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Brief communication

Analysis of *C9orf72* repeat expansion in 563 Japanese patients with amyotrophic lateral sclerosis

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Abstract

Recently, a hexanucleotide repeat expansion in *C9orf72* was identified as the most common cause of both sporadic and familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia in Western populations. We analyzed 563 Japanese patients with ALS (552 sporadic and 11 familial) using fluorescent fragment-length analysis of *C9orf72* and repeat-primed polymerase chain reaction analysis. Haplotype analysis was performed for 42 single nucleotide polymorphisms in patients with *C9orf72* repeat expansion. *C9orf72* repeat expansion was found in 2 patients with sporadic ALS (2/552 = 0.4%) and no patients with familial ALS (0/11 = 0%). In the probands' families, 1 primary progressive aphasia patient and 1 asymptomatic 76-year-old individual exhibited *C9orf72* repeat expansion. All of the patients with the *C9orf72* repeat expansion carried the 20-single nucleotide polymorphism consensus risk haplotype. The frequency of the *C9orf72* repeat expansion among Japanese patients is much lower than in Western populations. The existence of a 76-year-old asymptomatic carrier supported the notion of incomplete penetrance. The *C9orf72* mutation should be analyzed in sporadic ALS patients after determining their family histories not only of frontotemporal dementia but also of primary progressive aphasia.

Keywords: Amyotrophic lateral sclerosis; *C9orf72*; Incomplete penetrance; Sporadic; Aphasia; Frontotemporal dementia

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that primarily affects motor neurons in the spinal cord, brain stem, and cerebral cortex, typically leading to death within a few years. Five to ten percent of ALS cases are familial, and the remaining cases are believed to be sporadic (Valdmanis et al., 2009). A number of genes causing ALS with a dominant mode of inheritance have

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been discovered, such as *SOD1*, *TARDBP*, *FUS*, *VAPB*, *ANG*, *VCP*, *OPTN* (Ticozzi et al., 2011), and *UBQLN2* (Deng et al., 2011). Moreover, there is increasing clinical and pathological evidence for the hypothesis that ALS and frontotemporal dementia (FTD) constitute an overlapping continuum of diseases (Lomen-Hoerth et al., 2002; Neumann et al., 2006). Recently, the expansion of a noncoding GGGGCC hexanucleotide repeat in the *C9orf72* gene has been reported to be a major cause of both ALS and FTD (DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Renton et al., 2011) and the most common genetic abnormality in familial and sporadic forms of both ALS and FTD, particularly in Western populations (Chiò et al., 2012; DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Renton et al., 2011; Sabatelli et al., 2012; Stewart et al., 2012). In the present study, we describe the incidence and demographic and clinical features associated with the *C9orf72* mutation in a large cohort of Japanese ALS patients. We also perform haplotype analysis to investigate whether Japanese patients have the same risk haplotype as European patients (Gijselinck et al., 2012; Laaksovirta et al., 2010; Mok et al., 2012).

2. Methods

2.1. Subjects

We obtained a total of 760 DNA samples from the Japanese Consortium for Amyotrophic Lateral Sclerosis Research (JaCALS; Appendix A). A total of 563 (11 familial and 552 sporadic) patients were diagnosed with ALS according to the El Escorial revised criteria (Brooks et al., 2000) and classified as bulbar-onset, spinal-onset, FTD-ALS, or other (see Supplementary Table 1 for details). We had determined the family histories of ALS but not FTD or primary progressive aphasia (PPA) in all of the patients when they were enrolled as patients with sporadic ALS (SALS). We recruited 197 control subjects, none of whom had a medical or family history of neurodegenerative disorders. The mean age at onset of the patients with ALS was 60.4 ± 11.7 years (range 20–86), and the mean age at sampling of the controls was 60.6 ± 10.3 years (range 26–83). All of the subjects were unrelated Japanese individuals. Written informed consent was obtained from all of the subjects. The ethical committees at the participating institutions approved this study.

2.2. Fluorescent fragment-length analysis of C9orf72 and repeat-primed PCR analysis

The normal repeat number of the GGGGCC hexanucleotide was determined in all of the patients and control subjects using genotyping primers, as previously described (DeJesus-Hernandez et al., 2011). To provide a qualitative assessment of the presence of *C9orf72* repeat expansions, we performed repeat-primed polymerase chain reaction (PCR), as previously described (DeJesus-Hernandez et al., 2011).

2.3. Haplotype analysis

We genotyped 42 single nucleotide polymorphisms (SNPs) across 232 kilobase of Chromosome 9p21, which were first described as the founder haplotype in the Finnish ALS population (Laaksovirta et al., 2010), using primers (Supplementary Table 2) to determine whether our Japanese patients carried the haplotype associated with a risk of ALS. These 42 SNPs included the 20-SNP consensus risk allele that had recently been detected in genome-wide association studies in several populations (Mok et al., 2012). We also performed haplotype analysis with 4 microsatellites (D9S1121, D9S169, D9S270, and D9S104) flanking the *C9orf72* GGGGCC repeat, as previously described (Gijselinck et al., 2012) (Fig. 1).

3. Results

3.1. Detection of C9orf72 repeat expansion

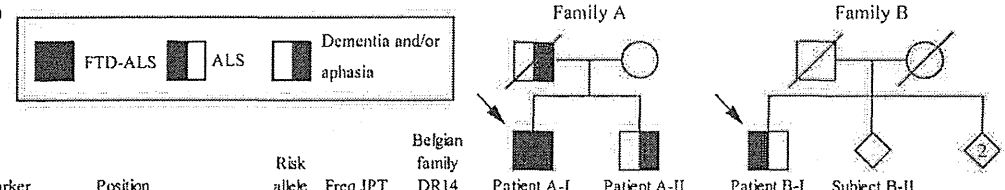
The *C9orf72* repeat expansion was found in 2 of 522 Japanese patients (2/552 = 0.4%) with SALS and none of the 11 patients (0/11 = 0%) with familial ALS (FALS) using repeat-primed PCR (Table 1). Patient A-I with a *C9orf72* mutation was classified as SALS in this study, but after detecting the mutation, we found that patient A-II (a brother of patient A-I) developed aphasia and dementia and had a *C9orf72* mutation (Fig. 1). The average repeat number based on fluorescent fragment-length analysis was 3.65 ± 2.43 (range 2–13 repeats) in 561 ALS patients without the *C9orf72* mutation. A subsequent analysis of 197 healthy controls did not detect any *C9orf72* mutation. The average repeat number was 3.69 ± 2.46 (range 2–14 repeats) in the 197 controls. The mean age at disease onset in patients with *C9orf72* mutation, including patient A-II, was 64.7 ± 6.1 years (range 57–72). The genotypes of all individuals with the *C9orf72* mutation were detected for the 20 SNPs spanning a 140-kilobase segment concordant with the recently identified risk haplotype on chromosome 9p (Mok et al., 2012) and 24 or 25 consecutive SNPs in the 42-SNP Finish risk haplotype (Laaksovirta et al., 2010) (Fig. 1, Supplementary Table 3).

3.2. Clinical presentations of individuals with C9orf72 mutation

3.2.1. Patient A-I (family A)

Patient A-I was a 65-year-old man who reported weakness in the left leg. The weakness progressed, and he developed fasciculation. At age 66, a neurological examination revealed dementia. His Mini Mental State Examination score was 23/30, and his Frontal Assessment Battery score was 13/18. He also exhibited dysarthria and weakness, atrophy, and fasciculation in the tongue and all 4 modalities. His tendon reflexes were diminished, and the plantar re-

A)



B)

Marker	Position	Risk allele	Freq JPT	Belgian family DR14	Patient A-I	Patient A-II	Patient B-I	Subject B-II
rs725804	27458939	A	A=0.081		C/C	C/C	C/C	C/C
rs10511816	27468461	T	T=0.105		T/G	T/G	G/G	G/G
rs1444533	27477874	A	A=0.553		A/A	A/A	A/G	A/G
rs1822723*	27478052	C	C=0.669		C/C	C/C	C/C	C/C
rs4879515*	27482235	T	T=0.645		T/T	T/T	T/T	T/T
rs895023	27483959	T	T=1.000		T/T	T/T	T/T	T/T
rs868856*	27489251	T	T=0.247		T/T	T/T	T/T	T/T
rs7046653*	27490967	A	A=0.250		A/A	A/A	A/A	A/A
rs2440622	27495418	A	A=0.988		A/A	A/A	A/A	A/A
rs1977661 *	27502986	C	C=0.651		C/C	C/C	C/C	C/C
rs903603*	27529316	C	C=0.341		C/C	C/C	C/T	C/T
rs10812610*	27533984	C	A=0.680		C/C	C/C	C/A	C/A
rs2814707*	27536397	A	A=0.058		A/A	A/A	A/G	A/G
rs3849942*	27543281	A	A=0.076		A/A	A/A	A/G	A/G
C9orf72	27546544..27573842			mutation	mutation	mutation	mutation	mutation
rs12349820	27553876	T	T=0.890		T/T	T/T	T/T	T/T
rs10122902*	27556780	G	G=0.738		G/G	G/G	G/G	G/G
rs10757665*	27557919	T	T=0.890		T/T	T/T	T/T	T/T
rs1565948*	27559733	G	G=0.600		G/G	G/G	G/G	G/G
rs774359*	27561049	C	C=0.070		C/C	C/C	C/T	C/T
rs2282241*	27572255	G	G=0.326		G/G	G/G	G/T	G/T
rs1948522*	27575785	C	C=0.837		C/C	C/C	C/C	C/C
rs1982915*	27579560	G	G=0.238		G/G	G/G	G/A	G/A
rs2453556*	27586162	G	G=0.448		G/G	G/G	G/A	G/A
rs702231*	27588731	A	A=0.634		A/A	A/A	A/A	A/A
rs696826*	27589657	G	G=0.983		G/G	G/G	G/G	G/G
rs2477518*	27599746	T	T=0.820		T/T	T/T	T/T	T/T
D9S270	28404862 .. 28404948			327	327/325	327/325	323/323	323/323
D9S104	28771379 .. 28771576			168	158/158	158/158	158/171	158/171

Fig. 1. (A) The pedigrees of the 2 families with *C9orf72* repeat expansion. To maintain confidentiality, several unaffected individuals who died early in families A and B are not shown. Probands are indicated by arrows. (B) The genotyping data of the single nucleotide polymorphisms (SNPs) and microsatellites. Twenty SNPs, which comprised a recently identified consensus risk haplotype (Mok et al., 2012), are shown with an asterisk. See Supplementary Table 3 for details of the analyses of 42 SNPs (Laaksovirta et al., 2010) and microsatellites (Gijselinck et al., 2012). Alleles possibly shared between our subjects and patients in Western populations are shown in bold. The genotypes of all 4 subjects with respect to the 20 SNPs were found to be concordant with the risk haplotype (Mok et al., 2012). All of the positions of SNPs and microsatellites were from NC_000009.11. Abbreviations: ALS, amyotrophic lateral sclerosis; Freq JPT, Frequency in Japanese in Tokyo from International HapMap project (International HapMap Consortium, 2003); FTD, frontotemporal dementia.

sponse was extensor on the left. He had neither dysphagia nor dyspnea. No sensory abnormalities were noted. Extensive screening for causes of motor neuropathy was negative. The diagnosis was clinically probable ALS-laboratory supported (Brooks et al., 2000) and FTD-ALS.

