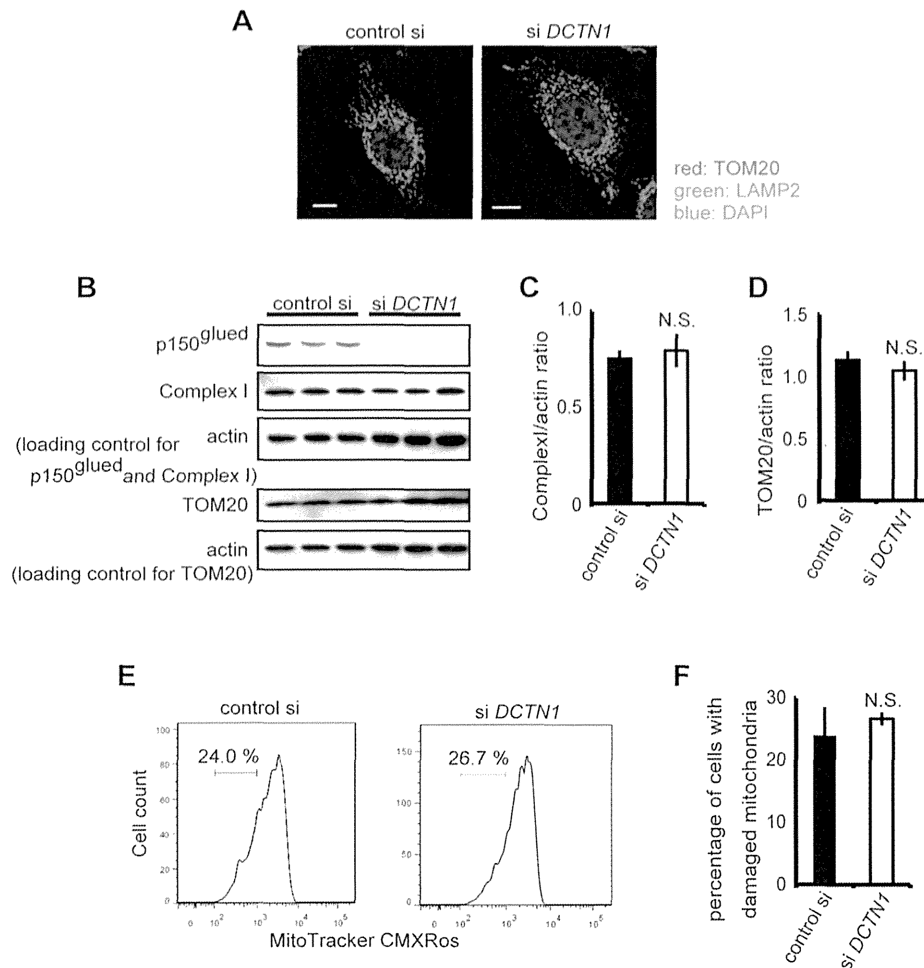

Figure 4. Depletion of p150^{glued} does not induce damaged mitochondria accumulation. (A) Control siRNA or DCTN1 siRNA transfected cells were fixed and co-stained with antibodies against LAMP2 (green) and TOM20 (red), and analyzed using confocal microscopy. Bars, 10 μm. (B) Control siRNA or DCTN1 siRNA transfected HeLa cells were analyzed by immunoblotting with antibodies against complex I, TOM20, and actin. (C, D) Densitometry analysis of complex I (C) and TOM20 (D) levels relative to actin was performed. (E, F) DCTN1 siRNA transfected HeLa cells were incubated with Mitotracker-Red CMXRos and intracellular fluorescence intensity was measured by flow cytometry. The histograms of MitoTracker-Red CMXRos fluorescence (E) and the percentages of cells with reduced mitochondrial potentials (F) are shown. The error bar indicates each standard deviation. Statistics are from three independent experiments: N.S., not significant.
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was kindly provided by Dr. Koji Yamanaka (RIKEN, Brain Science Institute, Wako, Japan).

RNA interference

CASP8, *CASP3*, and *DCTN1* siRNA were obtained as ON-TARGETplus *CASP8* siRNA SMARTpool (Dharmacon, Lafayette, CO, USA, L-003466-00), ON-TARGETplus *CASP3* siRNA SMARTpool (Dharmacon, L-004307-00), and ON-TARGETplus *DCTN1* siRNA (Dharmacon, J-012874-06), respectively. Non-targeting controls were also purchased from Dharmacon. Transfection of siRNA was performed using Lipofectamine 2000 according to the manufacturer's protocols (Invitrogen).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) for 15 min, permeabilized with 50 μg/mL digitonin in 1× phosphate-buffered saline (PBS) for 15 min, and incubated with 10% fetal bovine serum (FBS, Invitrogen) and 1% bovine serum albumin (BSA, Wako, Osaka, Japan) in 1× PBS for 30 min at room temperature. Cells were then incubated overnight with primary antibodies at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature. Cells were then mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). The following antibodies were used in this study: anti-FLAG (Sigma, F7425; 1:500), anti-α-tubulin (Sigma, T6199; 1:1000), anti-TOM20 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-11415; 1:500), and anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA, 9661; 1:800). Alexa Fluor 488- and 594-conjugated secondary antibodies (1:500) were from Invitrogen. For live-cell imaging, HeLa cells were grown on MatTek glass bottom dishes (MatTek Corp.) and transfected. After 24 hours, 200 nM MitoTracker Red CMXRos (Invitrogen) was added to the media for 15 min at 37°C, followed by a wash in PBS at 37°C. Images were acquired on a Zeiss LSM510 META

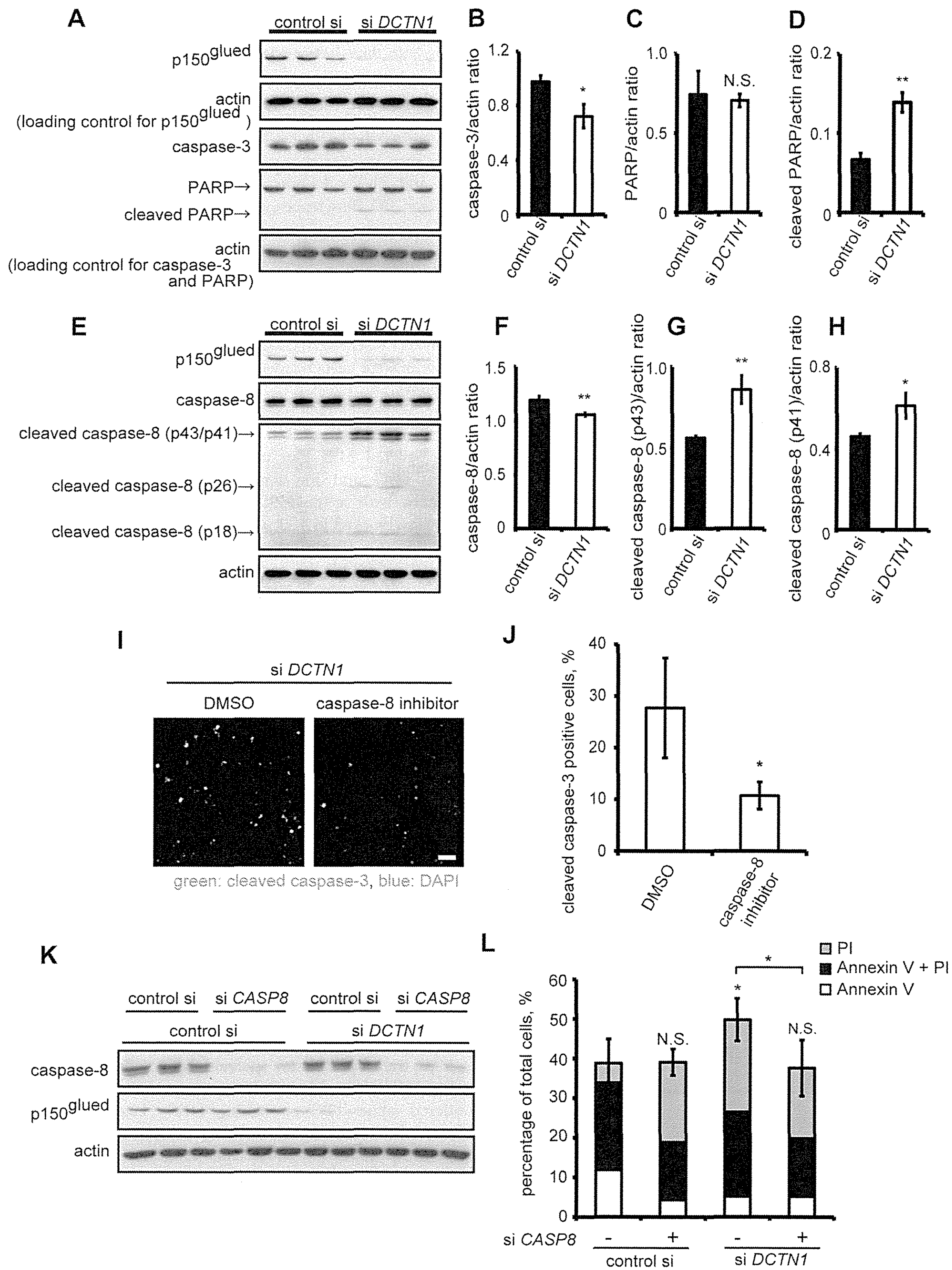

Figure 5. Depletion of p150^{glued} activates apoptotic pathways via caspase-8 cleavage. (A–H) HeLa cells were transfected with control scrambled siRNA or DCTN1 siRNA for 72 h, and immunoblotting analysis was performed with antibodies against caspase-3, PARP, cleaved PARP, and actin (G), or caspase-8, cleaved caspase-8, and actin (E) to monitor the effects on the apoptotic pathway. Densitometry analysis of each protein levels relative to actin was performed (B–D, F–H). (I,J) HeLa cells were transfected with DCTN1 siRNA and incubated with DMSO or 25 μM caspase-8 inhibitor for 48 h. Cells were fixed and stained with antibodies to cleaved caspase-3 (green) and DAPI (blue), and analyzed using fluorescence microscopy (I). The percentage of cleaved caspase-3-positive cells is shown (J). Bars, 100 μm (K,L). Twenty-four hours after transfection with control scrambled siRNA

or CASP8 siRNA, HeLa cells were transfected with control or DCTN1 siRNA. Forty-eight hours after DCTN1 siRNA transfection, cells were analyzed by immunoblotting to monitor the knockdown efficiency of caspase-8 and p150^{glued} (K), and stained with Annexin V and PI to assess rates of cell death using flow cytometry (L). The error bar indicates each standard deviation. Statistics are from three independent experiments: N.S., not significant; *,p<0.05; **,p<0.01; ***,p<0.001.
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confocal microscope (Zeiss, Oberkochen, Germany) using a 63× water-immersion objective lens (NA = 1.2). Images were magnified using Zeiss LSM510 v3.2 software. Colocalization was quantified using the colocalization plugin of ImageJ 1.43 (NIH).

