Table 3. Contribution Ratios of Each Element for Variance among the Patients with K-ALS, the Oshima Residents (O-resident) and the Controls

	Group	Mean	S.D.	p	Contribution ratio
Ca (mg/dL)	Control	9.38	0.34	< 0.01	0.05
	O-resident	9.25	0.35		
	K-ALS	8.94	0.32		
ionized Ca (mEq/L)	Control	2.72	0.12	< 0.01	0.19
	O-resident	2.7	0.12		
	K-ALS	2.46	0.03		
inorganic phosphorus (mg/dL)	Control	3.28	0.48	< 0.01	0.08
	O-resident	3.61	0.47		
	K-ALS	3.38	0.73		
intact PTH (pg/mL)	Control	34.08	15.42	< 0.01	0.16
	O-resident	49.48	19.13		
	K-ALS	36.71	9.32		
Cu (mg/dL)	Control	114.88	22.64	0.07	0.02
	O-resident	113.16	21.6		
	K-ALS	130.67	41.79		
Zn (mg/dL)	Control	105.02	19.92	< 0.01	0.60
	O-resident	66.23	10		
	K-ALS	56.33	16.12		
Cu/Zn	Control	1.14	0.34	< 0.01	0.23
	O-resident	1.74	0.47		
	K-ALS	2.68	1.7		
8-OHdG (ng/mL) (without smoking habit)	Control	6.24	3.03	< 0.01	0.19
	O-resident	11.19	8.6		
	K-ALS	19.62	8.4		
8-OHDdG (ng/mg creatinine) (without smoking habit)	Control	12.32	4.48	< 0.01	0.58
	O-resident	13.1	4.4		
	K-ALS	61.53	40.26		
Fe (mg/dL)	Control	104	41.43	< 0.01	0.06
	O-resident	84.84	29		
	K-ALS	57.67	44.93		
Albumin (mg/dL)	Control	4.25	0.27	< 0.01	0.10
	O-resident	4.19	0.26		
	K-ALS	3.71	0.58		

Table 4. Correlation Coefficients between Elements among the Patients with K-ALS and Residents Free from ALS

	serum Zn		8-OHdG/creatinine	
	K-ALS	Residents	K-ALS	Residents
	r	r	r	r
serum Ca	0.72 *	0.24 **	-0.46	-0.15
serum ionized Ca	0.58	0.14	-0.21	-0.14
serum Zn			-0.51	-0.13
serum Cu	-0.61 #	-0.04	0.64	0.32 **
intact PTH	0.57	-0.24 *	-0.74 #	0.11
inorganic P	0.67 #	-0.27 **	-0.28	0.14
Fe	0.76 #	0.31 **	-0.42	0.04
albumin	0.48	0.42 **	-0.66	-0.02
age	0.42	-0.179 *	0.029	0.095
clinical duration	-0.002		-0.103	
urinary 8-OHdG	-0.11	-0.25 *	-0.32	0.54 **
8-OHdG/creatinine	-0.51	-0.1		

#: $p < 0.05$ (one-side test), *: $p < 0.05$, **: $p < 0.01$.

area than in the controls. Increases in the levels of urinary 8-OHdG have been reported in patients with ALS/PDC (parkinsonism-dementia complex) in Hohara, another high-incidence area of ALS/PDC in the Kii peninsula (21), patients with spinal and bulbar muscular atrophy (22) and patients with Parkinson's disease (23). In the future, it is necessary to study whether increases in the levels of 8-OHdG and 8-OHdG/creatinine are specific to ALS patients in this area.

The Cu/Zn ratio is also regarded to be an oxidative stress marker (13, 14), and competition between Zn and Cu for absorption sites in the gut and an antagonistic correlation between Zn and Cu have been previously observed in healthy subjects and patients with various diseases (24, 25). Zn deficiency has been reported to affect DNA damage and DNA repair (26), decrease the antioxidant defense system and increase oxidative stress in the erythrocytes of rats (27). Marginal Zn deficiency impairs Ca utilization in rats (28), and coupled Ca and Zn dyshomeostasis increases oxidative stress in rat cardiac myocytes (29). Significant increases in the Cu ion levels have been found in the spinal cord in a mouse model of ALS. In addition, Cu-chelating drugs extended the lifespan of mice, while Cu nanoparticles easily enter the brain and exert heavy metal-induced neurotoxicity in experimental animals (30, 31). Interactions among Ca, Zn, Cu and other trace metals are important for mineral homeostasis (32), and imbalances among trace metals are toxic, leading to neuronal apoptosis (33).

In the present study, low serum Zn and Ca levels, high Cu/Zn ratios and high urinary 8-OHdG levels were commonly found in the patients with ALS in the K area and the Oshima residents. The serum Zn levels were negatively correlated with the serum Cu levels in the patients with ALS,

while the serum Cu levels exhibited a tendency towards a positive correlation with the urinary 8-OHdG/creatinine levels in both the patients with ALS and the residents free from ALS. Taken together, the relative Cu excess compared to the Zn levels may be associated with a risk of increasing oxidative stress in the patients with K-ALS and also possibly in the Oshima residents (13, 14), although the mechanism is unclear. It has been suggested that severely low levels of Ca and Zn in the river and drinking water are an environmental characteristic of this area that may have some sort of association with the lower levels of serum Ca and Zn and the higher oxidative stress markers observed in the residents and patients with ALS in the K area compared to that observed in the controls, although the causal relationship is unclear. Whether these environmental characteristics play a role in susceptible subjects with possible genetic vulnerabilities to ALS in this area (34-36) should be investigated in future prospective studies.

