Abstract

A case of amyloidosis with amyloid deposition detected only in skeletal muscles

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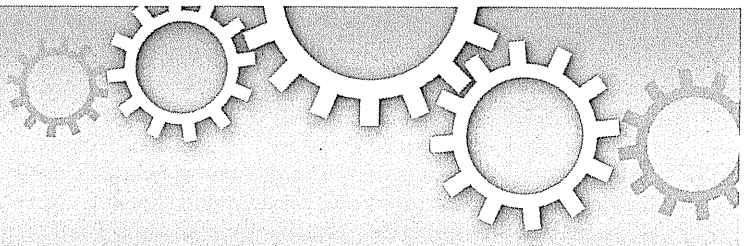
A 75-year-old man was admitted to our hospital with progressive weakness in the lower extremities for 7 months. Immunoelectrophoresis of serum detected IgA λ type M protein and bone marrow examination detected an increase in monoclonal plasma cells, thus leading to a diagnosis of IgA λ type multiple myeloma. Subsequent muscular CT scan showed severe fatty infiltration of vastus lateralis muscles, and histopathological examinations of biopsied muscle specimens an abundance of abnormal "ring-fiber-like" appearance, positive staining by Congo red and the presence of anti-λ light chain antibody. This led to a diagnosis of amyloid myopathy. No depositions were seen in rectal mucosa, cardiac muscle, or sural nerve. The results of double immunohistochemical staining using anti-dystrophin antibody and anti-λ light chain antibody suggested the possibility of direct injury by amyloid to muscle fibers. The case presented here was thus amyloidosis confirmed by deposition of amyloid only in muscles. In conclusion, when amyloidosis is suspected and there is evidence of muscle injury, muscle biopsy should be performed.

(Clin Neurol 2012;52:739-743)

Key words: amyloid myopathy, amyloidosis, multiple myeloma

SCIENTIFIC REPORTS



SUBJECT AREAS:
MITOPHAGY
PHOSPHORYLATION
CELL DEATH IN THE NERVOUS SYSTEM
UBIQUITIN LIGASES

Received
3 August 2012

Accepted
5 December 2012

Published
19 December 2012

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PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy

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Parkinson's disease genes *PINK1* and *parkin* encode kinase and ubiquitin ligase, respectively. The gene products PINK1 and Parkin are implicated in mitochondrial autophagy, or mitophagy. Upon the loss of mitochondrial membrane potential ($\Delta\Psi$m), cytosolic Parkin is recruited to the mitochondria by PINK1 through an uncharacterised mechanism – an initial step triggering sequential events in mitophagy. This study reports that Ser65 in the ubiquitin-like domain (Ubl) of Parkin is phosphorylated in a PINK1-dependent manner upon depolarisation of $\Delta\Psi$m. The introduction of mutations at Ser65 suggests that phosphorylation of Ser65 is required not only for the efficient translocation of Parkin, but also for the degradation of mitochondrial proteins in mitophagy. Phosphorylation analysis of Parkin pathogenic mutants also suggests Ser65 phosphorylation is not sufficient for Parkin translocation. Our study partly uncovers the molecular mechanism underlying the PINK1-dependent mitochondrial translocation and activation of Parkin as an initial step of mitophagy.

Mutations of the *PINK1* gene cause selective degeneration of the midbrain dopaminergic neurons in autosomal recessive juvenile Parkinson's disease (PD)[1]. The *PINK1* gene encodes a serine/threonine kinase with a predicted mitochondrial target sequence and a putative transmembrane domain at the N-terminus[2–5]. Loss of the *PINK1* gene in *Drosophila* results in the degeneration of mitochondria in cells with high energy demands, such as muscle and sperm cells, which is suppressed by the introduction of the *parkin* gene, another gene responsible for autosomal recessive juvenile PD[6–8]. The gene product Parkin encodes a RING-finger type ubiquitin ligase (E3) with a Ubl domain at the N-terminus[9–12].

A series of cell biological studies have provided strong evidence that there are important roles for PINK1 and Parkin in regulating mitochondrial homeostasis. PINK1 is constitutively proteolysed by the mitochondrial rhomboid protease, PARL, at the mitochondrial membrane of healthy mitochondria, resulting in processed forms of PINK1[13–16]. The processed PINK1 is rapidly degraded by the proteasome[2,17]. The reduction of $\Delta\Psi$m leads to the accumulation and activation of PINK1 in the mitochondria[17–19] through a currently unresolved mechanism[20]. The accumulation of PINK1 recruits Parkin from the cytosol to the mitochondria with decreased membrane potential, which stimulates Parkin E3 activity, promoting mitochondrial degradation via an autophagic event known as mitophagy[17,21–24]. The recruitment of cytosolic Parkin to the mitochondria upon disruption of $\Delta\Psi$m is believed to be the first step of mitophagy for the removal of damaged mitochondria. This recruitment is required for the kinase activity of PINK1[17,21–25]. Although two separate studies have proposed that Parkin is directly phosphorylated by PINK1[26,27], others have failed to detect Parkin phosphorylation by PINK1[21], suggesting that the kinase activity of PINK1 itself is relatively low. One reason biochemical analysis has been unable to obtain direct evidence is that recombinant human PINK1 purified from mammalian cultured cells or bacteria easily loses kinase activity, while insect PINK1 has significant autophosphorylation activity[28,29].

Very recently, Kondapalli, C. *et al.* reported that PINK1 directly phosphorylates Parkin at Ser65 in the Ubl domain[18]. However, the extent and consequences of Parkin phosphorylation by PINK1 in mitochondrial regulation are still not fully understood.

To address this issue, we attempted to independently monitor and compare the phosphorylation status of Parkin in wild-type and *PINK1*-deficient cells, thereby excluding the possibility of phosphorylations by uncharacterised kinases other than PINK1[30]. Here, we also report that Parkin is demonstrably phosphorylated at Ser65 in a PINK1-dependent manner. Furthermore, we show that this phosphorylation event is implicated in the regulation of mitochondrial translocation of Parkin and the subsequent degradation of mitochondrial surface proteins during mitophagy.

Results

Parkin is phosphorylated upon depolarisation in $\Delta\Psi$m. We used [^{32}P] orthophosphate to metabolically label mouse embryonic fibroblasts (MEFs) derived from *PINK1* deficient mice, in which HA-tagged Parkin together with FLAG-tagged wild-type or kinase-dead forms (triple mutant with K219A, D362A and D384A) of PINK1 were virally introduced (hereafter referred to as "PINK1-FLAG WT" or "KD/HA-Parkin/*PINK1*$^{-/-}$" MEFs) and then induced Parkin-mediated mitophagy via treatment with the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). As shown in Figure 1a, Parkin was specifically phosphorylated in CCCP-treated PINK1-FLAG WT/HA-Parkin/*PINK1*$^{-/-}$ MEFs, but not in PINK1-FLAG KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs. Phos-tag Western blotting, in which phosphorylated proteins appear as slower migrating bands[28], revealed that Parkin was phosphorylated within 10 min following CCCP treatment (Fig. 1b). Phosphorylation of Parkin reached its maximum level approximately 40 min after CCCP treatment and was sustained at least until 6 hr (Supplementary Fig. S1). Under these conditions, slower migrating bands of PINK1 also appeared, which very likely reflects the autophosphorylation of PINK1 when activated (Fig. 1b)[18]. The suppression of PINK1 accumulation by RNA interference suggested that$\Delta\Psi$m depolarisation-dependent activation of PINK1 along with PINK1 accumulation is a key element for Parkin phosphorylation (Fig. 1c). Every PINK1 deletion and pathogenic mutant we tested failed to stimulate Parkin phosphorylation effectively, strongly suggesting that intact PINK1 is required for this action (Fig. 1d and e). Importantly, human fibroblasts from a patient with *PINK1*-linked parkinsonism also lacked the activity to phosphorylate Parkin (Fig. 1f). The phosphorylated Parkin disappeared within 30 min during the recovery of $\Delta\Psi$m depolarisation by the removal of CCCP from the culture medium (Fig. 1g). Further analysis using phosphatase and proteasome inhibitors suggested that phosphorylated Parkin is at least partly degraded by proteasomal activity in the mitochondria (Supplementary Fig. S2).