Quantification of aggregate formation and cleaved caspase-3 positive cells

Aggregate formation and cleaved caspase-3 positive cells were assessed using a fluorescence microscope (Axio Imager 2, Zeiss) with a 40× objective. GFP-positive or FLAG-positive cells were selected and the population of cells with aggregates was counted. Quantification was based on at least three independent experiments, each carried out in triplicate, and 100–300 cells were counted in each slide. The scorer was blinded to the identity of the slides.

Cell viability assays

GFP-positive cells were scored 24 and 48 h after transfection for abnormal cell nuclei, according to previously reported criteria [30] using a fluorescence microscope (Axio Imager 2) with a 40× objective. Analysis was performed with at least three independent experiments, each carried out in triplicate, and 100–400 cells were counted on each slide. The scorer was blinded to the identity of the slides. Detection of apoptotic cells was also determined using an annexin V/propidium iodide (PI) detection kit (Invitrogen), according to the manufacturer's protocol. Briefly, cells were harvested and washed with 1× PBS 24 and 48 h after transfection. They were then incubated at room temperature with annexin V/Alexa350 and PI for 15 min and analyzed by flow cytometry (LSRFortessa, BD Biosciences, San Jose, CA, USA).

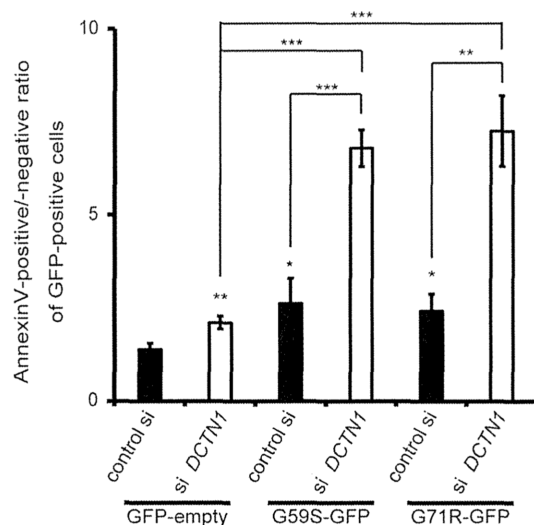

Figure 6. Depletion of p150^{glued} accelerates mutant p150^{glued}-induced cell death. Forty-eight hours after transfection with control scrambled siRNA or DCTN1 siRNA, HeLa cells were transfected with GFP-empty vector or mutant (G59S or G71R) p150^{glued} for 24 h. Cells were stained with Annexin V, and GFP-positive cells were analyzed by flow cytometry. The ratios of Annexin V-positive cells relative to Annexin V-negative cells were calculated. The error bar indicates each standard deviation. Statistics are from three independent experiments: *,p<0.05; **,p<0.01; ***,p<0.001.
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Flow cytometry analysis

Changes in the mitochondrial membrane potentials were assessed with MitoTracker Red CMXRos (Invitrogen). Twenty-four hours after transfection, PBS-washed cells were incubated in 50 nM MitoTracker Red CMXRos for 15 min at 37°C. After washing, cells were suspended in 1× PBS and were analyzed by flow cytometry.

Cleaved caspase-3 labeling was performed according to the flow cytometry protocol from Cell Signaling Technology. Briefly, HeLa cells were pelleted 24 h after transfection, fixed in 4% PFA for 10 min at 37°C and for 1 min on ice. The samples were permeabilized with ice-cold 90% methanol for 30 min on ice and then were blocked in 100 μL of incubation buffer (0.5 g BSA in 100 ml PBS) for 10 min at room temperature. Labeling of the samples was performed with anti-cleaved caspase-3 (Cell Signaling Technology, 9661; 1:800) antibodies for 1 h at room temperature. After incubation, the samples were washed and resuspended in incubation buffer containing Alexa 647-conjugated secondary antibody (Invitrogen-Molecular Probes; 1:500) for 30 min at room temperature. The samples were washed, resuspended in PBS and analyzed by flow cytometry. For flow cytometry analysis, 2,000–5,000 GFP-positive cells were analyzed for each sample and the experiments were performed at least in triplicate. Data were analyzed with CellQuest (BD Biosciences) and FlowJo software (Tree Star Inc., Ashland, OR, USA).

Western blotting

Cells transfected with *DCTN1* siRNA or GFP-tagged vectors for indicated times were lysed in cold RIPA buffer [25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] in the presence of protease inhibitors (Roche, Basel, Switzerland) for 20 min on ice. The lysates were centrifuged and the resulting supernatants mixed in NuPAGE LDS sample buffer (Invitrogen). The samples were resolved on 10–20% Tris-HCl gels (Bio Craft, Agra, India, MDG-297) in 1× Tris/Glycine/SDS buffer (Bio-Rad, Hercules, CA, USA) and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore). The membranes were blocked for 1 h in Tris-buffered saline (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 PBS-T three times followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse, rabbit, and guinea pig IgG (GE Health Care Biosciences, Pittsburgh, PA, USA). Immunoreactivity was assessed by chemiluminescence reaction using the ECL prime reagent (GE Health Care Biosciences). The antibodies used were as follows: anti-actin (Millipore, clone C4; 1:10000), anti-p150^{glued} (BD, 610473; 1:5000), anti-TOM20 (Santa Cruz, sc-11415; 1:1000), anti-complex I (Invitrogen, 459100; 1:1000), anti-caspase-3 (Cell Signaling Technology, 9665; 1:1000), anti-PARP (Cell Signaling Technology, 9542; 1:1000), anti-caspase-8 (Cell Signaling Tech-

nology, 4790; 1:500), and anti-cleaved caspase-8 (Cell Signaling Technology, 9496; 1:1000).

Electron microscopy

HeLa cells were plated on Thermanox plastic coverslips (Nunc, Penfield, NY, USA) and transfected with pAcGFP-empty, wild-type, G59S, and G71R p150^{glued} plasmids. Twenty-four hours after transfection, one set of cells was pre-fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C, and post-fixed with 2% OsO_4 in phosphate buffer for 1 h at 4°C. After fixation, they were dehydrated in a graded series of ethanol, placed in propylene oxide, and embedded in epoxy resin (Quetol 812, Nisshin-EM, Tokyo, Japan). Ultra-thin sections (90–100 nm) were cut using an ULTRACUT-UCT (Leica, Wetzlar, Germany) with a diamond knife. Sections were stained with 2% uranyl acetate in distilled water for 15 min followed by a lead staining solution for 5 min.

For immune electron microscopic analysis, another set of cells was pre-fixed in 4% PFA and 0.1% glutaraldehyde in phosphate buffer at 4°C, and post-fixed with 1% OsO_4 and 1.5% potassium ferricyanide in phosphate buffer for 1 h at 4°C. After fixation, they were dehydrated in a graded series of ethanol and embedded in LR White resin. Ultra-thin sections were cut, and samples were incubated in 3.8% sodium periodate for 1 h at room temperature. Samples were blocked with 2% BSA in PBS for 30 min at room temperature and then immunolabeled with primary anti-GFP antibody (Living Colors A.v. Peptide Antibody, Clontech, 632377; 1:10) followed by anti-rabbit immunogold (BB International, Cardiff, UK; 1:100). Afterwards, these samples were stained with 2% uranyl acetate in distilled water for 5 min followed by a lead staining solution for 1 min. All sections were examined with a JEM-1200EX (JEOL, Peabody, MA, USA) electron microscope at 80 KV.

Statistical analysis

Densitometry analysis was performed on immunoblots from three independent experiments using ImageJ 1.43. Differences among means were analyzed using 1- or 2-way ANOVA, followed, when results showed significant differences, by pair-wise comparisons between means using Tukey's Honestly Significant Difference Test. When only two groups were compared, the Student's t test was used. In all analyses, the null hypothesis was rejected at the 0.05 level. SYSTAT 13 software (Hulinks, Tokyo, Japan) was used for statistical calculations.