The authors state that they have no Conflict of Interest (COI).

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Frequency of the *C9orf72* hexanucleotide repeat expansion in patients with amyotrophic lateral sclerosis and frontotemporal dementia: a cross-sectional study

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Summary

Background We aimed to accurately estimate the frequency of a hexanucleotide repeat expansion in *C9orf72* that has been associated with a large proportion of cases of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).

Methods We screened 4448 patients diagnosed with ALS (El Escorial criteria) and 1425 patients with FTD (Lund-Manchester criteria) from 17 regions worldwide for the GGGGCC hexanucleotide expansion using a repeat-primed PCR assay. We assessed familial disease status on the basis of self-reported family history of similar neurodegenerative diseases at the time of sample collection. We compared haplotype data for 262 patients carrying the expansion with the known Finnish founder risk haplotype across the chromosomal locus. We calculated age-related penetrance using the Kaplan-Meier method with data for 603 individuals with the expansion.

Findings In patients with sporadic ALS, we identified the repeat expansion in 236 (7·0%) of 3377 white individuals from the USA, Europe, and Australia, two (4·1%) of 49 black individuals from the USA, and six (8·3%) of 72 Hispanic individuals from the USA. The mutation was present in 217 (39·3%) of 552 white individuals with familial ALS from Europe and the USA. 59 (6·0%) of 981 white Europeans with sporadic FTD had the mutation, as did 99 (24·8%) of 400 white Europeans with familial FTD. Data for other ethnic groups were sparse, but we identified one Asian patient with familial ALS (from 20 assessed) and two with familial FTD (from three assessed) who carried the mutation. The mutation was not carried by the three Native Americans or 360 patients from Asia or the Pacific Islands with sporadic ALS who were tested, or by 41 Asian patients with sporadic FTD. All patients with the repeat expansion had (partly or fully) the founder haplotype, suggesting a one-off expansion occurring about 1500 years ago. The pathogenic expansion was non-penetrant in individuals younger than 35 years, 50% penetrant by 58 years, and almost fully penetrant by 80 years.

Interpretation A common Mendelian genetic lesion in *C9orf72* is implicated in many cases of sporadic and familial ALS and FTD. Testing for this pathogenic expansion should be considered in the management and genetic counselling of patients with these fatal neurodegenerative diseases.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterised by rapidly progressive paralysis and death from respiratory failure, typically within 3 years of symptom onset. The disease is inherited in about 5% of cases, following a clear Mendelian pattern, whereas most cases are classified as sporadic because they seem to arise at random.[1] Substantial progress has been made in understanding the genetic underpinnings of familial ALS.[2] By contrast, the causes of sporadic or idiopathic ALS are far less well understood. Mutations in the known familial ALS genes—*SOD1*, *FUS*, and *TDP-43*—occur only rarely in sporadic cases (each accounting for less than 1·0% of cases);[3–5] genome-wide association studies have identified few risk loci, and these have proved difficult to replicate.[6]

Frontotemporal dementia (FTD) is a degenerative disorder of the frontal and anterior temporal lobes, and is a common form of dementia affecting individuals younger than 65 years. The syndrome is characterised clinically by initial behavioural disturbances, followed by cognitive decline leading to dementia and death within a median of 7 years from symptom onset. Akin to ALS and other neurodegenerative diseases, a large proportion (~60·0%) of these cases are categorised as sporadic, and the causes of this idiopathic form of disease are largely unknown.[7] A growing consensus

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suggests that ALS and FTD form part of a continuum of neurological diseases that share a common pathological background, consisting of TAR DNA-binding protein 43 (TDP-43)-positive inclusions within the CNS.[8]

We recently reported that a large hexanucleotide repeat expansion located within the non-coding portion of *C9orf72* is the cause of chromosome 9-linked ALS and FTD.[9,10] This genetic lesion accounted for a large proportion (~40·0%) of familial cases of ALS and FTD. The same mutation was present in nearly a quarter of apparently sporadic cases of ALS and FTD in the genetically homogeneous Finnish population, and in 4·1% of sporadic cases of ALS and 3·0% cases of sporadic FTD from the USA. However, these estimates were based on relatively small cohorts drawn from a small number of institutions.

These findings prompted us to aim to estimate the frequency of this *C9orf72* hexanucleotide repeat expansion more accurately, in a large cohort of European and US patients with sporadic ALS and sporadic FTD. We also examined the occurrence of this mutation in diverse non-white populations around the world.

Methods

Participants and study design

In this cross-sectional study, we screened 4448 patients diagnosed with ALS and 1425 patients diagnosed with FTD from 17 distinct regions worldwide. The appendix shows ethnic origin and clinical features of the patients. 3860 patients had sporadic ALS, 1022 had sporadic FTD, 588 had familial ALS, and 403 had familial FTD. Data for 401 Finnish patients with ALS, 233 other Europeans with familial ALS, 75 Finnish patients with FTD, 340 Dutch patients with FTD, and 420 English patients with FTD have been published previously.[10–12] All these cohorts were analysed to provide a comprehensive assessment of the global frequency of the expansion.

