promoter-regulatory region of human cytomegalovirus upstream of the FLAG epitope (E7398; Sigma-Aldrich, St. Louis, MO, USA). Because R319Q is located at the *CLCN6* 3'-terminus, we used a 5'-UTR fusion of FLAG. Finally, the *CLCN6* transcript variant 1–3 mutants carrying G250S and R319Q were created using the KOD-Plus-Mutagenesis Kit (SMK-101; TOYOBO, Osaka, Japan). The expression plasmids encoding either wild type *CLCN6* or *CLCN6* transcript variant 1–3 containing the two SNVs were introduced into COS-1 cells using Lipofectamine 2000 Reagent (Life Technologies). The subcellular localization of the recombinant proteins was analyzed by immunofluorescence using antibodies against FLAG (F7425, Sigma-Aldrich), protein disulfide isomerase (PDI) as an ER marker (RL90; Abcam, Cambridge, UK), and DAPI (P36931; Life Technologies). Cell lysates were analyzed by western blot using anti-FLAG antibody as previously described [11].

A patch clamp assay was performed to evaluate the physiological effects of the recombinant proteins (Supplemental Information).

Results

Molecular analyses

Whole exome sequencing produced an average of 1.76×10^8 sequence reads aligned to the reference genome (85.6% of which was properly mapped) with a mean coverage of 68.7 (S2 Table). The variants were filtered according to the flow chart shown in S1 Fig.; five SNVs and one insertion in six genes were selected as the candidate genes (Table 1). Among them, an SNV in the *CLCN6* coding region, chr1:11,887,176G>A was of particular interest because of its functional relevance and previously published linkage data [12]. Seven transcript variants are listed in the UCSC genome browser database (https://genome.ucsc.edu/), and the selected SNV has been identified in one of these transcript variants (transcript variant 1–3 [uc009vnf.2]: c.748G>A [p.G250S]) (Fig. 2B, Table 2). Sanger sequencing identified this SNV (Fig. 3) in all affected members of Family 1 members but not in the unaffected member (Fig. 1; II-4), confirming its segregation with the disease. None of the Family 1 members had mutations in *PRRT2*. The identified SNV was absent in 100 normal Japanese individuals.

Next, we performed a cohort study for *CLCN6* in 48 BPEI patients without *PRRT2* mutations and six patients who had convulsions associated with mild gastroenteritis. The Cohort 1 study identified a non-synonymous SNV in exon 10, c.956G>A (p.R319Q) in the members of Family 2 (Figs. 1 and 3). This SNV affected all four coding transcript variants by non-synonymous alteration R>Q or E>K (Fig. 2B, Table 2). Among the 100 normal Japanese controls, this SNV was identified in one individual. Although this SNV was not detected in the mother with a history of unconfirmed infantile seizures (Fig. 1; II-5), it was found in the father who

Table 1. Candidate genes selected by filtering.

Chromosome	Position*	Region	Gene name	Function	Reference	Alteration	PolyPhen2	SIFT
chr1	11,887,176	exon	*CLCN6*	non-synonymous SNV	G	A	0.619445	0.02
chr9	140,069,578	exon	*ANAPC2*	non-synonymous SNV	A	G	0.999	0
chr11	102,738,797	exon	*MMP12*	frameshift insertion	-	T	NA	NA
chr12	6,952,360	exon	*GNB3*	non-synonymous SNV	G	T	0.999	0
chr17	74,276,523	exon	*QRICH2*	non-synonymous SNV	T	C	0.98	0
chr22	50,927,689	exon	*MIOX*	non-synonymous SNV	G	A	1	0

*, genomic positions are referred to build19; SNV, single nucleotide variation; NA, not applicable

