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

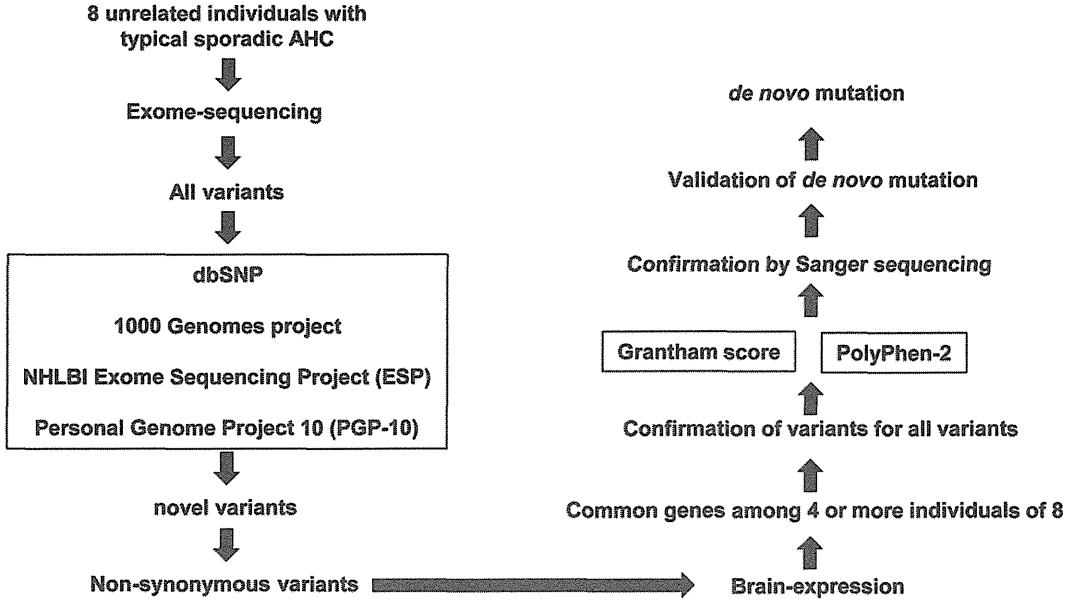

Figure 1. Pipeline for detection of novel *de novo* mutations. The pipeline was used to identify pathogenic mutations of alternating hemiplegia of childhood (AHC). All genetic variants detected by exome sequencing are sequentially filtered through the pipeline. First, variations are screened according 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.
doi:10.1371/journal.pone.0056120.g001

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.
doi:10.1371/journal.pone.0056120.t001

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

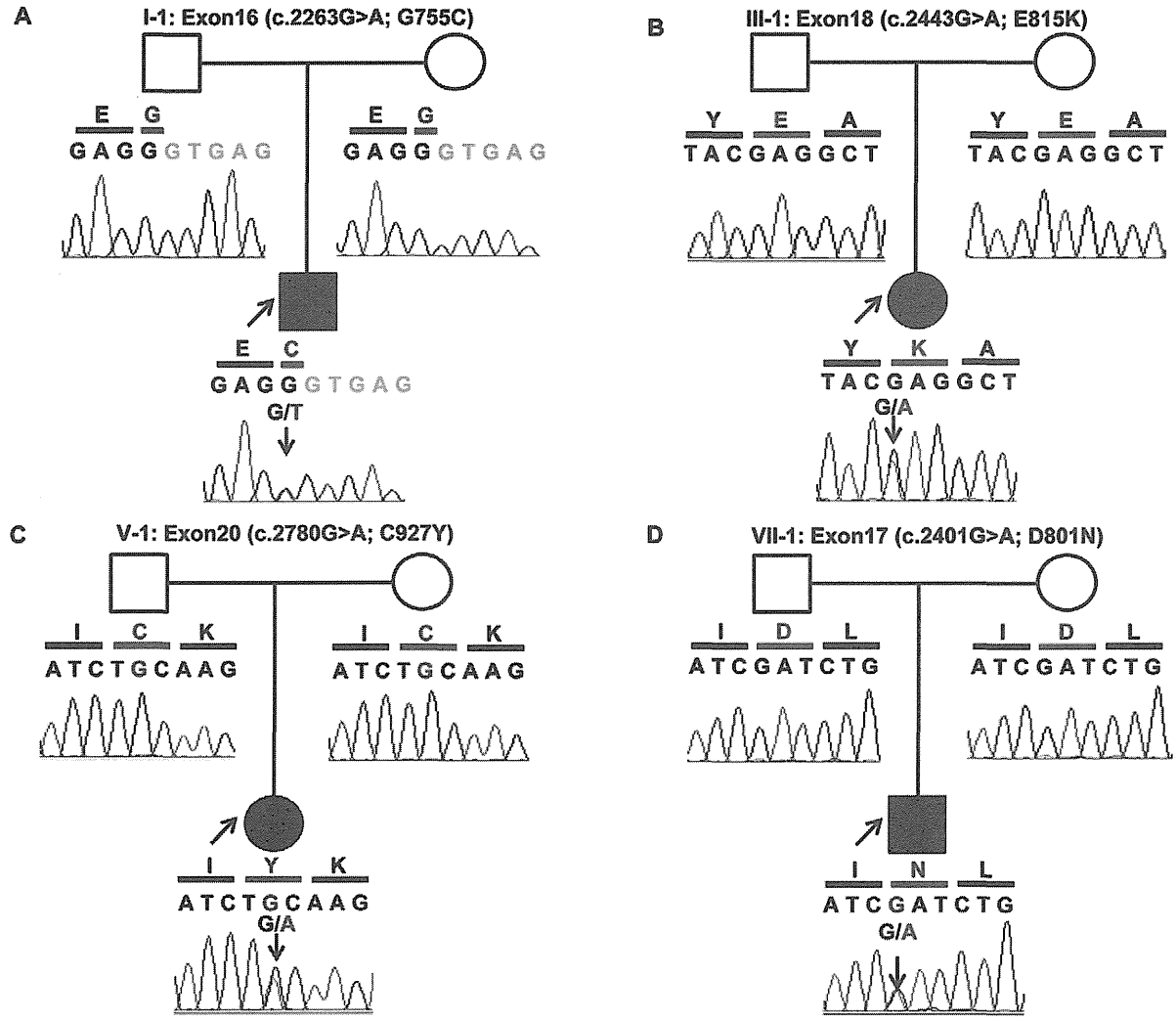

Figure 2. Chromatograms of four *de novo* mutations identified in *ATP1A3*. Data were obtained by Sanger sequencing during the confirmation process. In trio of each pedigree, black shadow represents the proband. In the chromatograms, *Black letters* show exonic nucleotide sequences, *gray letters* show 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.
doi:10.1371/journal.pone.0056120.g002