Phosphorylation of Ser65 in the Parkin Ubl domain primes the mitochondrial translocation of Parkin. To determine which residue(s) of Parkin are phosphorylated, we immunopurified HA-tagged Parkin from PINK1-FLAG WT or KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs treated with or without CCCP and performed mass spectrometric analysis for phospho-peptides (Supplementary Fig. S3). Although Phos-tag Western blotting of Parkin mainly detected a single band shift, which represents a single phospho-modification, the mass spectrometric analysis identified Ser9 or Ser10 and Ser65, Ser101 and Ser198 as phosphorylated residues of Parkin. Among these residues, only Ser65 phosphorylation increased (33-fold) in CCCP-treated PINK1-FLAG WT/HA-Parkin/*PINK1*$^{-/-}$ MEFs (Supplementary Fig. S3). Phos-tag Western blotting with mutant forms of Parkin, in which the identified phospho-serine residues are replaced with alanine, revealed that the band shift represents Ser65 phosphorylation (Fig. 2a). An *in vitro* kinase assay with recombinant insect PINK1, which has marked kinase activity[28], strongly suggested that PINK1 directly phosphorylates Parkin at Ser65 (Supplementary Fig. S4). The Ser65 residue lies in the Ubl domain and is highly conserved from human to *Drosophila* (Fig. 2b). We next examined whether phosphorylation of Ser65 is required for Parkin-mediated mitophagy. GFP-tagged Parkin WT, which was localised both in the cytoplasm and in the nuclei of mock (DMSO)-treated cells (0 hr, Fig. 2c and d), was translocated to the mitochondria and induced the perinuclear aggregation of mitochondria 2 hr after CCCP treatment, as previously reported (2 hr, Fig. 2c and d)[17,23]. Replacement of Ser65 with alanine (S65A) did not affect the subcellular localisation of Parkin in mock-treated cells when compared with that of GFP-Parkin WT (0 hr, Fig. 2c and d). However, GFP-Parkin S65A almost completely inhibited the mitochondrial translocation of Parkin and the perinuclear rearrangement of mitochondria 0.5 hr after CCCP treatment (0.5 hr, Fig. 2c and d) and showed delayed translocation in 2 hr (2 hr, Fig. 2c and d). The expression of a putative phosphomimetic Parkin S65E also showed a subcellular localisation similar to that of GFP-Parkin WT in both DMSO- and CCCP-treated cells (Fig. 2c). However, GFP-Parkin S65E exhibited a mild translocation defect, suggesting that S65E does not fully mimic the phosphorylated Ser65 (Fig. 2d).

Parkin Ser65 phosphorylation is not sufficient for mitochondrial translocation upon depolarisation of $\Delta\Psi$m. As PINK1-mediated Ser65 phosphorylation appeared to be required for efficient translocation of Parkin, we next examined whether well-characterised pathogenic Parkin mutants were subjected to phosphorylation upon CCCP treatment. In this experiment, we used three kinds of Parkin mutants based on the previous and current studies (Supplementary Fig. S5)[17,22,23]. The first group, V15M, P37L, R42P and A46P, had intact or weakly impaired mitochondrial translocation activity. The second group, T415N and G430D, had mildly impaired translocation activity. The third group, K161N, K221N and T240R, almost completely lacked translocation activity (Fig. 3a). Surprisingly, all of the mutants possessed comparable phosphorylation efficiencies to those of WT (Fig. 3b). This result suggests that Ser65 phosphorylation is not sufficient for the mitochondrial translocation of Parkin.

Biochemical fractionation of endogenous Parkin from SH-SY5Y cells detected only the phosphorylated form of Parkin in the mitochondrial fraction upon CCCP treatment (Fig. 3c), which strongly suggests that phosphorylation of Parkin is required for mitochondrial translocation. There was a slight difference in the gel mobility of phosphorylated Parkin between the cytosolic and the mitochondrial fractions and between CCCP-treated periods of time. These differences very likely reflect differences in the complexity of the contents of each fraction rather than in the phosphorylation status of Parkin because a single shifted band appears in the mixed fractions (Mito + Cyto in Fig. 3c; CCCP 30 min + 60 min in Supplementary Fig. S6).

Effect of Parkin Ser65 phosphorylation on the autophagic reaction. We next examined whether Ser65 phosphorylation is required for the subsequent autophagic reaction, in which various ubiquitin-proteasome- and autophagy-related proteins are involved, including the 26S proteasome, p97/VCP, p62/SQSTM1, LC3, ATG5 and ATG7[22,23,31–35]. Parkin has been reported to be involved in the ubiquitin-proteasome-dependent degradation of a variety of mitochondrial outer membrane proteins, including Mitofusin1 (Mfn1)[32], Mfn2[32], Miro1[36,37], Miro2[37], VDAC1[22] and Tom20[31]. Degradation of Mfn1, VDAC1 and Tom20 at the mitochondrial outer membrane was observed in PINK1 WT/GFP-Parkin/*PINK1*$^{-/-}$ MEFs 1 to 4 hr after CCCP treatment (Fig. 4a). While GFP-Parkin harbouring S65A or S65E mutations was also capable of inducing Mfn1, VDAC1 and Tom20 degradation, the efficiency was impaired, especially in Mfn1 and VDAC1 (Fig. 4a). Long-term time course analysis revealed that in cells expressing Parkin with S65A or S65E mutations, Mfn1 and VDAC1 cannot be degraded effectively, and the mitochondrial outer membrane was likely more intact as indicated by the sustained