Patients with ALS were diagnosed according to the El Escorial criteria,[13] and patients with FTD were diagnosed according to the Lund-Manchester criteria.[14] We classified patients' disease as familial in nature on the basis of a diagnosis of ALS or FTD in any other family member (irrespective of relationship), as reported at the time of sample collection. We based ethnic and racial classification on self-reports from patients at the time of sample collection. Case numbers

	Sporadic ALS			Sporadic FTD		
	n	Carriers	% (95% CI)	n	Carriers	% (95% CI)
Europe*						
Finnish	289	61	21·1% (16·5–26·3)	48	9	18·8% (8·9–32·6)
Swedish	..	..	..	6	0	0% (0·0–45·9)
English	916	62	6·8% (5·2–8·6)	543	31	5·7% (3·9–8·0)
German	421	22	5·2% (3·3–7·8)	..	..	..
Dutch	..	..	..	224	5	2·2% (0·7–5·1)
French	..	..	..	150	14	9·3% (5·2–15·2)
Italian	465	19	4·1% (2·5–6·3)	..	..	..
Sardinian	129	10	7·8% (3·8–13·8)	10	0	0% (0·0–30·8)
Moldovan	3	0	0% (0·0–70·8)	..	..	..
Total (Europe)	2223	174	7·8% (6·7–9·0)	981	59	6·0% (4·6–7·7)
USA						
White	890	48	5·4% (4·0–7·1)	..	..	..
Hispanic	72	6	8·3% (3·1–17·3)	..	..	..
Black	49	2	4·1% (0·5–14·0)	..	..	..
Native American	3	0	0% (0·0–70·8)	..	..	..
Total (USA)	1014	56	5·5% (4·2–7·1)	..	..	..
Rest of the world						
Middle Eastern*	1	0	0% (0·0–97·5)	..	..	..
Indian	31	0	0% (0·0–11·2)	31	0	0% (0·0–11·2)
Asian	238	0	0% (0·0–1·5)	10	0	0% (0·0–30·8)
Pacific Islander/Guam	90	0	0% (0·0–4·0)	..	..	..
Australian*	263	14	5·3% (2·9–8·8)	..	..	..
Overall	3860	244	6·3% (5·6–7·1)	1022	59	5·8% (4·4–7·4)

Data for Finnish (289 with ALS and 48 with FTD), English (333 with FTD), and Dutch (224 with FTD) patients were previously published,[10–12] but are included here to establish global frequencies. ALS=amyotrophic lateral sclerosis. FTD=frontotemporal dementia. *All self-reported as white.

Table 1: **Frequency of the pathogenic GGGGCC hexanucleotide repeat expansion of *C9orf72* in patients diagnosed with sporadic ALS or sporadic FTD classified by region**

	Familial ALS			Familial FTD		
	n	Carriers	% (95% CI)	n	Carriers	% (95% CI)
Europe*						
Finnish	112	52	46·4% (37·0–56·1)	27	13	48·1% (28·7–68·0)
Swedish	..	..	..	1	1	100·0% (2·5–100·0)
English	98	45	45·9% (35·8–56·3)	170	28	16·5% (11·2–22·9)
Irish	1	1	100·0% (2·5–100·0)	..	..	
German	69	15	21·7% (12·7–33·3)	29	4	13·8% (3·9–31·7)
Dutch	..	..	..	116	30	25·9% (18·2–34·8)
French	..	..	..	50	22	44·0% (30·0–58·7)
Italian	90	34	37·8% (27·8–48·6)	..	..	..
Sardinian	19	11	57·9% (33·5–79·7)	7	1	14·3% (0·4–57·9)
Total (Europe)	389	158	40·6% (35·7–45·7)	400	99	24·8% (20·6–29·3)
USA*	163	59	36·2% (28·8–44·1)	..	..	..
Rest of the world						
Middle Eastern*	2	0	0% (0·0–84·2)	..	..	..
Israeli*	14	3	21·4% (4·7–50·8)	..	..	..
Asian	20	1	5·0% (0·1–24·9)	3	2	66·7% (9·4–99·2)
Overall	588	221	37·6% (33·7–41·6)	403	101	25·1% (20·9–29·6)

Data for Finnish (112 with ALS and 27 with FTD), English (87 with FTD), German (41 with ALS), Italian (29 with ALS), US (163 with ALS), and Dutch (116 with FTD) patients were previously published,[10–12] but are included here to establish global frequencies. ALS=amyotrophic lateral sclerosis. FTD=frontotemporal dementia. *All self-reported as white.

Table 2: **Frequency of the pathogenic GGGGCC hexanucleotide repeat expansion of *C9orf72* in patients diagnosed with familial ALS and familial FTD classified by region**

listed for European countries and Australia and the Middle East refer to self-reported white individuals from that region. Italian data are from a population-based cohort that had been collected through the Piemonte ALS Registry, an ongoing population-based epidemiological study of ALS based in northwestern Italy.[15] The remaining cohorts were recruited through medical centres and from repositories in various countries.

We also screened 2585 neurologically healthy control individuals from Australia (213 patients), Finland (478), Germany (309), the Human Gene Diversity Panel (300), mainland Italy (354), Sardinia (87), and the USA (844) for presence of the pathogenic repeat expansion. 1167 of these individuals have been reported elsewhere.[10] None of the control individuals had been diagnosed with ALS, FTD, dementia, or any other neurodegenerative disease. Ethics committees from the respective institutions approved the study, and written informed consent was obtained from all patients and control individuals.

Procedures

We used our previously described[10] repeat-primed PCR assay to screen patients and control individuals for the presence of the chromosome 9p21 GGGGCC hexanucleotide repeat expansion (see appendix for technical details). The assay allows samples to be categorised into those that carry a pathogenic repeat expansion (>30 repeats) and those that carry only wild-type alleles (<20 repeats).