Supporting Information

Figure S1 Overexpression of mutant p150^{glued} disrupts p150^{glued} distribution and causes aggregate formation. (A) HeLa cells transfected with GFP-tagged wild-type or mutant p150^{glued} were fixed after 24 h and analyzed using confocal microscopy. Bars, 10 μm. (B) SH-SY5Y cells transfected with GFP-tagged wild-type or mutant (G59S or G71R) p150^{glued} were fixed and stained with an antibody against α-tubulin (red) after 24 h and analyzed using confocal microscopy. Bars, 10 μm. (C) HeLa cells transfected with 3xFLAG-tagged wild-type or mutant p150^{glued} were fixed and co-stained with antibodies against FLAG (green) and α-tubulin (red) after 24 h. Bars, 10 μm. (D) FLAG-positive cells were counted from three independent experiments. The percentage of FLAG-positive cells with aggregates is shown. The error bar indicates each standard deviation. Statistics are from three independent experiments. (E) Electron microscopy examination of HeLa cells transfected with GFP-tagged G59S or G71R p150^{glued}. Images on the right are magnified images of the boxed area from the left. Intracytoplasmic aggregate (a) is labeled. (F) HeLa cells were transfected with GFP-tagged wild-type or mutant (G59S or G71R) p150^{glued}, and cells were fixed and stained with anti-polyubiquitin antibody (FK2) after 24 h. (G) HeLa cells were co-transfected with FLAG-tagged TDP-43 and GFP-tagged wild-type or mutant (G59S or G71R) p150^{glued}, and cells were fixed and stained with antibody against FLAG after 24 h. Bars, 10 μm.
(TIF)

Figure S2 Mutant p150^{glued}-dependent apoptosis is not blocked by caspase-8 siRNA knockdown. (A, B) HeLa cells were transfected with control scrambled siRNA or caspase-8 siRNA for 72 h, and immunoblotting analyses were performed to monitor the knockdown efficiency of caspase-8 siRNA (A). Densitometry analysis of caspase-8 levels relative to actin was performed (B). (C, D). Twenty-four hours after transfection with control siRNA or caspase-8 siRNA, HeLa cells were transfected with GFP-empty or GFP-tagged G59S p150^{glued}. Forty-eight hours after transfection, cells were stained with Annexin V and PI, and GFP-positive cells were analyzed by flow cytometry. The error bar indicates each standard deviation. Statistics are from three independent experiments: N.S., not significant; ***,$p<0.001$.
(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: KI SS NF SK YT NH. Performed the experiments: KI SS YI HS. Analyzed the data: KI SS DY. Contributed reagents/materials/analysis tools: KI SS NF YL MK. Wrote the paper: KI SS NF.

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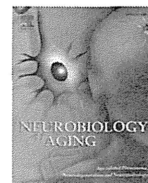

Brief communication

Clinicogenetic study of *GBA* mutations in patients with familial Parkinson's disease

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ABSTRACT

The glucocerebrosidase gene (*GBA*) is a known risk factor of Parkinson's disease (PD). We sequenced entire coding exons and exon/intron boundaries of *GBA* in 147 Japanese familial PD (FPD) patients from 144 families and 100 unrelated control subjects. Twenty-seven of 144 (18.8%) of index patients were heterozygous for known Gaucher disease mutations, suggesting that *GBA* heterozygous mutations are strongly associated with FPD (odds ratio = 22.9, 95% confidence interval = 3.1–171.2). The frequency was significantly higher in autosomal dominant PD (ADPD) compared with autosomal recessive PD. According to clinical assessments, PD patients with *GBA* mutations exhibited typical manifestations of PD or dementia with Lewy bodies (DLB), such as L-dopa responsive parkinsonism with psychiatric problems and/or cognitive decline. Interestingly, they also presented with reduced myocardial ^{123}I-metaiodobenzylguanidine uptake. Our findings suggest that heterozygous *GBA* mutations are strong risk factors in FPD, especially for autosomal dominant PD. Some patients with *GBA* heterozygous mutations develop clinical features of DLB. We speculate that *GBA* dysfunction may promote Lewy body formation, resulting in more severe PD or DLB phenotypes that are inherited in families.

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1. Introduction

Parkinson's disease (PD) is one of the most common neurodegenerative disorders. Patients develop disabled movement and complicating nonmotor symptoms, such as psychiatric disorders, cognitive dysfunction, olfactory nerve dysfunction, and sleep disorders (Weintraub and Burn, 2011). Cardinal features of PD are caused by marked loss of dopaminergic neurons in the substantia nigra, which is evidenced by the pathologic hallmark of Lewy bodies; however, PD is a more complicated and systemic disease. PD etiology was thought to be influenced mainly by the interactions between genetic and environmental factors. Nevertheless, recent developments in genetics have revealed that causative genes are involved in Mendelian-inherited parkinsonism (Hatano et al., 2009). Moreover, recent genome-wide association studies have also identified several common loci as genetic risk factors for PD (Hamza et al., 2010; Satake et al., 2009; Simon-Sanchez et al., 2009). During these studies and a subsequent meta-analysis, rare variants of the glucocerebrosidase gene (*GBA*; MIM#606463) have been found as risk factors of sporadic PD (SPD) (Aharon-Peretz et al., 2004; Sidransky et al., 2009). *GBA* is also known as the causative gene of Gaucher disease (GD), which is caused by a loss of function of hydrolytic enzyme activity and is inherited in an autosomal recessive pattern (Hruska et al., 2008; Tsuji et al., 1987).

Some genetic mutations in *GBA* were characterized as strong risk factors for SPD; however, there are few large studies of *GBA* mutations in familial PD (FPD) (Mitsui et al., 2009; Nishioka et al., 2010; Sidransky et al., 2009). Cosegregation was previously reported in a small number of families (Mitsui et al., 2009). One recent study emphasized an association between *GBA* mutations and cognitive impairment in PD (Alcalay et al., 2012).

In this study, we aimed to clarify the role of *GBA* mutations in PD, especially in FPD, by sequencing *GBA* in 147 FPD patients from 144 families and performing a comparative analysis of the clinical phenotype and severity to evaluate the association between *GBA* mutations and FPD.

2. Methods

2.1. Subjects

The study subjects comprised 147 FPD patients from 144 Japanese families and 100 Japanese controls (Table 1). The cohort of

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Table 1
Patient information

	n	Male/female	AAS (range)	AAO (range)
Patients	147	59/88	60.0 ± 12.8 (21–84)	50.8 ± 13.9 (13–81)
AD	85	35/50	57.7 ± 13.7 (21–84)	48.3 ± 14.2 (13–77)
AR	62	24/38	63.1 ± 10.8 (37–84)	54.3 ± 12.7 (25–81)
Controls	100	47/53	56.8 ± 16.3 (29–87)	

Key: AAO, age at onset; AAS, age at sampling; AD, autosomal dominant; AR, autosomal recessive.

Japanese patients consisted of 85 autosomal dominant PD (ADPD) and 59 autosomal recessive PD (ARPD). For the mode of inheritance to be considered autosomal dominant, there had to be affected family members in at least 2 consecutive generations; for autosomal recessive, we looked for affected siblings in the same generation. After detecting mutations, family members were analyzed to assess cosegregation. DNA samples were provided from various hospitals. Each patient submitted to a neurologic examination performed by expert neurologists and was given a diagnosis of PD on the basis of established criteria (Hughes et al., 1992). Diagnosis of dementia was given by the each clinician based on Mini Mental State Examination score (Folstein et al., 1975). All patients had good response to L-dopa. This study was approved by the ethics review committee of Juntendo University School of Medicine. All subjects provided informed and written consent prior to participation.

2.2. GBA mutation analysis

Genomic DNA was extracted from peripheral blood lymphocytes using standard protocols. Polymerase chain reaction was performed using previously reported primers to avoid amplifying the pseudogene (Mitsui et al., 2009). The purified polymerase chain reaction product obtained by ExoSAP IT (GE Healthcare, Salt Lake City, UT, USA) was subsequently used for dideoxy sequencing with BigDye Terminator Chemistry (Applied Biosystems, Foster City, CA, USA). The resulting products were loaded on ABI 310 or 3130 automated DNA sequence analyzers (Applied Biosystems) and analyzed with Sequencing Analysis Software v5.1 (Applied Biosystems). All exons and exon-intron boundaries were analyzed by direct sequencing.

To confirm Rec*Nci*I allele, we used TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) for TA cloning and separating alleles. After separating each allele, direct sequencing was performed as described above.

2.3. Clinical data analysis

To clarify the clinical features of patients with *GBA* mutations, 19 items (Table 2) concerning prominent PD symptoms were statistically compared between *GBA* mutation–positive and *GBA* mutation–negative groups. Other affected members with *GBA* mutations were also included.

2.4. Statistical analysis

Statistical analysis included the *t* test, Fisher's exact test, odds ratio, and its confidence interval, using GraphPad Prism version 5.0d (GraphPad Prism Software). The Hardy-Weinberg equilibrium test was performed using SNPAlyze v5.1 software (Dynacom, Chiba, Japan). In all statistical analyses, p values ≤ 0.05 were considered statistically significant.

3. Results

3.1. GBA mutations observed in FPD

In this study, we only observed heterozygous mutations; no individual had mutations in both alleles. We detected 6 nonsynonymous mutations: p.I(-20)V, p.G64V, p.R120W, p.D409H, p.L444P, and p.I489V; 1 nonsense mutation: p.W393X; 1 synonymous mutation: p.K466K; 1 frame-shift mutation: c.1447-1466delTGins; and 1 recombinant allele (Rec*Nci*I) in the Japanese population (Table 3). Among them, 5 mutations, p.R120W, p.D409H, p.L444P, c.1447-1466delTGins, and Rec*Nci*I, have been reported as causative mutations in GD patients. Patients with *GBA* mutation did not have causative *PARK2*, *PINK1*, and common *LRRK2* mutations.

p.I(-20)V is an amino acid change in the signal peptide region and was considered to be a single nucleotide polymorphism. The frequency of p.I(-20)V in patients with FPD (13 of 144, 9.0%) was not significantly different from the control subjects (10 of 100 = 10.0%, $p = 0.83$), and its genotype distribution was in Hardy-Weinberg equilibrium in both populations. Although p.K466K mutation was not detected in any control subjects, this synonymous mutation was also excluded for later clinical analyses because it is in a less important region of the final protein.

Two mutations, p.G64V and p.W393X (Fig. 1), were novel, and p.I489V was previously reported in SPD patients (Mitsui et al., 2009). The p.G64V mutation was caused by the substitution of the first amino acid of exon 4, and it seems to be segregated with PD (Figs. 1 and 2). In the family with p.W393X, no affected family member was confirmed for the mutation because of absent genomic DNA. The sequences around the region of mutation were interspecifically conserved in both mutations (Fig. 1).