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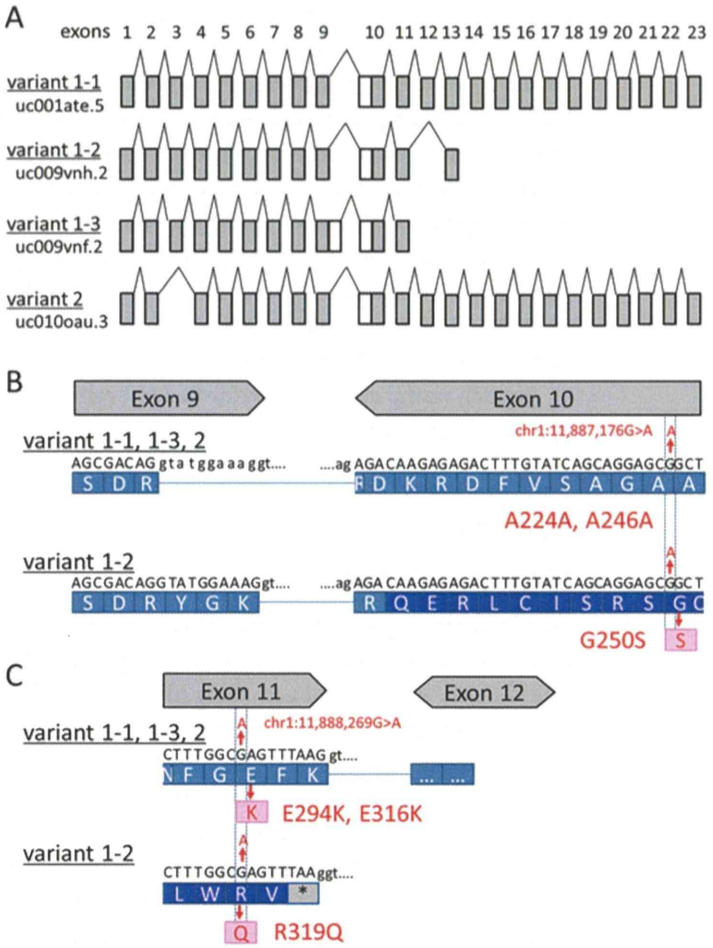

Fig 2. Exon usage and location of *CLCN6* transcript variants. (A) Exon usage of four coding transcript variants. (B) Schematic representation of the locations of the SNVs identified in this study for each *CLCN6* transcript variant. Two exon-intron boundaries are highlighted to clarify the complicated exon usage in the region.

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had FS, suggesting a possible linkage between *CLCN6* and FS. The Cohort 2 study of 48 unrelated FS patients identified another non-synonymous SNV, c.1159G>A (p.V387M) in exon 3 among Family 3 members. Although this SNV was not identified in 100 normal Japanese individuals, it is included in dbSNP build 138 as rs201349073, with an allele frequency of 0.092% (2/2179).

Thus, the incidence of *CLCN6* SNVs was 3% (3/102) in patients with BPEI and/or FS, which was higher than that in normal controls (1/100). However, statistical analysis by Fisher's exact test showed a p-value of 0.25, which did not suggest a significant difference.

Mutagenesis assay

Among the 20 clones produced by subcloning of the reverse transcription-PCR amplicons, one had sequence corresponding to that of transcript variant 1–3. The expression of FLAG-tagged CLCN6 was successfully confirmed in the transfected cells (Fig. 4A), where it was predominantly co-localized with PDI in the endoplasmic reticulum (ER). However, no differences in subcellular localization were detected between the wild type and mutants (Fig. 4A), and no differences in expression levels were observed by western blot (Fig. 4B). Patch-clamp analysis too

Table 2. *CLCN6* transcript variants and identified variants in this study.

Transcript variants	RefSeq annotation number	UCSC annotation number	Genome position	Length of amino acid	Coding exon counts	Type of RNA	SNVs identified in this study		
							1st SNV	2nd SNV	3rd SNV
Transcript variant 1–1	NM_001286	uc001ate.5	chr1:11,866,153–11,903,201	870	23	mRNA	c.738G>A (p.A246A)	c.946G>A (p.E316K)	c.1159G>A (p.V387M)
Transcript variant 1–2	NM_001286	uc009vnh.2	chr1:11,866,153–11,889,379	354	12	mRNA	c.738G>A (p.A246A)	c.946G>A (p.E316K)	NA
Transcript variant 1–3	NM_001286	uc009vnf.2	chr1:11,866,153–11,888,276	321	11	mRNA	c.748G>A (p.G250S)	c.956G>A (p.R319Q)	NA
Transcript variant 2	NM_001256959	uc010oau.3	chr1:11,866,153–11,903,201	848	22	mRNA	c.672G>A (p.A224A)	c.880G>A (p.E294K)	NA
Transcript variant 3–1	NR_046428	uc010oat.3	chr1:11,866,153–11,903,201	260	23	non-coding	NI	NI	NI
Transcript variant 3–2	NR_046428	uc009vng.2	chr1:11,866,153–11,888,276	309	11	non-coding	NI	NI	NI
Transcript variant 3–3	NR_046428	uc009vne.2	chr1:11,866,153–11,876,844	85	3	non-coding	NI	NI	NI

SNV, single nucleotide variant; NA, not affected; NI, not indicated

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did not reveal any significant functional difference between the wild type and mutant variants (S2 Fig.).