For haplotype analysis, we analysed genome-wide single-nucleotide polymorphism (SNP) data from 262 patients who carried the repeat expansion. We previously reported the identification in the Finnish population of a 42-SNP founder haplotype across the 232 kb block of chromosome 9p21 where the pathogenic hexanucleotide expansion was ultimately established.[16,17] In this study, we used a custom perl software script to compare unphased sample genotype data with the 42-SNP founder risk haplotype.[16]

We estimated mutation ages for all populations separately with the DMLE+ version 2.3 Bayesian linkage disequilibrium gene mapping package.[18] Mutation ages were iterated for 10 000 burn-in iterations and a further 10 000 iterations of the maximum-likelihood model. To obtain generalisable estimates of age of the repeat per population, we used median values of binned estimates passing the α threshold of 0·05 per iteration.

Statistical analysis

We calculated 95% CIs for proportions with the Clopper-Pearson exact method. We estimated penetrance of the GGGGCC hexanucleotide repeat expansion in relation to the patients' age on the basis of data available for 603 mutant-gene carriers with the Kaplan-Meier method using the survival package within R statistical software (version 2.9.0), but substituting patient age at symptom onset for survival time.[19] We assessed differences between groups with the χ^2 test for discrete variables such as sex, family history, and site of onset.

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See Online for appendix

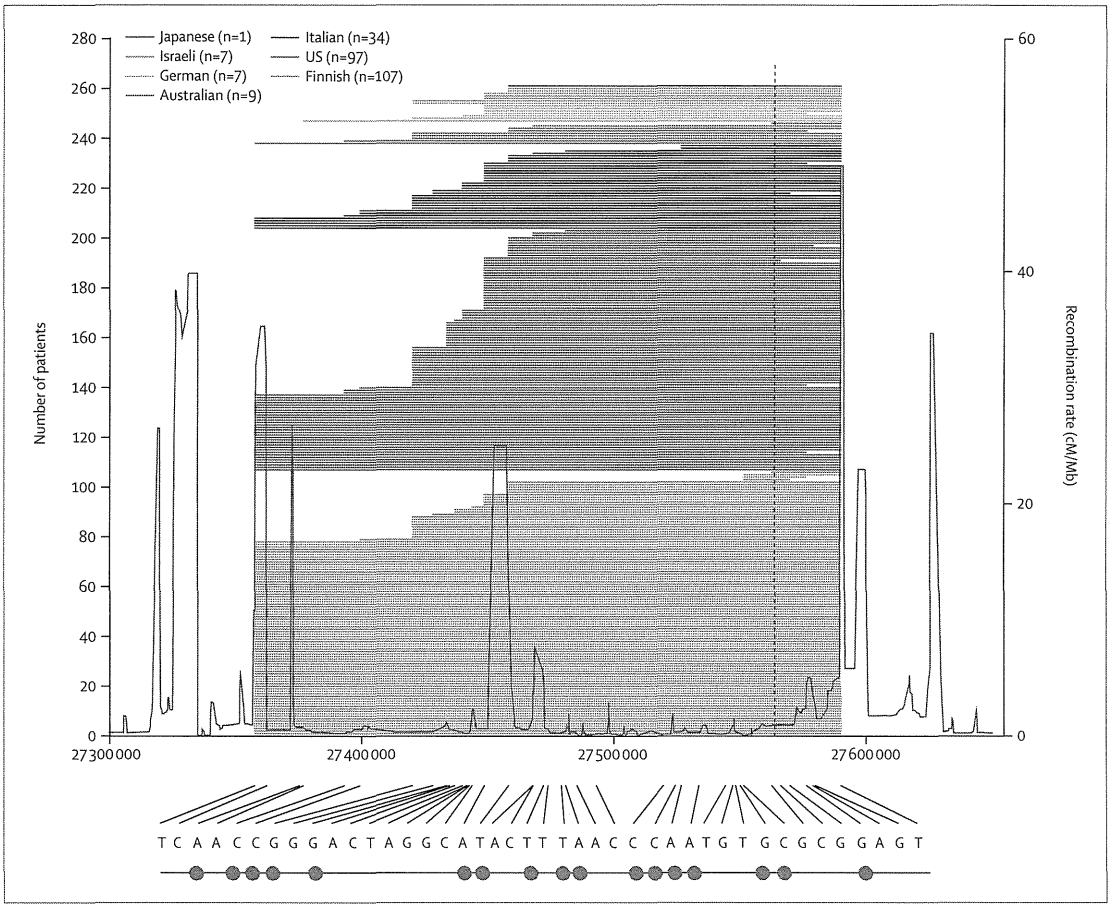

Figure 1: **Finnish risk haplotypes across the chromosome 9p21 region in 262 patients with amyotrophic lateral sclerosis and the *C9orf72* mutation**
The previously identified Finnish risk haplotype is shown below the graph (27 357 278–27 589 746 bp; NCBI build 36; 42 single nucleotide polymorphisms [SNPs]).[16] Underneath the haplotype is a binary representation of the same data, with red circles at SNP positions where the haplotype has the less common allele at that site. In the graph, individual patients are shown as horizontal lines showing the extent to which they share the risk haplotype. The vertical black dashed line shows the location of the *C9orf72* hexanucleotide repeat expansion. Recombination rates (centimorgans per megabase [cM/Mb]) from phase 2 Centre d'Etude du Polymorphisme Humain (CEPH) samples of HapMap are shown with a grey line.