The recombinant allele Rec*Nci*I was found in 1 patient and 1 control. p.L444P is more common, and p.R120W is less common in Japanese patients with GD, although p.R120W was frequently seen in Japanese FPD patients in the present study (9 of 144 = 6.3%, Table 3). However, p.L444P was the most frequent mutation seen in FPD patients (12 of 144 = 8.3%, Table 3).

In total, we found 31 FPD patients with heterozygous mutations that were reported in GD (p.R120W, p.D409H, p.L444P, c.1447-1466delTGins, and RecNciI) or unreported in GD (p.G64V,

Table 2
Comparison of clinical symptoms between *GBA*-positive and *GBA*-negative patients

Symptom	*GBA* mut (+)	*GBA* mut (−)	*p* value[a]
N	34	113	
Age at onset (mean ± SD)	49.1 ± 11.7	51.3 ± 14.5	
Resting tremor (%)	18 (52.9)	77 (68.1)	0.151
Bradykinesia (%)	27 (79.4)	99 (87.6)	0.265
Rigidity (%)	29 (85.3)	99 (87.6)	0.772
Gait disturbance (%)	28 (82.4)	95 (84.1)	0.795
Postural instability (%)	20 (58.8)	72 (63.7)	0.687
Wearing off (%)	20 (58.8)	49 (43.4)	0.112
Asymmetry at onset (%)	25 (73.5)	75 (66.4)	0.531
Orthostatic hypotension (%)	5 (14.7)	21 (18.6)	0.798
Incontinence (%)	7 (20.6)	11 (9.7)	0.131
Urinary urgency (%)	10 (29.4)	20 (17.7)	0.150
L-dopa-induced dyskinesia (%)	10 (29.4)	37 (32.7)	0.834
Sleep benefit (%)	3 (8.8)	21 (18.6)	0.288
Dystonia at onset (%)	4 (11.8)	11 (9.7)	0.750
Hyperreflexia (%)	5 (14.7)	17 (15.0)	1.000
Hallucination (%)	14 (41.2)	20 (17.7)	0.009[c]
Delusion (%)	8 (23.5)	6 (5.3)	0.004[c]
Other psychosis (%)	12 (35.3)	11 (9.7)	0.0009[d]
Dementia (%)	12 (35.3)	18 (15.9)	0.027[b]
Gaze palsy (%)	3 (8.8)	2 (1.8)	0.081

[a] Fisher's exact test.
[b] $p < 0.05$.
[c] $p < 0.01$.
[d] $p < 0.001$.

Table 3
Frequency of each *GBA* mutation found in this study

Mutations	FPD (n = 144)	AD (n = 85)	AR (n = 59)	Control (n = 100)	*p* Value[a] (FPD vs. control)	OR (95% CI) (FPD vs. Control)
Reported in GD						
p.R120W (%)	9 (6.3)	9 (10.6)	0 (0)	0 (0)	0.01	NA
p.D409H (%)	4 (2.8)	4 (4.7)	0 (0)	0 (0)	0.14	NA
p.L444P (%)	12 (8.3)	7 (8.2)	5 (8.5)	0 (0)	0.002	NA
indel (%)	1 (0.7)	1 (1.2)	0 (0)	0 (0)	NA	NA
RecNciI (%)	1 (0.7)	1 (1.2)	0 (0)	1 (1)	NA	NA
Unreported in GD						
p.G64V (%)	1 (0.7)	0 (0)	1 (1.7)	0 (0)	NA	NA
p.W393X (%)	1 (0.7)	0 (0)	1 (1.7)	0 (0)	NA	NA
p.I489V (%)	2 (1.4)	1 (1.2)	1 (1.7)	0 (0)	0.51	NA
Total (%)	31 (21.5)	23 (27.1)	8 (13.6)	1 (1)	<0.0001	27.2 (3.6–202.7)

Key: AD, autosomal dominant; AR, autosomal recessive; CI, confidence interval; FPD, familial PD; GD, Gaucher disease; indel, c.1447-1466delTGins; NA, not applicable; OR, odds ratio.
[a] Fisher's exact test. Italics denote novel mutations found in this study.

p.W393X, and p.I489V; Table 3). Interestingly, the frequency of *GBA* mutations among FPD patients was significantly higher than in the controls (31 of 144 = 21.5% vs. 1 of 100 = 1.0%, $p < 0.0001$, odds ratio = 27.2, 95% confidence interval = 3.6–202.7; Table 3). This cohort included 23 ADPD and 8 ARPD of a total of 31 FPD patients. When individual mutations were analyzed, the frequency of the p.R120W and p.L444P carriers was significantly higher in index PD patients than in the control subjects ($p = 0.01$ and $p = 0.002$, respectively).

3.2. Mode of inheritance

We compared the differences between groups with AD and AR modes of inheritance and found that the frequency of mutations reported in GD was significantly higher in the AD group than in the AR group (22 of 85 = 25.9% vs. 5 of 59 = 8.5%, $p = 0.009$). Four known mutations reported in GD (p.R120W, p.D409H, c.1447-1466delTGins, and Rec*Nci*I) were all found in the AD group. In 1 AR family (p.L444P) with 2 brothers affected with PD/dementia with Lewy bodies (DLB), the parents had been recorded as asymptomatic, but they died at relatively young ages during the war. Thus, the possibility that this family also should be included in AD family with cosegregation remains (family 28; Fig. 2).

In 2 families with p.L444P and 1 family with p.G64V, both affected siblings had the same mutations, suggesting cosegregation (families 25, 28, and 31; Fig. 2). The present study indicates the incomplete penetrance of p.R120W (families 7 and 9; Fig. 2), which is consistent with previous reports suggesting cosegregation (Mitsui et al., 2009). p.I489V was not considered to show cosegregation (family 35; Fig. 2).

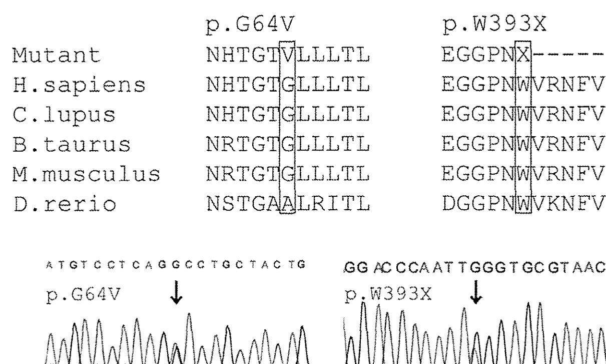

Fig. 1. Novel *GBA* mutations. Genomic sequence chromatogram and comparison of interspecific amino acids around the mutated site.

3.3. Clinical symptoms

In this study, we performed a comparative analysis between 2 groups: *GBA* mutation–positive (n = 34) and *GBA* mutation–-negative patients (n = 113). Clinical and demographic data are listed in Table 2 and Supplementary Table 1. The mean age at onset (AAO) was not significantly different ($p = 0.437$). Statistical analysis of 19 symptoms in PD revealed significant differences in four indexes of hallucination, delirium, dementia, and other psychosis (Table 2). Notably, psychiatric symptoms and/or cognitive decline were more common in *GBA* mutation–positive patients (see Supplementary Table 2). Nine patients with *GBA* mutations underwent cardiac ^{123}I-metaiodobenzylguanidine (MIBG) scintigraphy (see Supplementary Table 3), and all patients showed marked reduction of myocardial MIBG uptake.

4. Discussion

Recently, the largest multicenter analysis of *GBA* mutations has proved an association between *GBA* mutations and PD (Sidransky et al., 2009). To disclose the role of *GBA* in FPD, we performed a *GBA* mutational analysis for in 147 FPD patients from 144 families. The frequency of *GBA* mutations was 21.5% and 25.9% in FPD and ADPD, respectively, which are higher values than reported previously, even compared with SPD patients (Lesage et al., 2011; Mitsui et al., 2009; Sidransky et al., 2009). Therefore, our data suggest that *GBA* mutations play an important role in not only SPD but also FPD, and especially in ADPD. This finding implies that *GBA* could play a major role in FPD and ADPD.

Rare *GBA* mutations have been reported to be a strong risk factor of PD, with a robust odds ratio of 28.0 (Mitsui et al., 2009). Thus, an analysis for *GBA* mutations in FPD was essential to clarify the role of each *GBA* mutation in PD patients. Indeed, p.L444P and p.G64V cosegregated with PD in some families. However, compared with a previous report (Mitsui et al., 2009). p.R120W did not cosegregate in this study. Regarding an association with PD, some mutations remained controversial because of the small number of patients with each mutation and restricted genetic testing in the families. Considering allele frequency, it might be reasonable that *GBA* mutations are incompletely inherited with PD. The carrier frequency of *GBA* mutations was extremely low among the normal Japanese population in this study and in a previous report (Mitsui et al., 2009), whereas many controls among the Jewish population have p.N370S, which is associated with type I GD (Aharon-Peretz et al., 2004), and many controls among the UK population have p.E326K, which is a risk factor for PD (Duran et al., 2013). These racial differences might be derived from the differences of effects of each mutation and other genetic risk factors, which have different

