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Original Investigation

Lower Motor Neuron Involvement in TAR DNA-Binding Protein of 43 kDa–Related Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis

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IMPORTANCE TAR DNA-binding protein of 43 kDa (TDP-43) plays a major role in the pathogenesis of frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS). Although a pathological continuity between FTLD and ALS has been suggested, the neuropathological changes of the lower motor neuron (LMN) systems have not been assessed in TDP-43–associated FTLD (FTLD-TDP), to our knowledge.

OBJECTIVE To investigate a pathological continuity between FTLD-TDP and ALS by comparing their respective neuropathological changes in the motor neuron system.

DESIGN AND SETTING A retrospective clinical medical record review and a semiquantitative neuropathological evaluation of the cranial motor nerve nuclei and spinal cord were conducted at autopsy. We included 43 patients with sporadic FTLD-TDP, type A, B, or C, from 269 consecutively autopsied patients with TDP-43 proteinopathy. Patients were categorized as having FTLD without ALS, FTLD-ALS (onset of FTLD symptoms/signs preceded those of ALS), or ALS-FTLD (onset of ALS symptoms/signs preceded those of FTLD).

MAIN OUTCOMES AND MEASURES Neuronal TDP-43 pathological changes and neuronal loss.

RESULTS Forty-three patients were included in the clinical analysis, and 29 from whom spinal cords were obtained were included in the neuropathological analysis. Survival time was significantly shorter in the FTLD-ALS and ALS-FTLD groups than in the FTLD without ALS group ($P < .001$). At neuropathological examination, 89% of patients in the FTLD without ALS group showed aggregations of TDP-43 in the spinal motor neurons. The LMN loss was most severe in ALS-FTLD, followed by FTLD-ALS and FTLD without ALS. All the patients with type A or C FTLD-TDP were included in the FTLD without ALS group, and all those with type B pathological changes were in the FTLD-ALS or the ALS-FTLD group. Lower motor neuron loss and TDP-43–positive skeinlike inclusions were observed in all pathological subtypes.

CONCLUSIONS AND RELEVANCE The LMN systems of FTLD-TDP frequently exhibit neuropathological changes corresponding to ALS. Thus, a pathological continuity between FTLD-TDP and ALS is supported at the level of the LMN system.

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Frontotemporal lobar degeneration (FTLD) is a sporadic or familial neurodegenerative disease that clinically encompasses frontotemporal dementia, language disorder, and motor symptoms.[1] Immunohistochemical profiles show that approximately half of patients with FTLD present with tau-positive disease, but the other half primarily exhibit an accumulation of TAR DNA-binding protein of 43 kDa (TDP-43), referred to as FTLD-TDP.[2-5] Currently, the cortical TDP-43 pathological changes in sporadic FTLD-TDP are classified into 3 subtypes: A, B, and C.[6-8]

TAR DNA-binding protein of 43 kDa is also a major disease protein in amyotrophic lateral sclerosis (ALS), which is characterized by upper motor neuron and lower motor neuron (LMN) involvement.[9] The pathological features of LMN involvement in ALS include neuronal loss, gliosis, TDP-43-positive neuronal inclusions with skeinlike or round shapes and glial inclusions, and Bunina bodies.[10]

Some patients exhibit symptoms of both ALS and FTLD, and the cerebral cortices of patients with FTLD and ALS almost always show type B TDP-43 changes.[7,11-13] Thus, a pathological continuity between FTLD and ALS has been proposed based on brain TDP-43 pathological findings. Studies of the cerebral cortex, including the motor cortex, and subcortical gray matter have shown common TDP-43 pathological findings in FTLD, FTLD with ALS, and ALS.[12,14-17] However, the neuropathological features of LMN systems in FTLD-TDP have not been investigated comprehensively, particularly in the spinal cord, although characterization of these features is necessary to confirm the pathological relationship between FTLD and ALS.

In this study, we investigated LMN pathological findings in patients with sporadic FTLD-TDP who clinically demonstrated FTLD, FTLD with ALS, or ALS. We also investigated the correlation between TDP-43 pathological subtypes (type A, B, and C) and LMN involvement to further elucidate the continuity of FTLD and ALS.

Methods

Study Patients

We enrolled 269 consecutively autopsied patients with sporadic and adult-onset FTLD, FTLD with ALS, or ALS in which pathological aggregation of TDP-43 was confirmed at the Department of Neuropathology, Institute for Medical Science of Aging, Aichi Medical University, from 1988 to 2012. All patients had been clinically evaluated by neurological experts in the affiliated hospitals of Nagoya University School of Medicine or Aichi Medical University. Permission to perform an autopsy and archive the nervous system tissues for research purposes was obtained from family members after death. The clinical data on the included patients were obtained from case notes made at diagnosis and at an advanced stage of illness. We initially excluded 216 of the 269 patients because they did not present clinical FTLD symptoms. In 53 patients, FTLD or FTLD with ALS was diagnosed according to the diagnostic criteria of FTLD and ALS.[1,18] Moreover, we subclassified FTLD with ALS into FTLD-ALS (onset of FTLD symptoms/signs preceded those of ALS) and ALS-FTLD (onset of ALS symptoms/signs preceded those of FTLD) groups.

The enrolled patients were categorized into 3 groups: FTLD without ALS, FTLD-ALS, and ALS-FTLD. The FTLD symptoms were categorized into 2 groups: behavior-variant frontotemporal dementia and language impairments.[14] The LMN symptoms/signs were defined by progressive muscular weakness, muscular atrophy, fasciculation, or electromyographic findings. We excluded patients with Alzheimer disease-associated neurofibrillary pathological abnormalities that were more advanced than Braak stage IV,[19] those with argyrophilic grain disease, and those with invalid clinical data. Finally, 43 patients were included in the clinical analysis (11 with FTLD without ALS, 9 with FTLD-ALS, and 23 with ALS-FTLD). For comparison, we prepared 13 age-matched controls (mean [SD] age at death, 68.2 [6.9] years) who had no diagnosis of any neurodegenerative disease, dementia, or cerebrovascular disease.

Clinical Analyses

The information regarding sex, age at onset, disease duration, and duration between onset of FTLD and ALS was collected from clinical notes. Causes of death were classified as respiratory failure due to respiratory muscle weakness, pneumonia, or other. The last category comprised systemic diseases other than respiratory failure or pneumonia, including cancer, ileus, infections, and renal failure. Information on the subtypes of dementia, clinical data on motor symptoms, and electromyographic results were also collected.

Pathological Evaluations

For the neuropathological analysis, we excluded patients who had received a respirator/tracheotomy (n = 11) or whose spinal cord was not available (n = 3). In total, 29 patients were included in the neuropathological analysis and divided into 3 groups: FTLD without ALS (n = 9), FTLD-ALS (n = 8), and ALS-FTLD (n = 12). The tissues were fixed in 20% neutral-buffered formalin. The paraffin-embedded tissue blocks were cut at a thickness of 4.5 μm. We evaluated sections from the spinal cord and whole brain. The whole spinal cord was examined at each segmental level, but only the cervical cord was available in 3 patients and the sacral cord was not available in another 3.

