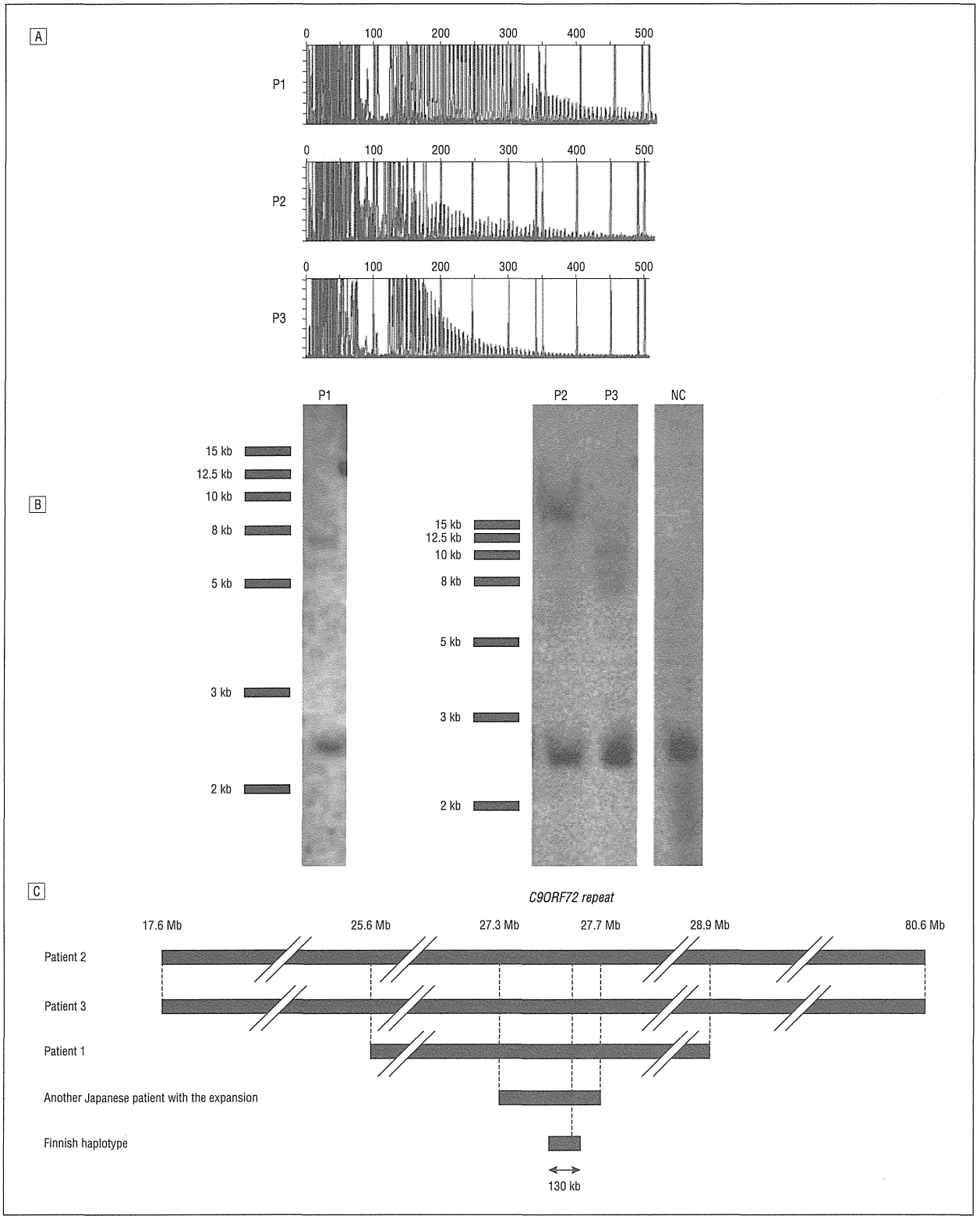

Figure 2. Mutational analyses of hexanucleotide repeat expansion in *C9ORF72*. A, Repeat-primed polymerase chain reaction analysis was performed as previously described.[8] Patients 1-3 show the characteristic sawtooth patterns with a 6-bp periodicity (blue lines). Red lines indicate DNA size markers. B, Southern blot hybridization analysis. Genomic DNA extracted from lymphoblastoid cell lines of patients 1 through 3 were subjected to Southern blot hybridization analysis, as described previously.[12] Patients 1-3 showed expanded alleles. C, Result of haplotype analysis. Physical positions are shown using the reference genome (NCBI36/hg18). An extended haplotype (Kii 9p-haplotype) spanning 3.3-63 Mb was shared by the 3 patients with ALS with the repeat expansions. A 410-kb region (defined by rs911602 and rs10511810) of the Kii 9p-haplotype was shared with that in another patient with the repeat expansion from another region of Japan.[14] We compared this haplotype with the Finnish haplotype; a 130-kb region (defined by rs10511816 and rs633583) was shared between the Kii 9p-haplotype and the Finnish haplotype. NC indicates negative control; P, patient.