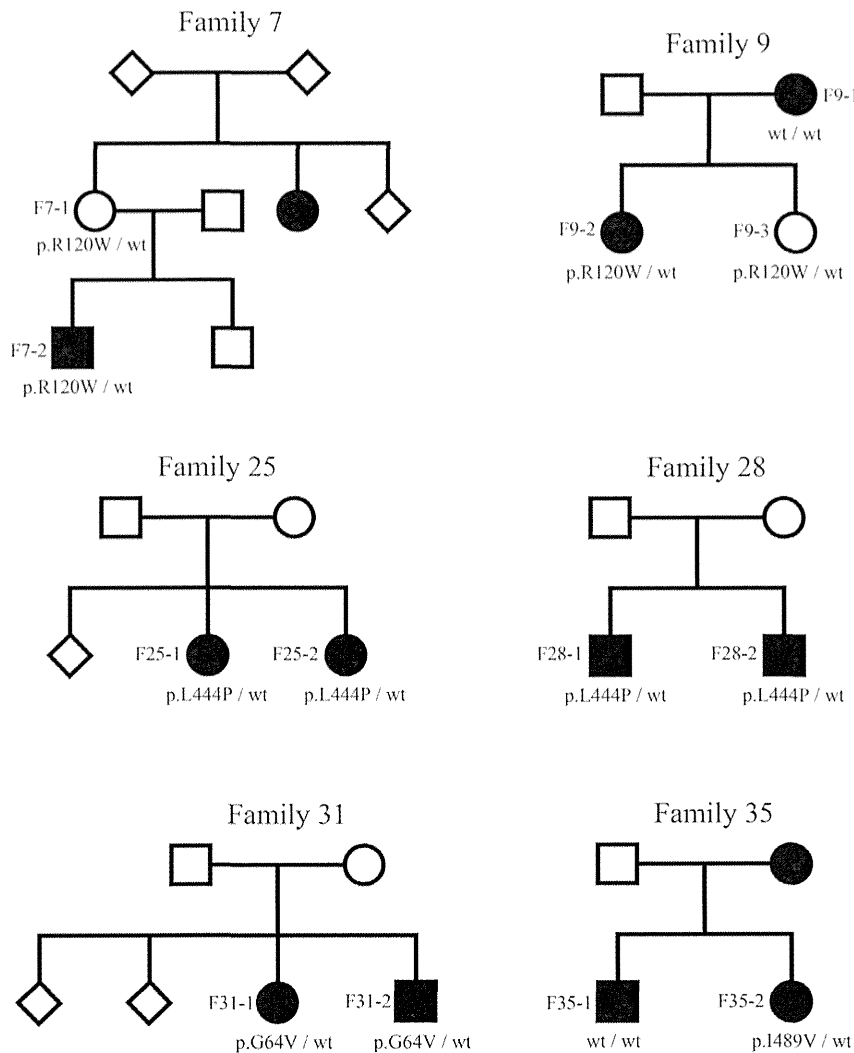

Fig. 2. Cosegregation study of *GBA*-positive families. Pedigrees of families with *GBA* mutations are shown. Affected Parkinson's disease patients are represented with a black symbol. The number of each family member represents his or her patient ID in Supplementary Table 1.

distributions due to founder effects (Lesage et al., 2011; Nishioka et al., 2010; Sidransky et al., 2009). Although racial or regional differences exist for each *GBA* mutation, our data emphasize that *GBA* mutations are more strongly associated with FPD rather than SPD.

A recent study reported a relatively high estimated penetrance ratio in *GBA* carriers, depending on age (7.6%, 13.7%, 21.4%, and 29.7% at 50, 60, 70, and 80 years, respectively). This result should lead to the consideration of *GBA* as a dominant causal gene with reduced penetrance (Anheim et al., 2012). Supporting this consideration, we detected heterozygous *GBA* mutations most frequently in ADPD patients. On the basis of our findings, we conclude that *GBA* is a strong and common risk factor but not a definite causal gene for ADPD and FPD.

In hereditary forms of PD, the frequency of heterozygous *GBA* mutations in ADPD (25.9%) was higher than that observed for ARPD (8.5%). Even allowing for classification bias by definition of ADPD and ARPD, the data imply that *GBA* mutations are strongly associated with ADPD, suggesting that heterozygous *GBA* mutations have a role in familial aggregation, especially in ADPD. In addition, heterozygous mutations have been identified among ARPD patients, suggesting the incomplete penetrance of forms even through the AR mode of inheritance.

Our PD patients with *GBA* mutations frequently developed psychiatric symptoms and/or cognitive decline. Our data support previous results in which PD patients with *GBA* mutations manifest exacerbated psychiatric symptoms and/or cognitive decline compared to those without *GBA* mutations (Alcalay et al., 2012; Sidransky et al., 2009; Winder-Rhodes et al., 2013). Some previous studies have reported the association of *GBA* mutations and DLB (Clark et al., 2009; Nalls et al., 2013; Tsuang et al., 2012). Our data further suggest that *GBA* heterozygous mutation carriers can develop clinical symptoms of PD and DLB. Accordingly, we found decreased cardiac MIBG uptake associated with *GBA* mutations. The heart-to-mediastinum ratio correlated with PD/DLB clinical severity; a decreasing ratio corresponded to an ascending Hoehn and Yahr stage (Nagayama et al., 2005). Thus, MIBG scintigraphy could be a useful biomarker for PD/Lewy body disease in patients with *GBA* mutations.

Lewy body diseases are alpha-synucleinopathies characterized by abnormal accumulation of alpha-synuclein in neuronal cytoplasm. Recently, biochemical analysis using cell and animal models demonstrated that some *GBA* mutations lead to increased alpha-

synuclein concentration (Cullen et al., 2011). *GBA* mutation may promote alpha-synuclein accumulation and Lewy body development via the aberrant lysosomal function, resulting in severe parkinsonism and cognitive decline associated with DLB (Mazzulli et al., 2011; Tsuang et al., 2012; Yap et al., 2013).

Collectively, our findings have major implications for the genetic and pathogenic mechanisms of *GBA*. GD is an autosomal recessive disorder caused by mutations in both *GBA* alleles, which leads to a loss of function or reduced enzyme activity. In contrast to GD, our FPD and ADPD patients carried 1 mutant allele and 1 wild-type *GBA* allele. Therefore, the pathogenic mechanism may be due to haploinsufficiency, dominant-negative effect, or toxic gain of function rather than a loss of function.

In conclusion, heterozygous *GBA* mutations play a greater role in FPD, especially in ADPD, and are likely to facilitate the development of PD and Lewy body diseases via different genetic and pathogenic mechanisms. Our findings suggest that further functional analyses for *GBA* should elucidate the pathogenesis of PD and Lewy body diseases.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2013.09.019.

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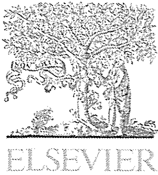

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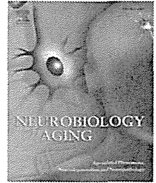

The evaluation of polyglutamine repeats in autosomal dominant Parkinson's disease

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ABSTRACT

We evaluated the contributions of various polyglutamine (polyQ) disease genes to Parkinson's disease (PD). We compared the distributions of polyQ repeat lengths in 8 common genes (*ATXN1, ATXN2, ATXN3, CACNA1A, ATXN7, TBP, ATN1*, and *HTT*) in 299 unrelated patients with autosomal dominant PD (ADPD) and 329 normal controls. We also analyzed the possibility of genetic interactions between *ATXN1* and *ATXN2*, *ATXN2* and *ATXN3*, and *ATXN2* and *CACNA1A*. Intermediate-length polyQ expansions (>24 Qs) of *ATXN2* were found in 7 ADPD patients and no controls (7/299 = 2.34% and 0/329 = 0%, respectively; $p = 0.0053 < 0.05/8$ after Bonferroni correction). These patients showed typical L-DOPA-responsive PD phenotypes. Conversely, no significant differences in polyQ repeat lengths were found between the ADPD patients and the controls for the other 7 genes. Our results may support the hypothesis that *ATXN2* polyQ expansion is a specific predisposing factor for multiple neurodegenerative diseases.
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1. Introduction

Several genes other than the "PARK" genes are suspected to be responsible for parkinsonism. Mutations of these genes sometimes confer symptoms that clinically mimic idiopathic Parkinson's disease (PD) and present radiological or pathologic findings characteristic of PD (Klein et al., 2009). These genes include the polyglutamine (polyQ) disease genes: *HTT* (Walker, 2007), *ATXN1* (Dubourg et al., 1995), *ATXN2* (Charles et al., 2007; Furtado et al., 2004; Gwinn-Hardy et al., 2000), *ATXN3* (Lu et al., 2004a; Subramony et al., 2002), *CACNA1A* (Kim et al., 2010), and *TBP* (Kim et al., 2009). Of these genes, it has been suggested that intermediate-length polyQ expansions in *ATXN2* and *TBP* are associated with PD (Charles et al., 2007; Furtado et al., 2004; Kim et al., 2009).

In addition, intermediate-length polyQ expansions (24–33 Qs) in *ATXN2* have recently been suggested as a risk factor for amyotrophic lateral sclerosis (ALS) (Chen et al., 2011; Elden et al., 2010). This observation has inspired several studies investigating how intermediate-length expansions of various polyQ disease genes contribute to neurodegenerative diseases other than those with which they were originally associated (Gispert et al., 2012; Lee et al., 2011b; Ross et al., 2011).

Based on these findings and the suggestion that polyQ diseases may share common pathogenic mechanisms (Al-Ramahi et al., 2007; Bertoni et al., 2011; Chen and Burgoyne, 2012), we hypothesized that polyQ disease genes in general might play a role in PD. We focused on autosomal dominant PD (ADPD) because polyQ neurodegenerative diseases generally have an AD mode of inheritance, and we compared the distribution of polyQ repeat lengths in 8 common genes between ADPD patients and normal controls.