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.

doi:10.1371/journal.pone.0056120.t003

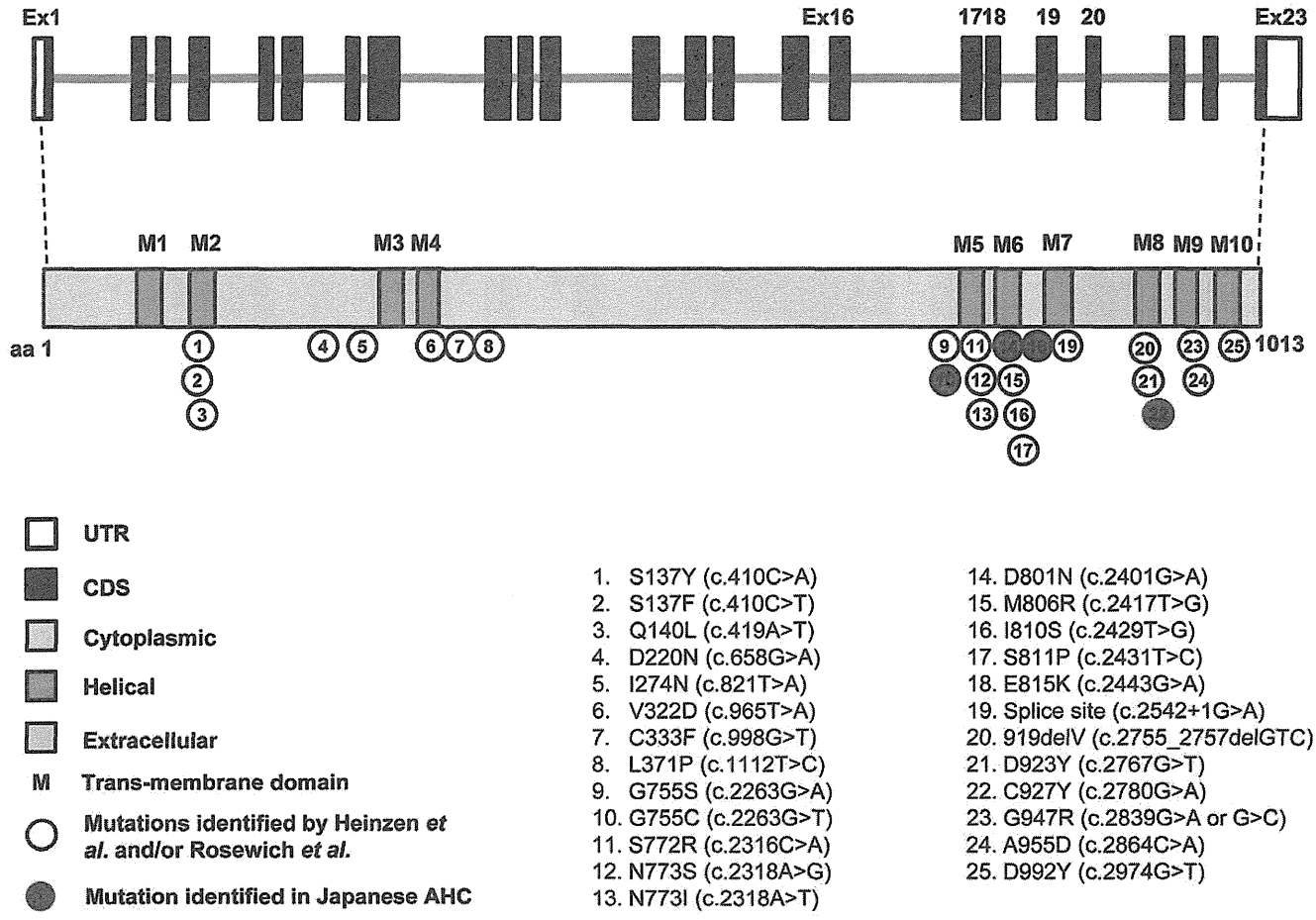

Figure 3. *ATP1A3* mutations and their protein domain structures. *Black lined circle:* Mutations reported recently [10,11]. *Red colored circle:* Mutations identified in the present study in a Japanese cohort with AHC. The *ATP1A3* gene consists of 23 exons that encode several domains in the ATP1A3 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.