For routine neuropathological examinations, the sections were stained with hematoxylin-eosin and Klüver-Barrera. Immunohistochemical studies were performed using a standard polymer-based method with the EnVision Kit or anti-goat immunoglobulin (Dako). As primary antibodies, we used antibodies to the following: anti-ubiquitin (ubiquitin, monoclonal mouse, 1:250; Millipore), anti-TDP-43 (TARDBP, polyclonal rabbit, 1:2500; ProteinTech), anti-phosphorylated TDP-43 (pTDP-43 ser409/410, polyclonal rabbit, 1:2500; CosmoBio), anti-phosphorylated tau (AT-8, monoclonal mouse, 1:4000; Innogenetics), anti-β-amyloid (β-amyloid 6F/3D, monoclonal mouse, 1:100; Dako), anti-CD68 (CD68, monoclonal mouse, 1:200; Dako), anti-cystatin C (cystatin C, polyclonal rabbit, 1:200; Dako), p62 N-terminal (p62N, polyclonal

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guinea pig, 1:100; Progen), anti-ubiquilin 2 (UBQLN-2 5F5, monoclonal mouse, 1:5000; Abnova), and anti-choline acetyltransferase (ChAT, polyclonal goat, 1:100; Millipore). Diaminobenzidine (Wako) was used as the chromogen.

Antigens were retrieved with trypsin for anti-CD68 immunohistochemistry and with 95°C 3 mmol/L citrate buffer at 95°C for 20 minutes, followed by 5-minute incubation in 98% formic acid for anti-p62N, anti-TDP-43, anti-pTDP-43, and anti-ChAT immunohistochemistry. To confirm the presence of TDP-43-positive inclusions within the cholinergic motor neurons, we performed double immunohistochemistry using anti-pTDP-43 and anti-ChAT antibodies. Spinal cord specimens were prepared from 3 patients with type A, 3 with type B, and 2 with type C. Initially, the specimens were immunostained with the anti-ChAT and anti-goat immunoglobulin antibodies and diaminobenzidine. The anti-ChAT antibody was inactivated in distilled water at 100°C for 20 minutes, followed by immunohistochemistry with pTDP-43 and violet pigmentation using a VIP Peroxidase Substrate Kit (SK-4600; Vector).

For the semiquantitative neuropathological analysis, 2 investigators (Y.R. and M.Y.) observed the specimens containing the facial and hypoglossal nuclei and the anterior horn of the spinal cord. They evaluated the severity of LMN neuropathological changes that are indicative of ALS (neuronal loss, gliosis, aggregation of macrophages, TDP-43-immunopositive neuronal inclusions, and Bunina bodies) and graded neuronal loss and gliosis using Klüver-Barrera and hematoxylin-eosin staining. The investigators also evaluated the aggregations of macrophages rather than rod-shaped microglia using anti-CD68 immunohistochemistry and identified Bunina bodies using hematoxylin-eosin staining and anti-cystatin C immunohistochemistry. They scored the severity of neuronal loss and gliosis as grade 0 (none), 1 (mild), 2 (moderate), or 3 (severe) (eFigure 1 in Supplement. The appearance of TDP-43-positive inclusions was scored as grade 0 (none), grade 1 (1-5 neuronal inclusions per 5 fields; ×20 objective), grade 2 (6-10 inclusions), or grade 3 (≥11 inclusions) using anti-pTDP-43 immunohistochemistry.

Pathological cortical TDP-43 subtypes were identified according to current neuropathological criteria, using specimens from the frontal lobes, temporal lobes, and hippocampus.[5] For FTLD-TDP, type A was defined as the presence of neuronal cytoplasmic inclusions predominantly in the neocortex layer 2 and short dystrophic neurites; type B, as a predominance of neuronal cytoplasmic inclusions in all cortical layers; and type C, as a predominance of long dystrophic neurites in layer 2 and cytoplasmic inclusions in the dentate granular cells of the hippocampus. Our patient series did not include type D, which is characterized by numerous short dystrophic neurites and neuronal intranuclear inclusions in association with valosin-containing protein gene mutations. We also evaluated pathological changes in the upper motor neuron systems that include the primary motor cortex and corticospinal tract (CST). We evaluated the presence or absence of neuronal loss and gliosis in the primary motor cortex and myelin pallor, as well as the aggregation of macrophages in the CST.

Immunohistochemical Screening of Hexanucleotide Repeat Expansion Sequence in Chromosome 9 Open Reading Frame 72

Our study focused on sporadic FTLD-TDP, and patients with familial histories of FTLD or ALS, dementia, or other neurodegenerative diseases were excluded. However, FTLD or ALS associated with chromosome 9 open reading frame 72 (C9ORF72) hexanucleotide expansion exhibits pathological aggregation of TDP-43 and, in some cases, low penetration,[20,21] although these mutations are extremely rare in Japan.[22] It was recently reported that the pattern of ubiquilin abnormalities in ALS and FTLD corresponds well with the presence of C9ORF72 hexanucleotide expansion.[23] The UBQLN-2-positive, p62-positive, but TDP-43-negative thick dystrophic neurites are abundantly present in patients with C9ORF72 hexanucleotide expansion, predominantly in the hippocampus and cerebellum. Because the materials for a genetic study were not available for a large proportion of our patients, we histologically screened C9ORF72 hexanucleotide expansion with the absence of UBQLN-2 and p62N-positive thick dystrophic neurites in the temporal lobes and cerebella of all patients.

Statistical Analysis

The Mann-Whitney test was applied to continuous variables between 2 groups, and the Kruskal-Wallis test was applied to the analysis of continuous variables among 3 groups. The χ^2 test was used for categorized variables among 3 groups. Spearman rank correlation coefficient analyses were applied to univariate correlations between the clinical groups and severity of pathological changes. Survival curves were constructed using the Kaplan-Meier method. The end point of clinical course was defined as death or the introduction of a respirator or tracheotomy. The significance level for all comparisons was set *at* $P < .05$. All statistical tests were 2 sided and were conducted using the PASW 18.0 program (IBM SPSS).

Results

Clinical Analysis

Patient characteristics are summarized in the **Table**. The mean (SD) time from symptom onset to death or respirator or tracheotomy administration was 50.5 (58.4) months across all patients. The survival time from symptom onset did not differ significantly between the FTLD-ALS and ALS-FTLD groups but was significantly shorter for the FTLD without ALS group than for the FTLD-ALS or ALS-FTLD group (**Figure 1** and Table; $P < .001$). The most common cause of death for the ALS-FTLD and FTLD-ALS groups was respiratory failure, but patients with FTLD without ALS commonly died of other systemic diseases ($P < .001$). Frequencies of dementia subtypes did not significantly differ between the clinical groups.