2. Methods

We conducted genetic analyses of *ATXN1, ATXN2, ATXN3, CACNA1A, ATXN7, TBP, ATN1*, and *HTT* in a Japanese cohort with ADPD and normal controls. In this study, we classified the mode of

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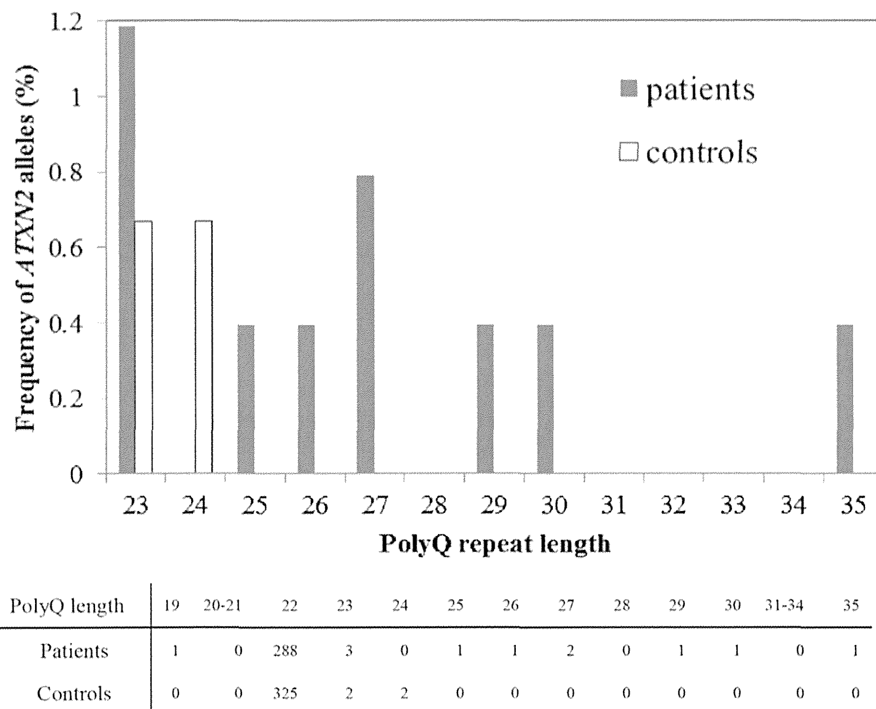

PolyQ length	19	20-21	22	23	24	25	26	27	28	29	30	31-34	35
Patients	1	0	288	3	0	1	1	2	0	1	1	0	1
Controls	0	0	325	2	2	0	0	0	0	0	0	0	0

Fig. 1. The distribution of polyglutamine (polyQ) repeat lengths of *ATXN2* in autosomal dominant Parkinson's disease patients and normal controls. The histogram shows only subjects with ≥23 repeats.

inheritance as autosomal dominant when a family included affected members in 2 consecutive generations. The diagnosis of PD was confirmed by the participating neurologists based on established criteria (Hughes et al., 1992).

We recruited the study subjects from the gene bank of our institution. We selected 299 unrelated patients with ADPD (169 women and 130 men; age at onset [AAO] = 57.7 ± 13.6-year-old [standard deviation], range 17–85 years) from families with unexplained pathogenesis, that is, those with no known pathogenic mutations in the *SNCA*, *PARK2*, *LRRK2*, and *VPS35* genes. A total of 329 healthy unrelated volunteers with no individual or family history of neurodegenerative disease (203 women and 126 men; age at examination = 57.5 ± 11.8-year-old [standard deviation], range 23–88 years) were examined as normal controls. Blood samples were obtained from the patients and controls, all of whom gave informed consent. Our institutional ethics committee approved the genetic study.

DNA was extracted from lymphocytes using standard methods. The polyQ repeat lengths in the polyQ disease genes were detected using capillary electrophoresis with fluorescent 5′-6-fluorescein amidite (FAM)-labeled forward primers. The primer sequences and polymerase chain reaction conditions are described in Supplementary Table 1. The polymerase chain reaction products were mixed with the LIZ-500 size standard (Applied Biosystems, Foster City, CA, USA) and processed on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems) for size determination. The sizes of the repeats were determined with GeneMapper 3.7 software (Applied Biosystems).

Statistical analysis was performed using JMP 8 software (SAS Institute, Cary, NC, USA). We evaluated the association between ADPD and the polyQ repeat lengths of each gene using 2-tailed Fisher exact tests, as previously described (Gispert et al., 2012; Lee et al., 2011a; Ross et al., 2011). A p value <0.05/8 after Bonferroni correction was considered significant (8 is for the number of genes investigated in the present study.).

3. Results

3.1. Molecular genetic analysis

The range of repeat lengths in *ATXN2* was between 19 and 35. Most patients (95.6% of patients with ADPD and 98.6% of the controls) had a repeat length of 22, as reported in previous studies (Lee et al., 2011a; Pulst et al., 1996). Of the 253 patients with ADPD, 7 harbored repeat lengths longer than 24, whereas none of the controls did (2.8% and 0%, respectively; $p = 0.0053$, Fig. 1 and Table 1).

No substantial differences in the repeat lengths in *ATXN1*, *ATXN3*, *CACNA1A*, *ATXN7*, *TBP*, *ATN1*, or *HTT* were observed between the ADPD patients and controls (Table 1 and Supplementary Fig. 1).

We supplementarily sequenced the entire coding exons and exon and/or intron boundaries of glucocerebrosidase gene (*GBA*) in

Table 1
Fisher exact tests of polyQ repeat lengths between ADPD patients and controls

PolyQ disease gene	PolyQ repeat length	Conventional normal range[a]	Difference between ADPD patients and controls?
ATXN1	21–36	6–44	No
ATXN2	19–35 25–35Qs: 2.3% of ADPD, 0% of control	14–31	Yes, $p = 0.0053$ (<0.05/8), OR = ∞
ATXN3	13–46	11–44	No
CACNA1A	5–18	4–18	No
ATXN7	1–10	4–19	No
TBP	30–40	25–42	No
ATN1	12–36	6–35	No
HTT	15–35	6–34	No

Key: ADPD, autosomal dominant Parkinson's disease; Q, glutamine.
[a] The consensus normal ranges of the polyQ repeat lengths associated with the corresponding disease (e.g., *ATXN1* for SCA1) (Hands et al., 2008; Sequeiros et al., 2010).

the 7 probands with intermediate *ATXN2* polyQ expansion, because rare *GBA* mutations have been considered to be a risk factor for PD (Li et al., 2013; Mitsui et al., 2009); no *GBA* mutation was found in these 7 probands.

3.2. Pedigree and clinical information for the 7 probands with ATXN2 *polyQ repeat lengths >24*

Fig. 2 shows the pedigrees of the 7 probands with *ATXN2* polyQ repeat lengths >24 and their families. In family A, AII-2 presented with resting tremor in the bilateral lower extremities and left-dominant bradykinesia, which were responsive to L-DOPA and selegiline. AIII-1, who experienced rigidity and resting tremor predominantly in the left extremities, presented with tongue and jaw tremor (Supplementary Table 2). All these signs were relieved by pramipexole. AIII-3 was reportedly initially diagnosed with essential tremor because her first sign was bilateral postural tremor. She underwent left and right thalamotomy at a 1-year interval. She showed hyperreflexia in the lower extremities, but this symptom was presumably because of cervical spondylosis, for which surgical decompression was performed. AIV-2 and AIV-3, who inherited an intermediate-length polyQ expansion of 35 Qs, were not affected at the time of this study.

In family B, BI-2 was affected at an older age than her offspring, although their genotypes were the same, and all had L-DOPA-responsive parkinsonism with laterality (Supplementary Table 2).

In family C, CII-2 was diagnosed with Parkinson's disease with dementia. Although her parents were consanguineous, her polyQ *ATXN2* lengths were heterozygous (29/22).

All other members of the 7 families showed L-DOPA-responsive parkinsonism with laterality and were free of motor neuron signs, cerebellar ataxia, and saccadic eye movement disorder. None was

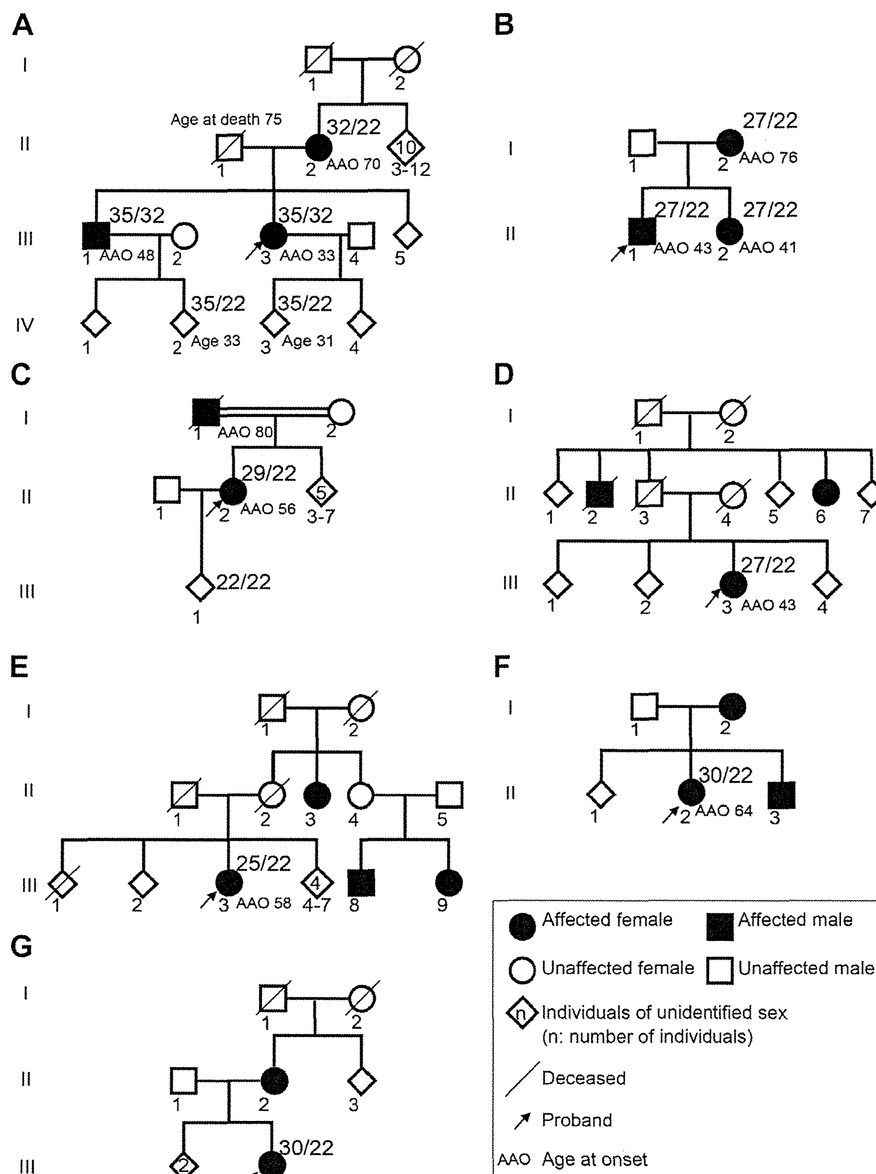

Fig. 2. The pedigrees of 7 families in which the proband has an *ATXN2* polyQ repeat length >24. *ATXN2* repeat lengths are listed previously and to the right of the pedigree symbols of the genotyped individuals.

reported to have any significant brain magnetic resonance imaging abnormality (Supplementary Table 2).