Table 1. Clinical Characteristics of Kii Patients With ALS With *C9ORF72* Repeat Expansions

	Patient 1	Patient 2	Patient 3
Age, y	Death at 74	71	Death at 49
Sex	Female	Female	Female
Age at onset, y	72	71	41
Age at examination, y	72	71	46
Family history	–	+	–
Initial symptom	Dysarthria	Leg weakness	Leg weakness
Cranial			
UMN signs	–	+	+
LMN signs	+	+	+
Upper limbs			
UMN signs	–	+	+
LMN signs	+	+	+
Lower limbs			
UMN signs	+	+	+
LMN signs	–	+	+
Dementia	+	–	–
Neuroimaging	Brain CT: mild cerebral atrophy	Normal	Normal
nEMG	Neurogenic changes	Neurogenic changes	Neurogenic changes
Other			Respirator-dependent after 6 y of illness

Abbreviations: ALS, amyotrophic lateral sclerosis; CT, computed tomography; LMN, lower motor neuron; nEMG, needle electromyography; UMN, upper motor neuron.

Because all the patients were singletons, we reconstructed the haplotypes using the homozygosity haplotype method.[15]

STATISTICAL ANALYSIS

The Fisher exact test was used to compare the frequencies of the repeat expansion in patients with ALS from Kii peninsula and those from other regions in Japan.

RESULTS

Patients with hexanucleotide expansion in *C9ORF72* were identified in the Kii peninsula of Japan. We screened a total of 37 patients with ALS and 16 patients with PDC identified in the Kii peninsula using repeat-primed polymerase chain reaction analysis. Three of the patients with ALS (patients 1-3) showed the characteristic sawtooth-like electrophoresis pattern (**Figure 2**A). Southern blot hybridization analysis of the genomic DNA from the 3 patients further confirmed the presence of expanded alleles (Figure 2B).

Interestingly, the 3 patients with ALS with the expansion were from the southernmost Kii peninsula neighboring the Koza River (Kozagawa and Kushimoto towns), which is 1 of the 2 disease foci. When confined to the southernmost Kii peninsula, 3 of the 15 patients with ALS (20%) showed the repeat expansion. In contrast, 30 patients from the Hohara district and its vicinity did not reveal the repeat expansion. Mutational analyses of the 40 probands with familial ALS and the 217 sporadic patients with ALS from other areas of Japan revealed only 1 patient with a family history of ALS, which were included as the summary data in the meta-analysis study.[14]

Table 2. Frequency of the *C9ORF72* Repeat Expansion in Patients With ALS

	Southernmost Kii Peninsula		Other Regions in Japan		*P* Value
Expansion	+	–	+	–	
Familial ALS	1	0	1	39	.048
Sporadic ALS	2	12	0	217	.003

Abbreviation: ALS, amyotrophic lateral sclerosis.

The clinical characteristics of the patients are shown in **Table 1**. Family history of ALS was present only in patient 2, whose sibling was also diagnosed as having ALS. There were no family histories of ALS and related disease in the other 2 patients. They showed both upper and lower motor neuron signs. Two of the patients had lower limb–onset ALS, whereas 1 patient had bulbar-onset ALS. Patient 1 showed moderate cognitive decline, and mild brain atrophy was detected on computed tomographic scans. None of the patients showed parkinsonism. There were no obvious inverse correlations between the age at onset and the size of expanded alleles, as determined by Southern blot hybridization analysis.

Haplotype analysis using a high-density single nucleotide polymorphism array revealed an extended shared haplotype spanning 3.3-63 Mb in the 3 patients with ALS, although the kinships among the 3 patients were not evident (Figure 2C). The findings strongly suggest that the expanded alleles in this region originated from a common founder. As just described, we found only 1 patient with the repeat expansion in *C9ORF72* in the 40 probands with familial ALS (2.5%) collected in other regions in Japan.[14] The haplotype of this patient with ALS shares a 410-kb segment with the Kii 9p-haplotype. When the Kii 9p-haplotype was compared with the Finnish haplotype, a common haplotype of 130 kb was observed.[14]

COMMENT

We identified the hexanucleotide repeat expansion in *C9ORF72* in the 3 patients from the southernmost Kii peninsula neighboring the Koza River. The frequency of patients with expanded alleles was 20% (3 of 15) in this area. In the study of the other cohort of ALS collected mainly in areas around Tokyo, we found only 1 patient with the repeat expansion in *C9ORF72* in the 40 probands with familial ALS (2.5%) and none in the 217 sporadic patients with ALS.[14] Although the number of patients examined in the southernmost Kii peninsula was small, virtually all the affected patients in this region were enrolled based on a continued epidemiologic study conducted by the authors (T.K. and S.Y.) in this region. Moreover, the difference in the frequency of patients carrying the repeat expansion in *C9ORF72* is statistically significant (**Table 2**). Thus, our findings in this study emphasize that patients with ALS with the repeat expan-

sion in *C9ORF72* are concentrated in the southernmost Kii peninsula with a founder effect.