With regard to motor symptoms/signs, 3 patients in the FTLD without ALS group had hyperreflexia, 1 had the Babinski sign, and 1 had spasticity, but none had a clinical diagnosis of progressive lateral sclerosis (PLS) according to the published diagnostic criteria of PLS.[24] Patients with FTLD-ALS or

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Table. Clinical and Demographic Patient Characteristics

Characteristic	Patient Group			P Value
	FTLD Without ALS (n = 11)	FTLD-ALS (n = 9)	ALS-FTLD (n = 23)	
Sex, No. female/male[a]	8/3	3/6	10/13	.16
Age at onset, mean (SD), y[b]	62.4 (9.4)	58.2 (11.3)	61.2 (9.5)	.79
Clinical duration without respirator or tracheotomy, median (range), mo[c]	84.0 (47.0-360.0)	28.0 (7.0-60.0)	22.0 (7.0-71.0)	<.001
Duration between FTLD and ALS, median (range), mo[d]		18.0 (4.0-48.0)	19.0 (0-60.0)	.92
Patients with tracheotomy, No. (%)[a]	0	0	2 (9)	.40
Patients with respirators, No. (%)[a]	0	1 (11)	8 (35)	.047
Duration with respirator or tracheotomy, median (range), mo		30.0	39.0 (1.0-141.0)	...
Causes of death, No. (%)[a]				
Respiratory failure	0	6 (67)	20 (87)	<.001
Pneumonia	3 (27)	3 (33)	3 (13)	.37
Other	8 (73)	0	0	<.001
Subtypes of dementia, No. (%)				
Behavior-variant FTD	7 (64)	7 (78)	19 (83)	.29
Language impairments	4 (36)	2 (22)	4 (17)	.62
Motor symptoms/signs, No. (%)				
Muscle weakness[a]	0	9 (100)	23 (100)	<.001
Muscle atrophy	0	7 (78)	22 (96)	<.001
Fasciculation	0	4 (44)	15 (65)	.002
Hyperreflexia	3 (27)	5 (56)	17 (74)	.009
Babinski sign	1 (9)	4 (44)	3 (13)	.08
Spasticity	1 (9)	0	1 (4)	.63
Electromyography, No. (%)				
Total examined	2 (18)	4 (82)	21 (91)	...
Active denervation[a]	0	3 (75)	12 (57)	.054

Abbreviations: ALS, amyotrophic lateral sclerosis; ALS-FTLD, onset of ALS symptoms/signs preceding those of frontotemporal lobar degeneration (FTLD); ellipses, not significant; FTD, frontotemporal dementia; FTLD-ALS, onset of FTLD symptoms/signs preceding those of ALS.

[a] χ^2 Test.

[b] Kruskal-Wallis test.

[c] Log-rank test.

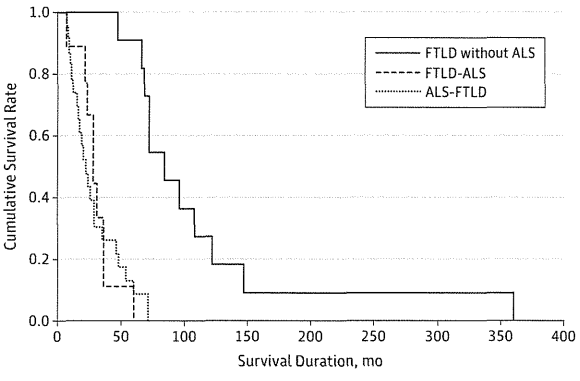
[d] Mann-Whitney test.

ALS-FTLD generally exhibited both upper motor neuron and LMN symptoms/signs except for 3 who exhibited only LMN symptoms/signs. Based on the electromyographic data, active denervation potentials (positive sharp waves and fibrillation potentials[18]) were identified in 3 patients with FTLD-ALS and 12 with ALS-FTLD but not in any of those with FTLD without ALS.

Pathological Evaluations of the LMN System

The results of semiquantitative pathological evaluations of the 3 clinical groups are summarized in **Figure 2**. In the FTLD without ALS group, 8 of 9 patients (89%) showed pTDP-43-positive neuronal inclusions. In addition, neuronal loss and gliosis in the spinal anterior horns were observed in 5 of 11 patients (45%) and Bunina bodies were present in 4 (36%). The pathological changes in LMN systems were most severe in the ALS-FTLD group, followed by the FTLD-ALS group, and were rather mild in the FTLD without ALS group. Among control patients, 1 had a pTDP-43-positive glial inclusion in the lumbar anterior horn, but this patient did not show neuronal loss, gliosis, or Bunina bodies (eFigure 2 in Supplement).

Figure 1. Survival by Clinical Group



Kaplan-Meier plot showing the survival rates of patients with frontotemporal lobar degeneration (FTLD) without amyotrophic lateral sclerosis (ALS) (solid line; n = 11), those in whom the onset of FTLD symptoms/signs preceded those of ALS (FTLD-ALS) (dashed line; n = 9), and those in whom the onset of ALS symptoms/signs preceded those of FTLD (ALS-FTLD) (dotted line; n = 23). Survival times were significantly shorter in patients with FTLD without ALS than in those with FTLD-ALS or ALS-FTLD ($P < .001$).

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Figure 2. Semiquantitative Evaluations of Pathological Changes by Clinical Group