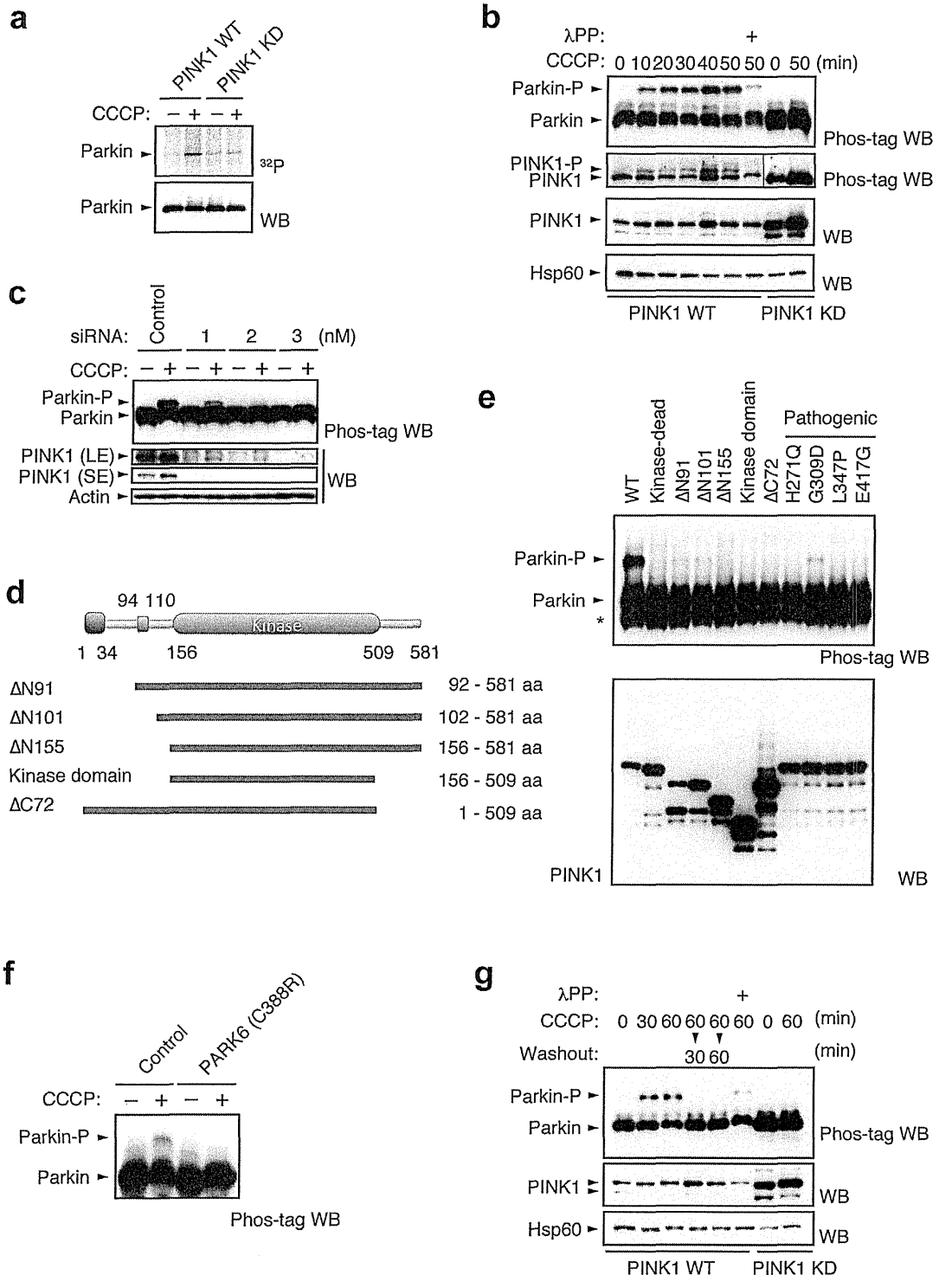

Figure 1 | PINK1-dependent phosphorylation of Parkin *in vivo*. (a) PINK1-FLAG WT or KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs were labelled with [^{32}P] orthophosphate and treated with 30 μM CCCP for 1.5 hr. Phosphorylated Parkin was detected by autoradiography (^{32}P). Immunoprecipitated HA-Parkin was detected by Western blotting (WB) with anti-Parkin. (b) PINK1-FLAG WT or KD/HA-Parkin/*PINK1*$^{-/-}$ MEFs were treated with or without 30 μM CCCP for the indicated periods of time. Cell lysate was subsequently separated on a Phos-tag gel, followed by WB with anti-PINK1 or anti-Parkin antibodies (Phos-tag WB). Phosphorylated bands of Parkin and PINK1 were confirmed by their disappearance with lambda protein phosphatase (λPP) treatment. Mitochondrial Hsp60 was used as a loading control. (c) Suppression of endogenous PINK1 expression inhibits Parkin phosphorylation. HeLa cells stably expressing non-tagged Parkin were treated with the indicated concentrations of stealth siRNA duplex against PINK1 (Invitrogen) with or without 10 μM CCCP for 1 hr. Long- (LE) and short-exposure (SE) blot signals for PINK1 were shown. Actin was used as a loading control. (d) Truncated PINK1 mutants used in this study. Putative mitochondria-targeting sequence, 1–34 aa; transmembrane domain, 94–110 aa; kinase domain, 156–509 aa. (e) Full-length PINK1 is required for Parkin phosphorylation. *PINK1*$^{-/-}$ MEFs stably expressing non-tagged Parkin were transfected with various PINK1 constructs with C-terminal FLAG-tags. PINK1 expression was confirmed with anti-FLAG-HRP. (f) Human fibroblasts from a normal control and a *PARK6* case with a homozygous C388R mutation[44] were transfected with Parkin and were treated with or without 30 μM CCCP for 1 hr. (g) Cells treated with CCCP up to 60 min as in (b) were further incubated with fresh culture medium without CCCP for the indicated periods of time (Washout).

