penetrance in a large group of individuals carrying the expansion.

Our data have implications for the clinical care of patients diagnosed with ALS and FTD. The clinical standard of care is to offer genetic testing to patients reporting a family history of ALS or FTD,[27] and to reassure patients classified as having sporadic disease that their relatives are not at increased risk of neurodegeneration. On the basis of an analysis of 191 Irish patients with ALS, Byrne and colleagues[28] suggested that genetic testing for the *C9orf72* repeat expansion is unnecessary in affected individuals without a family history of disease or substantial cognitive impairment. By contrast, we believe that genetic testing is a valuable technique for accurate diagnosis of the two disorders and in the decision-making process for patients and their families. The discrepancy between these two views might stem from differences in how sporadic and familial disease were defined in the two studies. Accumulation of sufficient data is an important step towards answering this key question for management of patients. In view of the large number of patients who carry the repeat expansion, investigators and clinicians should at least consider a focused debate on this issue.

Our paper has some limitations. First, the number of patients from some geographical regions was small and the mutational frequencies might change for those ethnic groups as additional patients are screened. Nevertheless, our data for more than 5000 patients with ALS or FTD provide a reasonable estimation of *C9orf72* global frequency. Second, although we have examined the chromosome 9p21 haplotype in a large and diverse cohort of individuals carrying the pathogenic expansion, additional testing of carriers might reveal other haplotypes, thereby indicating that the expansion arose on more than one occasion. Nevertheless, our data suggest that most expansion carriers share a common ancestor.[16,17] Third, we generated age-related penetrance estimates on the basis of data from retrospective cohorts, which potentially leads to overestimation of penetrance. Additional prospective studies examining family kindreds are necessary to confirm these estimates. Finally, case classification as familial or sporadic was done on the basis of clinical questioning at sample collection. The level of scrutiny might have varied between centres and countries, but re-collection of this information for existing cohorts was not feasible.

Contributors

EM, AER, KM, NN, AW, SR, JSS, YA, JOJ, DGH, SA, and JK did laboratory-based experiments and data analysis, and revised the report. ED, MSe, RP, RWO, KCS, HH, JDR, KEM, HP, KT, OA, MSa, GM, MC, FG, ACa, EE, GB, GLF, AMR, HL, LM, VED, and CD collected data from and characterised patients, and revised the manuscript. MAN analysed the data and revised the report. SM, JQT, VMVD, GDS, C-SL, T-HY, HI, YT, ST, ILB, AB, and PS supervised laboratory-based experiments, and revised the report. ACh, GR, JvS, NW, JH, PJT, PH, HRM and SP-B designed the study, supervised laboratory-based experiments, and revised the report. BJT designed the study, supervised laboratory-based experiments, did the data analysis, and drafted the report. The Chromosome 9-ALS/FTD Consortium, The French research network on FTLD/FTLD/ALS, and The ITALSGEN Consortium provided data and helped with data analysis.

Conflicts of interest

PT, PH, HW, SP-B, and BT have a patent pending on the clinical testing and therapeutic intervention for the hexanucleotide repeat expansion of *C9orf72*. JR is Director of the Packard Center for amyotrophic lateral sclerosis Research at Johns Hopkins (MD, USA). All other authors declare that they have no conflicts of interest.

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ORIGINAL CONTRIBUTION

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

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

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

Design: Case series.

Setting: University hospitals.

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

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

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

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

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

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

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

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

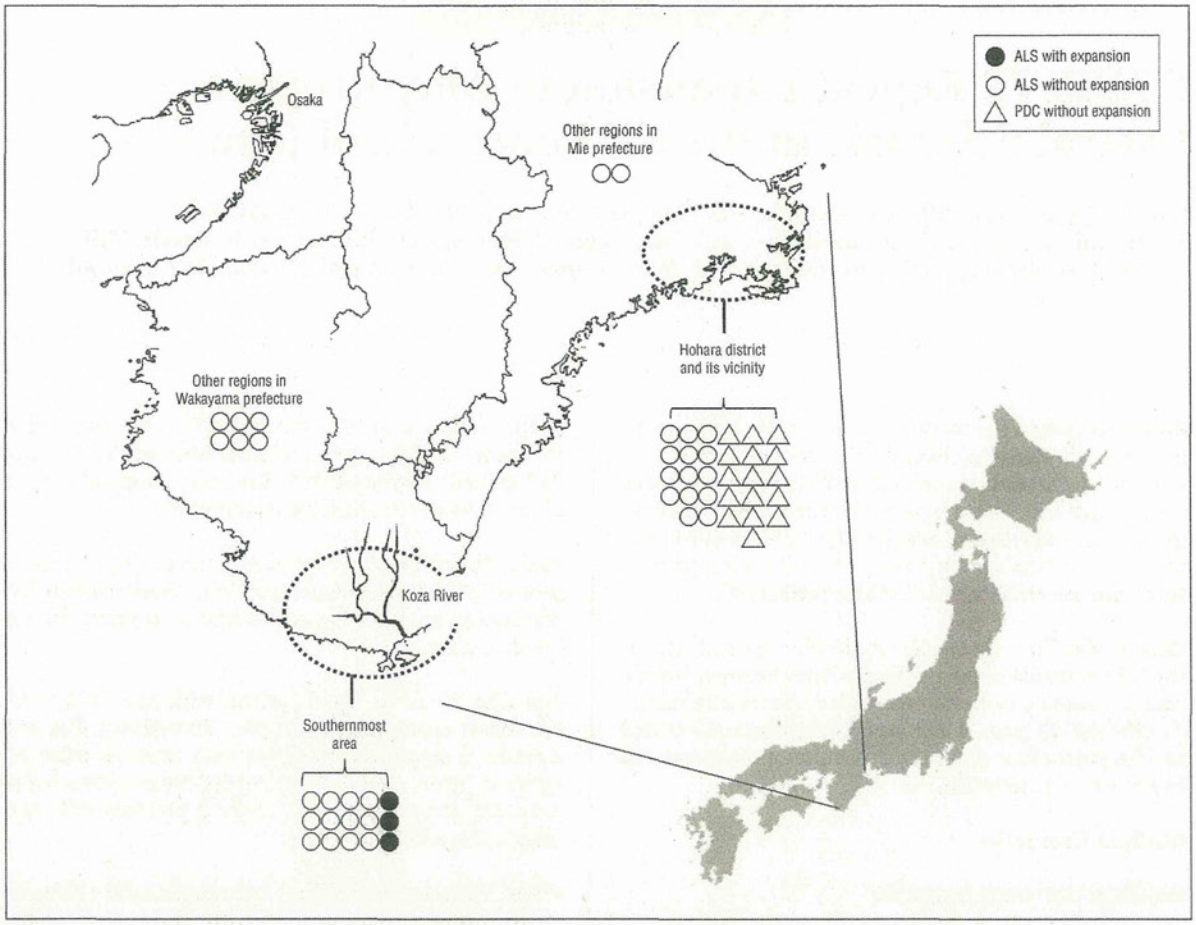

Figure 1. Map of Kii peninsula of Japan and distribution of patients with amyotrophic lateral sclerosis (ALS) and parkinsonism-dementia complex (PDC). The southernmost area neighboring the Koza River (Kozagawa and Kushimoto towns) and the Hohara district and its vicinity (Minamiise town and Shima city)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