Clinical information

In Family 1 (Fig. 1), the proband (III-1) was a girl who was first presented with unprovoked seizures at the age of 8 months and was subsequently diagnosed with BPEI. Her mother (II-2),

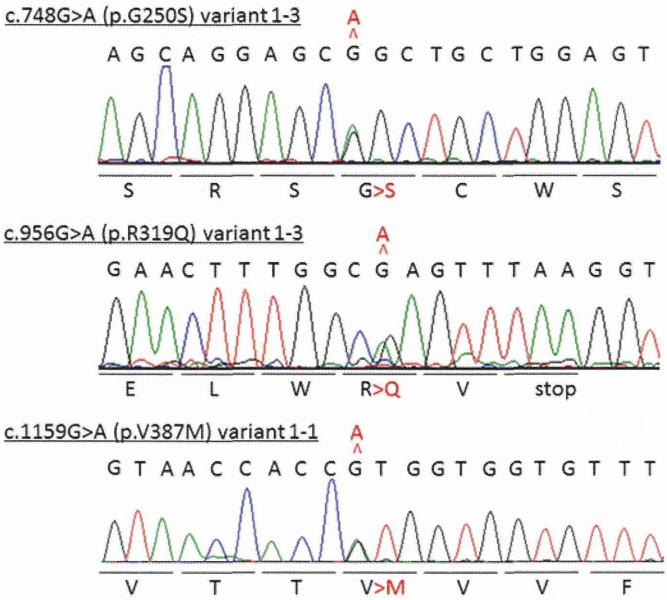

Fig 3. Electropherograms of the identified *CLCN6* variants confirmed by Sanger sequencing. Identified variants are shown in red.

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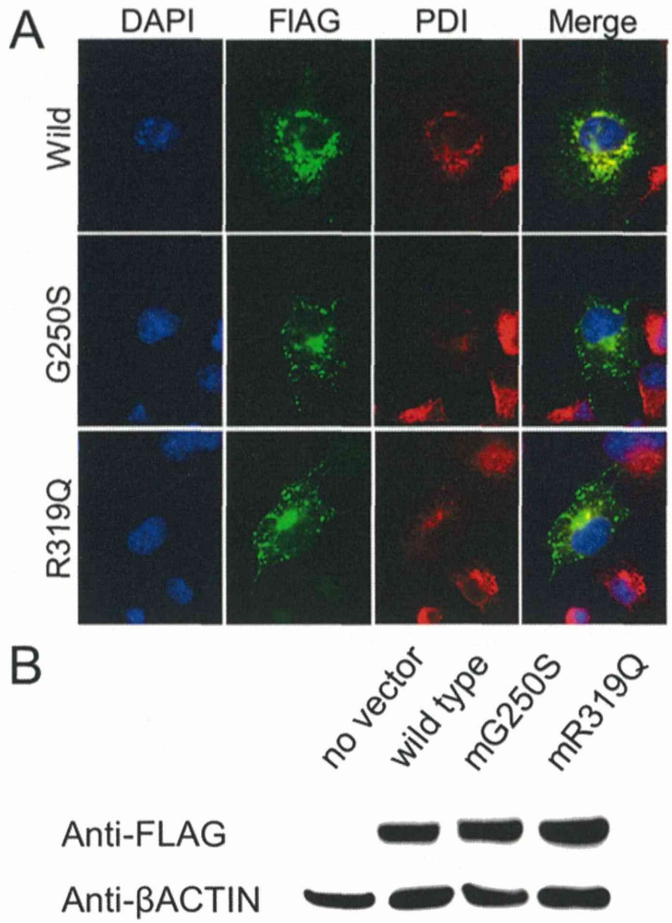

Fig 4. In vitro functional evaluation of SNVs effects. (A) Immunofluorescence staining of COS1 cells transfected with SNV-harboring *CLCN6* variants. Protein disulfide isomerase (PDI) is used as marker of the endoplasmic reticulum (ER). FLAG-tagged CLCN6 is merged with PDI, indicating CLCN6 localization in the ER. (B) Western blotting analysis of cell lysates shows no difference in CLCN6 expression.