The clinical features of the patients with the repeat expansion are indistinguishable from those with conventional ALS. Moderate cognitive decline was present in 1 patient, whereas none of them showed parkinsonism (Table 1). Because autopsy findings of patients with the repeat expansion are unavailable, further investigations will be certainly needed to address the relationship between the ALS with the repeat expansion in *C9ORF72* identified in the southernmost Kii peninsula and ALS/PDC identified in the Kii peninsula.

However, it should also be noted that the repeat expansion did not account for all the ALS cases, even in the southernmost Kii peninsula. It is also of interest that patients with the repeat expansion were not identified in the Hohara district or other areas of Wakayama and Mie prefectures. Taken together, our study demonstrates that the patients with the repeat expansion are concentrated in the southernmost Kii peninsula, but simultaneously raises the possibility of genetic heterogeneities even in these 2 regions in the Kii peninsula where ALS is prevalent.

In summary, we identified that the *C9ORF72* repeat expansion is concentrated in the patients with ALS in the Kii peninsula. Our finding suggests that the repeat expansion partly accounted for the high prevalence of ALS in the Kii peninsula of Japan.

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TBX1 Mutation Identified by Exome Sequencing in a Japanese Family with 22q11.2 Deletion Syndrome-Like Craniofacial Features and Hypocalcemia

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Abstract

Background: Although *TBX1* mutations have been identified in patients with 22q11.2 deletion syndrome (22q11.2DS)-like phenotypes including characteristic craniofacial features, cardiovascular anomalies, hypoparathyroidism, and thymic hypoplasia, the frequency of *TBX1* mutations remains rare in deletion-negative patients. Thus, it would be reasonable to perform a comprehensive genetic analysis in deletion-negative patients with 22q11.2DS-like phenotypes.

Methodology/Principal Findings: We studied three subjects with craniofacial features and hypocalcemia (group 1), two subjects with craniofacial features alone (group 2), and three subjects with normal phenotype within a single Japanese family. Fluorescence *in situ* hybridization analysis excluded chromosome 22q11.2 deletion, and genomewide array comparative genomic hybridization analysis revealed no copy number change specific to group 1 or groups 1+2. However, exome sequencing identified a heterozygous *TBX1* frameshift mutation (c.1253delA, p.Y418fsX459) specific to groups 1+2, as well as six missense variants and two in-frame microdeletions specific to groups 1+2 and two missense variants specific to group 1. The *TBX1* mutation resided at exon 9C and was predicted to produce a non-functional truncated protein missing the nuclear localization signal and most of the transactivation domain.

Conclusions/Significance: Clinical features in groups 1+2 are well explained by the *TBX1* mutation, while the clinical effects of the remaining variants are largely unknown. Thus, the results exemplify the usefulness of exome sequencing in the identification of disease-causing mutations in familial disorders. Furthermore, the results, in conjunction with the previous data, imply that *TBX1* isoform C is the biologically essential variant and that *TBX1* mutations are associated with a wide phenotypic spectrum, including most of 22q11.2DS phenotypes.

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Introduction

Chromosome 22q11.2 deletion syndrome (22q11.2DS) is a developmental disorder associated with characteristic craniofacial features with velopharyngeal incompetence, cardiovascular anomalies primarily affecting the outflow tracts, hypoparathyroidism and resultant hypocalcemia, and thymic hypoplasia leading to susceptibility to infection [1]. This condition is also frequently accompanied by non-specific clinical features such as developmental retardation [1]. Expressivity and penetrance of these features are highly variable and, consistent with this, chromosome 22q11.2 deletions have been identified in DiGeorge syndrome (DGS) and velocardiofacial syndrome (VCFS) with overlapping but different patterns of clinical features [1].

While multiple genes are involved in chromosome 22q11.2 deletions [2], *TBX1* (T-box 1) has been regarded as the major gene relevant to the development of clinical features in 22q11.2DS [3]. Indeed, heterozygous *TBX1* mutations have been identified in several deletion-negative patients with 22q11.2DS phenotype [2–8], and mouse studies argue for the critical role of *Tbx1* in the development of 22q11.2DS phenotypes [3]. However, the frequency of *TBX1* mutations remains rare in deletion-negative patients: Gong et al. identified only a few probable *TBX1* mutations after studying 40 patients with DGS/VCFS phenotypes