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

METHODS

SUBJECTS AND DNA EXTRACTION

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

REPEAT-PRIMED POLYMERASE CHAIN REACTION ANALYSIS

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

SOUTHERN BLOT HYBRIDIZATION ANALYSIS

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

HAPLOTYPE ANALYSIS

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

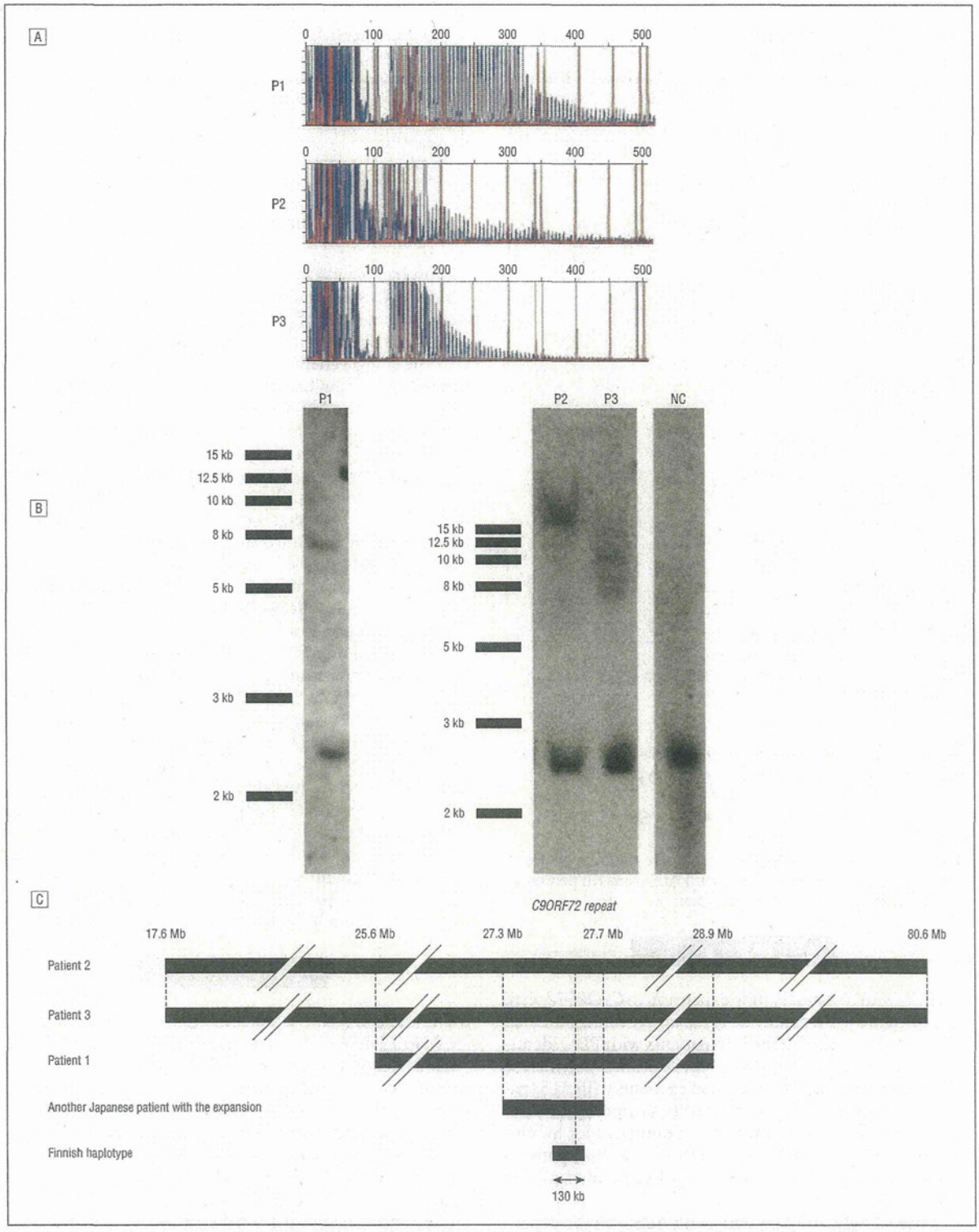

Figure 2. Mutational analyses of hexanucleotide repeat expansion in *C9ORF72*. A, Repeat-primed polymerase chain reaction analysis was performed as previously described.[8] Patients 1-3 show the characteristic sawtooth patterns with a 6-bp periodicity (blue lines). Red lines indicate DNA size markers.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

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

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

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

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

STATISTICAL ANALYSIS

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

RESULTS

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

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

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

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

Abbreviation: ALS, amyotrophic lateral sclerosis.

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

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

COMMENT

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

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

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

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

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

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ORIGINAL ARTICLE

Mutational analysis of familial and sporadic amyotrophic lateral sclerosis with *OPTN* mutations in Japanese population

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Abstract

Our objective was to elucidate the genetic epidemiology of familial amyotrophic lateral sclerosis (FALS) and sporadic ALS (SALS) with *OPTN* mutations in the Japanese population. Mutational analysis of *OPTN* was conducted in 18 FALS pedigrees in whom mutations in other causative genes have been excluded and in 218 SALS patients by direct nucleotide sequence analysis. Novel non-synonymous variants identified in ALS patients were further screened in 271 controls. Results showed that although no mutations were identified in the FALS pedigrees, a novel heterozygous non-synonymous variant c.481G>A (p.V161M) was identified in one SALS patient, who originated from the southernmost part of the Kii Peninsula. The mutation was not present in 271 controls. As the clinical feature, the patient carrying V161M showed predominantly upper motor neuron signs with slow progression. This study suggests that mutations in *OPTN* are not the main cause of ALS in the Japanese population.

Key words: *Motor neuron disease, amyotrophic lateral sclerosis, OPTN mutation, genetic analysis, V161M*

Introduction

Molecular genetic research on amyotrophic lateral sclerosis (ALS) has revealed a number of causative genes for familial ALS (FALS), which include *SOD1* (1), *ALS2* (2,3), *DCTN1* (4), *VAPB* (5), *CHMP2B* (6), *ANG* (7), *TARDBP* (8), and *FUS* (9,10). These genes collectively account for approximately 30% of FALS pedigrees (11). Mutations in these genes have also been identified in some sporadic ALS (SALS) patients, suggesting mutations with reduced penetrance or *de novo* mutations (12,13). Recently, hexanucleotide repeat expansion within the *C9ORF72* gene has been reported to be associated with a large proportion of cases of ALS and frontotemporal dementia (FTD) with wider European ancestry (14–16). Mutations in *UBQLN2* were also identified to cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia (17). *OPTN*, which was previously identified as the causative gene for rare autosomal dominant familial primary open-angle glaucoma (POAG), has been reported as the causative gene for autosomal dominant and autosomal recessive FALS (18). Subsequent genetic epidemiological studies on *OPTN* mutations in different cohorts have revealed that frequencies of mutations in patients with FALS and SALS vary among cohorts, from 0% to 4.35% (pedigree frequency) in those with FALS, and from 0% to 3.54% (case frequency) in those with SALS (18–23). Further analyses on larger cohorts of various ethnic backgrounds will be necessary to establish the genetic epidemiology and clinical characteristics of ALS and the genotype-phenotype correlations of ALS with *OPTN* mutations. We conducted further mutational analysis of *OPTN* in our cohorts to establish the molecular epidemiology of ALS in patients with mutations in *OPTN*.

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Materials and methods

Thirty-five FALS pedigrees, 218 SALS patients, and 271 controls, all of whom were from the Japanese population, were enrolled in this study. Of the 35 FALS pedigrees, 17 harbored causative mutations in other causative genes for FALS with the autosomal dominant mode of inheritance. The remaining 18 pedigrees consisted of 13 with the autosomal dominant mode of inheritance, two pedigrees with affected sibs with consanguinity, and three pedigrees with affected sibs without consanguinity. The 218 SALS patients, most of whom visited the University of Tokyo Hospital, included 33 from Yamagata Prefecture, on the northern part of Honshu island, and 15 from the Kii Peninsula, on the southern part of Honshu island. The mean age at onset of the SALS cohort was 58.9 years, and the male: female ratio was 3: 2. All of the genomic DNA samples were obtained from the participants of this study with their written informed consent, and this research was approved by the Institutional Review Board of the University of Tokyo.

Mutational analysis

Mutations in causative genes for FALS were analyzed employing a DNA microarray-based resequencing system as described elsewhere (24) or a direct nucleotide sequencing method conducted using a BigDye Terminator ver. 3.1 cycle sequencing kit on a 3100 ABI Prism Genetic Analyzer (Applied Biosystems). All the coding exons of *OPTN* (exons 4–16) were amplified by genomic PCR using specific primers for each exon recently reported (18) and further subjected to direct nucleotide sequence analysis.

Mutations in other causative genes for FALS, including *SOD1, ALS2, DCTN1, VAPB, CHMP2B, ANG*, and *TARDBP*, were firstly excluded employing a DNA microarray-based resequencing system. Secondary, mutational analysis of *FUS* employing a direct nucleotide sequencing method was performed. The remaining samples were subjected to mutational analysis of *OPTN* by direct nucleotide sequence analysis.