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The sponsors of the study had no role in study design, data collection, analysis, or interpretation, writing of the report, or in the decision to submit the paper for publication. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results
Table 1 and the appendix show the frequency of the *C9orf72* hexanucleotide repeat expansion in patients diagnosed with sporadic ALS and sporadic FTD from different geographical regions. Data for 289 patients with sporadic ALS and 605 with sporadic FTD have been reported elsewhere.[10–12] The pathogenic expansion was identified in 236 (7·0%) of 3377 white patients from the USA, Europe, the Middle East, and Australia, two (4·1%) of 49 black patients from the USA, and six (8·3%) of 72 Hispanic patients from the USA who were diagnosed with sporadic ALS. The rate of the pathogenic expansion was lower in sporadic FTD: 59 (6·0%) of 981 white patients from Europe carried the mutation. By contrast, the GGGGCC repeat expansion was not present in patients of Native American, Asian, or Pacific Islander origin who had sporadic disease (table 1), although this might reflect the smaller size of the cohorts screened in these populations.

In addition to sporadic cases, we screened 588 familial cases of ALS and 403 familial cases of FTD for the presence of the *C9orf72* repeat expansion (table 2, appendix). Of these, 345 patients with familial ALS and 230 with familial FTD have been reported elsewhere.[10–12] Overall, 221 (37·6%) of 588 patients with familial ALS and 101 (25·1%) of 403 patients with familial FTD carried the genetic lesion, reinforcing our previous findings that this mutation was responsible for an

unparalleled proportion of cases of these diseases.[10] We identified one Japanese individual diagnosed with familial ALS who carried the hexanucleotide repeat expansion. We also showed that one patient with familial FTD from Lund, Sweden, carried the expansion, suggesting that the chromosome 9p21 genetic lesion might be responsible for the geographical cluster of patients with FTD noted in that region.[20]

Of 2585 neurologically healthy control samples screened for the *C9orf72* repeat expansion, five (0·2%) were carriers: two were previously reported elderly individuals from Finland,[10] and the other three were individuals younger than 40 years from Germany and the USA (appendix).

Within Europe, the highest mutation frequency was noted in the Finnish population (21·1% of patients with sporadic ALS and 18·8% of patients with sporadic FTD).[10] About 6% of patients with sporadic ALS from Germany and England carried the expansion, whereas Italian patients with ALS had a lower rate (4·1%). 7·8% of patients with sporadic ALS from the genetically isolated island population of Sardinia had the mutation and the Dutch population had the lowest detected rate observed in European countries (2·2% of sporadic cases of FTD). White populations from Australia and the USA had an intermediate rate, with about 5·0% of patients with sporadic ALS carrying the pathogenic repeat expansion, perhaps because of the population and immigration histories of these countries.

Haplotype analysis suggested that every patient carrying the pathogenic GGGGCC repeat expansion also shared the Finnish founder risk haplotype, at least in part (figure 1). Furthermore, patients with sporadic and familial disease carried the same founder risk haplotype. These findings suggest that the pathogenic hexanucleotide repeat expansion in *C9orf72* might have occurred on one occasion in human history and subsequently disseminated throughout these populations. Analysis of haplotype sharing between these cases estimated the age of *C9orf72* repeat expansion to be about 1500 years old (representing a median of 100·5 generations [IQR 57·6–127·6], assuming a generation is 15 years old).

In analysis of age-related penetrance (figure 2), the pathogenic expansion was non-penetrant in carriers who were younger than 35 years of age, increasing to 50% penetrance by 58 years, and to almost full penetrance by 80 years. We noted no difference between disease penetrance according to familial status, ALS or FTD diagnosis, sex, or age of symptom onset in patients with ALS or FTD (appendix).

Table 3 shows clinical details of patients carrying the hexanucleotide repeat expansion. Patients with ALS and the pathogenic repeat expansion were more likely to be female (p=0·0008), have a family history of disease (p<0·0001), and to have bulbar-onset disease (p=0·0011) than were patients who did not carry the expansion. Patients with FTD carrying the repeat expansion were also more likely to have a family history of disease (p<0·0001) and to present with behavioural variant FTD (p<0·0001).

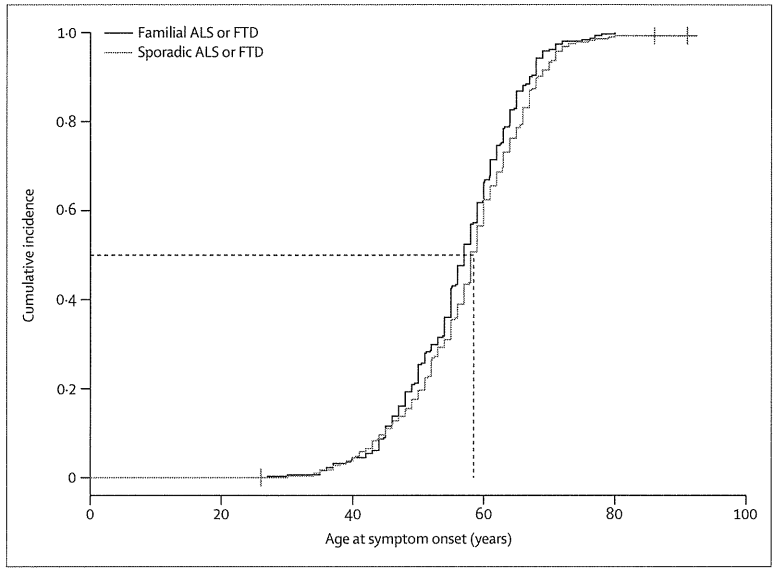

Figure 2: **Age-related penetrance of the GGGGCC hexanucleotide repeat expansion in *C9orf72***
Kaplan-Meier analysis of 603 mutant-gene carriers (212 patients with familial amyotrophic lateral sclerosis, 234 with sporadic amyotrophic lateral sclerosis, 99 with familial frontotemporal dementia, 53 with sporadic frontotemporal dementia, and five neurologically healthy controls). Age-related penetrance (ie, the proportion of mutant-gene carriers with manifestations of the disease by a given age) rose steadily, from 10% in patients younger than 45 years to almost 100% by the age of 80 years. The dotted lines shows the age at which 50% of the cohort developed symptoms. Vertical blue lines show censored events.

