PINK1-linked parkinsonism is associated with Lewy body pathology

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Phosphatase and tensin homolog-induced putative kinase 1 gene mutations have been associated with autosomal recessive early-onset Parkinson’s disease. To date, no neuropathological reports have been published from patients with Parkinson’s disease with both phosphatase and tensin homolog-induced putative kinase 1 gene copies mutated. We analysed the coding region of phosphatase and tensin homolog-induced putative kinase 1 gene in a large Spanish family with six members with parkinsonism. The phenotype was characterized by an early-onset (mean: 31.6, standard deviation: 9.6 years, range: 14–45 years), slowly progressive levodopa-responsive parkinsonism, initial gait impairment and psychiatric symptoms. We identified two segregating pathogenic phosphatase and tensin homolog-induced putative kinase 1 mutations that were either in homozygous or heterozygous compound state in all affected family members. We found an exon 7 deletion (g.16089_16383del293; c.1252_1488del) and a novel +1U1-dependent 5′ splice-site mutation in exon 7 (g.16378G＞A; c.1488 +1G＞A). Leukocyte-derived messenger RNA analysis showed that both mutations caused exon 7 skipping and c.1488 +1G＞A also lead to an in-frame transcript with a 33 base-pair deletion (p.L485_R497del) resulting from activation of a 5′ cryptic exon 7 splice site. Single photon emission computed tomography quantification of striatal dopamine transporter binding (123I-Ioflupane) revealed a posterior–anterior gradient similar to that of idiopathic Parkinson’s disease, but there was no correlation between striatal reduced uptake and disease duration. Post-mortem neuropathological examination of an early-onset Parkinson’s disease carrier of two heterozygous compound phosphatase and tensin homolog-induced putative kinase 1 mutations showed neuronal loss in the substantia nigra pars compacta, Lewy bodies and aberrant neurites in the reticular nuclei of the brainstem, substantia nigra pars compacta and Meynert nucleus, but the locus ceruleus and the amygdala were spared. This is the first...
Introduction

Parkinson’s disease is one of the most common neurodegenerative disorders with a prevalence of 1–2% in the population aged over 65 years (de Rijk et al., 2000). Parkinson’s disease is characterized by tremor, rigidity, bradykinesia and postural instability, resulting from the loss of dopaminergic neurons in the substantia nigra pars compacta and other brainstem nuclei.

Early-onset Parkinson’s disease is characterized by onset before the age of 50 years and good response to levodopa. Early-onset Parkinson’s disease is frequently inherited as an autosomal recessive condition associated with three main genes to date. Parkin (MIM#602544), phosphatase and tensin homolog-induced putative kinase 1 (PINK1) (MIM#608309) and DJ-1 (MIM#606324) genes are responsible for some early-onset Parkinson’s disease cases with a prevalence that varies across different studies (Kitada et al., 1998; Bonifati et al., 2003; Valente et al., 2004a). Parkin gene is responsible for 50% of autosomal recessive early-onset Parkinson’s disease and 15% of sporadic early-onset Parkinson’s disease (Lucking et al., 2000; Periquet et al., 2003), and DJ-1 mutations are responsible for 1% of early-onset Parkinson’s disease (Abou-Sleiman et al., 2003; Bandmann, 2004).

PINK1 gene was originally mapped in a Sicilian family with autosomal recessive parkinsonism (Valente et al., 2001). Subsequently, PINK1 mutations have been associated with early-onset Parkinson’s disease in several families and in 2–4% of sporadic early-onset Parkinson’s disease, being the second most frequent cause of early-onset Parkinson’s disease (Rohe et al., 2004; Chishti et al., 2006; Savettieri et al., 2008; Cazeneuve et al., 2009). Most PINK1 mutations are missense, but gene and exon rearrangements also have been described (Li et al., 2005; Ibanez et al., 2006; Marongiu et al., 2007; Camargos et al., 2009). PINK1 gene spans 1.8 kb and contains eight exons encoding for a 581-amino-acid protein that is ubiquitously expressed (Valente et al., 2004a). PINK1 protein has a C-terminal auto-regulatory region, a highly conserved putative serine-threonine kinase domain and a N-terminal mitochondrial targeting peptide. Dopaminergic neurons are especially dependent on PINK1, which protects neurons against stress-induced mitochondrial dysfunction (Valente et al., 2004a).

An 18F-dopa positron emission tomography study in PINK1-linked parkinsonism reported a more uniform loss of striatal dopamine terminal function than is found in idiopathic Parkinson’s disease (Khan et al., 2002). However, 123I-Ioflupane single photon emission computed tomography (SPECT) analysis in other PINK1 mutation carriers revealed a prominent dorsal loss of dopaminergic terminals (Kessler et al., 2005).

Although the presence of Lewy bodies in brainstem and neocortex and nigral neuronal cell loss has been described in brains from Parkinson’s disease carriers with only one heterozygous PINK1 mutation (Gandhi et al., 2006), no neuropathological reports of autosomal recessive PINK1-linked parkinsonism have been reported to date.