The variants identified by the mutational analysis were evaluated using databases of dbSNP (http://www.ncbi.nlm.nih.gov/SNP/index.html), 1000 Genomes Project (http://www.1000genomes.org/), and Exome Sequencing Project (https://esp.gs.washington.edu/). When novel non-synonymous variants not registered in these databases were identified, they were further screened in 271 controls by direct nucleotide sequence analysis. The effect of amino acid changes caused by identified novel variants was predicted using the PolyPhen-2 website (http://genetics.bwh.harvard.edu/pph2/).

Results

Of the 35 FALS pedigrees enrolled in this study, 17 harbored causative mutations in other causative genes for FALS including 14 *SOD1*, two *FUS*, and one *TARDBP*. The remaining 18 pedigrees were subjected to mutational analysis of *OPTN*. Five variants including four known SNPs and a novel synonymous variant in exon 16 were identified (Table I). We did not observe any causative mutations in *OPTN* in the FALS pedigrees in our cohort.

In the 218 SALS patients, seven variants including four known SNPs, two novel synonymous variants in exons 4 and 7, and one novel non-synonymous variant in exon 6 not registered in dbSNPs, 1000 Genomes Project, or Exome Sequencing Project were identified (Table II). Known causative mutations for ALS were not identified in the SALS patients. The novel heterozygous non-synonymous variant of c.481G >A in exon 6 substituting methionine for valine at amino acid position 161 (p.V161M) was identified in a SALS patient (Figure 1A, B). This novel variant of V161M was not present in 271 controls (542 chromosomes). Although the amino acid valine at position 161 was not necessarily highly conserved among species (Figure 1C), the PolyPhen-2 prediction was possibly damaging with a score of 0.913.

Interestingly, the patient with V161M mutation originated from the southernmost part of the Kii Peninsula, where the prevalence of ALS is high and patients with the ALS-parkinsonism-dementia

Table I. Summary of *OPTN* variants identified in 18 FALS patients.

Exon	SNP ID*	Base changes	Annotation	Amino acid changes	Number of pedigrees (Allele frequency)		Allele frequency (1000 Genomes)**
4	rs2234968	c.102G>A	Synonymous		3 homozygotes, 1 heterozygote#	(0.389)	0.182
5	rs11258194	c.293T>A	Non-synonymous	p.Met98Lys	1 heterozygote	(0.028)	0.110
10	rs523747	c.964A>G	Non-synonymous	p.Lys322Glu	18 homozygotes	(1.000)	1.000
16	rs75654767	c.1634G>A	Non-synonymous	p.Arg545Gln	2 heterozygotes#	(0.056)	0.028
16	Novel	c.1713C>T	Synonymous		1 heterozygote	(0.028)	0.000

*SNP ID is the single-nucleotide polymorphism identification obtained from dbSNP database.
**The allele frequencies in East Asian populations were obtained from 1000 Genomes Project (http://www.1000genomes.org/).
#One patient carried both the heterozygous c.102G>A variant and the heterozygous c.1634G>A variant.

Table II. Summary of *OPTN* variants identified in 218 SALS patients.

Exon	SNP ID*	Base changes	Annotation	Amino acid changes	Number of cases (Allele frequency)		Allele frequency (1000 Genomes)**
4	rs2234968	c.102G>A	Synonymous		3 homozygotes, 59 heterozygotes	(0.149)	0.182
4	Novel	c.147C>T	Synonymous		1 homozygote	(0.004)	0.000
5	rs11258194	c.293T>A	Non-synonymous	p.Met98Lys	17 heterozygotes	(0.039)	0.110
6	Novel	c.481G>A	Non-synonymous	p.Val161Met	1 heterozygote	(0.002)	0.000
7	Novel	c.630A>T	Synonymous		1 heterozygote	(0.002)	0.000
10	rs523747	c.964A>G	Non-synonymous	p.Lys322Glu	218 homozygotes	(1.000)	1.000
16	rs75654767	c.1634G>A	Non-synonymous	p.Arg545Gln	13 heterozygotes	(0.030)	0.028

*SNP ID is the single-nucleotide polymorphism identification obtained from dbSNP database.
**The allele frequencies in East Asian populations were obtained from 1000 Genomes Project (http://www.1000genomes.org/).

complex are clustered. We further conducted the mutational analysis of *OPTN* recruiting four additional patients with SALS in the same district. These patients, however, harbored neither the V161M mutation nor any other mutations in *OPTN*.

The clinical features of the patient with the V161M mutation are briefly presented as follows. The patient was a 35-year-old male at the time of diagnosis of ALS, who developed upper extremity weakness for one year. Weakness and atrophy predominantly in upper extremities gradually worsened. Neurological examination at the age of 39 years revealed tongue atrophy and fasciculation, attenuated tendon reflexes and muscle wasting in the upper extremities, and enhanced tendon reflexes in the lower extremities with bilateral extensor plantar reflexes. He became mechanical-ventilator-dependent at the age of 50 years. There was no evidence of parkinsonism or cognitive impairment at the age of 50 years. His medical history included unexplained vision loss of his right eye in his childhood. His father, who also originated from the southernmost part of the Kii Peninsula, was alive and did not show any symptoms indicative of motor neuron disease when the index patient was 35 years old. His mother, who originated from southeastern part of the Kii Peninsula, died of liver cirrhosis, but her age at death was not indicated.

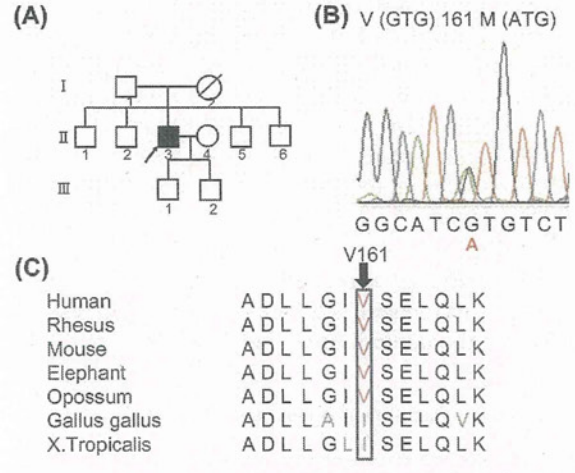

Figure 1. (A) Pedigree chart of patient with V161M variant in *OPTN*. Affected individuals are indicated by filled symbols. The proband is indicated by an arrow. Unaffected individuals are indicated by open symbols. Slashed symbols indicate deceased subjects. Ages at death are shown when information is available. Squares denote male family members and circles denote female family members. (B) Electropherogram of heterozygous *OPTN* c.481G>A (p.Val161Met) point mutation. (C) Conservation of *OPTN* amino acid sequences among different animal species. The valine residue at codon 161 is not necessarily highly conserved among different species (shown in red). Non-conserved amino acids are shown in green.

Discussion

In this study, we conducted a comprehensive mutational analysis of *OPTN* in a large cohort of Japanese FALS and SALS patients. Among our 35 FALS pedigrees, 17 families had mutations in other causative genes previously reported, as described in Results, and we did not find any causative mutations in *OPTN* in the remaining 18 pedigrees. On the other hand, among the 218 patients with SALS, we identified a patient carrying a novel non-synonymous mutation of *OPTN*.

Previous genetic studies on *OPTN* mutations in different cohorts have demonstrated that the frequencies of *OPTN* mutations are from 0% to 4.35% (pedigree frequency) in FALS (18–23) (Table IIIA). *OPTN* was initially identified as a causative gene for FALS in a consanguineous pedigree through homozygosity mapping followed by sequencing of candidate genes in the homozygous region. In our cohort, autosomal recessive inheritance was suggested in only five of the 35 FALS families, which may account for the fact that we did not identify any causative mutations in *OPTN* in the FALS families. Since the number of families enrolled in this study is limited, further extensive mutational analysis of larger cohorts of FALS will be necessary to establish the genetic epidemiology of FALS patients with *OPTN* mutations.