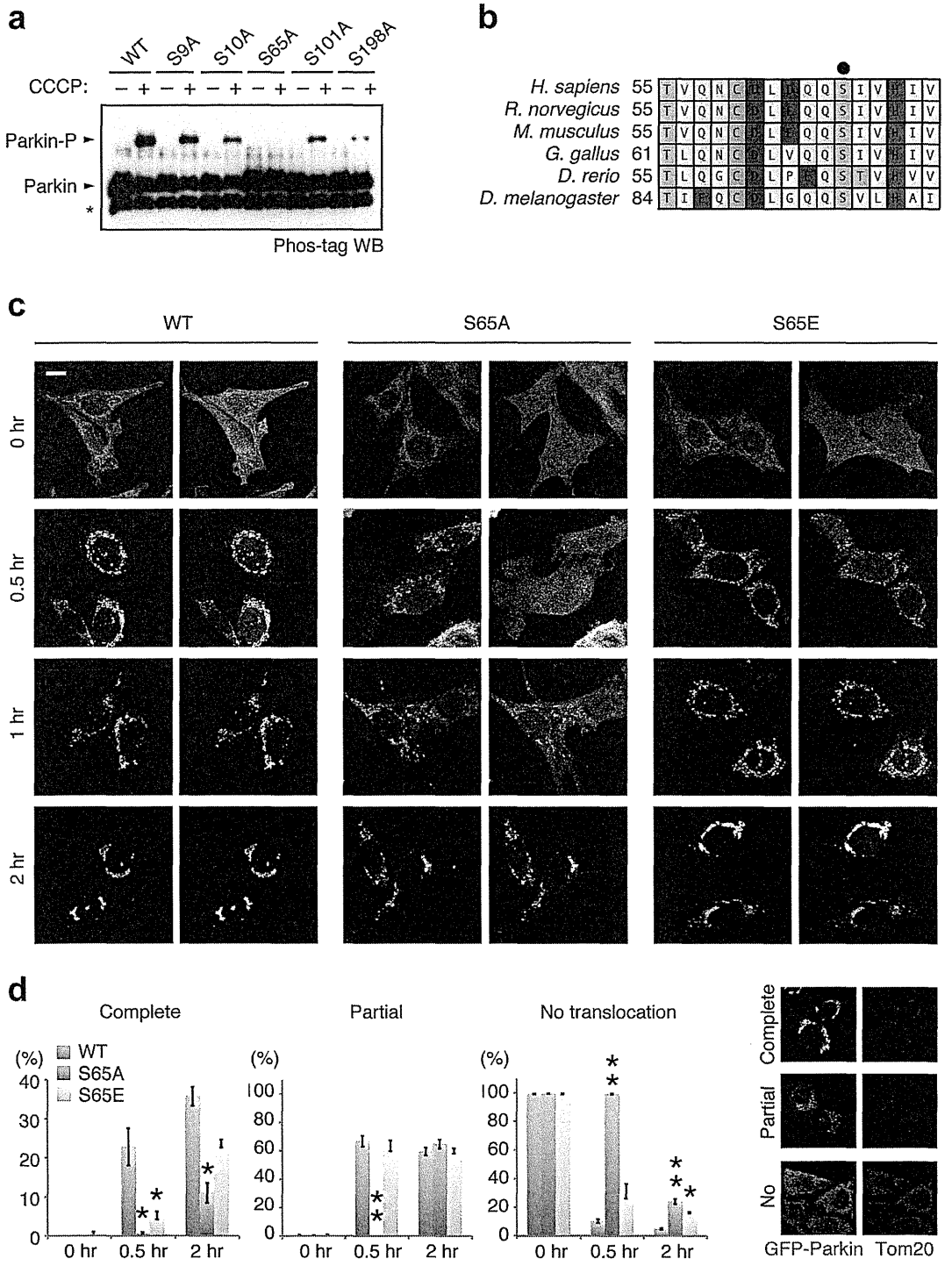

Figure 2 | Ser65 in the Ubl domain of Parkin is phosphorylated upon depolarisation of $\Delta\Psi$m. (a) Phos-tag Western blotting detected phosphorylation of Ser65. HeLa cells were transiently transfected with Parkin WT and a series of alanine mutants for the candidate phospho-residues followed by treatment with or without 20 μM CCCP for 1 hr. Cell lysates were analysed by Phos-tag Western blotting. An asterisk indicates degraded Parkin. (b) Alignment of the amino acid sequences surrounding Ser65 (marked by a black dot) from a variety of animal species. The numbers on the left correspond to the residue numbers of Parkin proteins. (c) Introduction of the S65A mutation delayed Parkin translocation to the depolarised mitochondria in PINK1 WT/GFP-Parkin/*PINK1*$^{-/-}$ MEFs. Cells retrovirally introduced with GFP-Parkin WT or its phospho-mutants (S65A and S65E) were treated with or without 30 μM CCCP for the indicated periods of time. GFP-Parkin and mitochondria were visualised with anti-GFP (green) and anti-Tom20 (red), respectively. Parkin signals are also shown as monochrome images. Scale bar = 10 μm. (d) Mitochondrial translocation efficiency of Parkin mutants. PINK1 WT/*PINK1*$^{-/-}$ MEFs stably expressing GFP-Parkin WT, S65A or S65E were treated as in (c). Cells expressing GFP-Parkin perfectly overlapped (Complete, examples are shown on the right), partially overlapped (Partial) or non-overlapped (No) with the Tom20 signal were counted. The data represent means ± SE from three experiments (n = 99–143 cells in each). ** $p < 0.01$, * $p < 0.05$ *vs.* WT at each time point.

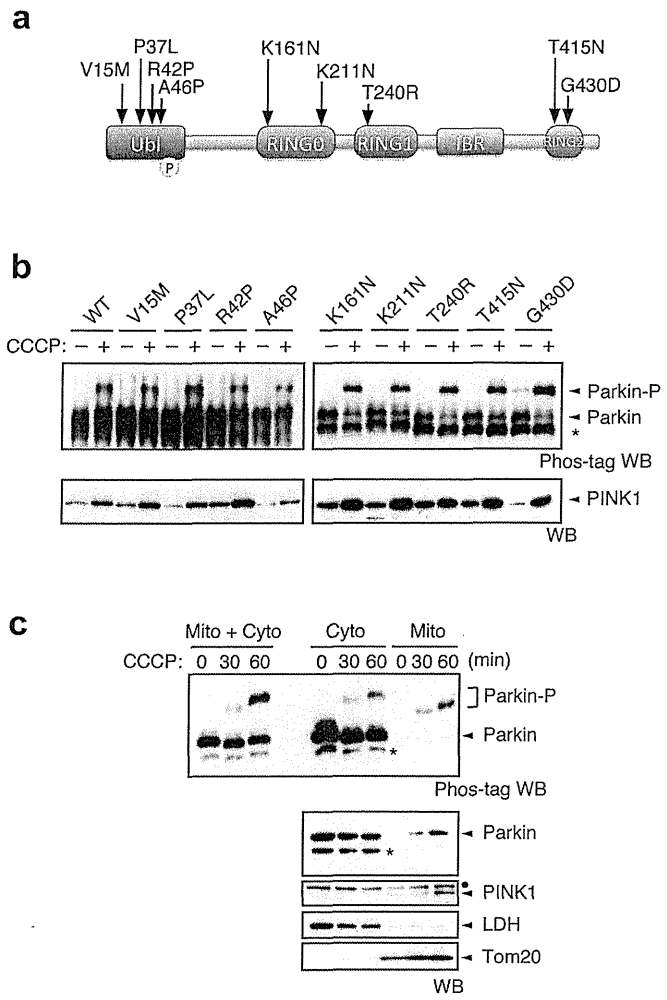

Figure 3 | Pathogenic mutants of Parkin are subjected to Ser65 phosphorylation. (a) Diagram of Parkin protein illustrating the pathogenic mutants used in this study. The Ser65 residue in the Ubl domain is shown as a yellow circle. RING, Ring-finger motif; IBR, in-between-Ring fingers domain. (b) Phos-tag Western blotting for Parkin and Western blotting for PINK1 were performed using Parkin WT and a series of pathogenic mutants as shown in Figure 2a. (c) Endogenous Parkin was also phosphorylated in SH-SY5Y cells after CCCP treatment. Post-nuclear cell lysates from SH-SY5Y cells treated with or without 10 μM CCCP for 30 and 60 min were fractionated into mitochondria-rich (Mito) and cytosolic (Cyto) fractions. These two fractions and their combination (Mito + Cyto) were subjected to Phos-tag or normal Western blotting analyses. Endogenous PINK1 was fractionated in the Mito fraction, as previously reported[45]. Lactate dehydrogenase (LDH) and Tom20 were used as cytosolic and mitochondrial marker proteins, respectively. Asterisks: putative cleaved Parkin; dots: non-specific bands.