3.2.2. *Patient A-II (family A)*

This patient was a 57-year-old man who presented with difficulty speaking. He was believed to have suffered from a mental disease after being imprisoned because of his involvement in a fatal car accident. At age 64, he was severely dysfluent and could barely speak. Logoclonia was particularly prominent. However, he did not exhibit any violent behavior or other behavioral abnormalities. He also did not display any clinical features of motor neuron disease. Brain magnetic resonance imaging revealed severe frontotemporal lobar atrophy. PPA was considered the most likely diagnosis.

3.2.3. *Patient B-I (family B)*

Patient B-I was a 72-year-old man who presented with gait disturbance and weakness in the proximal lower extremity muscle. His family history was negative for motor neuron disease and dementia (Fig. 1). The muscle weakness and atrophy progressed and spread to the other parts of his body despite treatment with intravenous gamma globulin. At age 74, he could not roll over while sleeping. A neurological examination showed marked muscle atrophy in his arms and shoulders and prominent fasciculation in his legs. The deep tendon reflexes were decreased in his limbs, and he had no pathological reflexes. Sensations in all 4 modalities were intact. At age 75, he developed dyspnea and dysphagia and started noninvasive positive pressure ventilation and intravenous hyperalimentation. He died of respiratory insufficiency at age 76. An autopsy was not performed. The diagnosis was clinically suspected ALS (Brooks et al., 2000).

Table 1
Frequencies of ALS patients with *C9orf72* and *SOD1* mutations in different countries

Study	Population	*C9orf72*			*SOD1*	
		Familial ALS	Sporadic ALS	Mean AAO (range), years	Familial ALS	Sporadic ALS
This study, 2012	Japanese (JaCALS)	0% (0/11)	0.4% (2/552)	64.7 (57–72)	NA	NA
Akimoto et al. (2011)	Japanese (JaCALS)	NA	NA	NA	NA	1.6% (4/255)
DeJesus-Hernandez et al. (2011)	Mixed[a]	23.5% (8/34)	4.1% (8/195)***	54.5 (41–72)	11.8% (4/34)	0% (0/195)
Renton et al. (2011)	Finish	46.4% (52/112)**	21.0% (61/290)***	53 (30–71)	NA	NA
Gijselinck et al. (2012)	Flanders-Belgian	46.7% (7/15)*	4.9% (6/122)***	54.5 (38–64)	0% (0/16)	0% (0/125)
Stewart et al. (2012)	Unknown[b]	27.4% (17/62)	3.6% (6/169)**	58.2 (39–82)	Total 8.2% (19/231)	
Byrne et al. (2012)	Ireland	40.8% (20/49)*	4.9% (19/386)***	56.3 (NA)	Total 0% (0/191)	
Cooper-Knock et al. (2012)	Northern England	42.9% (27/63)*	7.0% (35/500)***	57.3 (27–74)	Total 2.5% (14/563)	
Chiò et al. (2012)	Italian	37.5% (45/120)*	NA	59.0 (NA-80)	0% (0/141)	NA
	Sardinian	57.1% (12/21)**	NA	60.4 (NA)	NA	NA
	German	22.0% (9/41)	NA	56.4 (NA)	NA	NA
Majounie et al. (2012)	England	45.9% (45/98)**	6.8% (62/916)***	NA	NA	NA
	German	21.7% (15/69)	5.2% (22/421)***	NA	NA	NA
	Italian	37.8% (34/90)*	4.1% (19/465)***	NA	NA	NA
	Sardinian	57.9% (11/19)**	7.8% (10/129)***	NA	NA	NA
	USA White	US total 36.2% (59/163)*	5.4% (48/890)***	NA	NA	NA
	USA Hispanic		8.3% (6/72)***	NA	NA	NA
	USA Black		4.1% (2/49)	NA	NA	NA
	Australian	NA	5.3% (14/263)***	NA	NA	NA
	Israeli	21.4% (3/14)	NA	NA	NA	NA
	Indian	NA	0% (0/31)	NA	NA	NA
	Asian	5.0% (1/20)	0% (0/238)	NA	NA	NA
	Pacific islander/Guam	NA	0% (0/90)	NA	NA	NA
Sabatelli et al. (2012)	Italian	NA	3.7% (60/1624)***	58.6 (49–65)	NA	NA
	Sardinian	NA	6.8% (9/133)***	62.9 (58–63)	NA	NA

Key: AAO, age at onset; ALS, amyotrophic lateral sclerosis; JaCALS, Japanese Consortium of Amyotrophic Lateral Sclerosis Research; NA, not available.
[a] Mixed included 229 ALS patients from Mayo Clinic, Florida: White (212), Asian (1), Pacific Islander (1), and Black or African American (15).
[b] Unknown included 231 ALS patients from the ALS Clinic of Vancouver Coastal Health and the University of British Columbia (Vancouver General Hospital and GF Strong Rehabilitation Centre sites).
* $p < 0.05$, compared with our results (2-tailed, Yates's χ^2 test).
** $p < 0.01$, compared with our results (2-tailed, Yates's χ^2 test).
***$p < 0.001$ compared with our results (2-tailed, Yates's χ^2 test).

3.2.4. Subject B-II (family B)

Subject B-II, a sibling of Patient B-I, had a *C9orf72* mutation but did not have symptoms of dementia or motor neuron disease until age 76 (Fig. 1).

4. Discussion

We began this study considering patients without family histories of ALS to be SALS because our cohort included only family histories of ALS but not FTD or PPA. Although it may be difficult to describe the real frequency in SALS because 1 of the SALS patients had a family member who developed PPA, the frequencies of the *C9orf72* mutation in Japanese patients were 0.4% (2/552) in SALS and 0% (0/11) in FALS according to this classification. In contrast, the frequencies of the *C9orf72* mutation fall within the ranges of 21%–57% in FALS and 3%–21% in SALS in Western populations (Table 1), and the *C9orf72* mutation has been reported as the most common genetic cause of FALS and SALS in Western populations (Byrne et al., 2012; Chiò et al., 2012; Cooper-Knock et al., 2012; DeJesus-Hernandez et al., 2011; Gijselinck et al., 2012; Majounie et al., 2012; Renton et al., 2011; Sabatelli et al., 2012; Stewart et al., 2012). However, the *C9orf72* mutation in this study was not more frequent than the *SOD1* mutation in Japanese SALS patients (0.4% and 1.6%, Table 1) (Akimoto et al., 2011). Considering these data, the *C9orf72* mutation is more common than the *SOD1* mutation in Western populations but not in Japan, suggesting different genetic backgrounds. Our results may explain the association study of rs2814707 on 9p21.2, which was reported to be the most significantly associated SNP with SALS in Caucasian but not in Japanese and Chinese populations (Iida et al., 2011). A recent report revealed that the rate of expansion in Asian FALS and SALS was 5% (1/20) and 0% (0/238), respectively (Majounie et al., 2012). An analysis of the SNPs on chromosome 9p revealed that all 4 subjects with the *C9orf72* mutation and another Japanese subject from the previously mentioned report (Majounie et al., 2012) share a shorter region of the risk haplotype

than Western populations. Thus, the haplotype bearing the *C9orf72* mutation was only shared in a narrow region between Western and Asian populations, suggesting that the *C9orf72* mutation may be an old mutation in human migration history from Western to East Asia. This mutation was estimated to be approximately 1500 years old (Majounie et al., 2012).

Bulbar onset and cognitive impairment have been reported to be more common in ALS patients with the *C9orf72* repeat expansion (Chiò et al., 2012; Cooper-Knock et al., 2012; DeJesus-Hernandez et al., 2011; Sabatelli et al., 2012; Stewart et al., 2012). We did not find any patients with bulbar onset, but we identified 2 patients with dementia. Although the age at onset has been known to be lower in SALS patients with the *C9orf72* mutation than in those without this mutation (Sabatelli et al., 2012), our patients exhibited a relatively older age at onset (Table 1).

Although apparently sporadic patients with *C9orf72* mutation have been detected worldwide (Byrne et al., 2012; Cooper-Knock et al., 2012; Sabatelli et al., 2012), it was not known whether this phenomenon was due to incomplete penetrance or to spontaneous expansion of the GGGGCC hexanucleotide repeat from a nonpathogenic parental form (ie, a de novo expansion). In this study, we found a 76-year-old healthy individual with a *C9orf72* mutation (Subject B-II), as described in previous studies (Majounie et al., 2012; Renton et al., 2011). This discovery suggests not de novo expansion but incomplete penetrance, which explains the existence of apparently sporadic patients with the *C9orf72* mutation. Although it has been reported that the penetrance of the *C9orf72* mutation is almost full by 80 years by Kaplan–Meier analysis of 603 mutant gene carriers and 5 neurologically healthy individuals, further studies of family members of patients with the *C9orf72* mutation will be required to calculate the true penetrance and to improve genetic counseling.

Finally, we found a PPA patient with the *C9orf72* mutation after detecting the mutation in a SALS patient, suggesting the importance of collecting information regarding whether SALS patients have a family history of dementia or aphasia. Therefore, the possibility of *C9orf72* mutation should be investigated when clinicians meet with SALS patients after determining their family histories of FTD or PPA. Furthermore, our data supported Byrne and colleagues' suggestion that a family history of FTD should also be included in the revised definition of FALS (Byrne et al., 2012).

Disclosure statement

All of the authors disclose no conflicts of interest. The study was approved by the ethical committees of the participating centers. All participants gave written informed consent.

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Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2012.05.011.