In our SALS cohort, a novel heterozygous non-synonymous variant, V161M, was identified in a patient. Previous genetic studies on *OPTN* mutations in different cohorts have shown a number of heterozygous missense mutations in SALS patients (20,21) and that the frequencies of *OPTN* mutations are from 0% to 3.54% (case frequency) in SALS (18–23) (Table IIIB). When we assess the implication of the

Table IIIA. Summary of *OPTN* variants identified in FALS patients in previous and present studies.

Studies	Ethnicity	Variant	Number of pedigrees	Status
Maruyama H, et al.[18]	Japanese	exon 5 deletion	4	1 homozygote
		p.Q398X		1 homozygote
		p.E478G		2 heterozygotes
Belzil VV, et al.[19]	European	c.1242+1G>A_insA	2	1 heterozygote
		p.A481V		1 heterozygote
Del Bo R, et al.[20]	Italian	p.G23X	2	1 heterozygote
		p.K557T		1 heterozygote
Iida A, et al.[21]	Japanese	p.E478G	1	1 homozygote
Millecamps S, et al.[22]	Caucasian	p.R96L	1	1 heterozygote
Sugihara K, et al.[23]	Caucasian	None	0	
Present study	Japanese	None	0	

mutation identified in an isolated case without any family history, we need to carefully consider various possibilities including the possibilities of causative mutation with reduced penetrance and *de novo* mutation. Another possibility is that the variant might not necessarily be associated with a risk of ALS.

Hexanucleotide repeat expansion within the *C9ORF72* gene has very recently been reported to be frequent as a cause of ALS with wider European ancestry. Our recent study on the same cohort indicated that the frequency of the patients with the hexanucleotide repeat expansions is very low (16), suggesting that the result of our molecular epidemiology study of *OPTN* was not substantially affected by that of *C9ORF72* in our Japanese cohort.

Previous studies showed that the clinical phenotypes of patients with *OPTN* mutations are heterogeneous for both age of onset and disease duration, but are characterized by a relatively slow progression, lower-limb onset, and frequent upper motor neuron signs. The relatively slow progression after the onset and the presence of upper motor neuron signs observed in the patient with the V161M variant are consistent with the previous reports (18–23). However, this patient differed from those in previous reports to the extent that the onset site is the upper extremities. Further accumulation of clinical information is essential to delineate the phenotypic spectrum and to illustrate the genotype-phenotype correlations of ALS with *OPTN* mutations.

Of note, the patient originated from the southernmost part of the Kii Penisula including the Koza River, where the prevalence of ALS has been described to be higher than in other areas of Japan (25). Neither the causes of the high prevalence nor the genetic risk factors common to ALS patients in the region have been elucidated. Mutational analysis of four additional ALS patients residing in the same district (Koza River and its vicinity), however, revealed neither the V161M mutation nor other mutations. V161M does not appear to be very common among the patients with ALS in this district.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Table IIIB. Summary of *OPTN* variants identified in SALS patients in previous and present studies.

Studies	Ethnicity	Variant	Number of cases	Status
Maruyama H, et al.[18]	Japanese	p.Q398X	1	1 homozygote
Belzil VV, et al.[19]	European	None	0	
Del Bo R, et al.[20]	Italian	c.552+1delG	4	1 heterozygote
		p.T282P		1 heterozygote
		p.Q314L		1 heterozygote
		c.1401+4A>G		1 heterozygote
Iida A, et al.[21]	Japanese	p.A93P	2	1 heterozygote
		p.E478G		1 heterozygote
Sugihara K, et al.[23]	Caucasian	None	0	
Present study	Japanese	p.V161M	1	1 heterozygote

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ORIGINAL ARTICLE

Mutations in *KCND3* Cause Spinocerebellar Ataxia Type 22

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Objective: To identify the causative gene in spinocerebellar ataxia (SCA) 22, an autosomal dominant cerebellar ataxia mapped to chromosome 1p21-q23.
Methods: We previously characterized a large Chinese family with progressive ataxia designated SCA22, which overlaps with the locus of SCA19. The disease locus in a French family and an Ashkenazi Jewish American family was also mapped to this region. Members from all 3 families were enrolled. Whole exome sequencing was performed to identify candidate mutations, which were narrowed by linkage analysis and confirmed by Sanger sequencing and cosegregation analyses. Mutational analyses were also performed in 105 Chinese and 55 Japanese families with cerebellar ataxia. Mutant gene products were examined in a heterologous expression system to address the changes in protein localization and electrophysiological functions.
Results: We identified heterozygous mutations in the voltage-gated potassium channel Kv4.3-encoding gene *KCND3*: an in-frame 3-nucleotide deletion c.679_681delTTC p.F227del in both the Chinese and French pedigrees, and a missense mutation c.1034G>T p.G345V in the Ashkenazi Jewish family. Direct sequencing of *KCND3* further identified 3 mutations, c.1034G>T p.G345V, c.1013T>C p.V338E, and c.1130C>T p.T377M, in 3 Japanese kindreds. Immunofluorescence analyses revealed that the mutant p.F227del Kv4.3 subunits were retained in the cytoplasm, consistent with the lack of A-type K^+ channel conductance in whole cell patch-clamp recordings.
Interpretation: Our data identify the cause of SCA19/22 in patients of diverse ethnic origins as mutations in *KCND3*. These findings further emphasize the important role of ion channels as key regulators of neuronal excitability in the pathogenesis of cerebellar degeneration.

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Additional Supporting Information can be found in the online version of this article.

Spinocerebellar ataxia (SCA) is a clinically, pathologically, and genetically heterogeneous group of dominantly inherited neurodegenerative disorders characterized by progressive cerebellar ataxia variably associated with pyramidal, extrapyramidal, bulbar, spinal, and peripheral nervous system involvement. Thirty-two dominant SCAs (labeled SCA1–36) have been chromosomally mapped, and the genes causing 20 of these disorders have so far been identified.[1,2] The genetic etiologies of many SCAs have yet to be elucidated.[3,4]

Previously, we characterized a large Chinese pedigree with an autosomal dominant ataxia spanning 4 generations.[5] The disease locus was mapped to chromosome 1p21-q23 and was designated SCA22.[5] The locus of SCA22 overlaps with that of SCA19 on 1p21-q21, previously identified in a Dutch family.[6,7] SCA19 and SCA22 were therefore proposed to be allelic with a worldwide distribution.[7] Here, we report mutations in *KCND3* in the original SCA22 family as well as in 5 other SCA families of French, Ashkenazi Jewish, and Japanese origin with dominant ataxia.

Subjects and Methods

Subjects

In family A of Han Chinese origin (Fig 1A), the original SCA22 family, we enrolled 31 members, including 13 affected, 6 unaffected, 6 at-risk, and 6 married-in individuals. The age at onset of ataxia in this pedigree ranged from 13 to 46 years. Clinical severity of ataxia was evaluated longitudinally using the 40-point (0 being normal) validated Scale for the Assessment and Rating of Ataxia (SARA).[8,9]

In family B, of French origin (see Fig 1B), including 8 affected individuals, 4 at-risk relatives, and 1 spouse participated in the study. Age at onset ranged from 24 to 51 years. Pathological nucleotide expansions were excluded in SCA1, 2, Machado–Joseph disease/SCA3, 6, 7, 10, 12, 17, 31, 36 and ATXN1 genes, as were mutations in (SCA5, 11, 13, 14, 23, and 28 genes).

Family C, of Ashkenazi Jewish American origin (see Fig 1C), was ascertained through a proband (III-3) with ataxia with onset in her 50s. Samples from her and an affected relative were negative by commercial DNA testing, excluding SCA1, 2, 3, 5, 6, 7, 8, 10, 13, 14, and 17 and DRPLA (Athena Diagnostics, Worcester, MA). Four affected and 3 unaffected subjects (including 1 apparently unaffected obligate carrier), along with 2 spouses participated in the study.

In addition, we screened for mutations in the candidate gene in DNA from the index patients of 105 Chinese and 55 unrelated Japanese families with cerebellar ataxia, in whom mutations in SCA1, SCA2, Machado–Joseph disease/SCA3, SCA6, SCA7, SCA8, SCA12, SCA17, SCA31, and ATXN1 genes had been excluded.