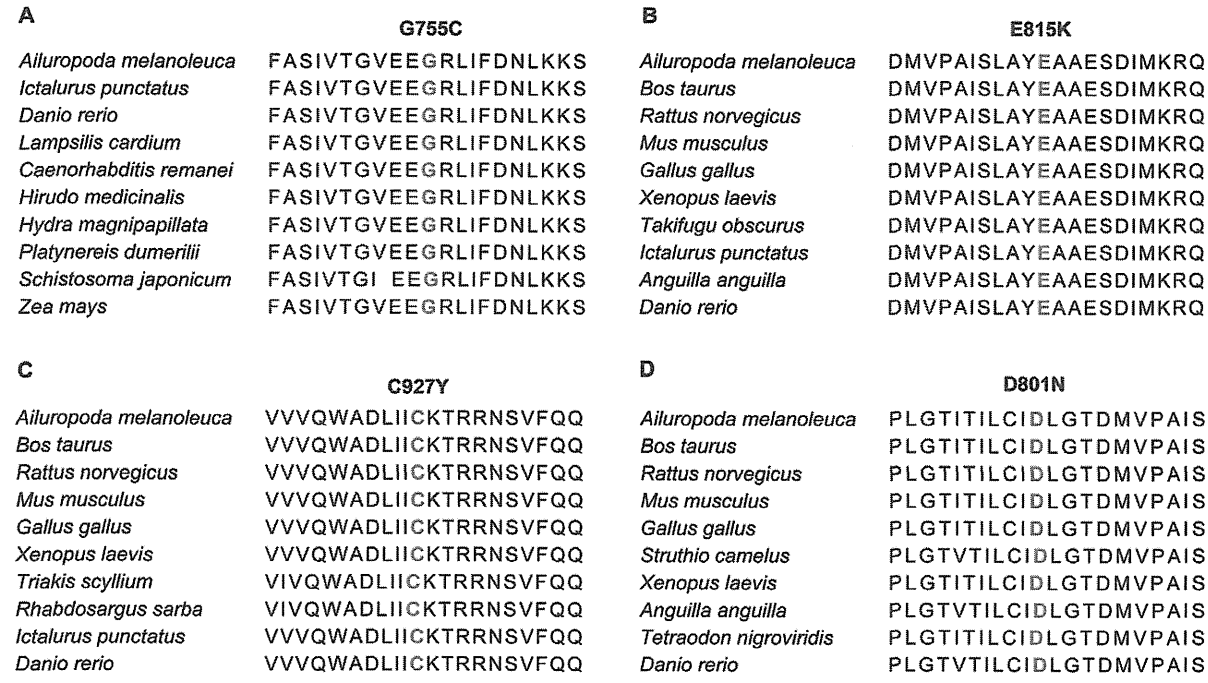

Figure 4. Homologous comparison of altering-protein. *Blue letters:* altering-protein by mutation, *red letters:* differential protein with human. (A) G755C changed by novel SNVs (c.2263G>T) of *ATP1A3* in Patient I-1. (B) E815K changed by novel SNVs (c.2443 G>A) of *ATP1A3* in Patients II-1, III-1, 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.
doi:10.1371/journal.pone.0056120.g004

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)

Acknowledgments

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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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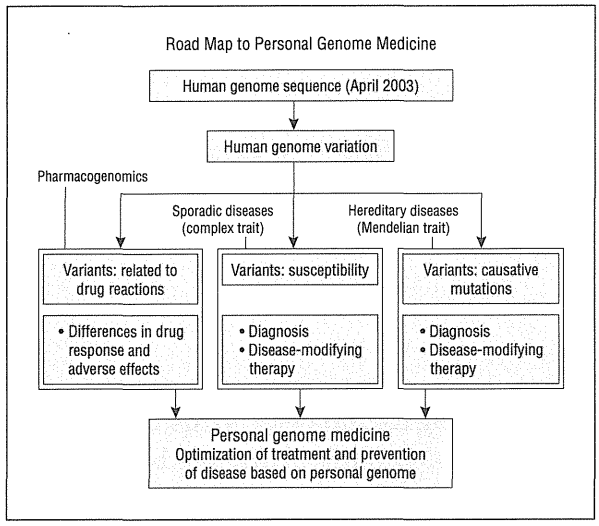

Figure 1. Diagram showing the road map to personal genome medicine. Since the completion of the human genome sequence in 2003, the research focus in human genetics has moved to how human genome variations affect human health. Human genome variations are considered to be associated not only with hereditary 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

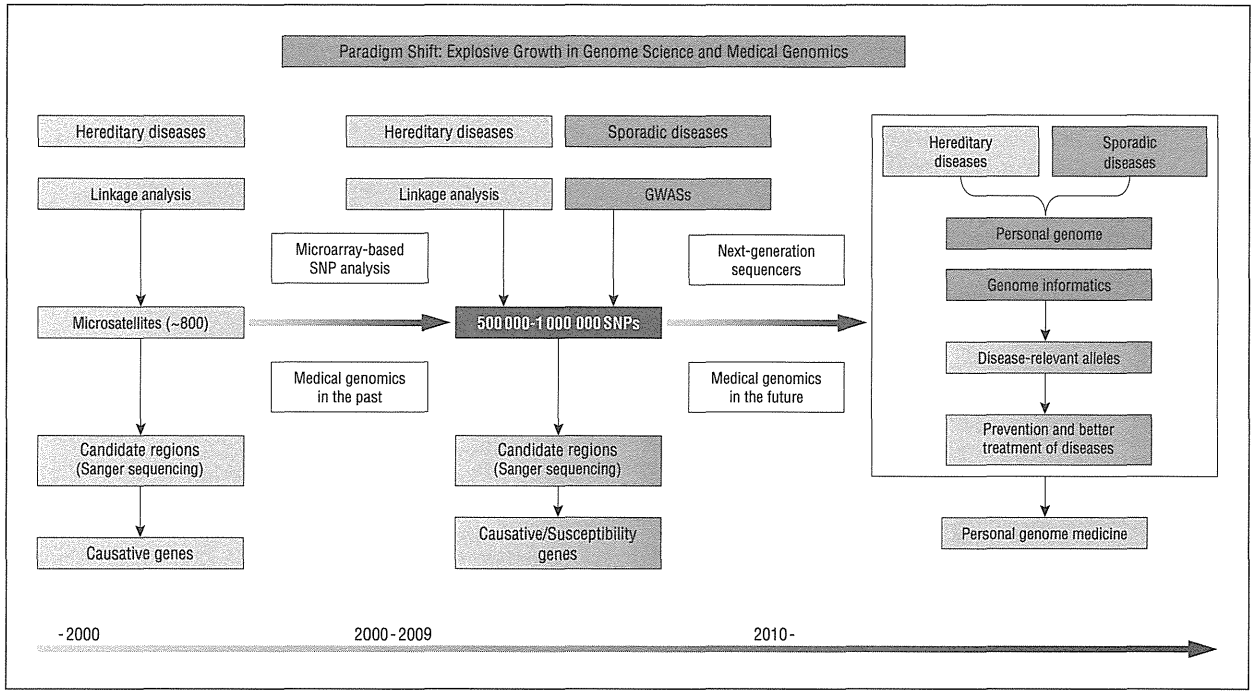

Figure 2. Diagram showing the paradigm shift (ie, the explosive growth in genome science and medical genomics). Over the past decade, genome-wide association studies (GWASs) using common single-nucleotide polymorphisms (SNPs) have been conducted to identify genomic variations in sporadic neurological 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