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Analyses of the *MAPT*, *PGRN*, and *C9orf72* mutations in Japanese patients with FTLD, PSP, and CBS

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ABSTRACT

Background: Mutations in the microtubule associated protein tau (*MAPT*) and progranulin (*PGRN*) have been identified in several neurodegenerative disorders, such as frontotemporal lobar degeneration (FTLD), progressive supranuclear palsy (PSP), and corticobasal syndrome (CBS). Recently, *C9orf72* repeat expansion was reported to cause FTLD and amyotrophic lateral sclerosis (ALS). To date, no comprehensive analyses of mutations in these three genes have been performed in Asian populations. The aim of this study was to investigate the genetic and clinical features of Japanese patients with *MAPT*, *PGRN*, or *C9orf72* mutations.

Methods: *MAPT* and *PGRN* were analyzed by direct sequencing and gene dosage assays, and *C9orf72* repeat expansion was analyzed by repeat-primed PCR in 75 (48 familial, 27 sporadic) Japanese patients with FTLD, PSP, or CBS.

Results: We found four *MAPT* mutations in six families, one novel *PGRN* deletion/insertion, and no repeat expansion in *C9orf72*. Intriguingly, we identified a *de novo* *MAPT* p.S285R mutation. All six patients with early-onset PSP and the abnormal eye movements that are not typical of sporadic PSP had *MAPT* mutations. The gene dosages of *MAPT* and *PGRN* were normal.

Discussion: *MAPT* p.S285R is the first reported *de novo* mutation in a sporadic adult-onset patient. *MAPT* mutation analysis is recommended in both familial and sporadic patients, especially in early-onset PSP patients with these abnormal eye movements. Although *PGRN* and *C9orf72* mutations were rare in this study, the *PGRN* mutation was found in this Asian FTLD. These genes should be studied further to improve the clinicogenetic diagnoses of FTLD, PSP, and CBS.

1. Introduction

Mutations in the microtubule-associated protein tau (*MAPT*) and the progranulin (*PGRN*) genes have been identified in families with frontotemporal dementia and parkinsonism linked to chromosome 17 [1–3]. Recently, two studies reported that the expansion of a noncoding GGGGCC hexanucleotide repeat in the *C9orf72* gene is

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a major cause of both frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) [4,5].

Each of these genes can be associated with multiple clinical entities. Patients with *MAPT* mutations may receive diagnoses of frontotemporal dementia (FTD), primary progressive aphasia (PPA), or progressive supranuclear palsy (PSP). Rarely, corticobasal syndrome (CBS) or FTD with ALS (FTD-ALS) may be manifested in these patients [6]. The clinical diagnoses of patients with *PGRN* mutations include FTD, PPA, and CBS [6]. *C9orf72* repeat expansion causes FTD, ALS, FTD-ALS [4,5], PPA [5,7], and CBS [8] phenotypes. Thus, due to the complicated and often overlapping genetic and phenotypic variability in these patients, an accurate diagnosis of these clinical entities before autopsy is often difficult for clinicians.

To date, few comprehensive screening studies of these three genes have been performed in Asian populations. The aims of this study are to characterize the roles of known and, more importantly, novel disease-causing genes and to investigate the genetic and clinical features of FTLD, PSP, and CBS patients with *MAPT*, *PGRN*, and *C9orf72* mutations. In this study, we also describe the abnormal eye movements that are generally not observed in sporadic PSP but occur in early-onset PSP patients bearing *MAPT* mutations.

2. Methods

2.1. Subjects

We studied 75 Japanese patients who were diagnosed with FTLD, PSP, and CBS with or without a family history of disease. FTLD was divided into three subclasses: behavioral variant FTD (bvFTD), FTD-ALS, and PPA. The clinical diagnoses were established according to the consensus criteria for FTD [9], PPA [10], PSP [11], and CBS [12]. The characteristics of the 75 analyzed patients (69 index patients) are shown in Table 1. This study was approved by the ethics committee of the Juntendo University School of Medicine. Each subject provided written informed consent. All of the subjects in the control cohort were Japanese individuals and were evaluated by neurologists to ensure that no subjects exhibited any clinical manifestations of neurodegenerative diseases.

2.2. Genetic analyses

For direct sequence analysis, each exon was amplified by polymerase chain reaction (PCR) using published primers for *MAPT* [13] and *PGRN* [2] in a standard protocol. Dideoxy cycle sequencing was performed using Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA). These products were loaded into ABI310 and 3130 automated DNA sequence analyzers and analyzed with DNA Sequence Analysis software (Applied Biosystems). To provide a qualitative assessment of the presence of an expanded $(GGGGCC)_n$ hexanucleotide repeat in the *C9orf72* gene, we performed repeat-primed PCR as previously described [4]. The normal repeat number of the GGGGCC hexanucleotide was determined in all of the patients using genotyping primers, as previously described [4]. The PCR products were analyzed on an ABI3130 DNA Analyzer and visualized using Gene Mapper software (Applied Biosystems).

Table 1
The clinical diagnoses and characteristics of 75 patients (69 index patients).

Clinical phenotype	No.	% of total	% of Male	Mean (SD) AAO (range, years)	Familial	Sporadic
FTLD	38	50.7	39.5	57.1 (±12.4), 36–78	21	17
bvFTD	29	38.7	34.5	54.5 (±12.6), 36–78	18	11
FTD-ALS	2	2.7	100	67.5 (±1.5), 66–69	1	1
PPA	7	9.3	42.9	65.0 (±7.4), 58–77	2	5
PSP	25	33.3	68.0	59.8 (±13.0), 40–76	18	7
CBS	12	16.0	33.3	58.4 (±9.52), 40–71	9	3
Total	75	100	48.0	58.2 (±12.3), 36–78	48	27
Index patients	69	92.0	46.4	58.9 (±12.4), 36–78	42	27
Relatives	6	8	66.7	50.3 (±6.6), 44–61	6	0

FTLD = frontotemporal lobar degeneration.
bvFTD = behavioral variant frontotemporal dementia.
FTD-ALS = frontotemporal dementia with amyotrophic lateral sclerosis.
PPA = primary progressive aphasia; PSP = progressive supranuclear palsy.
CBS = corticobasal syndrome; SD = standard deviation; AAO = age at onset.

2.3. Multiplex ligation-dependent probe amplification (MLPA)

To confirm the gene dosages of *MAPT* and *PGRN*, we performed MLPA using the SALSA MLPA P275-B1 MAPT-PGRN kit (MRC-Holland, Amsterdam, The Netherlands). The DNA detection/quantification protocol was provided by the manufacturer. The products were quantified using the ABI3130 Genetic Analyzer and Gene Mapper v3.7 (Applied Biosystems). The kit contains 32 probes, including 13 *MAPT* probes (located in exons 1–13) and 5 *PGRN* probes (located in exons 1, 3, 6, 10, and 12) located within other genes on chromosome 17q21. The MLPA data were analyzed as described previously [14].

2.4. Exon-trapping analysis

To determine whether a novel *MAPT* mutation was pathogenic, we performed an exon-trapping analysis. We used a wild-type construct and constructs containing the novel *MAPT* p.S285R or the IVS10+3 intronic mutation [15]. The *MAPT* sequences included exon 10, 34 nucleotides of the upstream intronic sequence and 85 nucleotides of the downstream intronic sequence. The PCR products were subcloned into the splicing vector pSPL3 (Invitrogen, Carlsbad, CA), and exon trapping was performed as described previously [15].

2.5. Paternity testing

Microsatellite analysis with 10 markers (D2S293, D3S3521, D4S2971, D5S495, D6S16171, D7S2459, D8S1705, D16S430, D18S450, and D20S842) was performed in Patient 1 and his parents to confirm paternity.

2.6. TA cloning

The novel *PGRN* heterozygous deletion/insertion found in this study, *PGRN* p.G338RfsX23 (c.1012_1013delGGinsC), was confirmed by cloning the PCR products into the pCR4-TOPO Vector using the TOPO TA Cloning kit (Invitrogen) and sequencing the two haplotypes of the heterozygote.

3. Results

3.1. Results of MAPT analysis

3.1.1. Genetic and molecular analyses of MAPT

In this study, we identified nine patients with *MAPT* mutations from six families. Four heterozygous missense mutations in *MAPT*, p.L266V, p.N279K, p.N296N, and the novel p.S285R (Supplementary Fig. 1), were identified by direct sequencing. None of the 182 normal Japanese controls included in this study had the *MAPT* p.S285R. In addition, we examined the amino acid sequences of the MAPT protein in other species and found that the site of the p.S285R mutation was highly conserved (see Supplementary Fig. 2). The novel p.S285R mutation in *MAPT* was detected in Patient 1 but not in his parents (Fig. 1A and Supplementary Fig. 1). The parentage of this patient and the DNA authenticity were confirmed using a microsatellite panel (see Supplementary Table 1). These results suggest that p.S285R is a *de novo* mutation. To investigate whether the p.S285R mutation is pathogenic, we performed an exon-trapping analysis. The p.S285R mutation produced a marked increase in the splicing of exon 10 (Fig. 1B) and resulted in the overproduction of tau isoforms that contain 4-repeat tau, such as IVS10+3 [15]. These results indicate that the p.S285R mutation is a novel, *de novo* pathogenic mutation. Previously, p.L266V, p.N279K, and p.N296N had been reported as pathogenic mutations [16–18].

Table 2 lists the clinical features of all of the *MAPT*- and *PGRN*-positive patients in this study, and Supplementary Fig. 3 shows Pedigrees C, D, E, F, and G. The average age at disease onset of patients with a single heterozygous *MAPT* mutation was 42.3 ± 2.9 (range: 37–46) years. MLPA analysis showed no gene dosage abnormalities (multiplications or deletions) in *MAPT* in this cohort.