accumulation of PINK1 (Fig. 4b). The impaired degradation cannot be explained simply by the delayed translocation of Parkin mutants because both mutants completed the mitochondrial translocation by the 6 hr time-point (data not shown and see Fig. 4c). In contrast, the profiles of Parkin expression and autoubiquitination in Parkin S65A- or S65E-expressing cells were comparable with those of WT (Fig. 4b). We also examined whether Ser65 mutations affect the accumulation of proteasome (Fig. 4c) and p62 (Supplementary Fig. S7) at the mitochondria during mitophagy via the immunostaining of the proteasome subunit alpha type 7 (α7) and p62. However, there was no evidence that Ser65 mutations inhibit or delay the recruitment of proteasome and p62 to the mitochondria. Finally, we tested whether the Parkin Ubl domain itself is indispensable for the mitochondrial translocation and the substrate degradation (Supplementary Fig. S8). Interestingly, Parkin mutant lacking the Ubl domain (ΔUbl) showed a mild delay in the mitochondrial translocation, slowed the mitochondrial reorganization to the perinuclear region (Supplementary Fig. S8b and c) and impaired the degradation of mitochondrial outer membrane proteins (Supplementary Fig. S8d). These results suggest that proper regulation of the Parkin Ubl domain through the Ser65 phosphorylation is required not only for efficient translocation to mitochondria as an initial step of mitophagy, but also for the degradation of mitochondrial outer membrane proteins during mitophagy through an as yet unknown mechanism.

Discussion

A series of *Drosophila* genetic and cell biological studies have clearly demonstrated that PINK1 is required for Parkin-mediated mitochondrial maintenance. The mitophagy of damaged mitochondria is a well-characterised event in which PINK1 and Parkin are involved. However, how PINK1 regulates Parkin is largely unclear. This study has shown that Ser65 in the Ubl domain of endogenous Parkin is phosphorylated in an activated PINK1-dependent manner. In addition to mitochondrial accumulation of PINK1, ΔΨm depolarisation-dependent PINK1 autophosphorylation has been reported to be an important element for PINK1 activation and Parkin recruitment[19,29]. Consistent with these observations, our investigation of PINK1 siRNA suggests that a lower level of PINK1 is able to phosphorylate Parkin after ΔΨm depolarisation (Fig. 1c, compare lanes 1 and 4). Our domain analysis of PINK1 demonstrates that intact PINK1 is required for CCCP-dependent Parkin phosphorylation, and the lack of phosphorylation in fibroblasts from a *PARK6* patient implies relevance to the pathogenesis of PD.

The biological significance of this phosphorylation event is suggested by the fact that replacement of Ser65 with alanine or glutamic acid impairs the mitochondrial translocation of Parkin and/or the subsequent mitophagy process. Our observation that maximal phosphorylation of Parkin occurs within 1 hr of CCCP treatment supports the idea that Ser65 phosphorylation is required for the early step of Parkin translocation. In contrast, PINK1 accumulation appears to last at least 6 hr (Fig. 4c and Supplementary Fig. S1b). The difference in time course between PINK1 accumulation and Parkin phosphorylation could be explained by the observation that phosphorylated Parkin is degraded by proteasomal activity. The biochemical evidence that only the phosphorylated form of endogenous Parkin is present in the mitochondrial fraction also implies that Parkin phosphorylation is an essential event for its mitochondrial translocation and subsequent activation (Fig. 3c and Supplementary Fig. S6). Overexpression of PINK1 and Parkin itself leads to mitochondrial translocation of Parkin independently of ΔΨm depolarization, which suggests that excessive amounts of PINK1 and Parkin do not faithfully reflect endogenous reactions. Our study using *PINK1*$^{-/-}$ MEFs stably co-expressing PINK1 and GFP-Parkin might also be saddled with such a problem. We believe that the endogenous observation in which phosphorylated Parkin is accumulated in mitochondria is a more reliable proposal as a molecular model. The delay of exogenous GFP-Parkin S65A in the mitochondrial translocation would indicate that modification of Ser65 is important for Parkin translocation at least. At the same time, another important finding is that pathogenic mutants that lose their translocation activity are also phosphorylated (Fig. 3b), raising the possibility that phosphorylation of Parkin at Ser65 is insufficient for translocation. Thus, Ser65 phosphorylation likely leads to other events in mitochondrial translocation, such as the association or dissociation of protein(s) involved in the mitochondrial translocation of Parkin or the modification of Parkin itself for activation at a different site(s).

Both the S65A and S65E Parkin mutants cannot undergo efficient mitophagy, as indicated by the incomplete degradation of