	FTLD Without ALS									FTLD-ALS								ALS-FTLD												P Values	r Values
Patients	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29		
Clinical duration, mo	47	66	84	108	147	360	68	96	122	22	23	28	28	31	36	36	60	7	8	9	10	16	20	22	24	26	54	60	71		
Neuronal loss																															
Facial nuclei	+	-	-	-	+	-	-	-	-	++	NA	+	+	+	+	++	+	+	++	+	+	++	++	NA	++	+	+++	+++	+	<.01	0.730
Hypoglossal nuclei	-	-	-	-	-	-	-	-	-	+++	NA	++	+++	++	+	+++	++	+++	+	+	+++	+++	+++	+++	++	+++	+++	+++	+++	<.01	0.823
Anterior horn of Cx	+	++	-	-	+	-	-	-	+	++	++	+	++	++	+	++	+	+++	++	++	++	++	++	NA	+++	+++	++	+++	++	<.01	0.804
Anterior horn of Tx	+	+	NA	+	+	-	NA	-	+	+	++	++	++	++	++	+	++	++	+	+++	++	++	+++	++	++	+++	+++	+++	++	<.01	0.768
Anterior horn of Lx	-	-	NA	+	+	-	NA	-	-	+	+	+	+	+	+	++	+	++	++	+++	+	++	++	NA	++	+++	++	++	++	<.01	0.856
Anterior horn of Sx	-	-	NA	+	-	-	NA	-	-	+	NA	+	+	+	+	++	+	++	++	NA	NA	++	++	NA	++	++	+++	NA	+	<.01	0.880
Gliosis																															
Facial nuclei	++	-	-	-	+	-	-	-	-	++	NA	+	+	+	+	++	+	++	++	+	++	++	++	NA	+	++	+++	+++	++	<.01	0.768
Hypoglossal nuclei	-	-	-	-	-	-	-	-	-	+++	NA	++	++	++	+	+++	++	+++	+	+	+++	+++	+++	+++	++	+++	+++	+++	++	<.01	0.828
Anterior horn of Cx	+	++	-	-	+	-	-	-	+	++	++	+	+++	++	+	++	+	+++	+	++	++	++	++	NA	++	+++	++	+++	+++	<.01	0.730
Anterior horn of Tx	+	+	NA	-	+	-	NA	-	+	+	++	+	++	++	++	+	++	++	+	+++	++	++	+++	++	+	+++	+++	+++	++	<.01	0.740
Anterior horn of Lx	-	-	NA	+	+	-	NA	-	-	+	+	+	++	+	+	++	+	++	++	+++	++	++	++	NA	+++	+++	++	++	++	<.01	0.878
Anterior horn of Sx	-	-	NA	+	-	-	NA	-	-	+	NA	+	++	+	+	++	+	++	++	NA	NA	++	++	NA	+++	+++	+++	NA	++	<.01	0.904
pTDP-43-positive neuronal inclusions																															
Facial nuclei	+	-	-	-	+	-	-	-	-	++	NA	+	+	+	+	+	+	+	++	+	++	+++	+	NA	+	++	+	+	++	<.01	0.729
Hypoglossal nuclei	-	+	+	-	-	-	+	-	-	+	NA	+	+	+	+	++	+	+	+	-	+	+++	+	-	+	+	-	+	+	.08	0.348
Anterior horn of Cx	+	+	+	+	+	-	+	+	-	++	++	+	+	+	+	+	++	++	+++	+	+++	++	++	NA	+	+	+	+	+	<.01	0.511
Anterior horn of Tx	+	+	NA	-	+	-	NA	+	+	+	+	++	+	+	+	+	+	+	+	+	++	+	++	+	+	+	+	+	+	<.05	0.403
Anterior horn of Lx	+	+	NA	+	-	-	NA	+	+	+	+++	++	++	+	+	++	++	+	++	++	++	+	+++	NA	+	+	+	++	+	<.05	0.412
Anterior horn of Sx	+	-	NA	-	-	-	NA	+	-	++	NA	++	+	+	+	+	+	+	+	NA	NA	+	+++	NA	-	+	+	NA	+	<.05	0.458
Aggregation of macrophages																															
Facial nuclei	++	-	+	-	+	-	-	-	-	++	NA	+	-	++	+	++	+	++	+	+	+	+	++	NA	+	++	++	+	+	<.01	0.518
Hypoglossal nuclei	+	-	-	-	-	-	-	-	-	+	NA	-	+	++	+	+	-	+++	+	++	+	+	+++	-	++	+++	+	++	-	<.01	0.634
Anterior horn of Cx	+	+	-	+	+	-	-	+	+	++	+	+	+	++	+	++	++	+	+	+	+	-	++	NA	+	+	+	-	-	0.78	0.073
Anterior horn of Tx	+	+	NA	-	-	-	NA	+	+	++	+	++	+	++	+	+	+	+	+	++	++	+	++	+	+	+	+	+	+	<.05	0.435
Anterior horn of Lx	-	++	NA	+	-	-	NA	-	-	+	-	-	+	+	+	++	+	+++	++	+++	++	+	+++	NA	+	+++	++	++	++	<.01	0.721
Anterior horn of Sx	-	+	NA	+	-	-	NA	-	-	+	NA	-	+	+	+	+	+	+++	++	NA	NA	+	++	NA	+	+++	++	NA	+	<.01	0.751
Bunina bodies	+	+	-	+	+	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+		
Brain TDP-43 disease type	A	A	A	A	A	A	C	C	C	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B		

Findings shown include the severity of neuronal loss, gliosis, phosphorylated TAR DNA-binding protein of 43 kDa (pTDP-43) pathological changes, and aggregations of macrophages and the presence of Bunina bodies in the lower motor neuron systems. The severity of each pathological change was graded as 0 (none [–, not colored]), 1 (mild [+, green]), 2 (moderate [++, yellow]), or 3 (severe [+++, red]). Neuropathological changes became increasingly severe in those in whom amyotrophic lateral sclerosis (ALS) symptoms/signs preceded those of frontotemporal lobar degeneration (FTLD; ALS-FTLD), as well as the FTLD-ALS (FTLD symptoms/signs preceding those of ALS) and FTLD without ALS groups (Spearman rank order). Cx indicates cervical cord; Lx, lumbar cord; NA, not assessed; Sx, sacral cord; TDP-43, TAR DNA-binding protein of 43 kDa; and Tx, thoracic cord.

According to cortical TDP-43 pathological findings,[5] 29 patients were classified into 3 subtypes: A (n = 6), B (n = 20), or C (n = 3). Patients with FTLD without ALS showed type A or C disease, whereas those with FTLD-ALS or ALS-FTLD all showed type B disease (Figure 2 and **Figure 3**). For all the subtypes, the LMN system showed neuropathological changes that were indicative of ALS, including pTDP-43-positive neuronal and glial inclusions, neuronal loss, and gliosis. In patients with type A disease (**Figure 4**A-H), the severity of neuronal loss and gliosis in LMN systems ranged from none to moderate. Five patients (83%) in this group had pTDP-43-positive, skeinlike cytoplasmic and/or nuclear inclusions (Figure 4B and C), and 4 (67%) had Bunina bodies (Figure 4E and F) in the LMNs.

All 20 patients in the type B group (Figure 4I-L) showed neuronal loss, gliosis, and pTDP-43-positive skeinlike cytoplasmic inclusions in the LMN systems, and 18 (90%) had Bunina bodies. Among the 3 patients with type C disease (Figure 4M-P), 1 (33%) had mild loss of the LMNs (Figure 4M), and all 3 (100%) had pTDP-43-positive skeinlike cytoplasmic inclusions in the LMNs (Figure 4N). Unlike patients with the other subtypes, those with type C disease lacked Bunina bodies. Moreover, thick dystrophic neurites were prominent in the spinal anterior horn in patients with type A or C disease but rarely present in those with type B disease (Figure 4G and O). These dystrophic neurites were larger in diameter (8-12 μm) than those found in the cortices. In a double immunohistochemical analysis, pTDP-43-positive inclusions were found within the cytoplasm of ChAT-positive neurons in patients with type A, B, and C disease (Figure 4H, L, and P).

Pathological Evaluations of the Upper Motor Neuron System

In the primary motor cortex, neuronal loss and gliosis were evident in 5 patients with FTLD without ALS (56%), 2 with FTLD-ALS (25%), and 3 with ALS-FTLD (25%). Myelin pallor in the CST was evident in 6 patients with FTLD without ALS (67%), 1 with FTLD-ALS (12%), and 2 with ALS-FTLD (17%). Aggregations of macrophages in the CST were evident in 4 patients with FTLD without ALS (44%), 5 with FTLD-ALS (62%), and 6 with ALS-FTLD (50%).

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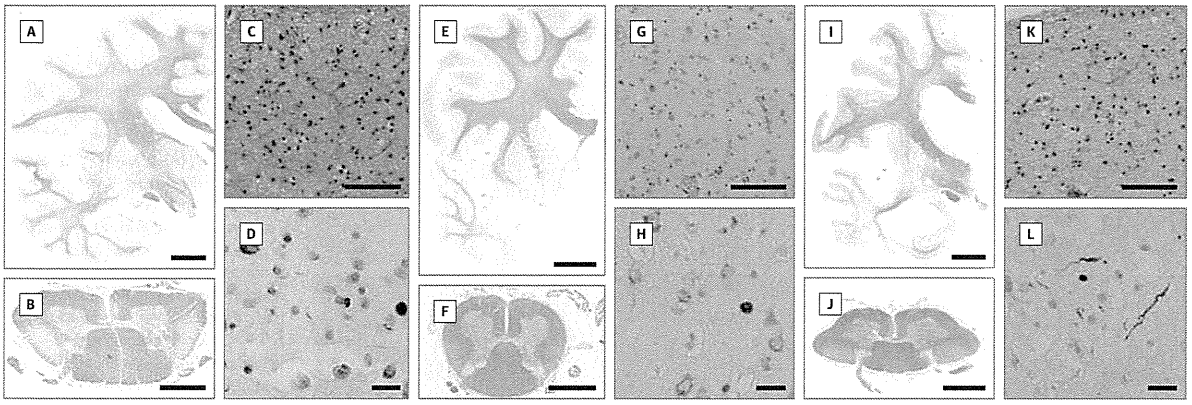
Figure 3. Semimacroscopic Appearances and Brain Pathological Findings in Patients With Type A, B, and C Pathological Changes