Written informed consent was obtained from all subjects according to study protocols approved by the institutional review boards of Taipei Veterans General Hospital, the Paris-Necker Ethics Committee, University of Michigan, and University of Tokyo. Genomic DNA was isolated from peripheral blood leukocytes following a standard protocol.[10]

Genetic Studies

Linkage Analysis. (Linkage analysis in family A to 1p21-q23 has been previously reported.[5]) In families B and C, genome scans were performed using Illumina (San Diego, CA) LINKAGE_12 microarrays (6,090 single nucleotide polymorphism [SNP] markers). Genotypes were determined using Beadstudio (Illumina) and analyzed with MERLIN 1.0.[11]

In family B, linkage analysis was run under a 0.85 penetrance model with equal allele frequencies, similar recombination fractions between males and females, and a disease frequency of 0.0005.

In family C, linkage analysis (run with 0.85 penetrance due to the presence of an unaffected obligate carrier female in the pedigree) identified ~200Mb regions on 8 different chromosomes with LOD scores between 0 and 2.0, reaching maximal LOD score of 1.97 on chromosome 1. To further narrow the regions, DNA of the most distantly related affected subjects (IV-6 and IV-7; see Fig 1C) was hybridized to Illumina Human660W-Quad high-density SNP BeadChips. PLINK[12] was used to identify chromosomal regions with large (>1,000kb) shared haplotypes.

(EXOME SEQUENCING.) Exomes were captured and enriched using either the Agilent SureSelect Human All Exon 50Mb kit (Agilent Technologies, Santa Clara, CA; families A and B) or the Nimblegen SeqCap EZ v1 (Roche, Indianapolis, IN; family C). The enriched samples were sequenced on the Illumina HiSeq2000 (Illumina) platform.

In families A and C, 2 affected subjects were sequenced, whereas in family B, DNA from 1 affected subject (III-6), the married-in parent (II-7), and 1 unaffected control subject were sequenced (see Fig 1). Only variants in the linked region and shared by both affected subjects sequenced (families A and C) or absent from the 2 controls (family B) were considered. Variants present in dbSNP, the 1000 Genomes Project,[13] the exome variant server (http://evs.gs.washington.edu/EVS/), or previously sequenced individuals without SCA were excluded. Variants were further filtered for those predicted to be functionally damaging, that is, nonsynonymous and splice variants. The details of exome sequencing, variants filtering, and analyses are available in the Supporting information.

Molecular analyses of KCND3

Mutational analysis of exons and their flanking introns of *KCND3* was conducted by polymerase chain reaction followed by direct nucleotide sequence analysis as previously described.[14]

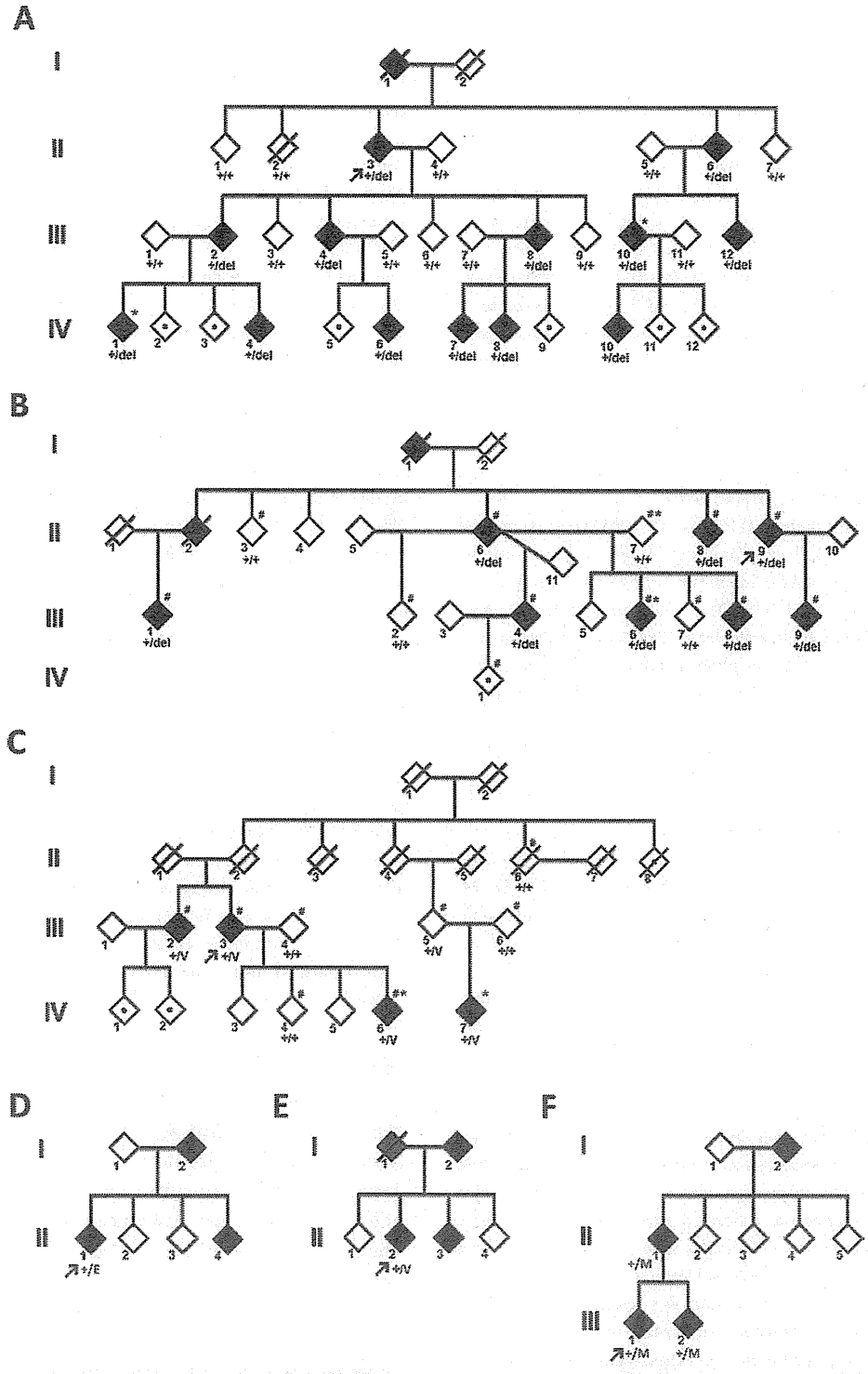

FIGURE 1: Pedigree charts of the families A, B, C, D, E, and F. The gender of the family members is obscured for privacy. The proband is denoted by an arrow. Filled diamonds represent affected members, grayed diamonds represent members with information suggesting but not confirming spinocerebellar ataxia, open diamonds indicate unaffected individuals, and those with a dot within an open diamond denote at-risk individuals. / = deceased; * = members who underwent whole exome sequencing; # = individuals included in linkage analysis; + = wild-type allele; del = allele with F227del; V = allele with G345V; E = allele with V338E; M = allele with T377M.

Expression Plasmids

A human Kv4.3 expression clone pE-11.GFPIre.hKv4.3L.WT was a generous gift of Dr J. M. Nerbonne (Washington University, St. Louis, MO).[15,16] The coding region of *KCND3* was subcloned into the pFLAG-CMV-5a vector (Sigma-Aldrich, St Louis, MO). The mutation c.679_681delTTC was introduced by site-directed mutagenesis using the Quick-Change method (Stratagene, Santa Clara, CA) and verified by bidirectional sequencing. The human Kv channel interacting protein 2 (KChIP2) expression clone was purchased from Open Biosystems (Thermo Scientific, Lafayette, CO). We constructed a plasmid expressing the integral membrane protein myelin protein zero (P0) fused to DsRed to mark the cell surface as previously described.[17] The endoplasmic reticulum (ER) markerp-DesRed-ER was purchased from Clontech (Mountain View, CA). All constructs were verified by sequencing.

Cell Culture and Transfection

Human embryonic kidney (HEK)-293T cells were maintained in high glucose Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37°C under 5% CO_2. Cells were grown on glass coverslips in 6-well multiwell plates. Transient transfection was performed using the calcium phosphate precipitation method.[18] HEK293T cells were transiently transfected with plasmids (in 1:1:1 ratio, 200ng each) expressing Kv4.3 (either wild-type [WT] or the F227del mutant), KChIP2, and either P0-DsRed (to mark the cell surface) or DsRed-ER (to mark the ER).