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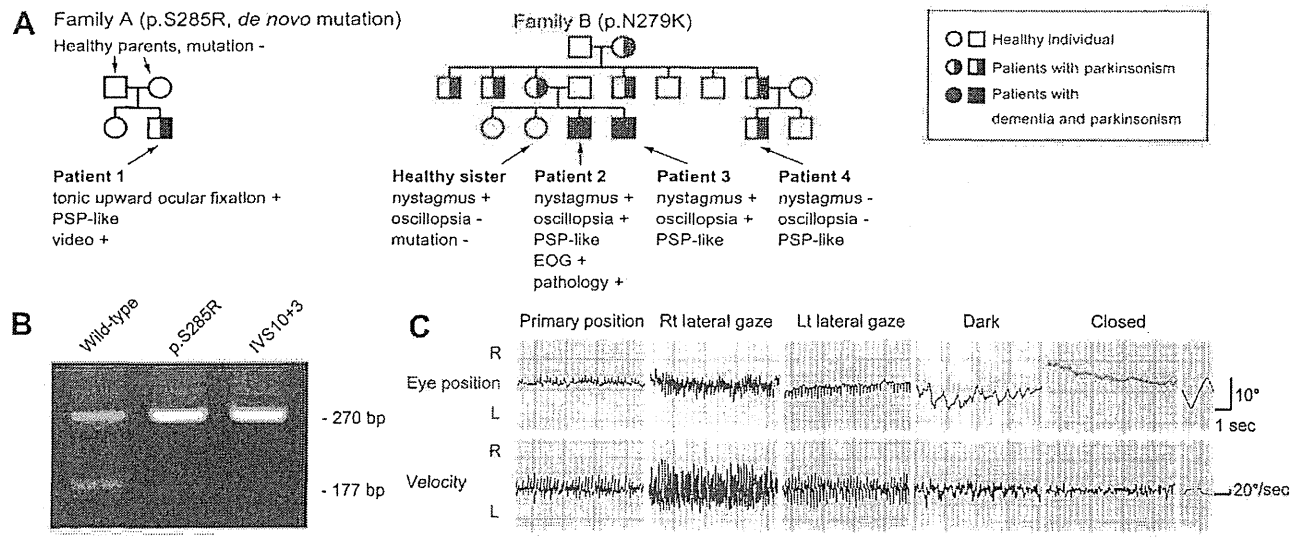

Fig. 1. (A) The pedigrees of families A and B. (B) Exon-trapping analysis for the effects of the *MAPT* p.S285R mutation on exon 10 splicing. (C) Horizontal electro-oculogram recordings in Patient 2.

3.1.2. Clinical presentations of MAPT-positive patients with the abnormal eye movements that are generally not observed in patients with sporadic PSP

3.1.2.1. Patient 1 (MAPT p.S285R). This patient was a 46-year-old man who presented with difficulty speaking and breathing. The patient had no family history of dementia or movement disorders (Fig. 1A). A physical examination revealed gait disturbance, limb bradykinesia, and frequent falling. At age 47, the patient exhibited palilalia and a mild obsession with eating. The patient's Mini-Mental State Examination (MMSE) score was 28/30, but his Frontal Assessment Battery score was 12/18. The patient exhibited a slowing of saccadic eye movements with a relative preservation of smooth pursuit, vertical supranuclear gaze palsy, and tonic upward ocular fixation (see Video Supplement); when the patient's eyes opened after closing, they remained fixated upward and could not be moved voluntarily to the primary position (i.e., Bell's phenomenon remained). To overcome this disability, the patient extended his neck, which resulted in a reflex downward movement of the eyes (the vestibulo-ocular reflex), and next he slightly flexed his neck to a neutral position with his eyes in the primary position. Later, the patient developed bradykinesia and postural instability with frequent falling. L-dopa/benserazide (up to 900 mg/day) was ineffective. The patient's condition gradually deteriorated, and he developed dementia, retrocollis, vertical and horizontal supranuclear palsy, and bradykinesia. At age 49, the patient died of suffocation from the aspiration of food material. No autopsy was performed. The clinical diagnosis was probable PSP.

3.1.2.2. Patient 2 (MAPT p.N279K). This patient was the older brother of Patient 3 (Fig. 1A). Patient 2 was a 42-year-old man who exhibited oscillopsia, micrographia, and a shuffling gait. This patient reported having had nystagmus without oscillopsia since childhood. A neurological examination revealed marked horizontal nystagmus. The patient's pupils were isocoric, and his visual acuity was normal. The patient presented with rigidity, bradykinesia, and postural tremor in the upper limbs. Electro-oculography revealed horizontal pendular nystagmus in the primary position and in all gaze directions (Fig. 1C). L-dopa/benserazide at 200 mg/day mildly alleviated his parkinsonism. Two years later, the patient developed prominent postural instability and became prone to falling. Upward and downward gaze palsy and apraxia of eyelid opening were also noted. At that time, the clinical diagnosis was possible PSP with a family history of dementia and parkinsonism. The patient's cognitive function deteriorated gradually. At age 52, he was bedridden and required a gastrostomy. The patient died of pneumonia at age 54. A postmortem pathological examination of the brain revealed mild atrophy of the frontal lobe and the tegmentum of the midbrain and pons. Microscopic analysis showed severe degenerative changes in the substantia nigra and the subcortical nuclei. Immunohistochemistry using anti-phosphorylated tau (p-tau) antibodies revealed numerous tau-positive neuronal and glial inclusions in the frontotemporal cortex, white matter, and the subcortical nuclei (see Supplementary Fig. 4). These p-tau deposits reacted with anti-4-repeat tau antibodies but not with anti-3-repeat tau antibodies.

3.1.2.3. Patient 3 (MAPT p.N279K). This patient was the younger brother of Patient 2 (Fig. 1A). At age 44, Patient 3 noticed clumsiness in his right hand and oscillopsia. The patient reported having nystagmus since childhood. A neurological examination revealed large, horizontal pendular nystagmus in the primary position and in all gaze directions. The patient's visual acuity, pupils, and light reflexes were all normal. Mild bradykinesia and rigidity in the neck and the right upper limb were noted. Postural tremor in both hands and the tongue and postural instability were observed. Treatment with 600 mg/day of L-dopa/carbidopa was not effective. The patient's oscillopsia gradually worsened, and eventually he was unable to read printed materials. At age 47, the patient developed upward and downward gaze palsy, slowing of saccades, and apraxia of eyelid opening. The patient had prominent postural instability and was prone to falling. The patient's first clinical diagnosis was possible PSP with a family history of dementia and parkinsonism. The patient died at age 56. An autopsy was not performed.

3.1.2.4. Patients 5, 6, and 7 (MAPT p.N279K). The clinical presentations of these three patients have been described previously [19]. All three patients had clinical diagnoses of possible PSP (Table 2) and visual grasping [19,20].

3.2. Results of PGRN analysis

3.2.1. Genetic Analyses of PGRN

We identified one patient with a *PGRN* mutation (Table 2, Supplementary Fig. 3). One novel heterozygous deletion/insertion

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Table 2
Clinical features of patients with *MAPT* and *PGRN* mutations.

Family	A	B			C	D		E	F	G
Patient	1	2	3	4	5	6	7	8	9	10
Gene	*MAPT*									*PGRN*
Genotyping	Heterozygous									
Nucleotide change	c.853A > C	c.837T > G	c.837T > G	c.837T > G	c.837T > G	c.837T > G	c.837T > G	c.796C > G	c.888T > C	c.1012_1013delGGinsC
Amino acid change	p.S285R	p.N279K	p.N279K	p.N279K	p.N279K	p.N279K	p.N279K	p.L266V	p.N296N	p.G338RfsX23
Exon	10	10	10	10	10	10	10	9	10	9
Mode of inheritance	*de novo*	AD	AD	AD	NA	AD	AD	AD	AD	AD
Age at onset, years	46	42	44	46	41	42	43	37	44	59
Age at evaluation, years	47	47	45	50	44	44	45	38	49	61
Age at death, years	49	54	56	alive	51	54	51	alive	alive	alive
Sex	M	M	M	M	F	F	F	F	M	F
Clinical syndromes	PSP	PSP	PSP	PSP	PSP	PSP	PSP	bvFTD	PSP	PPA
Clinical features										
Initial symptoms	P	P	P	P	P	P	P	dementia	P	aphasia
Personality/behavior changes	−	+	−	−	−	−	−	+	+	−
Mini mental state examination score	28/30	NA	NA	28/30	NA	NA	NA	0	24/30	29/30
Hasegawa dementia scale-revised[a]	NA	18/30	NA	NA	21/30	28/30	30/30	0	21/30	29/30
Nonfluent spontaneous speech	−	−	−	−	−	−	−	−	−	+
Apraxia of eyelid opening	−	+	+	+	+	+	+	−	−	−
Abnormal eye movements										
Supranuclear gaze palsy	+	+	+	+	+	+	+	−	+	−
Tonic upward ocular fixation	+	−	−	−	−	−	−	−	−	−
Oscillopsia with CN	−	+	+	−	−	−	−	−	−	−
Visual grasping	−	−	−	−	+	+	+	−	−	−
Parkinsonism										
Bradykinesia	+	+	+	+	+	+	+	−	+	−
Rigidity	−	+	+	+	+	+	+	−	+	−
Tremor	−	+	+	−	−	−	−	−	−	−
Postural instability	+	+	+	+	+	+	+	−	+	−
Response to L-dopa	−	partial[b]	−	partial[b]	partial[b]	partial[b]	partial[b]	NA	+	NA
Pyramidal sign	+	−	NA	−	+	−	+	+	+	−
Features of motor neuron disease	−	−	−	−	−	−	−	−	−	−
Reference					[19]	[19]	[19]			

AD = autosomal dominant.
P = parkinsonism; NA = not available.
CN = congenital nystagmus; PSP = progressive supranuclear palsy.
bvFTD = behavioral variant frontotemporal dementia; PPA = primary progressive aphasia.
[a] The Hasegawa dementia scale-revised is a brief dementia screening scale. The maximum score of the Hasegawa dementia scale-revised is 30 points. There was a significant difference in the mean score between the demented and non-demented subjects when the cut-off point was set at 20/21 [31].
[b] A partial response to L-dopa indicates that L-dopa was effective only in the early stages.

mutation in *PGRN*, p.G338RfsX23 (c.1012_1013delGGinsC), was detected by direct sequencing and TOPO TA cloning sequencing (Supplementary Fig. 1). None of the 182 normal Japanese controls included in this study had the *PGRN* p.G338RfsX23 (c.1012_1013delGGinsC) mutations. The age at disease onset of the patient with the heterozygous *PGRN* deletion/insertion was 59 years. Novel *PGRN* variants with unknown significance, p.R18Q and p.N118del, are listed in Table 3. MLPA analysis showed no gene dosage abnormalities in *PGRN*.

3.2.2. A clinical presentation of a novel PGRN mutation

3.2.2.1. Patient 10 (PGRN p.G338RfsX23, c.1012_1013delGGinsC). This patient, a 59-year-old woman, developed word-finding difficulties and underwent surgical clipping at age 54 for an unruptured

Table 3
Novel variants with unknown significance.

Gene	Nucleotidechange	Amino acid change	Exon	Amino acid conservation	Mean AAO (years)	Frequency		*P* value	Clinical diagnosis
						Patients *N* (%)	Controls *N* (%)		
PGRN	c.56G > A	p.R19Q	1	not conserved	66	1/69 (1.4)	0/186 (0)	0.605	PSP ($n = 1$)
PGRN	c.352_354delAAC	p.N118del	4	not conserved	53	3/69 (4.3)	3/272 (1.1)	0.187	bvFTD ($n = 3$)

AAO = age at onset.
PSP = progressive supranuclear palsy.
bvFTD = behavioral variant frontotemporal dementia.