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maternal aunt (II-3), and maternal grandfather (I-1) had a history of infantile seizures, but the other aunt (II-4) had no seizure history. The proband (III-2) of Family 2 was a girl diagnosed with BPEI (Fig. 1). Her elder brother (III-1) also had BPEI, while her father (II-2) had experienced one simple FS. The detailed case histories are available in the S1 Supporting Information.

Discussion

In this study, whole exome sequencing for a three-generation family with *PRRT2* mutation-negative BPEI showed six SNVs: five non-synonymous alterations and one frameshift change in six genes (Table 1). Among them, *CLCN6* located on 1p36.22 was considered the most promising candidate based on previous findings suggesting a linkage between BFIE and the 1p36.12-p35.1 locus [12]. *CLCN6* belongs to a family of chloride channels (CLCs) involved in a multitude of physiologic processes ranging from basal cellular functions such as cell volume control and acidification of intracellular vesicles to more specialized mechanisms [13], including regulation of electrical excitability, transepithelial transport, electroneutrality, and ionic homeostasis [14]. In mammals, the CLC family comprises nine members that differ in biophysical properties, cellular compartmentalization, and tissue distribution [15]. Among

them, four members have been associated with inherited disorders. The mutations in the voltage-sensitive chloride channel genes *CNCNKB*, *CLCN1*, *CLCN5*, and *CLCN7* have been linked to Bartter syndrome, myotonia congenita, Dent disease, and osteopetrosis, respectively [16,17,18,19]. Furthermore, variations in *CLCN1*, *CLCN2*, and *CLCN4* have been reported in patients with idiopathic epilepsy and epileptic encephalopathy [20,21,22,23]; however, the association of some variants with disease pathogenesis is still controversial [24].

Although *CLCN6* and *CLCN7* form a distinct branch of the CLC gene family, sharing 45% sequence homology with each other [15], CLCN6 is the least well-characterized mammalian CLC protein [25]. *CLCN6* mRNA is expressed in many tissues, including the brain and kidney [15], and CLCN6 has been reported to co-localize with the markers of ER or endosomes [26,27]. Knock-out of *CLCN6* in mice did not result in increased lethality or produce a strong phenotype [25], but moderate neuronal pathology, resembling that in mild forms of human neuronal ceroid lipofuscinosis (NCL), has been observed [28]. However, genetic analysis of 75 NCL patients identified only two heterozygous mutations in *CLCN6* [25].

On the other hand, a genome-wide association study (GWAS), conducted to identify potential genetic modifiers of cardiac hormonal response, showed a link between the N-terminal signal peptide of pro-B-type natriuretic peptide (NT-proBNP) and *CLCN6* variants. However, it did not exclude the possibility that the identified *CLCN6* variants may simply be a marker for unobserved causal variants in the neighboring gene locus [29]. Thus, phenotypic correlation of *CLCN6* with human diseases has not been confirmed.

In this study, we tested the hypothesis that *CLCN6* is another gene responsible for BPEI onset by analyzing samples from BPEI patients without *PRRT2* mutations by whole-exome sequencing. Because both *CLCN6* SNVs identified in Families 1 and 2 commonly affected transcript variant 1–3, the functional relevance of these SNVs was analyzed in vitro; however, no definite difference was observed between the cells expressing wild type and mutant variants. Therefore, we do not have sufficient evidence to suggest that these *CLCN6* SNVs have a significant pathological impact.