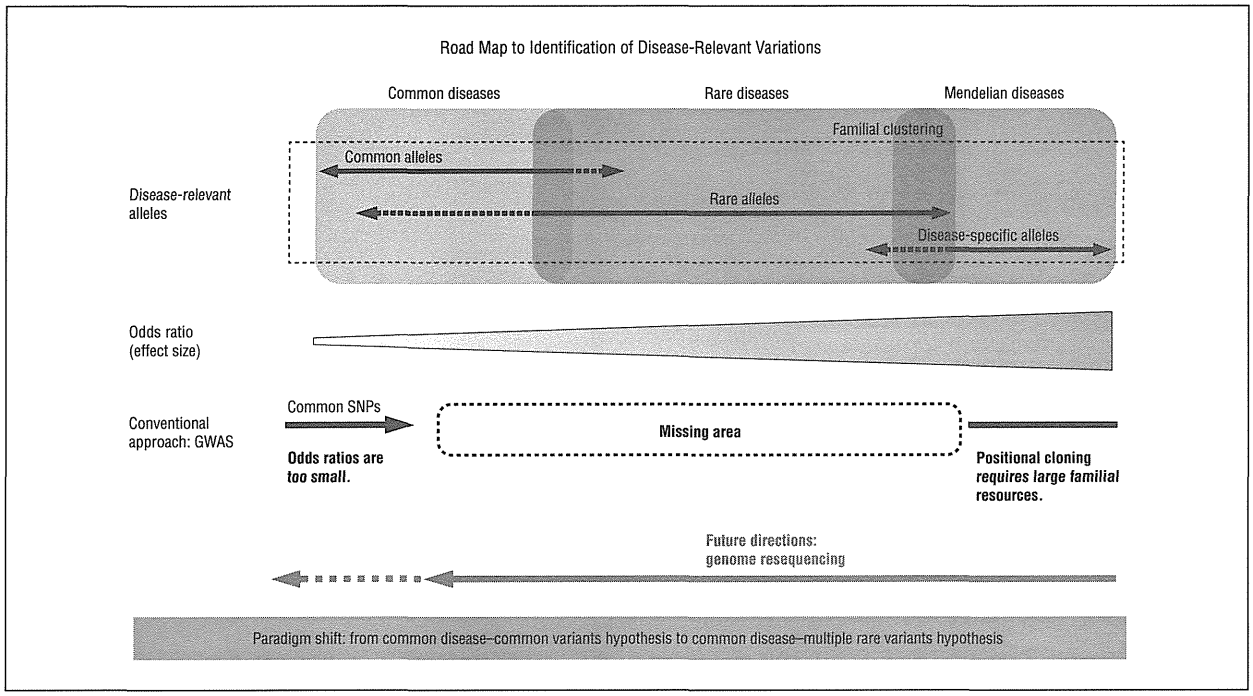

Figure 3. Diagram showing the road map to the identification of disease-relevant variations. Shifting the paradigm from the common disease–common variants hypothesis to the common disease–multiple rare variants hypothesis will lead to the elucidation of the molecular bases of sporadic neurological diseases. 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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Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in *SLC25A13*

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ABSTRACT

Citrin deficiency is an autosomal recessive disorder caused by mutations in the *SLC25A13* gene and has two disease outcomes: adult-onset type II citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency. The clinical appearance of these diseases is variable, ranging from almost no symptoms to coma, brain edema, and severe liver failure. Genetic testing for *SLC25A13* mutations is essential for the diagnosis of citrin deficiency because chemical diagnoses are prohibitively difficult. Eleven *SLC25A13* mutations account for 95% of the mutant alleles in Japanese patients with citrin deficiency. Therefore, a simple test for these mutations is desirable. We established a 1-hour, closed-tube assay for the 11 *SLC25A13* mutations using real-time PCR. Each mutation site was amplified by PCR followed by a melting-curve analysis with adjacent hybridization probes (HybProbe, Roche). The 11 prevalent mutations were detected in seven PCR reactions. Six reactions were used to detect a single mutation each, and one reaction was used to detect five mutations that are clustered in a 21-bp region in exon 17. To test the reliability, we used this method to genotype blind DNA samples from 50 patients with citrin deficiency. Our results were in complete agreement those obtained using previously established methods. Furthermore, the mutations could be detected without difficulty using dried blood samples collected on filter paper. Therefore, this assay could be used for newborn screening and for facilitating the genetic diagnosis of citrin deficiency, especially in East Asian populations.

1. Introduction

Citrin deficiency is an autosomal recessive disorder that results from mutations in the *SLC25A13* gene [1] and causes two diseases: adult-onset type II citrullinemia (CTLN2; OMIM #603471) and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; OMIM#605814) [1–4]. The clinical appearance of these diseases is variable and ranges from almost no symptoms to coma, brain edema, and severe liver failure requiring transplantation [5–8]. In a study of patients with NICCD, only 40% of individuals were identified by newborn screenings to have abnormalities, such as hypergalactosemia, hypermethioninemia, and hyperphenylalaninemia [9]. Other patients were referred to hospitals with suspected neonatal hepatitis or biliary atresia, due to jaundice or discolored stool [9]. Hypercitrullinemia was not observed in all patients [9]. Mutation analysis of *SLC25A13* is indispensable because of the difficulties associated with the chemical diagnosis of citrin deficiency. The *SLC25A13* mutation spectrum in citrin deficiency is heterogeneous, and more than 31 mutations of *SLC25A13* have been identified to date [1,10–18]. However, there are several predominant mutations in patients from East Asia. As shown in Table 1, 6 prevalent mutations account for 91% of the mutant alleles in the Japanese population [12,19]. Five additional mutations also occur within a 21-bp cluster in exon 17 (Table 1 and Fig. 1D). The six prevalent mutations, together with the five mutations in exon 17, account for 95% of the mutant alleles in Japan [12,19].

Several different methods, such as direct sequencing, PCR restriction fragment length polymorphism (PCR-RFLP), and denaturing high performance liquid chromatography (DHPLC), are currently used for the detection of mutations in *SLC25A13* [1,10–14,19]. However, these methods are too complex for clinical use. Direct sequencing is a standard but cumbersome method. The PCR-RFLP method is

Abbreviations: CTLN2, adult-onset type II citrullinemia; FRET, fluorescence resonance energy transfer; HRM, high resolution melting; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; Tm, melting temperature.

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Table 1
Seven primer/probe sets and 11 targeted mutations of *SLC25A13*.