Immunofluorescence Staining

Forty-eight hours after transfection, cells were fixed in 4% paraformaldehyde for 30 minutes, permeabilized with 0.2% Tween-20 for 30 minutes, and blocked with 1% bovine serum albumin for 30 minutes before incubation in primary antibody mouse anti-Kv4.3 at a dilution of 1:1,000 (ab99045; Abcam, Cambridge, UK) overnight at 4°C. Bound primary antibodies were detected using Alexa 488-conjugated goat antimouse immunoglobulin G (a11001; Invitrogen, Grand Island, NY).

Confocal Imaging

Images were captured with a Zeiss (Thornwood, NY) LSM 5 Pascal Laser Scanning Confocal system mounted on an Axiovert 200M inverted fluorescence microscope with a 63× oil immersion objective. Confocal imaging was performed on expression studies from 6 independent transfections. All images were processed and analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

The midlevel optical section was selected for quantification of the surface expression of Kv4.3, with P0 staining to define the plasma membrane. A blank region was selected to calculate the average pixel intensity as the background, which was subtracted from all images. In the channel of the cell surface marker P0 signal, 20% of the maximum pixel intensity was set as the threshold to define the cell contour. Surface expression of Kv4.3 was calculated as the amount of Kv4.3 signal in the plasma membrane region normalized by the total signal intensity inside the cell contour.

Electrophysiological Recordings

HEK-293T cells were transfected with either WT or p.F227del Kv4.3 cloned into a Green fluorescence protein plasmid (pGFP) Ire vector, together with KChIP2 in a 1:1 ratio. Cells transfected with the vectors alone plus KChIP2 were used as controls. GFP-positive HEK-293T cells were used for electrophysiological recordings 48 hours after transfection. HEK cells were transferred to bath solution containing (in millimolars) NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2.2, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) 10, and glucose 5; pH was adjusted to 7.3 with HCl. Transfected cells were visually selected by green fluorescence expression for recording under bright-field optics (BX51WI; Olympus, Tokyo, Japan). Patch pipettes (2–5MΩ) were pulled from borosilicate glass capillaries (outer diameter, 1.5mm; inner diameter, 0.86mm; Harvard Apparatus, Holliston, MA), heat-polished, and then filled with internal solution containing (in millimolars) K-gluconate 120, KCl 24, ethyleneglycoltetraacetic acid 0.2, and HEPES 10; pH was adjusted to 7.3 with KOH. Using a Multiclamp 700B amplifier (Molecular Devices, Union City, CA), whole cell patch recordings were made at 22 to 24°C. Pipette capacitance was compensated in cell-attached configuration, and patched cells were held in the voltage-clamp configuration at −90mV with series resistance (R_S) compensation (~80–90%, lag, ~0.5 milliseconds; R_S before compensation, 10–25MΩ). Potassium currents were evoked by voltage pulses (−80 to +70mV, 500 milliseconds; 10mV increments). Leakage and capacitive currents were subtracted using a P over −4 procedure. Membrane capacitances were determined from readout values (6–60pF) of membrane capacitance compensation on the patch amplifier. Potassium equilibrium potential (−86mV) was determined by the Nernst equation. Signals were low-pass filtered at 4kHz (4-pole Bessel) and sampled at 10kHz using the Digidata 1440 interface (Molecular Devices). Data acquisition was performed using the pClamp 10.2 software (Molecular Devices).

Results

Linkage Identifies SCA19/22 on 1p21-q23 as a Common Dominant Ataxia Locus

Families B and C with dominant adult onset ataxia were ascertained in France and in the USA in efforts to identify novel ataxia loci. Genome-wide low-density SNP chips followed by linkage analysis were used to identify candidate chromosomal regions. In family B, putative or uninformative linkage to 11 candidate regions was considered, including 6 regions reaching the maximal expected value of LOD score of 2.8 according to the pedigree structure (chromosomes 1, 2, 8, 9, and 14). In family C, high-density SNP chips and PLINK were used to identify shared haplotypes between the most distant relatives. Only 2 large shared haplotypes matched the linkage peaks, a 62Mb-long haplotype on chromosome 1 and a 33Mb haplotype on chromosome 15. The shared

haplotype with the highest LOD score on chromosome 1 overlapped the SCA22 region.

Exome Sequencing Identifies KCND3 Mutations in Families with Dominant Ataxia

In family A, after analysis and filtering, 11 heterozygous coding variants that mapped to 1p21-q23 were shared by both affected subjects and were not present in thousands of control samples in various databases (Supplementary Table 1). Sanger sequencing of these variants in the remaining 23 family members identified only 1 variant, c.679_681delTTC in *KCND3*, that completely segregated with the disease phenotype (Fig 2A). This mutation was not found in 500 normal Taiwanese-Chinese controls.

In family B, of 1,648 nucleotide variants present in the affected subject but absent in her married-in parent and the control, only 3 variants were heterozygous, absent, or rare in the Exome Variant Server and predicted to be damaging (Supplementary Table 2). Among these variants, c.679_681delTTC p.F227del in *KCND3* (see Fig 2B) was the strongest candidate, because mutations in the 2 other genes were known to have non-neurological phenotypes (detailed in the Supporting Information). This mutation cosegregated with the disease in the family and was absent from 152 French control chromosomes and in public database.

In family C, exome sequencing identified a large number of shared variants between the 2 affected individuals. After filtering by linkage and shared haplotypes, variants and that were predicted to be damaging, only 1 passed all filters, c.1304G>T p.G345V in *KCND3* (see Fig 2C; Supplementary Table 3). Conventional sequencing confirmed segregation in the family.

Mutation Screening in Chinese and Japanese Families with Hereditary Spinocerebellar Ataxias

No mutation in *KCND3* was found from any index patient of 105 Chinese families with hereditary ataxia.

Three missense mutations in *KCND3* were identified in 3 families among 55 Japanese families with ataxia (see Fig 1D): c.1013T>C p.V338E in exon 1 was found in family D, c.1034G>T p.G345V in exon 1 in family E, and c.1130C>T p.T377M in exon 2 in family F. None of these mutations was present in 96 Japanese controls or public databases.

SCA22-Associated Mutations Alter Highly Conserved Amino Acid Residues

All of the ataxia-associated mutations affect amino acids in Kv4.3 that are highly conserved across a wide variety of species, from zebrafish, frog, platypus, and mouse to humans (see Fig 2E). In silico analysis predicted deleterious consequences from the residue changes (Supplementary 4).

Clinical Features of Patients with KCND3 Mutations

Characteristically, the patients in the families with SCA19/22 all have a very slowly progressive cerebellar ataxia. In family A (Supplementary Table 5), II-3 has had difficulty walking for 35 years, with an onset at 46 years, and still manages to ride a 3-wheel motorcycle. III-2, at the age of 57 years (25 years after the onset of disease), has had a very slow progression of ataxia, with an average deterioration of only 0.3 SARA score point per year over the past 5 years. III-8 has been mildly ataxic for 31 years and yet only has a SARA score of 9 points at the age of 48 years. There has been no cognitive impairment, myoclonus, tremors, focal weakness, sensory loss, cogwheel rigidity, visual impairment, retinopathy, or ophthalmoplegia in any of the affected members. Brain magnetic resonance imaging (MRI) featured mild cerebellar atrophy (Supplementary Fig, A). Their electrocardiograms showed sinus rhythm with normal QT intervals. There was no arrhythmia on the 24-hour Holter monitor recordings. The echocardiograms were also unremarkable.

In family B (Supplementary Table 6), age at onset ranged between 24 and 51 years, and most patients have been seen at least twice to evaluate disease evolution. Progression was slow, as reflected by only 1 wheelchair user after 43 years of disease duration. Cerebellar ataxia was associated with impaired vibration sense at the ankles (3 of 8), with upward ophthalmoplegia (1 of 8) or with diplopia (1 of 8). Hyper-reflexes without positive Babinski sign was present in 3 of 8. Mild cogwheel rigidity was noticed in 2 patients at ages 61 and 77 years. In the absence of pyramidal involvement, urinary urgency/incontinence was seen in 5 of 8. Cerebellar atrophy was present in 5 patients with cerebral MRI (Supplementary Fig, B). The index case had sensory neuropathy.