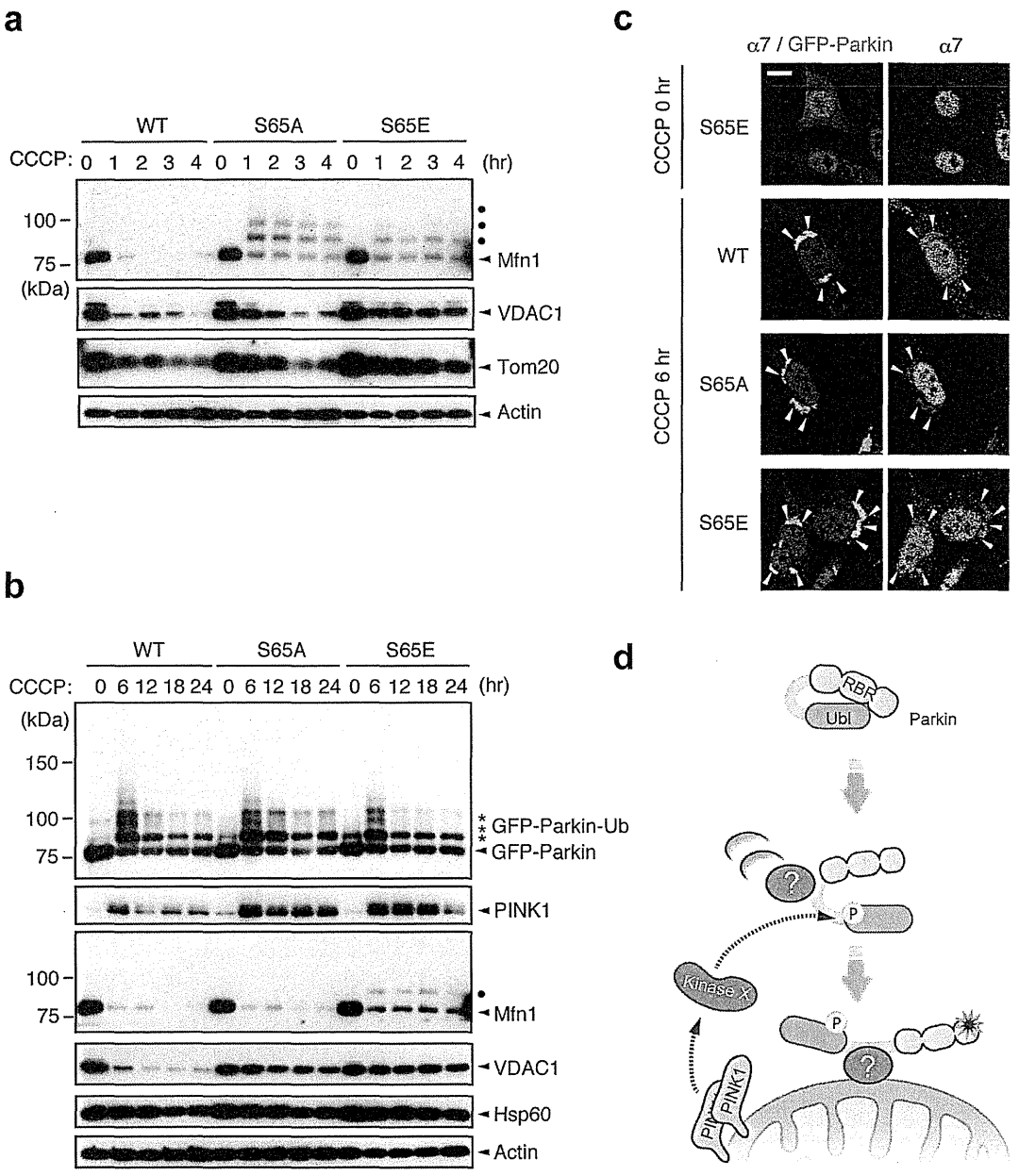

Figure 4 | Ser65 phosphorylation affects the subsequent autophagy reaction. (a) CCCP-dependent degradation of mitochondrial outer membrane proteins in PINK1 WT/*PINK1*$^{-/-}$ MEFs expressing WT or mutant forms of GFP-Parkin. Mfn1, VDAC1 and Tom20 were used as markers of mitochondrial outer membrane proteins. Actin: a loading control. Dots: ubiquitinated Mfn1. (b) Long-term time-course analysis of CCCP-dependent mitochondrial protein degradation. The degradation of outer membrane proteins was impaired in cells expressing GFP-Parkin S65A or S65E mutations. Hsp60 was used as a marker of mitochondrial matrix proteins. (c) S65A and S65E mutations do not affect proteasome recruitment to the mitochondria during mitophagy. PINK1 WT/*PINK1*$^{-/-}$ MEFs expressing WT or mutant forms of GFP-Parkin (green) were treated with 30 μM CCCP for 3 or 6 hr. Cells were stained with anti-proteasome subunit alpha type 7 (α7, red). α7-immunoreactivity was enriched in the nuclei of all three cell genotypes under normal conditions, as displayed in the representative image of S65E (CCCP 0 hr), and overlapped with the aggregated mitochondria (arrowheads) 6 hr after CCCP treatment irrespective of genotype. Similar results were obtained 3 hr after CCCP treatment. Scale bar = 10 μm. (d) Model for Parkin translocation and activation. The Parkin Ubl domain masks C-terminal RING-IBR-RING (RBR) domains for E3 activity[46]. A Parkin phosphorylation event at Ser65 (P), combined with unknown factor(s) (?), stimulates the mitochondrial translocation of Parkin, releasing the RBR domains from autoinhibition by the Ubl domain.

the mitochondrial outer membrane proteins. Because inhibition of the degradation of the mitochondrial outer membrane proteins by proteasome inhibitors is reported to block mitophagy[32,35], it may be that the modification of Parkin Ser65 has a greater than expected impact on the mitophagy process. Although our study does not demonstrate that the S65E mutant behaves exactly like the phosphorylated form of Parkin, the S65E mutant does translocate to the mitochondria in a similar way to WT, although with slightly impaired efficiency, suggesting that S65E has at least some properties that are similar to phosphorylated Parkin. Currently, it is unknown why S65E also inhibits the later processes of mitophagy. One possible explanation is that rapid degradation of phosphorylated Parkin is required for the proper progression of mitophagy, and S65E may not be degraded effectively. However,

there is no evidence that S65E is more stable than WT, as shown in Figure 4c.

Very recently, Kondapalli *et al.* proposed a model to explain the biological significance of Ser65 phosphorylation, in which Ser65 phosphorylation relieves autoinhibition of Parkin E3 activity by the Ubl domain[18]. This model may explain the depolarised $\Delta\Psi$m-dependent activation of Parkin. However, our data indicated that the Parkin S65A mutant is also autoubiquitinated (Fig. 4b) and that the ΔUbl mutant showed mild translocation defect and impaired substrate degradation (Supplementary Fig. S8). Moreover, if this is the case, the E3 activity of Parkin pathogenic mutants lacking mitochondrial translocational activity but harbouring intact E3 activity *in vitro* (such as K161N and K211N, which are subjected to the Ser65 phosphorylation) should be activated in the cytosol[38]. However, our previous data indicate that K161N and K211N are not activated by CCCP treatment[23]. Thus, it is conceivable that another step is required for depolarised $\Delta\Psi$m-dependent activation of Parkin E3. In addition, the Ubl domain might not only autoinhibit its E3 activity but also contribute to the mitochondrial translocation and the substrate degradation through an as yet unknown mechanism. We believe that an appropriate way to estimate Parkin E3 activity in the context of mitophagy is to evaluate the ubiquitination and degradation of substrates in cells with depolarised $\Delta\Psi$m. Mfn1 is a well-characterised direct substrate of Parkin[32], and Parkin-dependent poly-ubiquitination modification of Mfn1 can be detected by Western blotting upon $\Delta\Psi$m depolarisation[32,39,40]. Parkin S65A and S65E appear to ubiquitinate Mfn1, as poly-ubiquitinated forms of Mfn1 were observed (Fig. 4b). However, they cannot degrade it effectively, which suggests that the process of substrate degradation is also impaired in these mutants.

Kondapalli *et al.* have also shown that *T. castaneum* PINK1 (TcPINK1) directly phosphorylates human Parkin at Ser65[18]. We confirmed their finding using recombinant TcPINK1 produced from the same construct (Supplementary Fig. S4). The replacement of MBP-Parkin Ser65 with alanine completely abolished PINK1-mediated phosphorylation, indicating that Ser65 is the sole phosphorylation site *in vitro*. However, experiments in cultured cells showed that the replacement of Ser9, Ser10, Ser101 and Ser198 with alanine affects the Ser65 phosphorylation efficiency (Ser9, ~35% reduction; Ser10, ~76% reduction; Ser101, ~65% reduction; Ser198, ~92% reduction) (Fig. 2a). These residues might be priming phosphorylation sites for Ser65 phosphorylation.

Because PINK1 is believed to be activated in the mitochondria, a topological inconsistency arises from our cell-based data that cytosolic Parkin lacking the mitochondrial translocation activity is phosphorylated. Therefore, it is possible that PINK1 indirectly regulates Parkin phosphorylation. One possible explanation for this is the presence of another cytosolic kinase(s) regulated by PINK1 (Fig. 4d). Alternatively, because mitochondria are a dynamic organelle, cytosolic Parkin adjacent to the moving and fragmented mitochondria with depolarised $\Delta\Psi$m might be phosphorylated incidentally. The issue as to whether or not PINK1 directly phosphorylates Parkin in cells remains to be solved.

In conclusion, this study has suggested that PINK1-dependent Parkin phosphorylation at Ser65 accelerates the mitochondrial translocation of Parkin and showed that the introduction of mutations at this site also affects subsequent mitophagy processes. Concurrently, our data provide the possibility that there is an elaborate multi-step mechanism for the mitochondrial translocation of Parkin upon the loss of $\Delta\Psi$m (Fig. 4d), the clarification of which awaits further study.