Primer/probe set	Mutation		Location	Nucleotide change	Effects of mutations	Allele frequency*[19]	References
A	Mutation [I]	:851del4	exon 9	c.851_854delGTAT	p.R284fs(286X)	33.2%	[1]
B	Mutation [II]	:g.IVS11+1G>A	intron 11	c.1019_1177del	p.340_392del	37.6%	[1]
C	Mutation [III]	:1638ins23	exon 16	c.1638_1660dup	p.A554fs(570X)	3.4%	[1]
D	Mutation [IV]	:S225X	exon 7	c.675C>A	p.S225X	5.3%	[1]
E	Mutation [V]	:g.IVS13+1G>A	intron 13	c.1231_1311del	p.411_437del	8.2%	[1]
F	Mutation [XIX]	:IVS16ins3kb	intron 16	c. aberrant RNA	p.A584fs(585X)	4.6%	[19]
G	Mutation [VI]	:1800ins1	exon 17	c.1799_1800insA	p.Y600X	1.3%	[10]
	Mutation [VII]	:R605X	exon 17	c.1813C>T	p.R605X	0.90%	[10]
	Mutation [VIII]	:E601X	exon 17	c.1801G>T	p.E601X	1.2%	[11]
	Mutation [IX]	:E601K	exon 17	c.1801G>A	p.E601K	0.30%	[11]
	Mutation [XXI]	:L598R	exon 17	c.1793T>G	p.L598R	0%	[15]
						Total 95.1%	

* The frequency of each mutant allele among Japanese patients with citrin deficiency.

complicated and can lead to genotyping errors, due to incomplete digestion by the restriction enzymes. DHPLC is time-consuming and requires expensive equipment. Thus, there is a strong need for the development of a simple test for these mutations.

The goal of this study was to establish a rapid and simple test for the detection of the 11 most common *SLC25A13* mutations. We adopted the HybProbe format (Roche) for the detection of the mutations using real-time PCR followed by a melting-curve analysis with adjacent hybridization probes [20,21]. This assay can be completed in less than 1 h and has the advantage of being a closed-tube assay. The fundamental process for detecting point mutations using the HybProbe assay is presented in Fig. 1A. The 11 prevalent mutations contain not only point mutations but also include a 4-bp deletion and insertions of 1-bp, 23-bp and 3-kb genomic fragments (Table 1 and Fig. 1). Careful design of the PCR primers and HybProbes enabled us to test for these various *SLC25A13* mutations.

2. Methods

2.1. Subjects

CTLN2 and NICCD were diagnosed, as previously described [9,10,19,22–24]. Genomic DNA of the patients was obtained from peripheral blood leukocytes using the DNeasy blood kit (Qiagen Inc., Valencia, CA, USA). Genomic DNA was purified from filter paper blood samples using the ReadyAmp Genomic DNA Purification System (Promega, Madison, WI, USA). Mutations in these DNA samples were analyzed at Kagoshima University using a combination of PCR with or without restriction enzyme digestion or by direct sequencing, as previously described [1,10–14,19]. Another set of samples was obtained from 420 healthy volunteers (mainly from Miyagi prefecture in the northeastern region of Japan) at Tohoku University. Genomic DNA from leukocytes was extracted, as described above.

2.2. Detection of seven prevalent mutations in SLC25A13 using the HybProbe assay

HybProbe probes comprise a pair of donor and acceptor oligonucleotide probes designed to hybridize adjacent to their target sites in an amplified DNA fragment [20,21]. The donor probes are labeled at their 3′ end with fluorescein isothiocyanate (FITC), whereas the acceptor probes are labeled at their 5′ end with LC Red640; these acceptor probes are phosphorylated at their 3′ end to prevent extension by the DNA polymerase. When two probes hybridize to the amplicon, the fluorescent dyes are located within 5 bases of each other, which allows fluorescence resonance energy transfer (FRET) between the excited FITC and the LC Red640; this process emits light that can be quantified by real-time PCR. Following PCR amplification, a melting-peak analysis is performed. The melting peak is produced by the reporter probe, which has a lower melting temperature (Tm) than the other probe, called the anchor probe. As the reporter melts from the target, the fluorophores are separated, and the FRET ceases. The Tm of the reporter probe determines the reaction

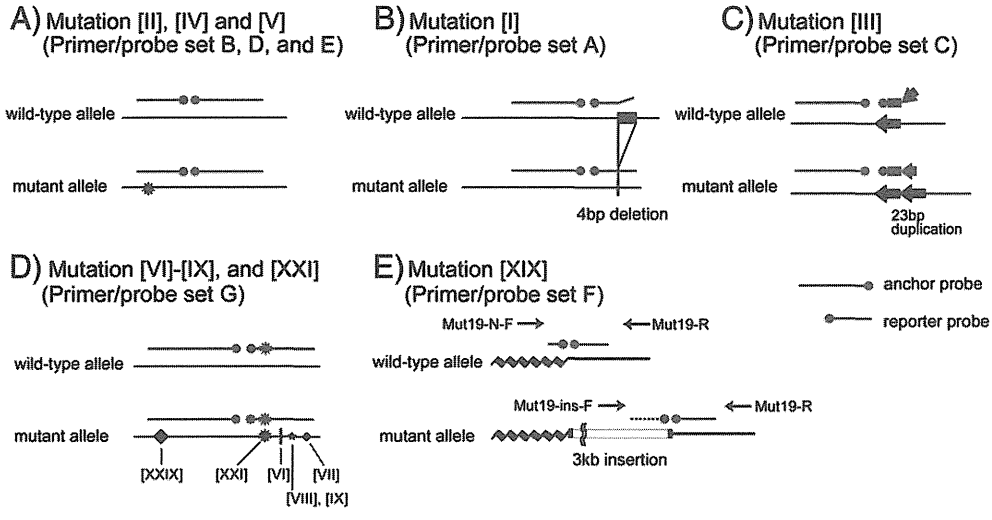

Fig. 1. Principle of *SLC25A13* mutation detection by melting-curve analysis with the HybProbe assay. In primer/probe sets A–E, and G, PCR was performed with a pair of primers, whereas in primer/probe set F, two forward primers and one common reverse primer were used for the amplification of both wild-type and mutant alleles. Note that mutation [XXIX], located on the anchor probe of primer/probe set G, is a non-target mutation.

specificity (i.e., binding of the probe to a perfectly matched sequence rather than to regions with sequence mismatches).

Seven primer/probe sets were designed for this study. Fig. 1 shows a schematic diagram of the strategy for mutation detection using these primer/probe sets. Tables 1 and 2 list the primer/probe sets and corresponding sequences and primer concentrations that were used to target the 11 mutations. Primer/probe sets A, B, C, D, E, and F were designed to detect mutations [I], [II], [III], [IV], [V], and [XIX], respectively. Primer/probe set G was designed to detect the five mutations clustered on exon 17: mutations [VI], [VII], [VIII], [IX], and [XXI] (Fig. 1D). All primers and probes were synthesized based on the NCBI reference SLC25A13 gene sequence (GenBank accession no. NM_014251) with the exception of mutation [XIX]:IVS16ins3kb, which was designed according to [19].