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aneurysm of the left middle cerebral artery. The patient's mother suffered from dementia, but the details of her disease were unknown. The patient substituted words for names of people and objects. Two years after the onset of symptoms, the patient became severely disfluent. However, she did not show any violent behavior, personality changes, or other behavioral abnormalities. The patient scored 29/30 on the MMSE. On the frontal assessment battery, she scored 13/18. The patient's time to complete the Trail Making Test (TMT) A was 70 s, and she could not finish the TMT B within five minutes. Her spontaneous speech production was characterized by slow and hesitant speech, frequently interrupted by long word-finding pauses. Her motor speech abilities were within the normal limits, and no apraxia of speech was noted. No parkinsonism was observed. The patient's clinical diagnosis was PPA with a family history of dementia.

3.3. Results of C9orf72 analysis

We identified no patients with expanded hexanucleotide repeats in *C9orf72* in this study. In 75 patients, the average repeat number based on fluorescent fragment-length analysis was 3.77 ± 2.56 (range 2–11 repeats). We have previously reported that an analysis of 197 Japanese healthy controls did not find any *C9orf72* mutation. The average repeat number was 3.69 ± 2.46 (range 2–14 repeats) in the 197 controls [21].

4. Discussion

We identified five *MAPT* mutations, including a novel *de novo* mutation and a novel *PGRN* mutation, and we found no *C9orf72* mutations in our 75 patients. More mutations were found in *MAPT* than in the other two genes evaluated in this study. The infrequent observation of *PGRN* and *C9orf72* mutations might be partly due to the small number of FTLD patients included ($n = 38$) because the majority of *PGRN* and *C9orf72* mutations have been described in patients with FTLD. In contrast to most other mutation screening studies, we performed MLPA analysis to ensure that exonic or larger deletions or multiplications of *MAPT* and *PGRN* would be identified. Therefore, our data also show that multiplications of *MAPT* and exonic or genomic deletions in *PGRN* are rare in Asian populations. Although mutations were detected in FTLD and PSP patients, we did not find any mutations in our CBS patients. A further larger study and investigation of the other genes are needed to clarify the genetic background of Japanese patients with CBS.

The *MAPT* p.S285R mutation, which we found in this study, is a novel *de novo* mutation. To the best of our knowledge, this report is the first description of an adult sporadic case of a *de novo MAPT* mutation associated with dementia and parkinsonism. All six patients (Patients 1, 2, 3, 5, 6, and 7) with PSP and the distinct eye movements described in the present study (such as tonic upward ocular fixation, oscillopsia with congenital nystagmus, and visual grasping) harbored *MAPT* mutations. Below, we discuss these abnormal eye movements, which are generally not observed in patients with sporadic PSP.

In Patient 1 (*MAPT* p.S285R), we observed tonic upward ocular fixation, which is a loss of downward saccades resembling an acquired ocular motor apraxia [22]. This condition is characterized by a loss of voluntary control of saccades and pursuit, whereas reflex movements—in particular, the vestibulo-ocular reflex—were preserved. Acquired ocular motor apraxia is usually the result of bilateral frontal or frontoparietal infarcts. Therefore, tonic upward ocular fixation due to a *MAPT* mutation might share "supranuclear" cerebral lesions in common with ocular motor apraxia. Brainstem functions, including the vestibulo-ocular reflex and Bell's phenomenon, were preserved in Patient 1.

In Patients 2 and 3 (*MAPT* p.N279K), pendular nystagmus was present since childhood and was suppressed with eyelid closure. These features are consistent with congenital nystagmus [23]. Most patients with congenital nystagmus do not complain of oscillopsia, despite having nearly continuous eye movement [23]. Notably, Patients 2 and 3 noticed oscillopsia when they developed parkinsonism. In these siblings, cerebral lesions caused solely by a *MAPT* mutation were unlikely to be the cause of their nystagmus; however, the co-existence of congenital nystagmus and the *MAPT* mutation might have caused the oscillopsia. This notion is supported in part because the patients had a sister who remained healthy – even in her late 60s – and did not complain of oscillopsia, despite having obvious pendular nystagmus (Fig. 1A). Thus, *MAPT* mutations might impair the visual-motion processing pathways that would normally suppress oscillopsia in patients with common congenital nystagmus. Visual grasping, which was first described by Ghika et al. [20], was observed in Patients 5, 6, and 7 (*MAPT* p.N279K) [19].

Although PSP is a rare manifestation of *MAPT* mutation [24], and the routine screening of sporadic PSP for mutations in *MAPT* is not recommended because of low yield [25], it is recommended that screening be considered for families in which there is an autosomal dominant history of a PSP syndrome, particularly when there are accompanying features suggestive of bvFTD [24]. The clinical difference from sporadic PSP might sometimes be difficult to detect, especially in patients without a family history [26–28]; however, an important case report indicated that an age at disease onset under 50 years combined with the absence of early falling may indicate a possible *MAPT* mutation in clinically diagnosed PSP, even in the absence of a positive family history [26]. Consistent with this observation, our eight *MAPT*-positive patients with PSP phenotype were younger than 50 years at disease onset (Table 2). We further suggest that it may be useful to test for *MAPT* mutations in early-onset PSP patients with the abnormal eye movements that are not typical of sporadic PSP. In fact, we identified the novel *de novo* mutation p.S285R in Patient 1 and p.N279K in Patient 5, who had no family history, after focusing on these clinical phenotypes.

To the best of our knowledge, the *PGRN* mutation has not been previously described in Asian populations [29]. We detected a novel *PGRN* mutation, p.G338RfsX23 (c.1012_1013delGGinsC), and thus showed that *PGRN* mutations may exist in Asian populations. This mutation introduces a premature termination codon at the same site as the p.G333VfsX28 (c.998delG) mutation, which was reported previously, and produced a PPA phenotype in all of the affected individuals [30]. The PPA phenotype of p.G338RfsX23 (c.1012_1013delGGinsC) in our study is remarkably similar to that of p.G333VfsX28 (c.998delG), especially in the manifestation of word-finding and object-naming difficulties and the lack of memory or personality changes during the first few years after symptom onset. We believe that the mutant RNA in both cases is most likely subjected to nonsense-mediated decay, similar to other *PGRN* mutations [2].

In summary, based on these findings, we recommend genetic testing for *MAPT* mutations not only in familial patients but also in sporadic patients, especially early-onset PSP patients with the abnormal eye movements that are generally not observed in sporadic PSP. Although *PGRN* and *C9orf72* mutations were rare in this study, we determined that the *PGRN* mutation does exist in Asian patients with FTLD (PPA). Based on the clinical information, screening for *MAPT*, *PGRN*, and *C9orf72* mutations should be further undertaken to improve the diagnosis of specific clinical entities of neurodegenerative disorders.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.parkreldis.2012.06.019.

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RESEARCH ARTICLE

VPS35 Mutation in Japanese Patients with Typical Parkinson's Disease

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ABSTRACT: Vacuolar protein sorting 35 *(VPS35)* was recently reported to be a pathogenic gene for late-onset autosomal dominant Parkinson's disease (PD), using exome sequencing. To date, *VPS35* mutations have been detected only in whites with PD. The aim of the present study was to determine the incidence and clinical features of Asian PD patients with *VPS35* mutations. We screened 7 reported nonsynonymous missense variants of *VPS35*, including p.D620N, known as potentially disease-associated variants of PD, in 300 Japanese index patients with autosomal dominant PD and 433 patients with sporadic PD (SPD) by direct sequencing or high-resolution melting (HRM) analysis. In addition, we screened 579 controls for the p.D620N mutation by HRM analysis. The p.D620N mutation was detected in 3 patients with autosomal dominant PD (1.0%), in 1 patient with SPD (0.23%), and in no controls. None of the other reported variants of *VPS35* were detected. Haplotype analysis suggested at least 3 independent founders for Japanese patients with p.D620N mutation. Patients with the *VPS35* mutation showed typical tremor-predominant PD. We report Asian PD patients with the *VPS35* mutation. Although *VPS35* mutations are uncommon in PD, the frequency of such mutation is relatively higher in Japanese than reported in other populations. In *VPS35*, p.D620N substitution may be a mutational hot spot across different ethnic populations. Based on the clinical features, *VPS35* should be analyzed in patients with PD, especially autosomal dominant PD or tremor-predominant PD. © 2012 *Movement* Disorder Society

Key Words: Parkinson's disease; *VPS35*; autosomal dominant; hotspot; mutation.

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive motor disturbances manifested by tremor, rigidity, akinesia, and postural instability. Neuropathologically, PD is characterized by selective loss of dopaminergic neurons in the substantia nigra and the presence of cytosolic inclusions called Lewy bodies (LBs) in the remaining neurons. The pathogenesis of PD is multifactorial, including genetic–environmental interaction. PD is a common disease in the elderly, with an incidence of about 1%–2% in individuals older than 60 years.[1] Among PD patients, approximately 5%–10% have a positive family history of PD,[2] and among these, the Mendelian forms of PD can contribute to the elucidation of the molecular pathways that lead to the degeneration and death of dopaminergic neurons.

Mutations in the vacuolar protein sorting 35 (*VPS35*) gene have recently been identified in families with autosomal dominant late-onset PD (MIM 601501).[3,4] Patients with *VPS35* mutations present with tremor-predominant dopa-responsive parkinsonism.[3,4] VPS35, a key component of the retromer cargo-recognition complex, is thought to associate with sorting cargos into the tubular endosomal network for retrieval to the trans-Golgi network.[5] Therefore, pathogenic mutations of *VPS35* may cause disruption of the retrograde transport system and contribute to dopaminergic neuronal cell death in PD. One missense mutation has been reported to be pathogenic for PD.[3,4] Mutation of c.1858G>A (p.D620N) was identified in 3 Austrian families and 1 family each in Switzerland, the United States, Tunisia, and the United Kingdom, as well as 1 family and 1 patient with sporadic PD (SPD) among Yemenite Jews from Israel.[3,4,6] In addition, several variants, such as p.M57I, p.I241M, p.P316S, and p.R524W, have been reported in Europe and the United States as potentially pathogenic for PD.[3,4]

Although multipopulation screenings for *VPS35* mutations were preformed in recent reports, there is still no report of PD patients with *VPS35* mutations of Asian ancestry.[3,4,6–8] In the present study, we screened Japanese patients with autosomal-dominant PD (ADPD), Japanese patients with SPD, and control subjects for mutations of *VPS35*, with a special focus on 7 reported nonsynonymous variants that were found in patients with PD, including the p.D620N. Here, we report 3 families and 1 SPD patient with the p.D620N mutation in *VPS35* and describe their clinical features.