4. Discussion

We investigated the distributions of the polyQ repeat lengths of 8 common polyQ disease genes (*ATXN1, ATXN2, ATXN3, CACNA1A, ATXN7, TBP, ATN1*, and *HTT*) in patients with ADPD. PolyQ repeat lengths >24 in *ATXN2* were significantly more common in the patients than in the controls. To the best of our knowledge, there have been only 2 similar studies investigating the distribution of *ATXN2* polyQ repeat lengths in PD patients and controls to date (Gispert et al., 2012; Ross et al., 2011). Although both previous studies failed to prove any significant difference, one (Gispert et al., 2012) showed that PD patients tended to have longer repeat lengths, consistent with our results. In the other previous study (Ross et al., 2011), the controls might have included some number of presymptomatic patients because the mean age of the controls was lower than that of the PD patients.

In reference to the recent studies concerning the effect of polyQ repeat length on neurodegenerative disease, we screened for a threshold of the normal *ATXN2* polyQ repeat length around a range from 24 to 34 (Charles et al., 2007; Chen et al., 2011; Elden et al., 2010; Gispert et al., 2012; Lee et al., 2011a, 2011b; Ross et al., 2011). The distribution of our patients differed significantly from that of controls only when the cutoff was set to 25. This may be much lower than the threshold for *ATXN2*-related PD adopted by previous studies (Charles et al., 2007), but it is possible that the cutoff for *ATXN2* polyQ repeat length and its influence on PD may vary from population to population, as is the case for ALS, as indicated in a previous study (Lee et al., 2011b). Such variation of the threshold would be consistent with the observation that previous reports of *ATXN2*-associated PD have mainly been from East Asian populations (Charles et al., 2007; Klein et al., 2009; Lu et al., 2004b; Sun et al., 2011; Wang et al., 2009). Additional factors, such as cis- and trans-acting genetic elements, non-allelic genetic modifiers, and stochastic and environmental factors (Charles et al., 2007; Pulst et al., 2005), might have enhanced the toxicity of *ATXN2* intermediate-length polyQ expansion in our population.

We described the details of family members with *ATXN2* intermediate-length expansions (>24 Qs, Fig. 2 and Supplementary Table 2). These patients generally manifested typical PD phenotypes without motor neuron signs, cerebellar ataxia, or saccadic eye movement disorder, as was stated in previous reports (Furtado et al., 2004; Klein et al., 2009). A correlation between the association of AAO and polyQ repeat length was not clearly present or absent in our patients with repeat lengths of *ATXN2* > 24, as previously observed (Furtado et al., 2002, 2004; Payami et al., 2003; Sun et al., 2011). For example, in family A, members of the third generation had earlier AAOs than did their mother. However, there was a gap between the AAOs of AIII-1 and AIII-3, even though their genotypes were the same. In addition, AIII-1 and AIII-3 had 2 allele expansions (35/32 Qs) instead of a single allele expansion, which might have caused their early onsets (Ragothaman et al., 2004). The 35Q alleles may have been inherited "as is" from AII-1, who reportedly had no neurologic disorder, although it is also possible that an expansion occurred upon transmission. Thus, AAOs might be affected by features other than polyQ repeat length, such as genetic and epigenetic factors.

In the present study, we did not find any association between the ADPD phenotype and the repeat lengths of polyQ disease genes other than *ATXN2*. This result implies that the contribution of *ATXN2* to ADPD is because of the specific effects of this gene rather than the presence of the polyQ expansion itself, as reported in a previous study of ALS (Lee et al., 2011a). This result might appear to be inconsistent with recent reports suggesting that the intermediate polyQ expansion of *TBP* is likely to be a risk factor for PD (Kim et al., 2009; Wu et al., 2004; Xu et al., 2010; Yun et al., 2011). However, because those reports did not provide significant evidence, and because all of these studies were performed in East Asian patients, further evidence should be accumulated.

As a supplementary analysis, we also applied a multiple logistic regression including the product terms *ATXN1* × *ATXN2*, *ATXN2* × *ATXN3*, and *ATXN2* × *CACNA1A* to screen for some interactions among these polyQ disease gene combinations, based on previous studies showing the possibility of interaction among these polyQ genes (Al-Ramahi et al., 2007; Jardim et al., 2003; Lessing and Bonini, 2008; Pulst et al., 2005). However, no significant difference was detected between the PD patients and controls (with a threshold p-value of 0.05, Supplementary Table 3).

In conclusion, an intermediate-length polyQ expansion of *ATXN2* is likely to contribute to the pathogenesis of ADPD, either directly causing the PD phenotype or modifying the effects of unknown genes on the PD phenotype. Our results add to the recent finding that intermediate-length polyQ repeat expansions of *ATXN2* may be a contributing factor in multiple neurodegenerative diseases.

Disclosure statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2014.01.022.

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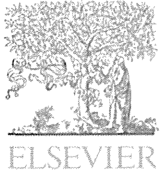

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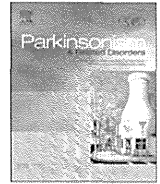

Short communication

EIF4G1 gene mutations are not a common cause of Parkinson's disease in the Japanese population

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ABSTRACT

Pathogenic mutations in the *EIF4G1* gene were recently reported as a cause of autosomal dominant parkinsonism. To assess the frequency of *EIF4G1* mutations in the Japanese population we sequenced the entire gene coding region (31 exons) in 95 patients with an apparent autosomal dominant inherited form of Parkinson's disease. We detected three novel point mutations located in a poly-glutamic acid repeat within exon 10. These variants were screened through 224 Parkinson's disease cases and 374 normal controls from the Japanese population. We detected the poly-glutamic acid deletion in exon 10 in two additional patients with sporadic Parkinson's disease. Although the *EIF4G1* variants identified in the present study were not observed in control subjects, co-segregation analyses and population-based screening data suggest they are not pathogenic. In conclusion, we did not identify novel or previously reported pathogenic mutations (including the p.A502V and p.R1205H mutants) within *EIF4G1* in the Japanese population, thus future studies are warranted to elucidate the role of this gene in Parkinson's disease.

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1. Introduction

Parkinson's disease (PD) is one of the most common movement disorders in the elderly. Pathogenic mutations that result in hereditary forms of PD/parkinsonism are reported in a number of genes and have subsequently directed both functional studies and the generation of disease model systems [1,2]. The nomination of each new gene for parkinsonism implicates disease pathways and provides a rationale for targeted therapeutic development [3,4]. Recently, two substitutions in the eukaryotic translation initiation factor 4-gamma 1 protein (*EIF4G1*, MIM#600495); p.R1205H and p.A502V were nominated to cause PD with autosomal dominant inheritance in a number of pedigrees [5]. Furthermore studies in a yeast model revealed the *EIF4G1* ortholog (TIF4632) is a suppressor of α-synuclein toxicity [6].

EIF4G1 is a protein scaffold subunit of the translation initiation complex, EIF4F, which binds the ribosomal 40S. A decrease in the levels of EIF4G1 protein in cells results in a reduction of overall protein synthesis linked to nutrient sensing [7]. Reported pathogenic EIF4G1 substitutions, p.R1205H and p.A502V, were shown to disrupt binding to EIF3E and EIF4E respectively, and result in impaired nutrient sensing and mitochondrial dysfunction [5,7]. Interestingly, over-expression of EIF4G1 protein has been implicated in cell proliferation as observed in some malignant disorders, especially inflammatory breast cancer [8]. This evidence supports a role for *EIF4G1* mutations in cell survival and potentially the neuronal damage observed in PD. Herein, we set out to examine the

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Table 1
Demographic of patients and control subjects.

Subjects	Ethnicity	No.	Male to female ratio	Mean age at onset (SD)
Autosomal dominant PD	Japanese	95	1:1.32	52.7 (±11.4)
Replication				
Autosomal dominant PD	Japanese	43	1:0.95	41.7 (±14.2)
Sporadic PD	Japanese	181	1:0.91	38.6 (±12.3)
Control subjects	Japanese	374	1:1.49	

PD; Parkinson's disease.
SD; standard deviation.

occurrence and frequency of mutations in the *EIF4G1* gene among PD patients of Japanese origin.

2. Subjects and methods

All individuals were collected at Juntendo University, Tokyo and at Mie University, Mie and were of Japanese ethnicity. Patients were diagnosed with PD based on the modified United Kingdom Parkinson's disease society brain bank criteria. DNA was extracted from peripheral blood by standard protocols. A series of 95 patients with autosomal dominant PD had an average of age at onset of 52.7 years ± 11.4 (SD) and a 1:1.32 male to female ratio were selected (Table 1). Family history was defined as one or more affected relatives within 2 degrees of relationship. All variants were then screened through a population-based patient-control series of 224 patients with PD including 43 probands (age at onset 41.7 ± 14.2 years old and male:female = 1:0.95) with possible autosomal dominant PD, 181 sporadic PD cases (age at onset 38.6 ± 12.3 years old and male:female = 1:0.91) and 374 normal controls (age at examination 57.8 ± 12.6 years old and male:female = 1:1.49). The ethical review boards at the Mayo Clinic, Juntendo University and at Mie University approved the study, and all participants provided informed written consent.