	Amyotrophic lateral sclerosis		Frontotemporal dementia	
	With expansion (n=465)*	Without expansion (n=3983)†	With expansion (n=160)‡	Without expansion (n=1265)§
Mean age at onset (range; SD)	56·8 (27·0–80·0; 9·1)	58·7 (4·0–93·0; 12·8)	57·5 (30·0–76·3; 8·3)	60·0 (23·0–87·0; 8·8)
Sex, male	232 (50·1%)	2251 (58·4%)	87 (54·4%)	683 (55·4%)
Positive family history	221 (47·5%)	367 (9·2%)	101 (63·1%)	302 (23·9%)
Presentation				
Bulbar	139 (33·1%)	933 (26·0%)	..	..
Limb	281 (66·9%)	2655 (74·0%)	..	..
Behavioural variant	..	..	106 (85·5%)	685 (65·6%)
Progressive non-fluent aphasia	..	..	11 (8·9%)	165 (15·8%)
Semantic dementia	..	..	7 (5·6%)	195 (18·6%)

Data are mean (range; SD) or n (%). *Data not available for age at onset for 19 patients and site of onset for 45 patients. †Data not available for age at onset for 305 patients, sex for 130 patients, and site of onset for 395 patients. ‡Data not available for age at onset for eight patients and site of onset for 36 patients. §Data not available for age at onset for 71 patients, sex for32 patients, and site of onset for 220 patients.

Table 3:* Demographic and clinical features of patients classified by diagnosis and by carrier status for the GGGGCC hexanucleotide repeat expansion in *C9orf72

Discussion

Our data show that the *C9orf72* hexanucleotide repeat expansion is the most frequent cause of sporadic ALS and sporadic FTD identified thus far, accounting for

Panel: Research in context

Systematic review

We searched Medline up to December, 2011, without language restrictions for relevant publications and selected studies that reported the GGGGCC hexanucleotide repeat expansion in *C9orf72* in pathogenesis of amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD). On the basis of these criteria, seven studies were identified for further assessment (appendix). The number of patients screened for the pathogenic repeat expansion and the phenotype and ethnic origin reported by these studies are summarised in the appendix.

Interpretation

We report the frequency of the *C9orf72* repeat expansion in a large cohort of patients with sporadic ALS and sporadic FTD. We also screened a large number of non-white patients for the expansion, and present frequency data for the mutation in these populations. We confirmed that the *C9orf72* repeat expansion explains a substantial proportion of sporadic ALS (~7·0%) and sporadic FTD (~6·0%) cases in white populations. We also noted that patients with sporadic and familial disease carrying the expansion share a founder risk haplotype, suggesting that these patients have a common ancestor and that the original mutational event that led to the repeat expansion occurred only once in the past. We provide initial estimates of age-related penetrance, showing that 50% of carriers manifest disease by 58 years of age, and that the mutation is fully penetrant by 80 years of age.

about 5·0–7·0% of cases in white Europeans, Americans, and Australians in our large cohort. These frequency rates were slightly higher than were estimates from smaller cohorts obtained at one institution.[9] Before identification of the genetic lesion underlying chromosome 9-linked ALS and FTD, mutations in the *SOD1* gene were the most common known genetic cause of sporadic ALS (accounting for 0·7% of cases in a population-based cohort),[3] whereas mutations in the *PGRN* gene were the most common known cause of sporadic FTD (3·0–4·0% in clinic referral series).[21] The high frequency of the pathogenic expansion in our cohort is consistent with previous genome-wide association studies that identified the association signal on chromosome 9p21 as the only replicable locus in the sporadic form of ALS and FTD.[16,22–24] Our findings confirm the importance of genetics in the pathogenesis of the idiopathic form of these neurodegenerative diseases.

Our haplotype data suggest that the pathogenic GGGGCC hexanucleotide repeat expansion in *C9orf72* arose from a one-off mutation event[16,17] that occurred about 1500 years ago. The geographical distribution of the mutation suggests that the mutation appeared in northern Europe and spread from there. Alternatively, the high frequencies in Finland and other isolated populations could be explained by the history of these communities. Finland and Sardinia are comparatively isolated regions, and have genetically homogeneous populations that originated from a small number of founders.[25] Genetic drift has had a large influence on allele frequencies in these populations and could explain the high occurrence of the mutation in these geographical isolates.

Recognition that all patients carrying the *C9orf72* repeat expansion share a common ancestor has important implications for the interpretation of global frequency data for this mutation. Although the hexanucleotide repeat expansion is common in white Europeans, it is also present in black and Hispanic populations in the USA and individuals from Israel. This finding probably reflects the scale and nature of past human migration and intermarriage between ethnic groups. Similarly, the relative absence of the pathogenic hexanucleotide repeat in India, Asia, and the Pacific Islands might be explained by the greater physical distances of these regions from Europe, and the consequent lack of admixture between populations. Notably, the one Japanese patient who we identified as a carrier of the *C9orf72* expansion carried the Finnish risk haplotype, reinforcing the notion that the expansion occurred on one occasion in the past.