Methods

Antibodies, plasmids and cell lines. Antibodies used in Western blot analysis were as follows: anti-Parkin (1 : 1,000 and 1 : 5,000 dilution for endogenous and exogenous Parkin, respectively; Cell Signaling Technology, clone PRK8), anti-PINK1 (1 : 1,000 dilution; Novus, BC100-494 or 1 : 1,000 dilution; Cell Signaling Technology, clone D8G3), anti-Mfn1 (1 : 1,000 dilution; Abnova, clone 3C9), anti-VDAC1 (1 : 1,000 dilution; Abcam, Ab15895), anti-Tom20 (1 : 500 dilution; Santa Cruz Biotechnology, FL-145), anti-FLAG-HRP (1 : 2,000 dilution; Sigma-Aldrich, clone M2), anti-GFP (1 : 5,000 dilution; Abcam, ab290), anti-Actin (1 : 10,000 dilution; Millipore, MAb1501), anti-LDH (1 : 1,000 dilution; Abcam, ab7639-1), anti-phospho-GSK3ß (1 : 1,000 dilution; Cell Signaling Technology, clone 5B3), anti-GSK3ß (1 : 1,000 dilution; Cell Signaling Technology, clone 27C10), and anti-Hsp60 (1 : 10,000 dilution; BD Biosciences, clone 24/Hsp60). Antibodies used in immunocytochemistry were as follows: FITC-conjugated anti-GFP (1 : 1,000 dilution; Abcam, ab6662), anti-Tom20 (1 : 1,000 dilution; Santa Cruz Biotechnology, FL-145), anti-Myc (1 : 500 dilution; Millipore, clone 4A6), anti-p62 (1: 500 dilution; Progen Biotechnik, GP62-C), anti-Parkin (1 : 1,000 dilution; Cell Signaling Technology, clone PRK8) and anti-proteasome α7 (1 : 250; a kind gift of Dr S. Murata at the University of Tokyo). cDNAs for human Parkin, PINK1 and its pathogenic and engineered mutants are as described in previous studies[23,41]. Parkin phospho-mutants were generated by PCR-based mutagenesis followed by sequencing confirmation of the entire gene. *PINK1*$^{-/-}$ MEFs, cultured as previously described[23], were retrovirally transfected with pMXs-puro harbouring non-tagged PINK1, PINK1-FLAG, non-tagged Parkin, HA-Parkin, GFP-Parkin and related cDNA; transfected cells were then selected with 1 μg/ml puromycin. HeLa cells maintained at 37°C in a 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and 1x non-essential amino acids (GIBCO) were retrovirally transfected with pMXs-puro harbouring non-tagged Parkin along with pcDNA3Hyg-mSlc7a1-VSVG and pcDNA3Hyg-mSlc7a1-FLAG (a kind gift of Dr N. Fujita at UCSD). Stable cell lines were selected with 1 μg/ml puromycin and cloned. Transient transfections of cultured cells were performed using Lipofectamine 2000 (Invitrogen) for plasmids and Lipofectamine RNAiMAX (Invitrogen) for stealth siRNA duplexes (Invitrogen), which were used according to the manufacturer's instructions.

Tissue culture. Skin biopsies were obtained from a *PARK6* case and a control without mutations in any known PD genes. The study was approved by the ethics committee of Juntendo University, and all participants gave written, informed consent. Dermal primary fibroblasts established from biopsies were cultured in high glucose DMEM supplemented with 10% foetal bovine serum, 1x non-essential amino acids, 1 mM sodium pyruvate (GIBCO), 100 μM 2-mercaptoethanol, and 1% penicillin–streptomycin at 37°C in a 5% CO_2 atmosphere.

Mapping of Parkin phosphorylation sites. *PINK1*$^{-/-}$ MEFs (6.0 x 10^7) expressing HA-Parkin and PINK1-FLAG were treated with or without 30 μM CCCP for 30 min. HA-Parkin (~500 ng in each) immunopurified with anti-HA-conjugated agarose beads was eluted with 8 M urea buffered with 50 mM Tris-HCl at pH 9.0. Samples from two independent experiments were digested with trypsin or chymotrypsin and analysed by nano-scale liquid chromatography-tandem mass spectrometry (Dionex Ultimate3000 RSLCnano and ABSciex TripleTOF 5600) followed by MASCOT searching and Mass Navigator/PhosPepAnalyzer processing for identification and label-free quantitation, respectively[42]. Determination of phosphosite localisation was performed based on the presence of site-determining ions[43].

Phosphorylation assay and mitochondrial fractionation. *PINK1*$^{-/-}$ MEFs harbouring HA-Parkin along with wild-type or a kinase-dead form of PINK1-FLAG were metabolically labelled with 175 μCi/ml of [^{32}P] orthophosphate in phosphate-free DMEM (GIBCO) with 10% FBS at 37°C for 3 hr. The medium was then replaced with fresh DMEM containing 10% FBS. Cells were treated with CCCP for 1.5 hr and were lysed on ice with lysis buffer containing 0.2% NP-40, 50 mM Tris (pH 7.4), 150 mM NaCl and 10% glycerol supplemented with protease inhibitor (Roche Diagnostics) and phosphatase inhibitor (Pierce) cocktails, and HA-Parkin and PINK1-FLAG were immunoprecipitated with anti-HA (Wako Pure Chemical, clone 4B2)- or anti-FLAG (Sigma-Aldrich, clone M2)-conjugated agarose beads. Immunoprecipitates were separated by SDS-PAGE and transferred onto a PVDF membrane. Autoradiography and Western blotting were performed to visualise proteins. Phos-tag Western blotting was performed as previously described[28]. Briefly, phospho-Parkin and phospho-PINK1 were separated on 8% gels containing 50 μM Phos-tag. Mitochondrial and cytosolic fractionations were performed as previously described, with some modifications[20]. The cytosolic fractions were further clarified by a second centrifugation at 105,000 *g* for 60 min to remove residual organelle membranes.

Immunocytochemical analysis. Cells plated on 3.5 mm glass-bottom dishes (MatTek) were fixed with 4% paraformaldehyde in PBS and permeabilised with 50 μg/ml digitonin for anti-Tom20 and anti-p62 staining or with 0.1% NP-40 for anti-α7 staining in PBS. Cells were stained with anti-Tom20 or anti-α7 antibodies in combination with FITC-conjugated anti-GFP antibody and were counterstained with DAPI for nuclei. Cells were imaged using laser-scanning microscope systems (TCS-SP5, Leica or LSM510 META, Carl Zeiss).

Statistical analysis. A one-way repeated measures ANOVA was used to determine significant differences between multiple groups unless otherwise indicated. If a significant result was achieved ($p < 0.05$), the means of the control and the specific test group were analysed using the Tukey-Kramer test.