The SNV identified in Family 2 was shared with the parent who had FS, but not with the other parent who had infantile seizures. There is no contradiction in this finding, given that 15% of BPEI patients have FS [30]. The SNV identified in Family 2 was also detected in one of the 100 control samples (1%; 1/100). We subsequently examined a relationship between *CLCN6* SNVs and FS in a cohort of FS patients and identified the third SNV in a patient who had a single FS attack. The third SNV identified in Family 3 was listed in the SNV database but with a very low incidence of 0.1%. Overall, the data indicate that the incidence of *CLCN6* SNVs in patients with BPEI and/or FS was 3% (3/102), which was not significantly higher than in the general population (1%). Because FS is a relatively common condition, occurring in 2–5% of infants in Europe and North America and in 6–9% of infants in Japan [31], the existence of the same *CLCN6* SNVs in the general population should not be a reason of discounting the relationship between *CLCN6* SNVs and BPEI and/or FS.

There are many mutations in the ion-channel genes that show low penetrance in segregation [32]. Indeed, *PRRT2* mutations are often shared with non-phenotypic carriers in families with a history of BPEI [7], suggesting that SNV-related clinical effects would not be significant in episodic disorders. Given that, in this study, *CLCN6* SNVs have been identified in patients with BPEI or FS, such SNVs may not be BPEI-specific but could have a milder association with convulsive disorders including BPEI and FS. The second SNV identified in Family 2 members with or without BPEI/FS produces a non-synonymous substitution in all *CLCN6* transcript variants; however, the first SNV identified in Family 1 members with BPEI results in a non-synonymous substitution only for transcript variant 1–3. Meanwhile, the third SNV identified in Family 3 members with FS produces a non-synonymous substitution only for the other

transcript variants. These results suggest that SNVs in different *CLCN6* transcript variants may be related to distinct phenotypes (i.e., BPEI and/or FS). Alternatively, it may be possible that the observed variants generally shift genetic predisposition toward seizures.

This study was aimed at identifying another gene responsible for BPEI, but SNVs in *CLCN6* were found in only a small proportion of BPEI patients. Thus, the data are inconclusive. Recent massive parallel sequencing for patients with sporadic epilepsy of unknown etiology identified SNVs in the chloride channel genes, *CLCN1* and *CLCN2* [33], suggesting an association of CLCs with epilepsy. In that study, *CLCN6* variants made up a small proportion of the patients but were not present in the controls (detailed results unavailable). Therefore, there is still a possibility that *CLCN6* variants are related to genetic susceptibility for convulsive disorders such as BPEI and FS. Further investigation is required to test this possibility.

Supporting Information

S1 Fig. Filtering steps in the selection of the variants extracted by whole exome sequencing.
(PDF)

S2 Fig. Wild-type and mutant hCLCN6 currents recorded in *Xenopus oocytes*. **(A)** Averaged current-voltage relationships for the oocytes injected with wild-type (WT, solid line; n = 10), G250S (dotted line; n = 8), or R318Q (dashed line; n = 6) *CLCN6* cDNA or water (H2O dot-dash line; n = 8). Oocytes were held at-20mV and stepped from-100mV to 100 mV for 800 msec every 10 sec in 20 mV increments. (B) Average peak currents at 100 mV for WT (n = 10), G250S (n = 8), R319Q (n = 6), and H2O (n = 8).
(PDF)

S1 Supporting Information. Supplemental information. Supplemental methods and results are included.
(PDF)

S1 Table. Primers used for *CLCN6* Sanger sequencing.
(PDF)

S2 Table. The result of the mapping of whole-exome sequencing data for family 1.
(PDF)

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Author Contributions

Conceived and designed the experiments: TY. Performed the experiments: TY KS NS YK T. Furukawa CJM. Analyzed the data: TY. Contributed reagents/materials/analysis tools: AI SA SY KI TK T. Fukasawa TO HE TT AS TS AO. Wrote the paper: TY. Contributed to statistical analysis: AS. Supervised the study: SP SEH LMD SH

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Case Report

A case of recurrent encephalopathy with *SCN2A* missense mutation

Tatsuya Fukasawa [a,*], Tetsuo Kubota [a], Tamiko Negoro [a], Makiko Saitoh [b], Masashi Mizuguchi [b], Yukiko Ihara [c], Atsushi Ishii [c], Shinichi Hirose [c]

[a] *Department of Pediatrics, Anjo Kosei Hospital, Aichi, Japan*
[b] *Department of Developmental Medical Sciences, Graduate School of Medicine, University of Tokyo, Japan*
[c] *Department of Pediatrics, School of Medicine, Fukuoka University, Fukuoka, Japan*