Real-time PCR and subsequent melting curve analyses were performed in a closed tube using a 20-μL mixture on a LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan). The PCR mixture contained 2.0 μL of genomic DNA (10–50 ng), 0.5 μM of forward primer, 0.5 or 0.1 μM of reverse primer, 0.2 μM of each sensor and anchor probe, and 10 μL of Premix ExTaq™ (Perfect Real Time) reagent (TaKaRa Bio Inc., Otsu, Japan).

The thermal profile conditions were identical for all seven assays and consisted of an initial denaturation step (30 s at 95 °C), followed by 45 amplification cycles with the following conditions: denaturation for 5 s at 95 °C and annealing and extension for 20 s at 60 °C. The transition rate between all steps was 20 °C/s. After amplification, the samples were held at 37 °C for 1 min, followed by the melting curve acquisition at a ramp rate of 0.15 °C/s extending to 80 °C with continuous fluorescence acquisition.

Table 2
Primers, probes and target amplicon sequences, target mutation sites, and primer concentrations.

Primer/probe set	Name	Sequences of PCR products, primer locations, probe sequences, and mutation sites (5′ to 3′)	Concentration (μmol/L)
A		GGCTATACTGAAATATGAGAAatgaaaaaagggatgtttttaaatttataatgtaaattgtaataaattggtatatttgttgcttgtgtttgtttttcccctacagac	
		gtatgaccttagcagacattgaacggattgctcctctggaagagggaactctgccCTTTAACTTGGCTGAGG (181 bp)	
	Mut1-F	GGCTATACTGAAATATGAGAA	0.5
	Mut1-R	CCTCAGCCAAGTTAAAG	0.5
	Mut1-UP	ATGTAAATTGTAATAAATTGGTATATTTGTTGCTTGTGTT-FITC	
	Mut1-DW	LC Red640-GTTTTTCCCCTACAGAC**GACC**-P	
B		GAATGCAGAACCAACGAtcaactggctcttttgtgggagaactcatgtataaaaacagctttgactgttttaagaaagtgctacgctatgaaggcttctt	
		tggactgtatagag**gt**tagtgccacatgctcaatacctgttaggtgaaataacactcaaaggtttggtttctcatcttagtgcctGACATGAATTAGCAAGACTG (205 bp)	
	Mut2-F	GAATGCAGAACCAACGA	0.5
	Mut2-R	CAGTCTTGCTAATTCATGTC	0.1
	Mut2-UP	ACCTAACAGGTATTGAGCATGTG-FITC	
	MUt2-DW	LC Red640-CACTAA**C**CTCTATACAGTCCA-P	
C		GCAGTTCAAAGCACAGTTATTtttatatagtgagaatgtgaccagactgagatggtgttgtgtctctcctgcaggtatgcctgcagcatctttagtg	
		accctgctgatgttatcaagacgagattacaggtg	
		gctgcccggg(**gagatta**caggtggctgcccggg)ctggccaaaccaCTTACAGCGGAGTGATAGAC (175 bp)	
	Mut3-F	GCAGTTCAAAGCACAGTTATT	0.5
	Mut3-R	GTCTATCACTCCGCTGTAAG	0.5
	Mut3-UP	ACCCCTGCTGATGTTATCAAGACGAGATTACAGGT-FITC	
	Mut3-DW	LC Red640-GCTGCCCGGG**GAGATTA**-P	
D		TCAATTTATTTGAGGCTGCtggaggtaccacatcccatcaagttagtttctcctattttaatggatttaattcgctccttaacaac	
		atggaactcattagaaagatctatagcactc	
		tggctggcaccaggaaagatgttgaagtGACTAAGGGTGAGTGAGAA (164 bp)	
	Mut4-F	TCAATTTATTTGAGGCTGC	0.5
	Mut4-R	TTCTCACTCACCCTTAGTC	0.5
	Mut4-UP	AATGGATTTAATT**C**GCTCCTTAACA-FITC	
	Mut4-DW	LC Red640-ATGGAACTCATTAGAAAGATCTATAGCACTC-P	
E		TGCACAAAGATGGTTCGgtcccacttgcagcagaaattcttgctggaggctgc**g**taagtacctttgaagctctcttcattgaaaagacttgtttcac	
		atatatatcactaccatggtcaacaggtgtggactaaggcttctgttTAACCACAGATCCTGCA (162 bp)	
	Mut5-F	TGCACAAAGATGGTTCG	0.5
	Mut5-R	TGCAGGATCTGTGGTTA	0.5
	Mut5-UP	GTGAAACAAGTCTTTTCAATGAAGAGAGCTTC-FITC	
	Mut5-DW	LC Red640-AAGGTACTTA**C**GCAGCCTC-P	
F	normal allele	GGAGCTGGTGGTATGGAAataatgtgttcttaactaactctttggtatcaggtaaatttttaaaatatctaattatatctgtgatttctc	
		catttttttaaagctcgtgtatttcgatcctcaccccagtttggt	
		gtaactttgctgacttacgaattgctacagcgatggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaatttt	
		GGCTGCTGCTAATGCTC (244 bp)	
	insertion allele	CCATCTTCCTCCTCCCTTggcagccccgccccccgatttctccatttttttaaagctcgtgtatttcgatcctcaccccagtttggt	
		gtaactttgctgacttacgaattgctacagcgatggttctacattgatttt	
		ggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (196 bp)	
	Mut19-N-F	GGAGCTGGTGGTATGGAA	0.5
	Mut19-ins-F	CCATCTTCCTCCTCCCTT	0.5
	Mut19-R	GAGCATTAGCAGCAGCC	0.5
	Mut19-UP	ACCAAACTGGGGTGAGGATCGAAATACACGAGCTTTAAAAAAATG-FITC	
	Mut19-N-DW	LC Red640-AGAAATCACAGATATAATTAGATATTT-P	
	Mut19-ins-DW	LC Red640-AGAAATCGGGGGGCGGGG-P	
G		TCTTAACTAACTCTTTGGTATCAGGTaaatttttaaaatatctaattatatctgtgatttctccatttttttaaagctcg	
		tgtatttcgatcctcaccccagtttggtgtaactttgct**g**actt**a(a)cg**aattgctacag**c**ga	
		tggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (217 bp)	
	Mut6-9, 21-F	TCTTAACTAACTCTTTGGTATCAGGT	0.5
	Mut6-9, 21-R	GAGCATTAGCAGCAGCC	0.5
	Mut6-9, 21-UP	TGTATTTCGATCCTCACCCCAGTTTGGTGTAACTT-FITC	
	Mut6-9, 21-DW	LC Red640-GC**G**GACTT**ACG**AATTGCTACAG**C**GA-P	

Upper case and underlined letters indicate the locations of primers and probes, respectively. Inserted DNA is shown in parenthesis. Nucleotides in boldface were used for mutation detection.
F: forward, R: reverse, UP: upstream, DW: downstream, N: normal allele, ins: insertion allele, FITC: fluorescein isothiocyanate, P: phosphate.