3. Genetic analysis

The 31 coding exons (exon 3–33, NM_198241.2) of *EIF4G1* were sequenced in 95 patients with apparent autosomal dominant PD. Primer pairs for coding regions of *EIF4G1* (exons 3–33) were used and are available upon request [5]. PCR products were purified from unincorporated nucleotides using Agencourt bead technology (Beverly, MA) with Biomek FX automation (Beckman Coulter, Fullerton, CA). Electropherograms were analyzed with SeqScape v2.1.1 using 3730 DNA Analyzer (ABI, Applied Biosystems, Foster City, CA, USA). In addition, real-time PCR was employed to investigate the role of exon dosage and gene copy number variation was analyzed as previously described [5]. We also screened any novel mutations identified and the previously reported mutants (EIF4G1 p.R1205H and p.A502V) in an additional 43 autosomal dominant PD patients, 181 sporadic patients and 374 controls. We performed allelic cloning using a TOPO® TA Cloning® Kit (Life Technologies, Carlsbad, CA, USA) followed by individual clone PCR and sequencing to assess the phase of three exon 10 variants identified in one PD patient.

4. Results

We identified novel *EIF4G1* mutations in one patient with autosomal dominant PD (J-19) among the 95 probands (Supplemental Fig. 1). Patient J-19 had three point mutations; [p.E463G, c.1388AG>GA] and [p.E465A, c.1394A>C] on the same allele, in exon 10 (Fig. 1(a)). However we found the three point mutations in two healthy siblings of patient J-19 (73-year old man and 67-year old woman). In addition, our sequencing analysis identified four novel synonymous variants; p.Q149Q (c.447A>G), p.K1206K (c.3618G>A), p.T1211T (c.3633G>A), and p.Y1488Y (c.4464C>T) which were observed independently each in a single autosomal dominant PD patient (n = 95). No *EIF4G1* gene copy number variations were observed in 95 probands.

Screening an additional 43 patients with autosomal dominant PD and 181 sporadic PD patients, identified two sporadic patient (ID#1558, 1601) with the same three point mutations as patient J-19. Furthermore, one of the two patients (ID#1601) had a known 9 bp deletion (rs111659103) in exon 10 (Fig. 1(b)); these exon 10 variants were not observed in our 374 normal control subjects, but rs111659103 is reported on the Exome Variant Server database with a carrier frequency of over 5%.

5. Discussion

Our comprehensive screening of the coding region of the *EIF4G1* gene in 95 probands from families with apparent autosomal dominant inheritance of PD did not detect any novel pathogenic mutations, nor the p.A502V or p.R1205H variants described in Chartier-Harlin et al. [5]. Several novel variants were identified, including a number of point mutations located in a poly-glutamic acid tract. However the location of the poly-glutamic acid tract

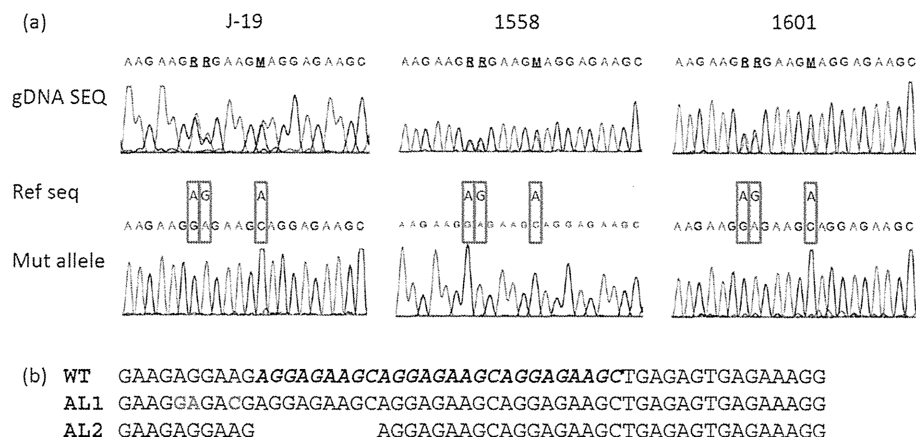

Fig. 1. (a) Genomic sequence of the *EIF4G1* exon 10 from the patients J-19, 1558 and 1601 showing three point mutations; [p.E463G, c.1388AG>GA], and [p.E465A, c.1394A>C], as well as the mutant haplotype from the TOPO TA cloning. The three mutations exist on same allele. (b) Diagrammatic representation of the wild-type sequence around the poly-glutamic acid repeat and highlighting the three point mutations (AL1; red) and 9 bp deletion (AL2; rs111659103) in *EIF4G1* exon 10 from sporadic patient 1601. A three unit perfect repeat (bold in WT allele) precludes determining which unit is rs111659103. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

does not appear to be in a region involved in complex formation or RNA-binding, therefore if these variants do have a functional influence the mechanism is yet to be determined. *In silico* analysis using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) predicts the [p.E463G, c.1388AG>GA] and [p.E465A, c.1394A>C] to be both possibly damaging and benign based on HumDiv and HumVar measures respectively. In addition, evidence from disease co-segregation analysis within families, *in silico* prediction and population frequency data from the Exome Variant Server database (rs111659103) does not support variants in the poly-glutamic acid tract as high penetrant pathogenic factors for parkinsonism.

These findings support the initial report describing a relatively low frequency of *EIF4G1* mutations in 4708 individuals with idiopathic PD (7/4708) [5]. Recently, independent studies examining the frequency of *EIF4G1* variation have identified both the p.A502V and p.R1205H variants in non-diseased individuals, thus demonstrating the importance of replication studies to resolve the role of *EIF4G1* variants in PD pathophysiology (Supplemental Table 1). In addition, findings from other *EIF4G1* gene screening studies suggest that mutations are rare in patients across multiple populations and that the pathogenicity of this gene in PD remains to be resolved (Supplemental Table 1). We conclude from our data that *EIF4G1* mutations are not a common cause of PD in patients of Japanese origin.

Given the technological advances in DNA sequencing approaches an ever increasing number of rare variants will be nominated as pathogenic in PD and related neurodegenerative disorders. As observed for *EIF4G1* variants, even large series of patients and controls may not be sufficient to confer definitive pathogenicity. There will need to be large collaborative consortia efforts as recently reported to determine the true nature of rare variants within the context of disease risk and clinical relevance [9,10]. In the absence of overwhelming genetic evidence we may have to rely on a functional readout (e.g. PINK1/PARKIN mutation effects on cellular mitophagy), although reliable disease-specific assays are still to be developed for PD-related genes [11,12]. With the noted caveats in mind further investigations are warranted to confirm the pathogenicity of *EIF4G1* variants in PD and to assess global prevalence and clinical relevance to disease.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.parkreldis.2014.03.004.

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NEUROPATHOLOGY

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Case Report

Autopsy case of spinocerebellar ataxia type 31 with severe dementia at the terminal stage

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Spinocerebellar ataxia type 31 (SCA31) is an autosomal dominant cerebellar ataxia commonly observed in Japan. However, few neuropathological examinations have been conducted. Here we report the case of a 76-year-old Japanese male SCA31 patient. He noticed dysarthria and difficulty walking at 65 years old. His symptoms subsequently deteriorated, although he could still walk with assistance at 70 years. At 73 years, when he could no longer walk, he was admitted to our hospital. He showed severe limb and truncal ataxia. His father and older brother had shown the same symptoms. Brain magnetic resonance imaging showed cerebellar atrophy of the anterior lobe and white matter hyperintensities. He was diagnosed with SCA31 by genetic analysis. Gradually, his cognitive functions and ability to communicate declined. He died of respiratory failure at the age of 76. Neuropathological examination revealed severe Purkinje cell loss that was accentuated in the anterior lobe of the cerebellum. Furthermore, the remaining Purkinje cells showed abnormal processes (that is, halo-like amorphous materials), as has been reported previously. Severe deposition of hyperphosphorylated tau-positive neurites, many senile plaques and amyloid angiopathy were observed in the neocortex. Our findings suggest that in SCA31, accelerated tau and amyloid pathology in the neocortex might induce dementia at the terminal stage.

Key words: autosomal dominant, dementia, pathology, SCA31, tau.

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INTRODUCTION

Spinocerebellar ataxia type 31 (SCA31), an autosomal dominant cerebellar degenerative disease, is clinically characterized by a slowly progressing pure cerebellar ataxia. Genetically, the insertion of TGGAA pentanucleotide repeats ranging in length from 2.5 to 3.8 kb between the *TK2* (thymidine kinase 2) and *BEAN* (brain expressed, associated with NEDD4) genes located on chromosome 16q22.1 has been reported.[1,2] Although SCA31 is the fourth most common form of autosomal dominant cerebellar degeneration in Japan,[3] few neuropathological examinations have been conducted.[4–6] Dementia has not been mentioned in previous reports. However, in a previous report on community-based prevalence of SCA in Tottori Prefecture, western Japan, we documented a high prevalence of dementia (58%) and a relatively high incidence (66%) of white matter changes with or without hypertension in a cohort of patients with SCA31 (called 16q-autosomal dominant cerebellar ataxia at the time) in small communities.[7] In other areas in Japan, the incidence of dementia in SCA31 was estimated from 0%[8] to 5%.[3] Why our cohort showed a higher prevalence of dementia is unknown. Here, we report clinical and pathological findings in a patient with SCA31 who exhibited severe dementia at the terminal stage and consider the cause of this dementia.

CLINICAL SUMMARY

The patient was a 76-year-old Japanese man at the time of death. His development was normal. He had no hypertension, diabetes mellitus or hyperlipidemia and no history of habitual drinking. He noticed dysarthria and difficulty walking at 65 years old. His symptoms gradually worsened and he could not walk without assistance at 70 years old. At 73 years, he could not walk and lived at home by crawling. That same year, he was admitted to our hospital. He