Patients and Methods

Subjects

The study was approved by the ethics committee of Juntendo University, and all subjects gave written informed consent to participate in the genetic research. The study subjects were 308 Japanese patients (300 index patients) with ADPD (age at disease onset [AAO; mean ± SD], 51.1 ± 11.7 years; range, 8–83 years; female/male [F/M] ratio, 1.35) and 433 Japanese SPD patients (AAO, 47.2 ± 12.9 years; range, 5–88 years; F/M ratio, 1.09) selected from the gene bank of Juntendo University. Some of the selected subjects had been confirmed negative for *SNCA*, *PARK2*, *PINK1*, *PARK7*, *LRRK2*, and *PLA2G6* mutations.[9–14] From the same gene bank, we also selected 579 healthy Japanese subjects without a family history of parkinsonism (age at sampling, 58.0 ± 9.3 years; range, 23–89 years; F/M ratio, 1.54). The criteria for the diagnosis of PD were adopted by the participating neurologists and were established based on the United Kingdom Parkinson's Disease Society Brain Bank.[15]

Genetic Analysis

Genomic DNA was extracted from peripheral blood using a standard protocol. Patients with ADPD and SPD were examined for the following 7 variants: p.M57I (exon 3), p.I241M (exon 7), p.P316S (exon 9), p.R524W (exon 13), p.D620N (exon 15), p.A737V (exon 16), and p.L774M (exon 17) of *VPS35* (RefSeq accession number NM_018206.4). PCR direct sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing kit and 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) or 3730 DNA Analyzer (Applied Biosystems). In addition, SPD patients and control subjects were also genotyped for c.1858G>A (p.D620N) mutation by high-resolution melting (HRM) analysis using LightScanner and LCGreen plus (Idaho Technology, Salt Lake City, UT). HRM analysis was performed using a previously described protocol[16] and the following primers: forward, GAGGATGGTTGGTCCTTGAA; reverse, TGCCAATGATCAAGGTGATG. All exons of *VPS35* were also analyzed in patients with the p.D620N mutation using the method described previously.[3]

Haplotype analysis of the *VPS35* flanking region was performed using 3130 Genetic analyzer and GeneMapper software (Applied Biosystems, Foster City, CA). To adjust the size of PCR products, we also genotyped Centre d'Étude du Polymorphisme Humain (CEPH) control samples (1331-01 and 1331-02) for comparison of haplotypes with previously reported patients carrying the p.D620N mutation. The sequences of the PCR primers were reported previously.[3]

Results

Detection of p.D620N Mutation

We detected the heterozygous missense p.D620N mutation in 3 unrelated patients with ADPD and 1

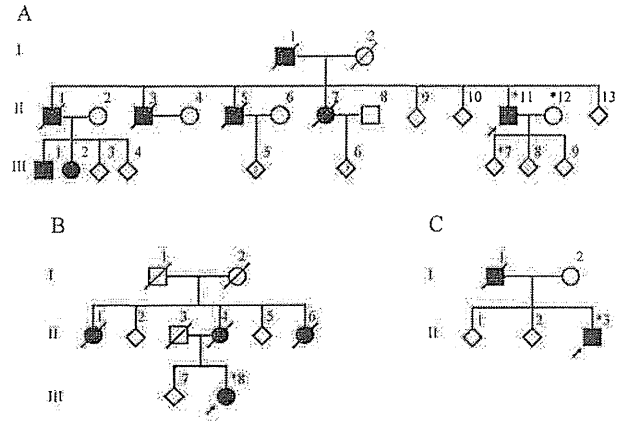

FIG. 1. Pedigrees of families with the *VPS35* p.D620N mutation (open symbol, unaffected family member; closed symbol, affected member; arrow, proband; asterisk, individual analyzed for the p.D620N mutation and/or haplotype; forward slash through symbol, deceased individuals; square, male; circle, female; diamond, unspecified sex).

patient with SPD (Fig. 1). The p.D620N has been reported previously as a pathogenic mutation for familial PD.[3,4,6] This mutation was not found in 1158 control chromosomes. Patients carrying the p.D620N mutation did not have any other variants in all exons of *VPS35*. In our population, the incidence of the p.D620N mutation was 1.0% (3 of 300) in ADPD and 0.23% (1 of 433) in SPD. The remaining variants analyzed in this study were not identified in any patients.

Haplotype analysis demonstrated that the Japanese patients with the p.D620N mutation had different genotypes from those of white patients with the same mutation.[3] One disease allele was detected by analyzing patient AII-11 and his relatives. Patients AII-11 and BIII-8 in this study carried at least the same single allele of microsatellites in the flanking region of *VPS35* (Table 1). On the other hand, patients CII-3 and D had a different genotype of D16S3105, with a locus mapped very close to *VPS35*, compared with the disease allele of AII-11 (Table 1, boldface).

TABLE 1. Haplotype analysis of VPS35 p.D620N mutation carriers

	Patient ID			
Microsatellite	AII-11	BIII-8	CII-3	D
D16S401	170	166/170	166/172	166/170
D16S3068	143	141/145	145/147	145/145
D16S753	272	272/268	268/276	264/268
VPS35 p.D620N	A	A/G	A/G	A/G
Chr16_45.333M	294	294/298	294/300	294/304
D16S3105	191	191/189	189/193	187/187
Chr16_45.615M	147	147/147	147/145	147/145
Chr16_45.806M	246	246/238	246/244	246/244
Chr16_45.835M	237	237/237	237/237	237/237
Chr16_45.855M	212	212/210	210/210	210/216
D16S3044	195	195/195	195/197	197/197

Both alleles are shown when markers of phase could not be determined.

TABLE 2. Clinical features of patients with p.D620N mutation

	Patient ID			
	AII-11	BIII-8	CII-3	D
Age at disease onset (y)	62	55	34	42
Disease duration (y)	15	2	7	21
Resting tremor	+	+	+	+
Bradykinesia	+	+	+	+
Rigidity	+	+	+	+
Gait disturbance	+	–	–	+
Postural instability	+	–	–	+
Clinical response to levodopa	+	+	+	+
Wearing off	+	–	+	+
Asymmetry at onset	+	+	+	+
Orthostatic hypotension	+	–	–	–
Incontinence	+	–	–	–
Urinary urgency	–	–	–	–
Levodopa-induced dyskinesia	+	–	+	+
Sleep benefit	+	–	+	Unknown
Dystonia at onset	–	–	–	–
Hyperreflexia	–	–	–	–
Hallucination	–	–	–	–
Other psychosis	–	–	–	–
Dementia	+	–	–	–
Gaze palsy	–	–	–	–
Brain MRI	WNL	WNL	WNL	WNL
Cardiac MIBG scintigraphy	H/M ratio (E/L), 2.38/2.68; washout ratio, 4.15%[a]	Not performed	Not performed	Not performed

[a]MIBG scintigraphy was performed when AII-11 was 76 years old.
WNL, within normal limit; H/M ratio, heart-to-mediastinum ratio; (E/L), early/late stage.

Clinical Presentation

Table 2 summarizes the clinical features of the 4 *VPS35* mutation-positive patients. Patient AII-11 was a 77-year-old man who developed right upper limb rest tremor at age 62. At age 75, he underwent gastrostomy for progressive dysphagia, then developed cognitive dysfunction without hallucination. Single-photon emission computed tomography of cerebral blood flow showed no reduction in blood flow in the basal ganglia. His father and 4 of 8 siblings were diagnosed with PD (Fig. 1A) and presented levodopa-responsive typical parkinsonism: upper limb tremor and small-step gait. His nephew and niece were also diagnosed with PD, and the nephew developed parkinsonism in his early fifties. Patients BIII-8 and CII-3 both developed upper limb rest tremor at ages 34 and 55, respectively. The mother and aunts of patient BIII-8

and the father of patient CII-3 also developed PD (Fig. 1B, C). Patient D, who developed upper limb rest tremor at age 42, had no family history of PD. She underwent subthalamic nucleus deep brain stimulation (STN-DBS) at age 60 because of disabling motor fluctuation and dyskinesia refractory to pharmacological treatment. All affected patients were born to nonconsanguineous parents.

Discussion

VPS35 has been reported as the pathogenic gene for ADPD, and only 1 mutation, p.D620N, has been reported in several unrelated white families. To our knowledge, there have been no reports of Asian PD patients with *VPS35* mutations.[3,8] Based on this background, we set out in this study to determine the incidence of *VPS35* mutations in Japanese patients with PD. We detected the heterozygous p.D620N mutation of *VPS35* in 3 ADPD families and 1 SPD patient with East Asian ancestry. On the other hand, we could not conclude the pathogenicity of 6 other variants that had been reported as potentially pathogenic for PD because none of the variants was detected in our patients with PD.

The frequency of the p.D620N mutation in Japanese patients was 1.0% in ADPD and 0.23% in SPD. Although the exact frequency among whites is undetermined, the frequency is relatively higher in Japanese patients compared with that reported in previous studies (0%–1.22%).[3,4,6,7,17] Moreover, the frequency in Japanese patients also differs greatly from those of other Asian populations such as Taiwanese patients and mainland Chinese patients (0%).[3,8] Although the mutation frequency was expected to be lower than that of other pathogenic genes for ADPD, such as multiplication of *SNCA*[9,18] and point mutation of *LRRK2*,[19–21] *VPS35* may be one of the most important genes in Japanese PD. Because we screened for only 7 reported variants, we cannot determine the exact frequency of *VPS35* mutations in ADPD; we would need to analyze all 17 exons of *VPS35* in ADPD patients to screen for other variants and to assess the incidence of all disease-associated *VPS35* mutations.[3,4] Furthermore, we would need to perform mutational analysis for SPD patients, in addition to ADPD, to identify Asian population-specific variants, such as *LRRK2* p.G2385R, associated with susceptibility for PD.[19]

Based on haplotype analysis reported in previous studies, the substitution of *VPS35* c.1858G>A (p.D620N) occurs from independent mutational events.[3] We were able to determine the chromosomal phase only in patient AII-11 (family A). The p.D620N mutation possibly shared a common founder between Japanese ADPD families A and B; however, it was inconclusive because the phase of patient BIII-8 was undetermined. On the other hand, the same p.D620N mutation probably occurred independently in patient CII-3 (family C) and patient D. By genotyping of D16S3105, which is located approximately 1.5 kb centromeric of *VPS35*, there were at least 3 different haplotypes in Japanese because families A and C and patient D (SPD) did not have the same alleles for this microsatellite. To determine the chromosomal phase of families B and C, detailed genetic analyses of other family members are needed in future studies. These results suggest the existence of 3 or more founders in Japanese patients, in addition to the reported white patients with the p.D620N mutation or de novo mutations, indicating that the p.D620N mutation site is a mutational hot spot in *VPS35* across different ethnic populations.