Findings in patients with type A (A-D) , B (E-H), and C (I-L) pathological changes. In a patient with type A pathological change, cerebral coronal sections showed cortical atrophy of the parasylvian region (A). Transverse section of the cervical cord showed marked myelin pallor in the corticospinal tract (B). Microscopically, the frontal cortices showed marked neuronal loss (C) and phosphorylated TAR DNA-binding protein of 43 kDa (pTDP-43)-positive neuronal inclusions and short dystrophic neurites (D). In a patient with type B pathological change, the cerebral cortex showed severe temporal atrophy (E), neuronal loss (G), and pTDP-43-positive neuronal inclusions (H). The corticospinal tract showed mild myelin pallor (F). In a patient with type C patholgoical change, the frontal and temporal cortices showed severe atrophy (I), marked neuronal loss (K), and pTDP-43-positive long dystrophic neurites (L). The corticospinal tract showed marked myelin pallor (J). Klüver-Barrera staining (A, B, E, F, I, and J), hematoxylin-eosin staining (C, G, and K), and pTDP-43 immunohistochemistry (D, H, and L) were performed. Scale bars represent 1 cm (A, E, and I), 3 mm (B, F, and J), 100 μm (C, G, and K), and 20 μm (D, H, and L). Original magnifications are ×1 (A, B, E, F, I, and J), ×200 (C, G, and K), and ×400 (D, H, and L).

Anti-UBQLN-2 and Anti-p62N Immunohistochemistry

No patients showed any cerebellar UBQLN-2-positive or p62N-positive structures. In the temporal lobes, UBQLN-2-positive structures were occasionally observed in 8 patients, but abundant, thick, and aggregatelike structures, which are found in patients with C9ORF72 expansions, were not observed (eFigure 3 in Supplement). We presumed that our patients did not have C9ORF72 expansions.

Discussion

Our study demonstrated that pTDP-43-associated pathological changes were common in the spinal anterior horns of the FTLD without ALS, FTLD-ALS, and ALS-FTLD groups. Neuronal loss and gliosis were most severe among the ALS-FTLD group, followed by the FTLD-ALS and then the FTLD without ALS groups. Our results clearly demonstrated the pathological continuum among TDP-43-associated FTLD and ALS, even at the LMN level.

Although the FTLD without ALS group that lacked LMN symptoms showed a loss of LMNs, the degree of neuronal loss and TDP-43 disease were generally mild in this group. Experiment data using ALS mouse models revealed that symptoms developed when approximately 29% of spinal motor neurons were lost.[25] Further investigation will be needed to clarify whether LMN involvement occurs in a later stage of illness or progresses very slowly compared with cerebral involvement in FTLD without ALS.

Our results revealed that the FTLD-TDP types A, B, and C were associated with neuropathological changes corresponding to ALS in the spinal motor neurons. The severity of neuronal loss and pTDP-43 disease in the spinal motor neurons may differ quantitatively among these neuropathological subtypes. Based on cortical TDP-43 pathological findings, patients in the type B group had severe neuronal loss and diffuse pTDP-43-positive neuronal inclusions, which were entirely identical to ALS, whereas these changes were mild in the type C group. In type A, LMN pathological findings were diverse regardless of clinical duration; their severity and extension may be heterogeneous among patients with type A disease, unlike those with type B or C disease. Indeed, type A disease has also been identified in the FTLD with ALS phenotype in sporadic or familial (C9ORF72 expansion or progranulin gene mutations) form.[2,5,21,26] Dystrophic neurites were prominent in the spinal anterior horn of patients with type A or C disease. In our patient series, Bunina bodies were observed in most patients with type A or B disease but were absent in those with type C disease, findings consistent with those of previous studies.[3,17]

Several studies have demonstrated that some patients with FTLD-TDP, particularly type C, showed marked CST degeneration.[3,11,17,27] We also observed a marked myelin pallor in the CST in 67% of patients with FTLD without ALS, 12% with FTLD-ALS, and 16% with ALS-FTLD (50% for type A, 15% for type B, and 100% for type C). Some patients showed neuronal loss or gliosis in the primary motor cortex to varying extents. Furthermore, patients with FTLD without ALS often exhibited severe degenerative changes in broad areas of the frontal cortices. The broad involvement of the frontal lobes might also contribute to the CST degeneration because CST fibers arise not only from the primary motor cortex but also from the premotor cortex and supplementary motor areas.[28]

Two limitations of our study is that the evaluation of slight or very mild muscle weakness was not completed and that there were few patients with electromyographic data in

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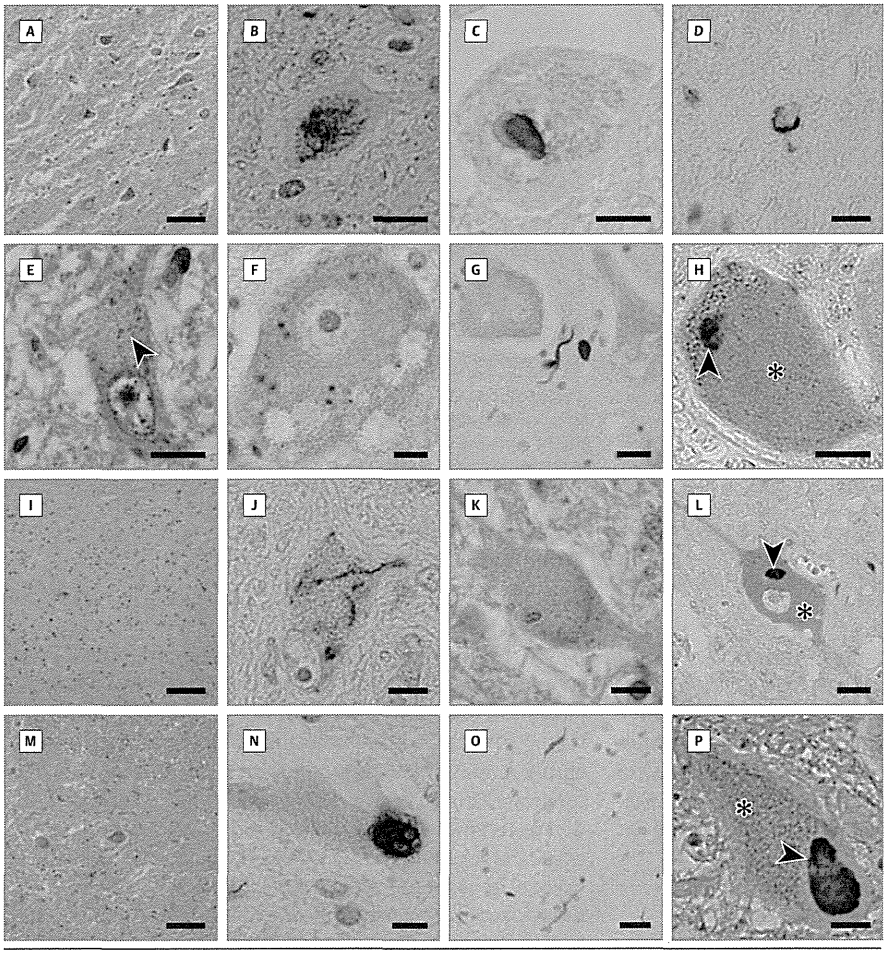
Figure 4. Pathological Findings of Spinal Motor Neuron in Subtypes of TAR DNA-Binding Protein of 43 kDa (TDP-43) Pathological Changes