The sharing of a common risk haplotype in the *C9orf72* region of chromosome 9p21 in patients with sporadic and familial ALS suggests that these apparently sporadic cases are actually cryptically related familial cases. This scenario might have occurred for several reasons, including unfamiliarity with the pedigree on the part of the patient or neurologist or because previous generations might have died at a young age before onset of neurological symptoms. The median age at onset in patients with the expansion was 57 years, and life expectancy in the USA began to exceed this point only in the early 1940s.[26] Furthermore, the incomplete penetrance of the mutation, in which not all individuals carrying the expansion manifest a clinical phenotype, might be a contributing factor in apparently sporadic disease. Indeed, we have reported symptom onset in the ninth decade of life in patients carrying the expansion and also encountered two elderly, neurologically healthy individuals with the expansion. Thus, the penetrance of this mutation seems to be complete only at a late stage of life, which is an observation of particular relevance for genetic counselling of healthy individuals carrying the expansion. The molecular biological substrate underlying this variability in age at onset is unclear: it might be driven by differences in expansion lengths between patients, by age-related methylation across the locus, or by genetic factors elsewhere in the genome.

We compared our results with those of previous studies that reported the frequency of the *C9orf72* hexanucleotide repeat expansion in the pathogenesis of ALS and FTD (panel). Data were available from seven studies (appendix). Our study screened one of the largest cohorts of cases of ALS and FTD assessed to date, and also provides an initial report of the frequency of the pathogenic repeat expansion in non-white patients, a detailed examination of the haplotype across the locus, and an initial estimate of age-related disease

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

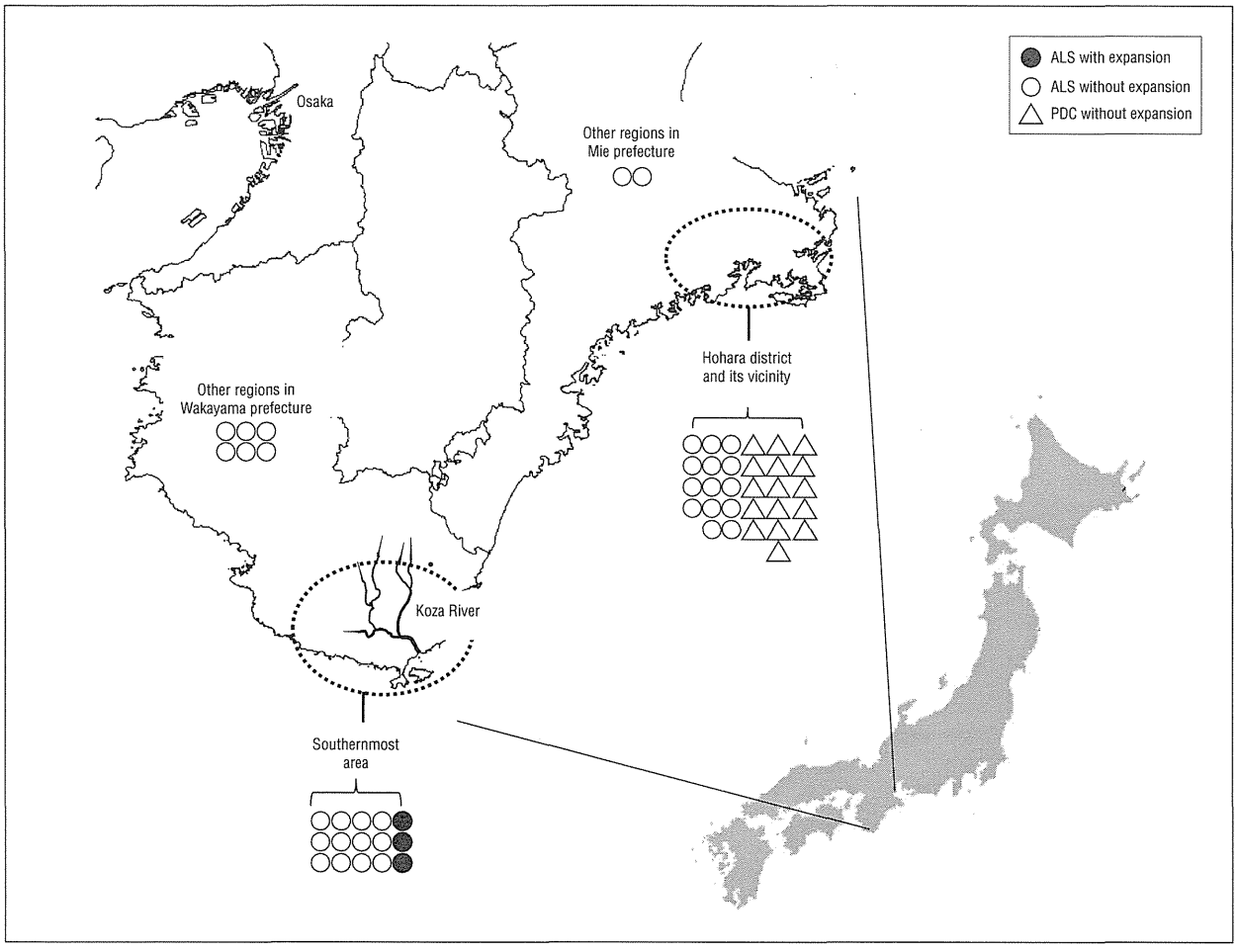

Figure 1. Map of Kii peninsula of Japan and distribution of patients with amyotrophic lateral sclerosis (ALS) and parkinsonism-dementia complex (PDC). The southernmost area neighboring the Koza River (Kozagawa and Kushimoto towns) and the Hohara district and its vicinity (Minamiise town and Shima city) 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]