In family C, the proband (III-3) is tripping and falling slightly more frequently now (once or twice each month), 10 years after the beginning of imbalance and slurred speech at the age of 55 years, which have only slightly progressed in the absence of any visual problem or dysphagia. Neurological examination revealed breakdown of the smooth pursuit with saccades, mild dysarthria, difficulty with heel-to-shin test, mildly wide-based gait, and difficulty with tandem walk. One of the 4 children, 1 of the siblings, and 1 of the first cousins also have had imbalance (Supplementary Table 7). Brain MRI revealed cerebellar vermian atrophy.

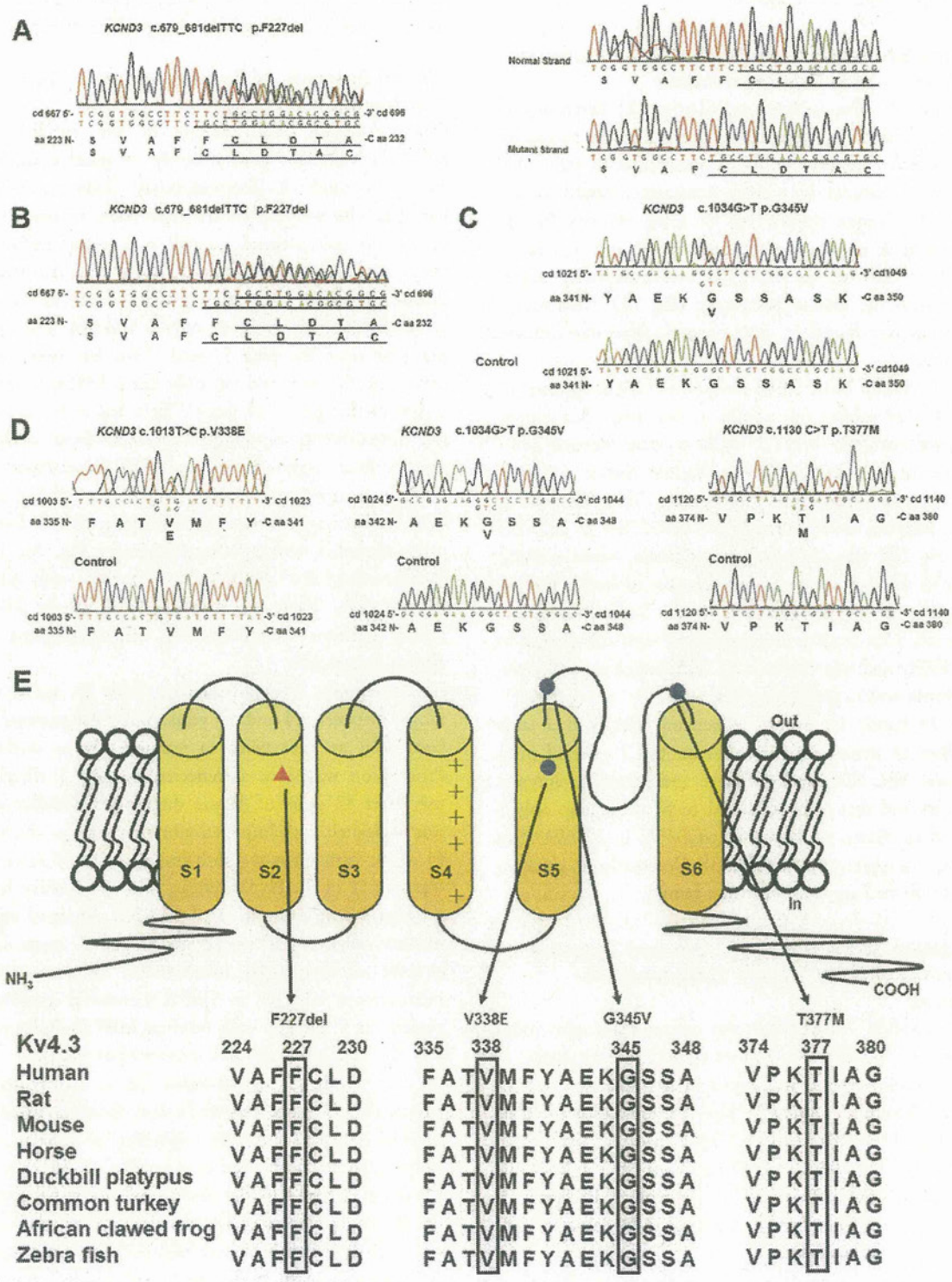

FIGURE 2: *KCND3* mutations and Kv4.3 membrane topology. (A–D) The electropherograms of *KCND3* heterozygous mutations. (E) Predicted Kv4.3 topology and locations of 4 mutations: red triangle, c.679_681delTTC (p.F227del); blue circles, c.1013T>C p.V338E, c.1034G>T p.G345V, and c.1130C>T p.T377M. The mutated residues are evolutionarily conserved, as shown by aligning protein sequences of Kv4.3 orthologs in various organisms.

In families D, E, and F, the age at onset was late, and the clinical progression of ataxia was also slow in the majority of the affected (Supplementary 8).

Immunofluorescence Studies

The immunofluorescence staining pattern of HEK-293T cells expressing either WT or p.F227del mutant Kv4.3 was characterized to address the question of channel protein localization. Cells expressing WT Kv4.3 displayed robust cell surface Kv4.3-specific staining, whereas virtually all cells expressing the mutant Kv4.3 demonstrated diffuse cytoplasmic Kv4.3-specific staining without discernible cell surface signal, suggesting impaired plasma membrane targeting of the mutant F227del Kv4.3 protein, which appeared to be abnormally retained in the cytoplasm and colocalized with an ER-specific marker (Fig 3). We observed no evidence of Kv4.3 in the mock-transfected cells. The expression of the cell surface marker P0 enabled us to define the cell membrane and quantify the subcellular localization of Kv4.3 using an unbiased approach. The ratio of cell surface expression for the p.F227del Kv4.3 was significantly lower than that observed in the WT Kv4.3 ($n = 10$; mean $\pm$ standard error of the mean, 0.28 ± 0.04 vs 0.61 ± 0.06; $p = 0.0014$).

Electrophysiological Recordings

In whole cell voltage-clamp recording on transfected HEK-293T cells, potassium currents were evoked by voltage pulses. In the control cells (KChIP2 + GFP), HEK-293T cells exhibited endogenous noninactivating outward currents (Fig 4). In contrast, large transient outward currents were observed in the WT Kv4.3-transfected HEK293T cells (WT Kv4.3 + KChIP2 + GFP). The outward currents recorded from p.F227del Kv4.3-transfected HEK293T cells (p.F227del + KChIP2 + GFP) were comparable to the endogenous potassium currents recorded from mock-transfected cells. After normalizing the current amplitude for cell size, WT Kv4.3-transfected cells had a significantly higher current density compared to p.F227del-transfected cells (WT: 0.52 ± 0.14 Nano-Ampere/pico-Faraday (nA/pF), $n = 8$; p.F227del: 0.09 ± 0.02 nA/pF, $n = 7$; $p < 0.0005$, Mann–Whitney test). The difference in current densities between p.F227del-transfected and control cells (p.F227del: 0.09 ± 0.02 nA/pF, $n = 17$; control: 0.09 ± 0.01 nA/pF, $n = 3$; $p = 0.67$, Mann–Whitney test) was not statistically significant. Given that the cell surface expression level of p.F227del Kv4.3 was minimal, the finding of low current densities by electrophysiological recordings is consistent with a notion of a defect in channel expression.