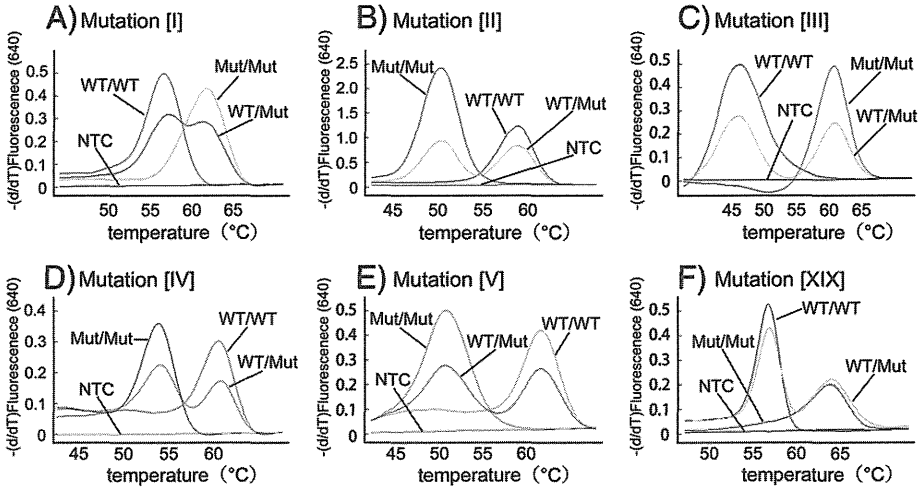

Fig. 2. Typical melting curves used in the detection of mutations [I–V] and [XIX]. Each assay using primer/probe sets A–F is displayed in a separate graph (A–F). WT: wild-type allele, Mut: mutant allele, NTC: no DNA template control.

2.3. Validation of the mutation detection system

After establishing the protocol for detecting the 11 prevalent mutations, 50 DNA samples from patients' blood were sent from Kagoshima University to Tohoku University for the validation of this system in a single-blind manner. Similarly, 26 DNA samples purified from paper-filter blood samples were analyzed in the same manner as the blood DNA samples.

2.4. Estimation of the carrier frequency

For the estimation of the heterozygous carrier frequency, 420 genomic DNA samples from healthy volunteers were screened using the HybProbe analysis for the 11 prevalent mutations. All detected mutations were confirmed by direct sequencing.

2.5. Ethics

This study was approved by the Ethical Committees of Tohoku University School of Medicine and Kagoshima University. Written informed consent was obtained from all participants or their guardians.

3. Results

3.1. Development of the mutation detection system

In primer/probe sets B, D, and E, the reporter probes were designed to be complimentary to the wild-type allele (Fig. 1A). To allow for an improved detection of the mutations, primer/probe sets A and C were designed to be complementary to the mutant allele (Figs. 1B, C). In the primer/probe set F, two forward PCR primers, which were specific to the wild-type and the mutant alleles, were used with a common reverse primer for the co-amplification of the wild-type and 3-kb insertion alleles (Fig. 1E). Two reporter probes, which had a common anchor probe, were used for the detection of the wild-type and mutant alleles. Because the two reporter probes had different melting temperatures, we were able to identify the allele that was amplified. Fig. 2 shows representative results of the melting curve analyses using the primer/probe sets A–F, in which all of the mutant alleles generated distinct peaks corresponding to the wild-type alleles.

In the primer/probe set G, we used a reporter probe that was complementary to the mutant [XXI] allele (Fig. 1D). All five mutations in exon 17 were successfully differentiated from the wild-type allele (Figs. 3A–E). The [XXIX] mutation is an additional mutation in exon

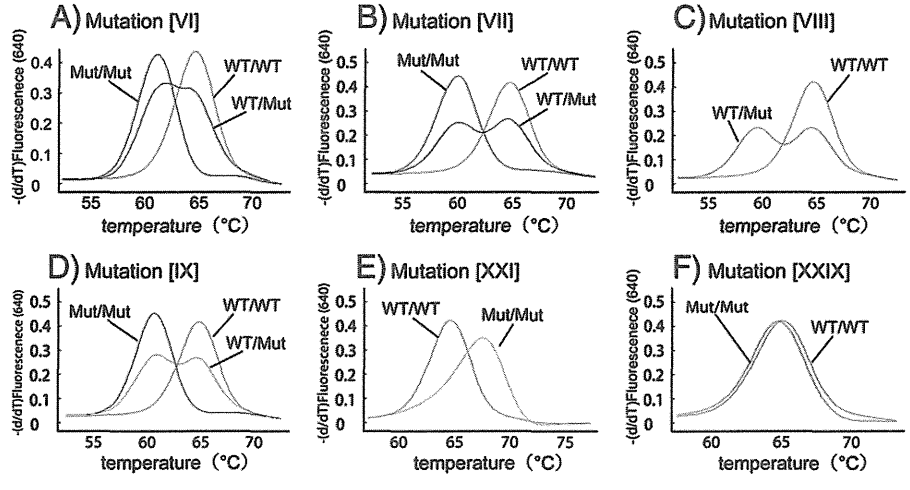

Fig. 3. Typical melting curves used in the detection of mutations [VI–XI], [XXI], and [XXIX] on exon 17. Genotyping was performed using primer/probe set G. Each melting curve for a target mutation is displayed in a separate graph (A–F). Note that mutation [XXIX] (F) is a non-target mutation on the anchor probe. WT: wild-type allele, Mut: mutant allele.

17 that is not listed in Table 1. The [XXIX] mutation is located in the anchor-probe binding site and not on the reporter-probe binding site (Fig. 1D). To examine the effect of mutations on the anchor probe, we genotyped a patient with a heterozygous [XXIX] mutation using primer/probe set G (Fig. 3F). We found no change in the melting curves between the wild-type allele and the [XXIX] allele, thereby suggesting that point mutations within the anchor probe sequence have little effect on the melting curve analysis.