Patients with type A (A-H), type B (I-L), and type C (M-P) pathological changes. A patient with type A pathological change showed mild neuronal loss (A), phosphorylated TDP-43 (pTDP-43)–positive skeinlike cytoplasmic inclusions (B), nuclear inclusions (C), and glial inclusions (D), Bunina bodies (E [arrow] and F) in the spinal anterior horn, and dystrophic neurites (G). In a patient with type B pathological change, neuronal loss (I), pTDP-43–positive skeinlike cytoplasmic inclusions (J), and Bunina bodies (K) were markedly observed. In a patient with type C pathological change, the spinal anterior horn showed mild neuronal loss (M), pTDP-43–positive skeinlike cytoplasmic inclusions (N), and dystrophic neurites (O). Double immunohistochemistry for choline acetyltransferase (ChAT) and pTDP-43 revealed cytoplasmic inclusions (violet [arrows]) present within the cytoplasm of a ChAT-positive spinal motor neuron (brown [asterisks]) of patients with type A (H), B (L), or C (P) pathological change. Hematoxylin-eosin staining (A, E, I, K, and M), pTDP-43 immunohistochemistry (B, C, D, G, J, N, and O), cystatin-C (F), and double immunohistochemical analysis for pTDP-43 and ChAT (H, L, and P) were performed. Scale bars represent 100 (A, I, and M), 20 (G, L, and O), and 10 (B-F, H, J, K, N, and P) μm. Original magnifications are ×100 (A, I, and M), ×400 (G, L, and O), and ×1000 (B-F, H, J, K, N, and P).

the FTLD without ALS group. However, our clinical data demonstrated that patients with FTLD without ALS had significantly longer survival times than those with FTLD-ALS or ALS-FTLD. These prognostic data correspond well to previous results.[29,30] In addition, the causes of death differed considerably between the FTLD without ALS group and the FTLD-ALS and ALS-FTLD groups. Respiratory failure was observed in patients with FTLD-ALS or ALS-FTLD but not in those with FTLD without ALS, and respiratory failure was strongly associated with severity of LMN loss. These results support the view that classification of FTLD based on the presence of LMN involvement was applicable in this study.

In conclusion, the LMN systems of FTLD-TDP generally show neuropathological changes that are indicative of ALS, although the severity of pathological changes differs among clinical phenotypes or subtypes of cortical TDP-43 disease. A pathological continuity between FTLD-TDP and ALS is supported by evidence of LMN involvement.

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A Case of α-*Synuclein* Gene Duplication Presenting With Head-Shaking Movements

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ABSTRACT

Background: *PARK4* is a candidate locus for familial Parkinson's disease (PD), combined with multiplication of the α-*synuclein* gene (*SNCA*). The eventual phenotype is dependent on the copy number of *SNCA*. Mutations in *leucine-rich repeat kinase 2* (*LRRK2*) are also causative of parkinsonism. This report describes a man who presented at our hospital complaining of a stagger after running and difficulty in handling the mouse of a personal computer, having suffered tremors since his twenties. Nine months after treatment and discharge, he developed titubation and began to drag his right foot.

Methods: We examined the patient's family pedigree for *SNCA* dosage, using quantitative polymerase chain reaction. We also screened this pedigree for mutations in *parkin* and *LRRK2*, using gene-sequencing techniques.

Results: We identified the proband, his sister, and his paternal uncle as carrying a duplication of *SNCA*. In addition, we found that the proband and his mother carried the G2385R variant of the *LRRK2*, a strong risk factor for PD in Asians and the rare V1450I variant, although only the proband showed symptoms of parkinsonism. No mutations were found in *parkin*.

Conclusions: The combination of *SNCA* gene duplication and *LRRK2* G2385R variant may explain the early onset of disease in this patient. ©2012 *Movement* Disorder Society

Key Words: head shaking; familial Parkinson's disease; alpha-synuclein gene duplication; leucine-rich repeat kinase 2

Parkinson's disease (PD) is a progressive disorder of the nervous system, with a significant number of patients having a family history of the disease. In 1900, Golbe et al. reported on a large kindred, where PD was inherited in an autosomal-dominant fashion.[1] Later, Polymeropoulos et al. identified a mutation in the α-*synuclein* gene (*SNCA*) of this family.[2] Alpha-synuclein is a major component of Lewy bodies and Lewy neurites. Abnormalities in *SNCA* include not only point mutations, but also variable copy numbers. In addition, *PARK4* has been reported on as a candidate locus for familial PD in cases with multiple copies of *SNCA*.[3] Reports show that patients with homozygous *SNCA* duplications suffer earlier ages of PD onset and death and present with more-severe cognitive impairment, compared with patients carrying heterozygous *SNCA* duplications.[4,5] These data suggest that the PD phenotype is dependent on the *SNCA* copy number.

Patients with heterozygous *SNCA* duplications present with various clinical features. Dementia has been observed in some families, although cognitive decline does not appear to be prominent in this cohort of patients.[6] Familial PD with heterozygous *SNCA* duplications shows 33% to 50% penetrance. It would seem likely that *SNCA* duplications alone are not enough to trigger PD, and that an additional risk factor is required. Mutations in *leucine-rich repeat kinase 2* (*LRRK2*) cause parkinsonism in *PARK8*-linked fami-

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

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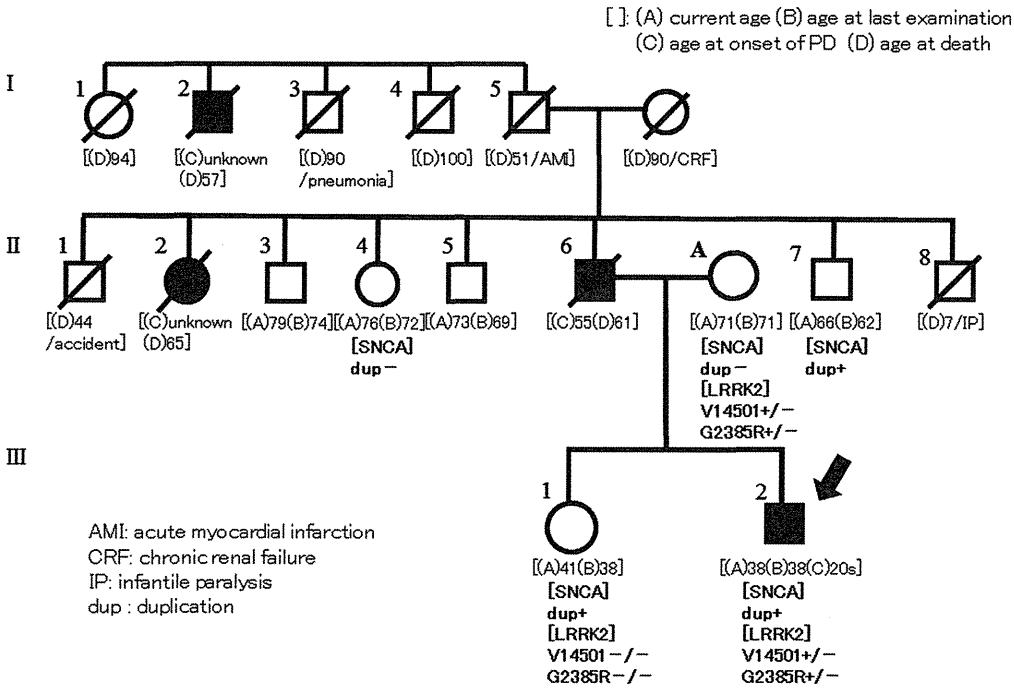