According to previous reports, the average AAO of patients with the *VPS35* mutation was 50–60 years (50.6 ± 7.3 years),[3] with a distinctive feature of a slightly younger AAO compared with patients with idiopathic PD. In our study, the AAO was nonspecific with a wide range between 30–70 years. Because the family history of patient D was unknown, she was categorized as SPD. With regard to *VPS35* mutation penetrance, it is incomplete from the results of a previous report.[3] Therefore, although the frequency is low, patients with p.D620N mutation could be found among SPD patients.

The clinical symptoms of our patients with *VPS35* mutation closely resembled the idiopathic PD form, with tremor-dominant dopa-responsive parkinsonism. Psychiatric problems were inconspicuous; however, dementia may occur in patients with a long disease course, similar to patient AII-11, who had PD for 15 years. Our patients with *VPS35* mutations had normal brain MRI and cardiac MIBG scintigraphy. There have been no definite pathological mutations of *VPS35* in the spectrum of LB disorders. On the basis of these results, patients with *VPS35* mutation could show comparatively benign disease course without widespread LBs pathology.[22,23]

VPS35 assembles into the retromer cargo-recognition complex that associates with the cytosolic face of the endosomes. The retromer mediates the retrograde transport of transmembrane cargo from the endosomes to the trans-Golgi network.[5] The p.D620N mutation of *VPS35* might cause impairment of interaction with other components of the retromer complex and impaired retrograde trafficking of recycling proteins,[4] similar to α-synuclein and LRRK2, which are involved in vesicle trafficking.[24,25] Mutations in familial PD genes, including *VPS35*, may cause disruption of intracellular trafficking and lead to neurodegeneration. These findings suggest that impairment of intracellular trafficking systems is associated with the pathogenesis of PD. Although the association between the p.D620N mutation of *VPS35* and PD remains unknown, further functional studies might shed light on the pathogenesis

of *VPS35* mutation and the effects of interaction with other known pathogenic gene products on PD.

In conclusion, we have reported Asian PD patients with the *VPS35* p.D620N mutation. The p.D620N substitution may be a mutational hot spot across different ethnic populations. The frequency of *VPS35* mutation was low in ADPD; however, it is relatively high in Japanese patients compared with that reported in other populations.[3,4,6–8] Based on the clinical features of patients with *VPS35* mutation, *VPS35* should be analyzed in patients with PD, especially ADPD or tremor-predominant PD. ■

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LRRK2 I2020T mutation is associated with tau pathology

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ABSTRACT

Mutations in *the leucine-rich repeat kinase 2 (LRRK2)* gene are the most common cause of autosomal-dominant familial Parkinson's disease (FPD). The variable pathological features of *LRRK2*-linked FPD include Lewy bodies, degeneration of anterior horn cells associated with axonal spheroids, neurofibrillary tangles (NFTs) and TAR DNA-binding protein of 43 kDa (TDP-43) positive inclusion bodies. Furthermore, abnormal hyperphosphorylation of microtubule associated protein tau, in part generated by catalysis of protein kinases, has been reported to be involved in progressive neurodegeneration in a number of diseases, including FPD. Thus, we examined six patients carrying the *LRRK2 I2020T* mutation, a pathogenic mutation associated with PARK8, and found abnormal tau phosphorylation depositions in the brainstem. Additionally, we found LRRK2 I2020T enhanced tau phosphorylation in cultured cells co-expressing LRRK2-I2020T and 3 or 4-repeated tau. This is the first report describing the relationship between hyperphosphorylation of tau and LRRK2 I2020T.

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disease, characterized by rigidity, bradykinesia, resting tremor and postural instability. Mutations in *leucine-rich repeat kinase 2* (*LRRK2*) have been identified as the causative gene for *PARK8*-linked PD [1,2]. LRRK2, also known as PARK8, is a large protein of 2527 amino acids, with a molecular weight of approximately 280 kDa. LRRK2 contains multiple protein domains, including a leucine-rich repeat (LRR) domain, a ROC domain, a COR domain, a MAPKKK domain and a WD40 domain [2,3]. Various intracellular functions of LRRK2 have been reported, with alterations in its kinase activities thought to be critical for neuronal degeneration [4–7]. Interestingly, the *LRRK2 I2020T* mutation is located within the kinase domain and is also associated with altered kinase activity [6,8,9]. However, molecular studies have not shown a robust association between neuronal cell death and altered LRRK2 kinase activity, and the pathogenic mechanism of the *LRRK2 I2020T* mutation remains unknown.

Patients with *LRRK2* mutations show pleomorphic neuropathologies, which are not unique to PD and show overlap with other neurodegenerative diseases. These include nigral degeneration with or without Lewy bodies (LB) [2,10–14], also observed in diffuse LB disease [2,12,13], anterior horn cell degeneration associated with axonal spheroids, similar to amyotrophic lateral sclerosis [2], and neurofibrillary tangles (NFTs), also observed in progressive supranuclear palsy (PSP) [2,11,14,15] and Alzheimer's disease (AD) [2,12,13]. Notably, PD cases with G2019S [15], Y1699C [11] or I1371V [16] *LRRK2* mutations, have shown varied tau pathology. Similarly, Li et al. reported that tau was hyperphosphorylated in brain tissues from LRRK2-R1441G overexpressing mice, compared with LRRK2 wild type (WT) mice [17]. In addition, G2019S overexpressing mice [18] and Drosophila [19], exhibited tau alterations including mislocalization and hyperphosphorylation. Therefore, we investigated the relationship between the *LRRK2 I2020T* mutation and tau phosphorylation. We examined brain tissue from the Sagamihara family, a Japanese kindred originally reported to be linked to the *PARK8* locus [20], and found abnormally increased deposits of phosphorylated tau in the brainstem. Additionally, we showed that LRRK2 I2020T enhances tau phosphorylation in cultured cells co-expressing both LRRK2-I2020T and 3 or 4-repeated tau.

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However, there was no direct interaction between mutant LRRK2 and tau proteins. Our results indicate that the presence of the pathological I2020T mutation causes hyperphosphorylation of tau and may participate in the pathogenesis of PD and other tau-associated neurodegenerative diseases. Our findings contribute to the understanding of PARK8 pathogenesis.

2. Material and methods

2.1. Subjects

We examined the brains of six patients who came to autopsy. The clinical findings of patients A-E have been reported previously [20,22,23]. In this report patient A represents case 3, B case 4,C case 5 D case 9 E case 10 from the previous report [23]. All patients showed a good response to levodopa developing motor complications in the later stages of their disease, consistent with idiopathic PD. None had marked autonomic or cognitive dysfunction.

Patient F was a 68-year-old female. At 51 years of age, she developed clumsiness in the legs and gait disturbance, and was diagnosed with PD. Treatment with levodopa resulted in a marked improvement of her symptoms. She developed "wearing-off" motor fluctuations at age 57. By 64 years, she had developed visual hallucinations; by age 65, she was unable to walk without assistance. At She died at age 68 of multiple organ failure caused by pneumonia. You have said this already above. This patient was genetically determined to have the I2020T amino acid substitution in LRRK2.

2.2. Immunohistochemistry

Autopsy was performed within 6 h after death in each case. Brain sections were fixed in formalin and representative areas were embedded in paraffin and sectioned. Brain sections were stained with hematoxylin-eosin (H&E) for histological examination. For immunohistochemistry, sections of all patients were deparaffinized and incubated with the following primary antibodies: rabbit polyclonal antibody against ubiquitin (Dako; 1:800), and mouse monoclonal antibodies against phosphorylated α-synuclein (#64; Wako; 1:10,000) and phosphorylation-dependent tau (AT8; Innogenetics, 1:10,000). Primary antibodies were incubated overnight at 4 °C and then visualized by the avidin-biotin-peroxidase complex method. In addition, brain sections were stained with three repeat (3R) or four repeat (4R) tau-specific antibodies (RD3; 1:3000 or RD4; 1:1000 respectively; Upstate) [24], after pretreatment with potassium permanganate and oxalic acid to eliminate non-specific staining [25].

2.3. Construct preparation

pRK5-FLAG-LRRK2-WT and LRRK2-I2020T mutant vectors were prepared as described previously [21]. Three or 4 repeat tau cDNA was amplified from human adult brain using reverse transcript PCR and cloned into Myc-pcDNA 3.1(−). The rabbit polyclonal anti-LRRK2 antibody with synthetic peptides at the C-terminal end (2510-2527 aa) of human LRRK2 was generated as described previously [21]. Monoclonal mouse anti-human PHF-tau antibodies (clone AT-180 and clone AT-270), and tau antibody (clone HT-7) were from Innogenetics. Secondary antibodies conjugated to horseradish peroxidase were from GE HealthCare Bio-Sciences.

2.4. Cell Culture and transfection

COS-1 cells were grown in Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum (Sigma–Aldrich) and 1% penicillin/streptomycin (Invitrogen) under an atmosphere of 5% CO_2 at 37 °C. COS-1 cells were transiently transfected with LRRK2 and tau vectors using FuGENE HD Transfection Reagent (Roche Diagnostics) according to the manufacturer's protocol.

2.5. Immunoblotting

After 96 h, cells were lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% nonidet P-40, 0.25% DOC, 400 μM Na_3VO_4, 400 μM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM sodium pyrophosphate and protease inhibitors (Complete Mini, EDTA-free; Roche Diagnostics). To detect LRRK2, the samples were resolved on 3–8% NuPAGE Tris-acetate polyacrylamide gels (Invitrogen) in 1 × NuPAGE Tris-Acetate SDS running buffer and transferred onto polyvinylidene fluoride (PVDF) membrane. The membranes were blocked for 1 h in PBS containing 0.05% Tween-20 (PBS-T) and 5% non-fat milk (BD Difco) and then incubated overnight at 4 °C with the primary antibody. The membranes were washed with PBS-T three times followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (1:4000) and immunoreactivity assessed by chemiluminescence reaction using Western Lightning ECL (Perkin Elmer-Cetus). To detect tau, samples were resolved on 10% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen) in 1 × NuPAGE MOPS SDS running buffer and transferred onto PVDF membrane. The membranes were blocked for 1 h in TBS containing 0.05% Tween-20 (TBS-T) and 5% non-fat milk (BD Difco) and then incubated overnight at 4 °C with the primary antibody. The membranes were washed with TBS-T buffer three times followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse IgG (1:2000). The remaining steps were as described above. Blots were quantified using Image J software analysis.