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Abstract

Voltage-gated sodium channels regulate neuronal excitability, as well as survival and the patterning of neuronal connectivity during development. Mutations in *SCN2A*, which encodes the Na^+ channel $Na_v1.2$, cause epilepsy syndromes and predispose children to acute encephalopathy. Here, we report the case of a young male with recurrent acute encephalopathy who carried a novel missense mutation in the *SCN2A* gene. He was born by normal delivery and developed repetitive apneic episodes at 2 days of age. Diffusion-weighted imaging revealed high-intensity areas in diffuse subcortical white matter, bilateral thalami, and basal nuclei. His symptoms improved gradually without any specific treatment, but he exhibited a motor milestone delay after the episode. At the age of 10 months, he developed acute cerebellopathy associated with a respiratory syncytial viral infection. He received high-dose intravenous gammaglobulin and methylprednisolone pulse therapy and seemed to have no obvious sequelae after the episode. He then developed severe diffuse encephalopathy associated with gastroenteritis at the age of 14 months. He received high-dose intravenous gammaglobulin and methylprednisolone pulse therapy but was left with severe neurological sequelae. PCR-based analysis revealed a novel *de novo* missense mutation, c.4979T>G (p.Leu1660Trp), in the *SCN2A* gene. This case suggests that *SCN2A* mutations might predispose children to repetitive encephalopathy with variable clinical and imaging findings.

Keywords: *SCN2A*; Mutation; Encephalopathy

1. Introduction

Although the precise pathomechanism of acute encephalopathy remains to be elucidated, multiple genetically determined factors might play a role. Mutations in genes such as Ran-binding protein 2 (*RANBP2*) [1], toll-like receptor 3 (*TLR3*) [2], and neuronal sodium channel alpha1-subunit (*SCN1A*) [3], as well as polymorphisms in genes such as carnitine palmitoyltransferase II (*CPT2*) [4] and adenosine A2A receptor (*ADORA2A*) [5], are risk factors for multiple syndromes of acute encephalopathy. The etiology and pathophysiology of these syndromes are variable, and the correlation between genotype and phenotype is complex.

Here, we report a patient who presented with recurrent episodes of encephalopathy during the neonatal period and infancy. He had a novel point mutation in the *SCN2A* gene. This case suggests that *SCN2A* mutations might predispose neonates and infants to repetitive encephalopathies with variable clinical phenotypes.

* Corresponding author at: Department of Pediatrics, Anjo Kosei Hospital, 28 Higashihirokute Anjo-cho, Anjo-shi, Aichi-pref 446-8602, Japan. Tel.: +81 566 75 2111; fax: +81 566 76 4335.
E-mail address: fukasawa@kosei.anjo.aichi.jp (T. Fukasawa).

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2. Case report

The patient was born by normal delivery from non-consanguineous parents at 38 weeks of gestation, weighing 2610 g. His Apgar score was 10 after both 1 and 5 min. There was no family history of neurological disorders. He developed repetitive apneic episodes and fatigue at the age of 2 days. At the age of 5 days, diffusion-weighted imaging (DWI) revealed high intensity areas (HIAs) in the diffuse subcortical white matter, bilateral thalami, and basal nuclei (Fig. 1). Examination of the cerebrospinal fluid (CSF) and blood showed no abnormalities. His symptoms improved gradually without any specific treatment. After this episode, a head magnetic resonance imaging (MRI) scan showed diffuse cerebral atrophy, and the patient exhibited motor milestone delay.