FIG. 1. Pedigree of patient III-2. Black boxes represent affected patients. Five members (II-4, II-A, II-7, III-1, and III-2) were examined clinically by a neurologist, and blood samples were collected. III-2 was affected, and II-7 and III-1 were carriers of the *SNCA* duplication.

lies as an autosomal-dominant form of familial PD.[7] The most common mutation in *LRRK2* is G2019S, found in Caucasian and North African patients.[8] Conversely, variants that represent risk factors for sporadic PD are distributed over a wide domain. Moreover, mutations in *SNCA* and *LRRK2* have been identified as strong risk factors for PD in two genome-wide association studies (GWAS).[9] The G2385R variant of *LRRK2* constitutes a specific risk factor in Asian sporadic PD, with a frequency of 10%.

It is probable that altered *SNCA* and *LRRK2* together could drive both the development and severity of PD symptoms. In fact, previous reports have observed genetic mutations of *LRRK2* in combination with other genes, such as *parkin* and the *GRB10-interacting GYF protein 2* (*GIGYF2*), in patients with PD.[10] However, there is discordance in the influence of multiple genetic variants on the clinical phenotype of PD. Both *SNCA* multiplication and *LRRK2* mutations are causative genetic events associated with autosomal-dominant familial PD at a higher frequency than any other genetic events.

Here, we detail the case of a patient with *SNCA* duplication and the *LRRK2* G2385R variant, presenting with head-shaking movements. The patient's pedigree shows familial PD inherited in an autosomal-dominant manner. Head tremors have not previously been reported on in cases with *SNCA* duplication.

Patients and Methods

Patients

The proband (III-2; Fig. 1) had experienced tremors in his right hand since his twenties. The tremor then appeared in his left hand a few years later. At the age of 33, he first visited our hospital, complaining of a stagger after running and difficulty in handling the mouse of a personal computer.

His father (II-6), paternal aunt (II-2), and paternal uncle (I-2) had developed parkinsonism in their fifties.

Genetic analysis

Each subject provided written informed consent. Genomic DNA was extracted from peripheral blood using a QIAamp DNA Blood Maxi Kit (QIAGEN, Valencia, CA). All participants were examined for gene dosage of *SNCA* by quantitative polymerase chain reaction, as described previously.[6] We also screened all the exons and exon/intron boundaries of *LRRK2* for mutations in the proband (III-2; Fig. 1), using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Screening of the proband's mother and sister was limited to exons 31, 41, and 48 of *LRRK2*. When

novel or rare variants of *LRRK2* were detected, direct sequencing of samples from 114 unrelated healthy subjects was performed (mean age at sampling: 56.0 ± 15.4 years; range, 23–98; female/male ratio: 1.05). This study was approved by the ethics committee at Juntendo University School of Medicine (Tokyo, Japan).

Results

Clinical Findings

Neurological examinations revealed cogwheel rigidity, bradykinesia, and tremors at rest. These signs were more pronounced in the right limbs. Scores of the Mini–Mental State Examination and frontal assessment battery were normal (30 and 18, respectively), but the times taken in the Trail-Making Test were 1 minute and 59 seconds in part A and 2 minutes and 20 seconds in part B, suggestive of a mild decline in attentiveness. Our patient displayed no orthostatic hypotension. Brain T2-weighted MRI yielded normal results. Voxel-based specific regional analysis for Alzheimer's disease (VSRAD), using T1-weighted three-dimensional (3D) MRI, showed atrophy of cerebral gray matter, localized to the left-side Brodmann area 10 and the left cerebellum (Fig. 2). The ratio of cardiac radiolabeled metaiodobenzylguanidine (MIBG) accumulation in the region of interest in the heart to that of the mediastinum (H/M ratio) was reduced (early, 1.58; delayed, 1.26).

Our patient was started on ropinirole hydrochloride at 0.75 mg/day, and this was increased over a period of 4 weeks to a maintenance dose of 3 mg/day. His bradykinesia and tremors improved slightly, and he was discharged from our hospital. Nine months after discharge, he developed "yes-yes" titubation at a frequency of 1 Hz and began to drag his right foot (see Video, Segment 1). His titubation stopped in the decubitus position with anteflexion of the neck. Gait disturbance improved when walking backward. Because his bradykinesia was progressive, the dose of ropinirole hydrochloride was increased to 6 mg/day, and levodopa/carbidopa was added at a dose of 400 mg (four times a day). The titubation and foot dragging showed some degree of improvement with L-dopa/carbidopa. Six months after starting L-dopa/carbidopa, horizontal head movement emerged along with the "yes-yes" titubation (see Video, Segment 2). At this time, we noted an "on-off" phenomenon, with an on-period of 2 hours. During the off-period, both bradykinesia and titubation were exacerbated. Entacapone was added at a dose of 400 mg/day, prolonging the on-period to 3 hours, but the patient was unable to go to the bathroom unaided during the night. Zonisamide was then started at a dose of 25 mg/day, and within 2 months, he was able to complete this task.

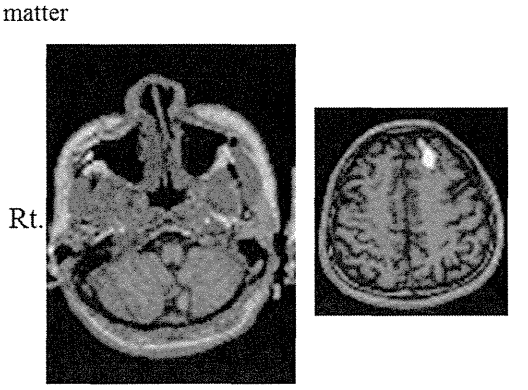

FIG. 2. VSRAD showed atrophy of cerebral gray matter localized to the left-side Brodmann area 10 and left cerebellum. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

An MIBG examination of the proband's sister (III-1) showed a normal H/M ratio.

Mutation Screening

The proband (III-2), his sister (III-1), and paternal uncle (II-7) were identified as having *SNCA* duplications. The G2385R variant of *LRRK2*, a strong risk factor for PD in Asians,[11] and the rare variant, V1450I[12] (which was absent from 114 healthy subjects), were present in the proband (III-2) and his mother (II-A), but within the family, only the proband showed symptoms or signs of parkinsonism.

We also screened the *parkin* gene for mutations, but found none. No analyses for other genes related to dystonia were performed.