Discussions

Using exome sequencing and mutational analyses, we identified in 6 unrelated families of diverse ethnic origins with autosomal dominant cerebellar ataxia heterozygous mutations in the voltage-gated potassium channel Kv4.3-encoding *KCND3* gene. A 3-nucleotide in-frame deletion leading to p.F227del cosegregated with ataxia in family A of Han Chinese origin and family B of French origin. A missense mutation leading to p.G345V was found in family C of Ashkenazi Jewish origin and family E of Japanese origin. Two other missense mutations leading to p.V338E and p.T377M were identified in 2 other Japanese families. All of the amino acids involved are evolutionarily conserved in all vertebrates. The mutations were not observed in the Exome Variant Server, 10 Chinese and 32 French non-SCA subjects, or 1,248 chromosomes of French, Chinese, or Japanese controls. Our findings provide strong genetic support for *KCND3* being the causative gene in SCA19/22.

Kv4.3 is highly expressed in the brain, in particular in the cerebellar Purkinje cells, granule cells, basket cells, stellate cells, a subset of γ-aminobutyric acidergic deep neurons, and Lugaro cells adjacent to the somata of Purkinje cells.[19–23] Kv4.3 may play an important role in the development of cerebellum.[23,24] Of note, Kv4.3 is also expressed in the heart, and rare missense mutations in the cytoplasmic C-terminus have been implicated in Brugada syndrome,[25] which is a hereditary condition with cardiac arrhythmia predisposed to sudden cardiac death. There was no mention of neurological disorders in these patients. None of the patients in this report had cardiac symptoms or signs.

KCND3 encodes Kv4.3, an alpha subunit of the *Shal* family of the A-type voltage-gated K^+ channels which are important in membrane repolarization[19] in excitable cells. Similar to other voltage-gated K^+ channels, Kv4.3 forms homo- or heterotetramers with members of the *Shal* subfamily channels. Each alpha subunit has 6 transmembrane segments (S1–S6) and a re-entrant loop linking S5 and S6 (see Fig 2D). S1 through S4 form the voltage sensing domain, whereas S5, S6, and the re-entrant loop form the ion selective pore. F227 resides in S2 and is conserved between Kv4 and Kv1 of the *Shaker* family, which, similar to *Shal*, also carries a voltage-dependent potassium current. F227 is equivalent to F223 in Kv1.2 and F280 in *Shaker*. Functionally, F227 has been suggested to be a high-impact residue that indirectly coordinates the omega pathway, which is formed by S1–S3 helices and S4 movement in response to membrane voltage changes.[26,27] Thus, p.F227del may lead to a shift in the downstream residues in S2 lining the ion permeation pathway to interfere with the normal movement

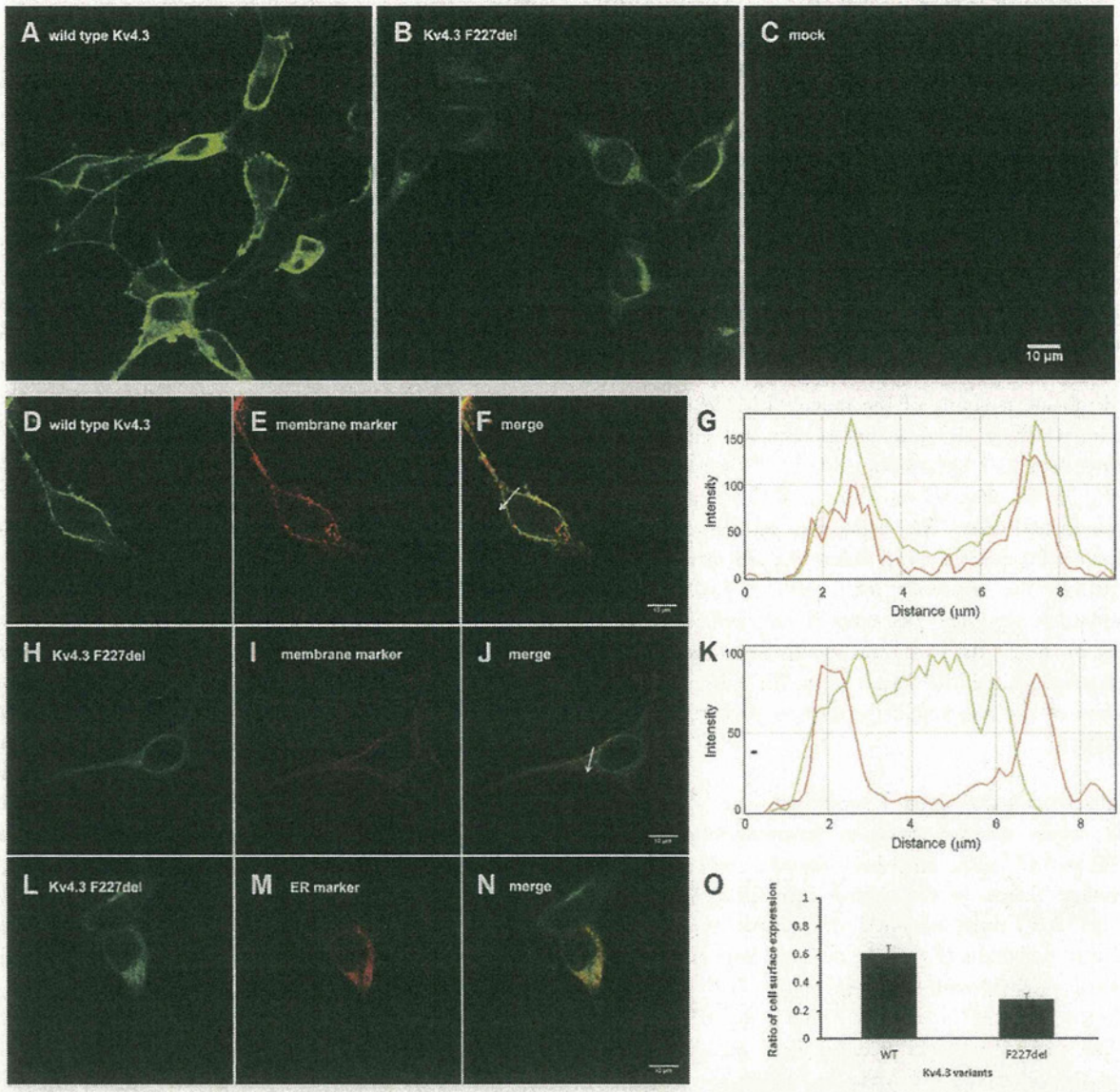

FIGURE 3: Confocal images demonstrating impaired cell surface expression and cytoplasmic retention of the mutant F227del Kv4.3. HEK-293T cells were transiently transfected with wild-type (WT; A) or p.F227del mutant (B) human Kv4.3, or empty vector (C) and immunostained with Kv4.3-specific antibody and green fluorophore-labeled secondary antibodies. Green fluorophore-labeled WT Kv4.3 (D) was expressed in the plasma membrane (E), colocalizing with DsRed-labeled membrane protein P0 (F). The spatial profile of fluorescent intensity of WT Kv4.3 (green) and P0 (red) along the arrow imposed on the images is shown in (G). The x-axis displays the distance relative to the start point of the arrow, and the y-axis displays the fluorescence intensity. F227del Kv4.3 (H) was deficient in targeting to the plasma membrane (I, J). The spatial profile of fluorescent intensity of F227del Kv4.3 (green) and P0 (red) along the arrow imposed on the images is shown in (K). Instead, F227del Kv4.3 was retained in the cytoplasm (L) and colocalized with an endoplasmic reticulum-specific marker (M, N). The ratio of cell surface expression for the p.F227del Kv4.3 was significantly lower (O) than that observed in the WT Kv4.3 (mean ± standard error of the mean, p.F227del: 0.28 ± 0.04, n = 10; WT Kv4.3: 0.61 ± 0.06, n = 10; $p = 0.0014$, paired t test). Scale bar = 10μm.

of S4. Empirically, we were surprised not to be able to detect any channel activity or changes in the biophysical properties of the channel (see Fig 4). Instead, we observed intracellular retention, suggesting that p.F227del causes a loss of channel function by interfering with proper plasma membrane targeting and incorporation into a functional tetrameric channel complex (see Fig 3).

V338 resides in S5, and both G345 and T377 are in the putative outer vestibule of the channel between transmembrane segments S5 and S6. Efforts are underway to continue to characterize the functional consequences of mutations in *KCND3*.

Following *KCNA1* (Kv1.1, NM_000217) and *KCNC3* (Kv3.3, NM_004977), *KCND3* (Kv4.3,