2.6. Immunoprecipitation

Cell lysates were centrifuged at 15,000× *g* for 20 min at 4 °C and the resulting supernatant fluid was incubated with Anti-FLAG M2 Agarose (Sigma–Aldrich) overnight at 4 °C. The resin was separated by centrifugation, washed three times with lysis buffer and then boiled in Laemmli sample buffer. Finally, each sample was analyzed by SDS-PAGE followed by immunoblotting.

2.7. Statistical analysis

Three group comparisons were analyzed by UNI-ANOVA followed by Turkey's multiple comparison tests (SPSS). All values were expressed as mean ± SEM. A *P* value less than 5% denoted a statistically significant difference among the groups.

3. Results

3.1. Variable tau pathology in PD associated with LRRK2 I2020T mutation

A previous pathological study of *LRRK2 I2020T* patients reported an apparent loss of nigral neurons without LBs, with the exception of onecase with LBs. However extensive immunohistochemical analysis of phosphorylated tau was not performed.

The pathological features of patients A-E have been described previously [23]. The additional new patient (patient F) shared neuropathological features with patients A-E, as follows [23]. Macroscopic examination revealed marked discoloration of the substantia nigra (SN) (Fig. 1a), with a well preserved locus coeruleus (LC) (Fig. 1b). This region-specific contrast in neuropathology was confirmed following microscopic examination, with marked neuronal loss, gliosis and extraneuronal melanin present in SN (Fig. 1c), in contrast to well preserved neurons with minimal gliosis in LC (Fig. 1d). Of note, the dorsal motor nucleus of the vagus nerve (DVN) appeared predominantly normal. In addition, we observed Marinesco bodies, ubiquitin-positive intranuclear inclusions, in the surviving neuromelanin-containing SN neurons (Fig. 1e).

Characteristics of the tau-positive lesions are summarized in Table 1. Patient B and E had tau-positive lesions restricted to the brainstem, namely SN, LC and the trochlear nucleus (Fig. 2a). In patients C and D, abnormal phosphorylated tau depositions were observed not only in the brainstem but also in the hippocampus and amygdala. Senile plaques were not found in any regions. In patients A and F, there were no tau-positive lesions. Immunohistochemistry with isoform-specific antibodies, determined that the tau-positive lesions contained both 3R and 4R tau (Fig. 2b, c). Overall, these results show that the I2020T mutation causes autosomal-dominant PD with a pleomorphic pathology, as observed with other LRRK2 mutations.

3.2. LRRK2 is associated with hyperphosphorylation of tau

Based on our pathological findings in *LRRK2 I2020T* patients, we hypothesized that mutant LRRK2 may be involved in hyperphosphorylation of tau. To determine the effect of LRRK2 I2020T on tau phosphorylation, we co-transfected COS-1 cells with LRRK2-WT or I2020T and 4R tau. Levels of phosphorylated tau and total tau were assessed by western blotting using antibodies, which recognize tau phosphorylation, AT-180 at Thr231 and AT-270 at Thr181 (Fig. 3c, d). Neither LRRK2-WT nor I2020T changed expression levels of total tau protein (Fig. 3c, d). However, significantly increased levels of phosphorylated 4R tau were detected in cells with overexpressed LRRK2-I2020T, but not WT (AT-180: 100.0 ± 1.2% [mean ± SEM] with WT vs. 118.5 ± 1.5% with I2020T, $p < 0.001$; AT-

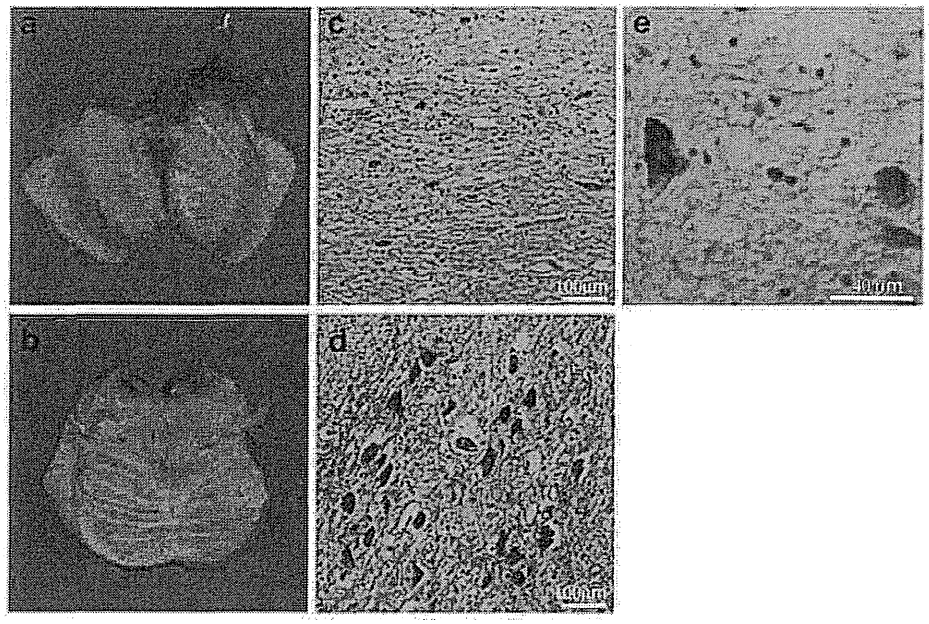

Fig. 1. Neuropathology of patient F, a LRRK2 I2020T carrier from the original Japanese Sagamihara family. Marked discoloration of the substantia nigra (SN, a) and relative preservation of locus coeruleus (LC, b). Marked neuronal loss with gliosis in the SN (c, H&E) is in contrast with preserved neurons in LC (d, H&E). Marinesco bodies are abundant in the SN (e, ubiquitin immunostain). Bars: c, d:100 μm; e: 40 μm.

270: 93.7 ± 4.0% with WT vs. 113.8 ± 5.3% with I2020T, $p < 0.001$; Fig. 3c, d). Next, we determined if I2020T affects expression levels of phosphorylated 3R tau. LRRK2-I2020T induced a significant, albeit modest, increase in the level of phosphorylated 3R tau protein compared with WT (AT-180: 94.9 ± 2.4% with WT vs. 100.5 ± 6.5% with I2020T, n.s.; AT-270: 93.5 ± 1.2% with WT vs. 104.1 ± 2.5% with I2020T, $p < 0.01$; Fig. 3a, b). To investigate further the interaction between LRRK2 and tau, we performed immunoprecipitation experiments. There was no evidence of a direct interaction between either LRRK2-WT or I2020T mutant with 4R tau (Fig. 3e).

4. Discussion

Tau pathology has been identified in the brains of PD patients with *LRRK2* mutations, with reports of various forms of tau depositions of, for example PSP-like or AD-like distribution and pattern of age related changes [26,27]. In this study, we identified tau pathology in four patients with *LRRK2 I2020T* mutation; an increased amount of phosphorylated tau was associated with LRRK2 I2020T mutation compared to wild type in cultured cell models. In addition, we found that affected members of the Sagamihara family display a homogeneous pattern of neuronal loss, namely degeneration of the SN with relative preservation of LC and DVN. This is in sharp contrast to idiopathic PD, where involvement of LC and DVN is observed. We also identified Marinesco bodies in our patients. The presence of Marinesco bodies has been described in other *LRRK2*-linked PD patients with R1441C [2] and G2019S mutations [14]. Thus, mutant LRRK2 may possibly affect dopaminergic neurons by accelerating the formation of Marinesco bodies.

In contrast to the homogeneity of neuronal degeneration that we observed, deposits of α-synuclein were confirmed only in patient E, and tau-positive deposits in the brainstem nuclei also varied? among the subjects. In previous reported pathological findings of *LRRK2*-linked PD, the presence of LBs and tau deposits did not overlap, even in the same family, which is in agreement with our observations in the Sagamihara family. Cookson et al. reported that although clinical features of *LRRK2*-linked PD were similar to sporadic PD, the pathological findings varied, confounding the correlation between etiology and disease expression [29]. Similarly, all examined members of the Sagamihara family showed typical PD features irrespective of pathological deposits. In addition, we did not find a direct correlation between tau deposits and clinical symptoms. Tau-positive deposits were seen in the

Table 1
Summary of tau pathology in LRRK2 I2020T carriers from the Sagamihara family

	Patient A	Patient B	Patient C	Patient D	Patient E	Patient F
Hippocampus	–	–	+	+	–	–
Meynert	–	–	–	–	+	–
Amygdala	NA	–	–	++	NA	NA
IV	–	+++	++	–	NA	NA
LC	–	+	++	+	+	–
Central gray matter	–	–	++	–	–	–
SN	–	–	+	–	+	–
Braak stage	<1	<1	2	3	<1	<1

The severity and distribution of NFT pathology was estimated using Braak staging (Braak and Braak 1991) (– none; + mild; ++ moderate; +++ severe; n/a not applicable). Tau pathology was observed in 4 out of 6 patients. Two individuals (patient B and E) had tau-positive lesions restricted to the brainstem, with another two individuals (patient C and D), showing tau-positive lesions in the hippocampus as well as the brainstem. The remaining two patients (patient A and F) did not show tau-positive lesions in any brain regions. IV; trochlear nucleus, LC; locus coeruleus, SN; substantia nigra.

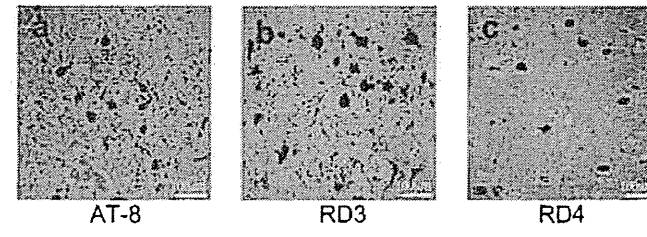

Fig. 2. Tau pathology in patient B, a LRRK2 I2020T carrier. Representative immunohistochemical analysis of tau in the trochlear nerve nucleus from Patient B. Sections are labeled with AT8 (a), RD3 (b) and RD4 (c). Bars: c, d:100 μm; e: 40 μm.