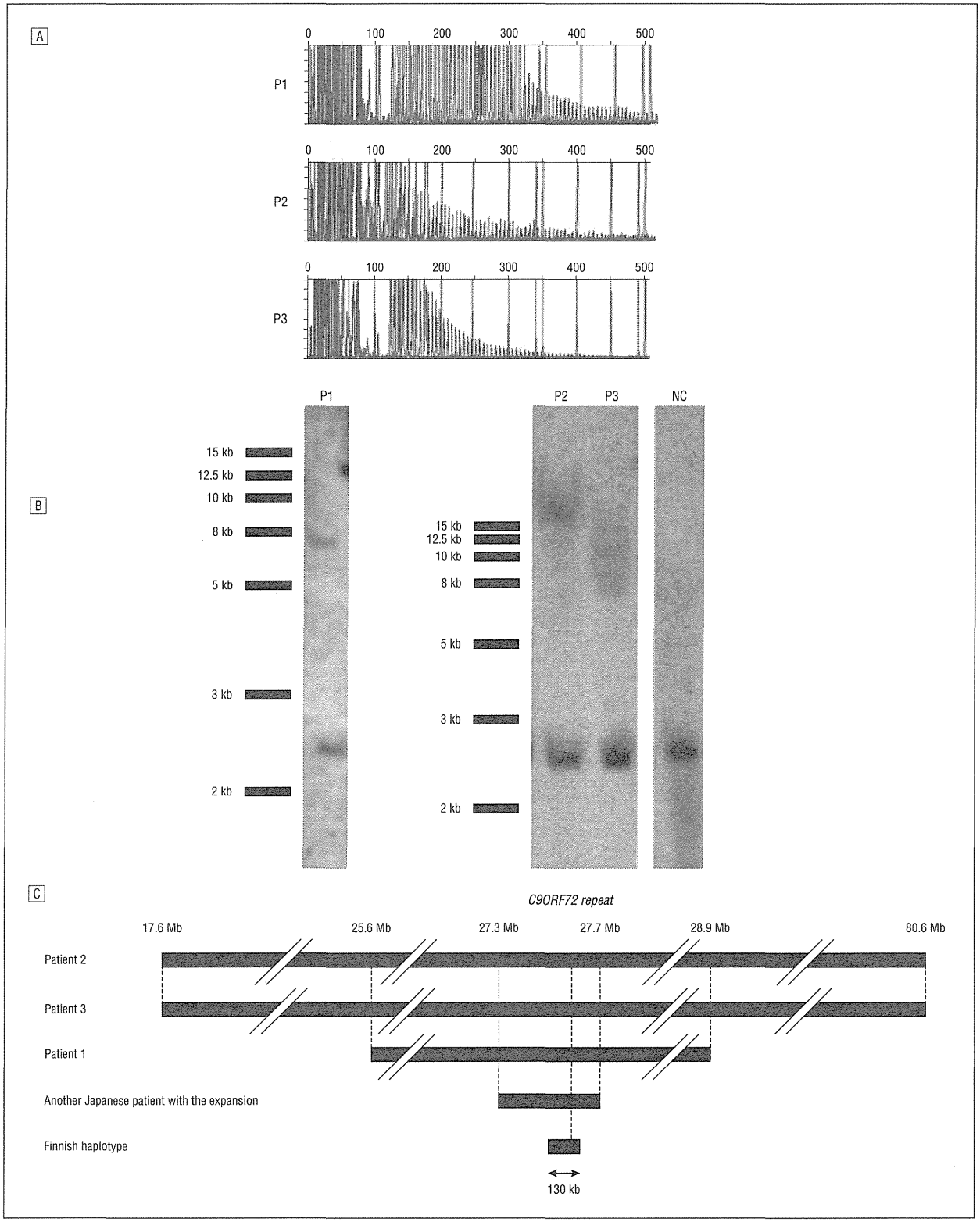

Figure 2. Mutational analyses of hexanucleotide repeat expansion in *C9ORF72*. A, Repeat-primed polymerase chain reaction analysis was performed as previously described.[8] Patients 1-3 show the characteristic sawtooth patterns with a 6-bp periodicity (blue lines). Red lines indicate DNA size markers. 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.

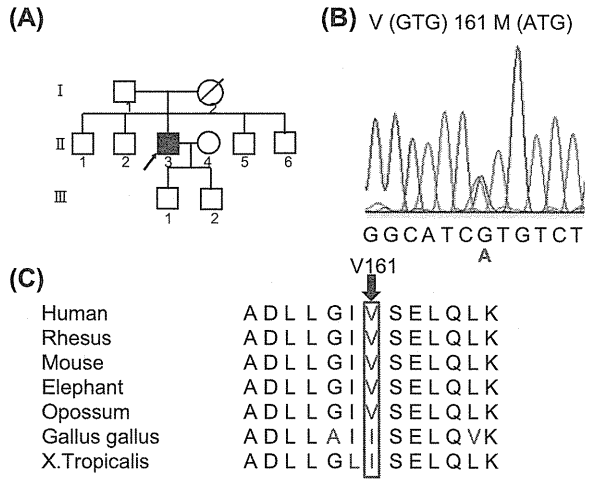

Figure 1. (A) Pedigree chart of patient with V161M variant in *OPTN*. Affected individuals are indicated by filled symbols. The proband is indicated by an arrow. Unaffected individuals are indicated by open symbols. Slashed symbols indicate deceased subjects. Ages at death are shown when information 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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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