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Acknowledgements

We thank: Drs K. Tanaka, N. Matsuda, K. Okatsu, T. Kitamura, S. Murata, N. Fujita, N. Furuya, M.M.K. Muqit and R.J. Youle for their generous supply of materials; T. Hasegawa and Y. Imaizumi for the preparation of human fibroblasts; and T. Imura for her technical help. This study was supported by the Naito Foundation, the Novartis Foundation, the Grant-in-Aid for Young Scientists (B) from MEXT in Japan (SK-F, YI), the CREST program of JST (NH) and Grant-in-Aid for Scientific Research on Innovative Areas (NH).

Author contributions

K.S., Y. Imai and N.H. designed the research; K.S., Y. Imai, S.Y., T.K. and Y. Ishihama performed the experiments; S.S. contributed new reagents/analytic tools; K.S. and Y. Imai analysed the data; and Y. Imai and N.H. wrote the paper. K.S. and Y. Imai contributed equally to this work.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Shiba-Fukushima, K. *et al.* PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy. *Sci. Rep.* **2**, 1002; DOI:10.1038/srep01002 (2012).

RESEARCH ARTICLE

VPS35 Mutation in Japanese Patients with Typical Parkinson's Disease

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

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

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Funding agencies: This work was supported by Strategic Research Foundation Grant-in-Aid Project for Private Universities, Grants-in-Aid for Scientific Research (80218510 [to N.H.] and 21591098 [to H.T.]), Grant-in-Aid for Young Scientists (22790829 [to M.F.] and 23791003 [to Y.L.]), Grant-in-Aid for Scientific Research on Innovative Areas (23111003 [to N.H.] and 23129506 [to M.F.]) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and Grant-in-Aid from the Research Committee on Muro Disease (Kii ALS/PDC; 21210301 [to Y.K.]), the Ministry of Health, Labor, and Welfare, and JST, CREST.

Relevant conflicts of interest/financial disclosures: Nothing to report.
Full financial disclosures and author roles may be found in the online version of this article.

Received: 5 March 2012; **Revised:** 11 July 2012; **Accepted:** 17 July 2012
Published online in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/mds.25145

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

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

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

Patients and Methods

Subjects

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

Genetic Analysis

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

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

Results

Detection of p.D620N Mutation

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

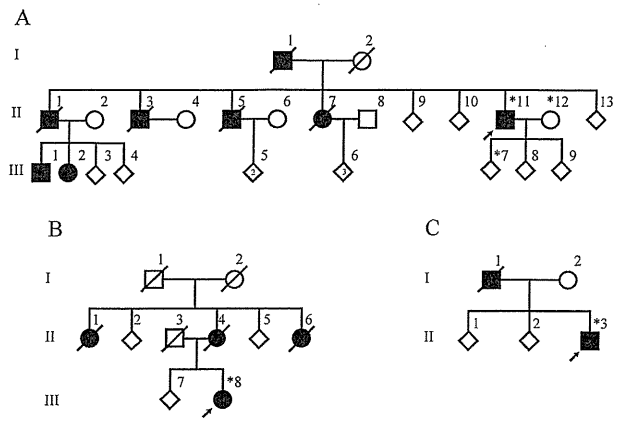

FIG. 1. Pedigrees of families with the *VPS35* p.D620N mutation (open symbol, unaffected family member; closed symbol, affected member; arrow, proband; asterisk, individual analyzed for the p.D620N mutation and/or haplotype; forward slash through symbol, deceased individuals; square, male; circle, female;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

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

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

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

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

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

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

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

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

Clinical Presentation

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

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

Discussion

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

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

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

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

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

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

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

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

Acknowledgments: We thank all the participants in this study.

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Parkinsonism and Related Disorders 19 (2013) 15–20

Contents lists available at SciVerse ScienceDirect

Parkinsonism and Related Disorders

journal homepage: www.elsevier.com/locate/parkreldis

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

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ARTICLE INFO

Article history:
Received 26 April 2012
Received in revised form
16 June 2012
Accepted 19 June 2012

Keywords:
MAPT
PGRN
C9orf72
De novo
Abnormal eye movements

ABSTRACT

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

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

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

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

1. Introduction

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

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1353-8020/$ – see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.parkreldis.2012.06.019

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.

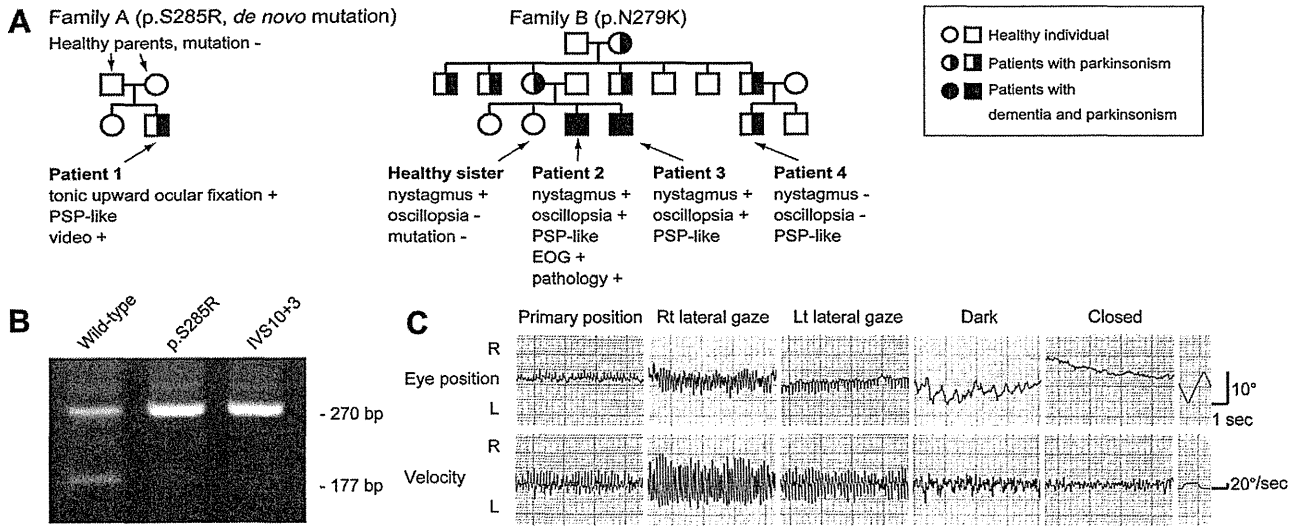

Fig. 1. (A) The pedigrees of families A and B. (B) Exon-trapping analysis for the effects of the *MAPT* p.S285R mutation on exon 10 splicing. (C) Horizontal electro-oculogram recordings in Patient 2.

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.

Acknowledgments

The authors thank all of the participants in this study. The authors also thank Dr. Mariely DeJesus-Hernandez for technical advice on the analysis of *C9orf72* repeat expansion. This work was supported by the Strategic Research Foundation Grant-in-Aid Project for Private Universities, Grants-in-Aid for Scientific Research, Grant-in-Aid for Young Scientists, and Grant-in-Aid for Scientific Research on Innovative Areas from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Grants-in-Aid from the Research Committee of CNS Degenerative Diseases and Muro Disease (Kii ALS/PDC), Grants-in-Aid from the Research Committee on CNS Degenerative Diseases and Perry Syndrome from the Ministry of Health, Labor and Welfare of Japan, Project Research Grants-in-Aid from Juntendo University School of Medicine, and CREST from the Japan Science and Technology Agency (JST).

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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