Discussion and Conclusion

The unique features of this case were the early age at onset, compared to previously reported cases with *SNCA* duplication, and the marked dystonia in the neck and right leg. Dragging of the right foot was the result of dystonia, because this symptom disappeared when the patient walked backward. The titubation followed a stereotypic pattern, appearing with specific postures, and disappearing with resumption of a resting posture. Both symptoms mirrored each other in fluctuations, and both improved during the on-period. From these results, we concluded that the titubation also represented a form of dystonia. Dystonia is often present in familial PD, particularly in patients carrying *parkin* mutations, but is uncommon in families with *SNCA* duplications. The histopathological basis for primary dystonia remains unknown, although the disease is generally regarded as a disorder of basal ganglia and its efferent connections to the thalamus and brainstem, which, in turn, implies

functional abnormalities in the cerebellum.[13] Reductions in cerebellothalamic connectivity correlate with increased motor-activation responses.[14] In this case, VSRAD using T1-weighted 3D MRI[15] showed atrophy in the left Brodmann area 10 and the left cerebellum. These abnormalities may be responsible for dystonia.

Although triplication of *SNCA* leads to early-onset PD with dementia, the complication of dementia in patients with *SNCA* duplication remains controversial.[6,17–19]

The initial symptoms of *SNCA* duplication manifest 15 years after those in *SNCA* triplication families (50 years of age versus 35, respectively).[6,16,18,20] Onset occurred at a younger age in this study, compared with past reports. In addition, both *SNCA* duplication and the *LRRK2* G2385R variant were identified within our patient, together representing a strong risk for sporadic PD in Asian patients. *LRRK2* is involved in cytotoxicity and expression of *SNCA* in cellular and animal models of PD.[21,22] One possibility is that the *LRRK2* G2385R variant is related to this rare phenotype of *SNCA* duplication. Because our study included only a partial analysis of *LRRK2*, further investigation of this gene and all other modifying genes in cases of younger onset PD with head-shaking movement appears warranted. The lack of any apparent cognitive decline in this case may be the result of the younger age of this proband.

Legend to the Video

Segment 1. The patient showed "yes-yes" titubation at a frequency of 1 Hz. No tremors were present in the limbs at rest. While walking, he dragged his right leg.

Segment 2. Six months after the start of therapy with L-dopa/carbidopa, horizontal movement was added to the "yes-yes" titubation. Dragging of his right leg disappeared while walking backward. ■

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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.

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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.

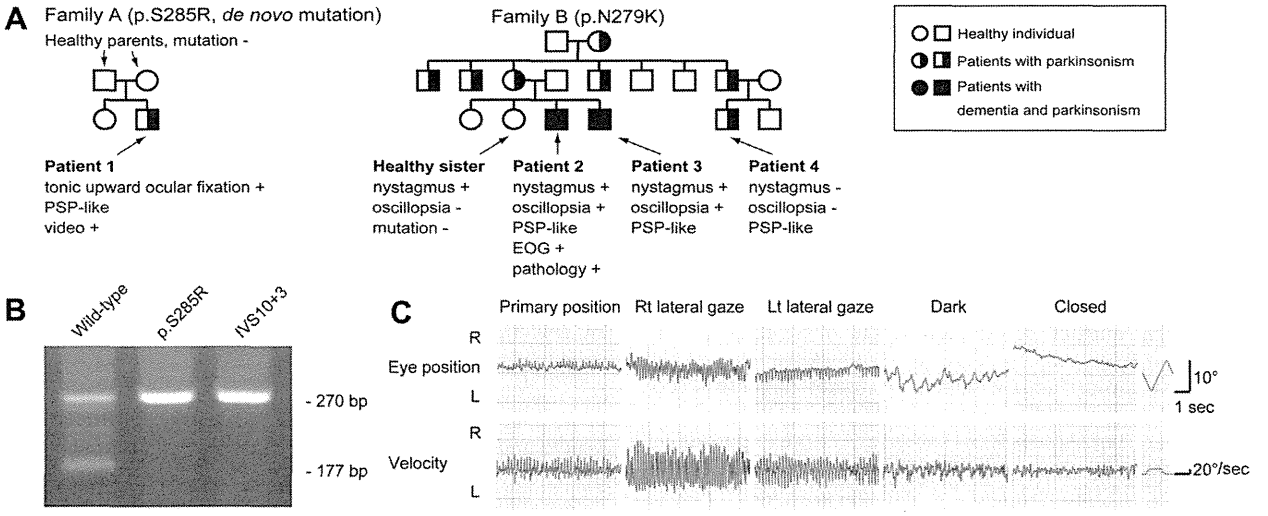

Fig. 1. (A) The pedigrees of families A and B. (B) Exon-trapping analysis for the effects of the *MAPT* p.S285R mutation on exon 10 splicing. (C) Horizontal electro-oculogram recordings in Patient 2.

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

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.

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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ATP13A2 deficiency induces a decrease in cathepsin D activity, fingerprint-like inclusion body formation, and selective degeneration of dopaminergic neurons

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ABSTRACT

Kufor-Rakeb syndrome (KRS) was originally described as an autosomal recessive form of early-onset parkinsonism with pyramidal degeneration and dementia. *ATP13A2* was identified as the causative gene in KRS. *ATP13A2* encodes the ATP13A2 protein, which is a lysosomal type5 P-type ATPase, and ATP13A2 mutations are linked to autosomal recessive familial parkinsonism.

Here, we report that normal ATP13A2 localizes in the lysosome, whereas disease-associated variants remain in the endoplasmic reticulum. Cathepsin D activity was decreased in ATP13A2-knockdown cells that displayed lysosome-like bodies characterized by fingerprint-like structures. Furthermore, an atp13a2 mutation in medaka fish resulted in dopaminergic neuronal death, decreased cathepsin D activity, and fingerprint-like structures in the brain. Based on these results, lysosome abnormality is very likely to be the primary cause of KRS/PARK9.
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1. Introduction

Parkinson's disease (PD) is one of the most common movement disorders, and it is caused by loss of dopaminergic neurons. The molecular mechanisms underlying neuronal degeneration in PD remain unknown; however, it is now clear that genetic factors heavily contribute to the pathogenesis of this disease [1]. In approximately 10% of patients with clinical features of PD, the disease state has a strict familial etiology.

PARK9-linked PD is an autosomal recessive early-onset disorder that is characterized by levodopa-responsive parkinsonism, supranuclear gaze palsy, pyramidal signs, and dementia; this condition is also called Kufor-Rakeb syndrome (KRS), being named for a consanguineous Jordanian family containing four members with this disorder [2]. Recently, *ATP13A2* was identified as the causative gene for KRS/PARK9. The *ATP13A2* gene comprises 29 exons that encode a lysosomal type 5 P-type ATPase with 10 transmembrane domains [3]. Thus far, eight mutations have been reported in just five families and in two additional unrelated patients, and no neuropathological examination of an autopsy case has been documented [3–8]. The function of the ATP13A2 protein remains largely unknown, but it is supposed that ATP13A2 might participate in autophagic protein degradation via the lysosomal pathway [9].

Here, we established and analyzed a cell culture model of ATP13A2 knockdown and Atp13a2 mutant medaka fish to elucidate the mechanisms underlying PARK9-associated pathology.

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