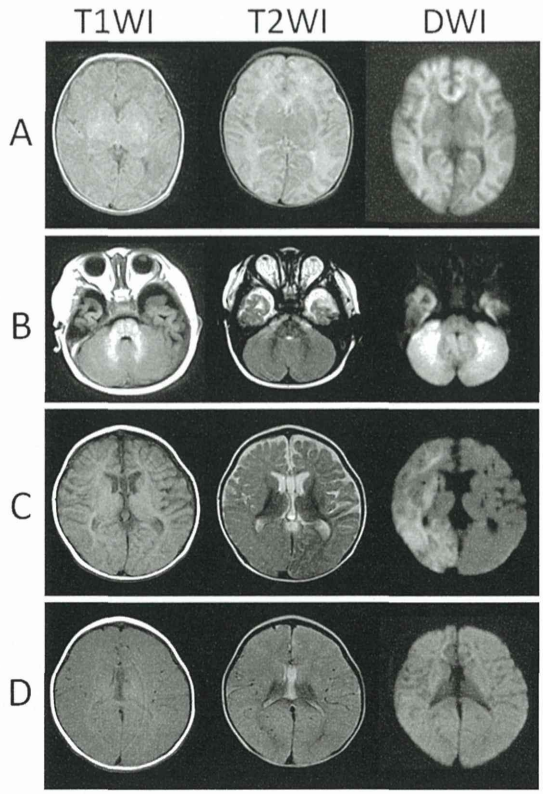

Fig. 1. Head magnetic resonance imaging (MRI) of the patient. (A) At the age of 5 days, diffusion-weighted imaging (DWI) revealed high-intensity areas (HIAs) in the subcortical white matter, bilateral thalami, and basal nuclei. T1-weighted imaging (T1WI) and T2-weighted imaging (T2WI) showed no obvious abnormal findings. (B) At the age of 10 months, DWI revealed HIAs in the bilateral cerebellar hemispheres but not in the cerebral hemispheres. T1WI and T2WI showed no obvious abnormal findings. (C) At the age of 14 months, DWI showed diffuse HIAs in the right hemisphere and left frontal lobe at the time of symptom onset. (D) Two days later, the diffuse HIAs had enlarged into the bilateral hemisphere, excluding the thalami. T1WI and T2WI revealed a blurred cortical-white matter junction and diffuse cerebral edema.

At the age of 10 months, he presented with fever, nasal congestion, and cough. Analysis of a sample taken from a nasopharyngeal swab for rapid respiratory syncytial virus using an antigen-detection assay was positive. The next day he developed nystagmus and disturbed consciousness. DWI revealed the presence of HIAs in the bilateral cerebellar hemispheres (Fig. 1). He received high-dose intravenous gammaglobulin and methylprednisolone pulse therapy, which gradually improved his symptoms. A head MRI showed cerebellar atrophy, but his electroencephalogram (EEG) showed no abnormal findings, and he had no seizures or obvious sequelae after this episode.

At the age of 14 months, he suffered an episode of fever, vomiting, and diarrhea, with repeated generalized tonic-clonic convulsions the same day. Examination of his CSF was normal. DWI revealed HIAs in the diffuse right hemisphere and left frontal lobe. He received high-dose intravenous gammaglobulin and methylprednisolone pulse therapy. However, 2 days later, DWI revealed the presence of diffuse HIAs in the bilateral hemisphere, except for in the bilateral thalami. After this episode, a head MRI showed severe cerebral atrophy, and his interictal EEG showed frequent spikes and sharp waves in the right fronto-temporal regions. He had severe neurological sequelae and spastic quadriplegia; he became bedridden and required tube feeding, and tonic seizures occurred several times a day.

Genetic analysis of 26 exons of the *SCN2A* gene using PCR revealed a missense mutation c.4979T>G (p.Leu1660Trp), located between segment 4 and 5 of domain IV (Fig. 2). Amino acid residue of 1660 leucine is well conserved in other sodium channels, as well as other species. Sorting Intolerant From Tolerant (SIFT) predicted that this mutation may be highly damaging to the structure of $Na_v1.2$. The score of SIFT was 0.00 (damaging) with Median Information of Content 3.39. This mutation has not been reported previously in the literature and was not detected in 80 normal individuals. Neither of his parents carried this mutation.

3. Discussion

The clinical course of this patient raised two important clinical issues.

First, some *SCN2A* mutations may cause repetitive encephalopathy. *SCN2A* encodes the voltage-gated sodium channel α2-subunit. Previous electrophysiological analyses demonstrated that different mutations have diverse effects on sodium channels. *SCN2A* mutations were associated with a variety of diseases: benign familial neonatal-infantile seizures, generalized epilepsy with febrile seizures, Dravet syndrome, some intractable childhood epilepsies [6], and acute encephalitis with refractory, repetitive, partial seizures [7]. Considering the diverse effects of *SCN2A* mutations on sodium

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