3.2. Validation

The genotypes determined at Tohoku University using the proposed method and those determined at Kagoshima University using a previously published method were identical for the 11 common mutations (Table S1 in supplementary material). We performed a similar test using DNA samples purified from filter-paper blood samples to determine if this method could be used for newborn screening. The genotypes determined in both laboratories were identical for all 26 DNA samples (Table S2 in supplementary material).

3.3. Frequency of eleven prevalent mutations

We found four heterozygous carriers of mutation [I], three of mutation [II], and two of mutation [V]. In addition, primer/probe set G detected one heterozygous mutation, which was confirmed as mutation [VIII] by direct sequencing. Altogether, 10 mutations were detected in 420 Japanese healthy controls.

4. Discussion

We developed a simple and rapid genetic test using real-time PCR combined with the HybProbe system for the 11 prevalent mutations in *SLC25A13*: mutations [I], [II], [III], [IV], [V], [VI], [VII], [VIII], [IX], [XIX], and [XXI]. This genetic test is a closed-tube assay in which no post-PCR handling of the samples is required. In addition, the genotyping is completed within 1 h. This test can utilize DNA samples purified from both peripheral blood and filter-paper blood. The reliability of the test was confirmed by genotyping 76 blind DNA samples from patients with citrin deficiency, including 50 peripheral blood and 26 filter-paper blood DNA samples. Because screening for the 11 targeted mutations would identify 95% of mutant alleles in the Japanese population [19], both, one, and no mutant alleles are expected to be identified in 90.4%, 9.3%, and less than 0.3% of patients, respectively. This genetic test would be useful not only in Japan but also other East Asian countries, including China, Korea, Taiwan and Vietnam, in which the same mutations are prevalent. Our test is expected to detect 76–87% of the mutant alleles in the Chinese population [12,19,25], 95–100% in the Korean population [12,19,26], 60–68% in the Taiwanese population [27,28], and 100% in the Vietnamese population [12,19]. If we were to prepare a primer/probe set for mutation [X]:g.IVS6+5G>A [12], which is prevalent in Taiwan, the estimated sensitivity would exceed 90% in the Taiwanese population [27,28].

Recently, the high resolution melting (HRM) method was reported to be suitable for the screening of mutations in the diagnosis of citrin deficiency [28]. HRM analysis is a closed-tube assay that screens for any base changes in the amplicons. The presence of SNPs anywhere on the amplicons can affect the melting curve, thereby suggesting that HRM is not suitable for screening for known mutations, but rather, is best suited to screening for unknown mutations. When we detected one heterozygous prevalent mutation, we performed HRM screening for all 17 exons of *SLC25A13*. After HRM screening, only the HRM-positive exons were subjected to direct sequencing analysis. Several mutant alleles were identified using this approach.

The frequency of homozygotes, including compound heterozygotes, presenting *SLC25A13* mutations in the population at Kagoshima (a prefecture in the southern part of Japan) has been calculated to be 1/17,000 based on the carrier rate (1/65) [19]. The prevalence of NICCD has been also reported to be 1/17,000–34,000 [29]. In this study, the carrier rate in Miyagi (a prefecture in northern Japan) was 1/42 (95% confidential interval, 1/108–1/26), thereby yielding an estimated frequency of patients with citrin deficiency of 1/7,100. Our result, together with the previous report [19], suggests that a substantial fraction of the homozygotes or compound heterozygotes of SLC25A13 mutations was asymptomatic during the neonatal period.

The early and definitive diagnosis of citrin deficiency may be beneficial for patients with citrin deficiency by encouraging specific dietary habits and avoiding iatrogenic worsening of brain edema by glycerol infusion when patients develop encephalopathy [30,31]. Because the screening of blood citrulline levels by tandem mass analysis at birth does not detect all patients with citrin deficiency, the development of a genetic test would be welcomed. In this study, we demonstrated that genomic DNA extracted from filter paper blood samples was correctly genotyped, thereby indicating the feasibility of newborn screening using this genetic test. If 100,000 babies in the northern part of Japan were screened by this method, we would detect 14 homozygotes or compound heterozygotes with *SLC25A13* mutations and 2400 heterozygous carriers. In 2400 heterozygous carriers, we would expect to observe only 1 to 2 compound heterozygotes with one target and one non-target mutation. The estimated frequency of babies with two non-target mutations is 0.04/100,000. Our genetic method would therefore allow us to screen newborn babies efficiently. If we performed this genetic test in a high-throughput real-time PCR system, such as a 384- or 1,536-well format, the cost per sample could be lowered.

In conclusion, we have established a rapid and simple detection system using the HybProbe assay for the 11 prevalent mutations in *SLC25A13*. This system could be used to screen newborns for citrin deficiency and may facilitate the genetic diagnosis of citrin deficiency, especially in East Asian populations.

Supplementary materials related to this article can be found online at doi:10.1016/j.ymgme.2011.12.024.

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

Prevalence and Clinical Features of Costello Syndrome and Cardio-Facio-Cutaneous Syndrome in Japan: Findings From a Nationwide Epidemiological Survey

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Costello syndrome and cardio-facio-cutaneous (CFC) syndrome are congenital anomaly syndromes characterized by a distinctive facial appearance, heart defects, and intellectual disability. Germline mutations in *HRAS* cause Costello syndrome, and mutations in *KRAS*, *BRAF*, and *MAP2K1/2* (MEK1/2) cause CFC syndrome. Since the discovery of the causative genes, approximately 150 new patients with each syndrome have been reported. However, the clinico-epidemiological features of these disorders remain to be identified. In order to assess the prevalence, natural history, prognosis, and tumor incidence associated with these diseases, we conducted a nationwide prevalence study of patients with Costello and CFC syndromes in Japan. Based on the result of our survey, we estimated a total number of patients with either Costello syndrome or CFC syndrome in Japan of 99 (95% confidence interval, 77–120) and 157 (95% confidence interval, 86–229), respectively. The prevalences of Costello and CFC syndromes are estimated to be 1 in 1,290,000 and 1 in 810,000 individuals, respectively. An evaluation of 15 adult patients 18–32 years of age revealed that 12 had moderate to severe intellectual disability and most live at home without constant medical care. These results suggested that the number of adult patients is likely underestimated and our results represent a minimum prevalence. This is the first epidemiological study of Costello syndrome and CFC syndrome. Identifying patients older than 32 years of age and following up on the patients reported here is important to estimate the precise prevalence and the natural history of these disorders.

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