In this report, we studied a large Spanish family with slow progressive parkinsonism and found two segregating PINK1 mutations, an exon 7 deletion and a splicing mutation, owing to a substitution at the +1U1-dependent 5’ splice site in exon 7. Furthermore, we performed 123I-Ioflupane brain SPECT imaging in seven affected members of the family and we describe for the first time the neuropathological examination of the brain from an early-onset Parkinson’s disease individual carrier of two heterozygous compound PINK1 mutations.

Materials and methods

Clinical characteristics

A large early-onset Parkinson’s disease Spanish family (Family 476) comprising 49 members in five consecutive generations was investigated (Fig. 1). Thirty-one individuals participated in the study and 11 individuals underwent a neurological examination conducted by experienced neurologists (J.M.A., O.L., C.M., M.A.P. and P.P.). Clinical diagnosis of Parkinson’s disease was carried out according to standard criteria (Hughes et al., 1992). Demographic and clinical data are shown in Table 1. Written consent was obtained from all individuals and the study was approved by the University of Navarra School of Medicine Ethical Committee.

Genetic analysis

DNA was available from 6 affected and 25 healthy family members. All eight PINK1 exons and their flanking regions were amplified using published primers (Valente et al., 2004a) and sequenced in both directions in affected and non-affected individuals on an ABI3130xl automated sequencer (Applied Biosystems, Foster City, CA, USA). In order to test the effects at expression level of the PINK1 mutations found, blood samples from IV:3, IV:6, IV:11, IV:12, V:1 and V:2 individuals were collected into Tempus™ vacuette blood
Table 1 Clinical features of affected family relatives with PINK1 mutations

<table>
<thead>
<tr>
<th>Individual identification</th>
<th>III:2</th>
<th>III:3</th>
<th>IV:3</th>
<th>IV:5</th>
<th>IV:6</th>
<th>IV:9</th>
<th>IV:11</th>
<th>V:2</th>
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<td>38</td>
<td>36</td>
<td>39</td>
<td>36</td>
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<td>38</td>
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<td>8</td>
<td>9</td>
<td>3</td>
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<td>1</td>
<td>NA</td>
<td>NA</td>
<td>8</td>
<td>3</td>
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<tr>
<td>c.1488 +1G &gt; A</td>
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<td>+/–</td>
<td>+/+</td>
<td>+/–</td>
<td>+/+</td>
<td>+/–</td>
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<td>+/–</td>
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<tr>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Postural tremor</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>NA</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Rigidity</td>
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<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Postural instability</td>
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<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>Psychosis</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>Asymmetrical</td>
<td>Asymmetrical</td>
<td>–</td>
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<td>Levodopa response</td>
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<td>NAa</td>
<td>Good</td>
<td>NAa</td>
<td>Good</td>
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<tr>
<td>Response to dopaminergic agonists</td>
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<td>NA</td>
<td>Good</td>
<td>NAa</td>
<td>NAa</td>
<td>Good</td>
<td>Good motor response</td>
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<tr>
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<td>Normal</td>
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<tr>
<td>Lower limb reflexes</td>
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<td>NA</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

NA = not available; + = present; – = absent; ↓ = reduced; ↑ = increased; UPDRS = Unified Parkinson’s Disease Rating Scale.
a Treatment not received.
b Intolerance to dopaminergic agonists.
RNA tubes for total mRNA isolation using Tempus™ reagents according to manufacturer’s protocol in an ABI Prism™ 6100 Nucleic Acid PrepStation™ (Applied Biosystems, Foster City, CA, USA). Leukocyte-derived PINK1 cDNA was amplified using Superscript™ III and reverse transcriptase polymerase chain reaction (Invitrogen Life Technologies, Carlsbad, CA, USA). Primer pairs were designed along exons 1–2, 4–5, 6–8 and 7–8 to PCR amplified PINK1 cDNA fragments (Supplementary Table S1).

DNA and mRNA variants were numbered according to standard nomenclature (http://www.hgvs.org/mutnomen/, den Dunnen and Antonarakis, 2000) based on Ensembl accession #OTTHUMG00000002841 and #OTTHUMT00000007954, respectively. The virtual effect of variants on splicing was assessed using the Human Splicing Finder program (http://www.umd.be/SSF/).

**SPECT analysis**

Individuals III:3, IV:3, IV:5, IV:6, IV:9, IV:11 and V:2 underwent 123I-Ioflupane SPECT examination. SPECT acquisition protocol is displayed in the online supplementary data. Tracer-specific 123I-Ioflupane uptake binding ratio (123IUBRROI) was calculated over three consecutive transverse slices showing the most intense nigrostriatal dopamine transporter binding, using the following formula modified from Isaias and colleagues (2006):

\[
\text{AsymROI} = \frac{<\text{Frontal cortex ROI}_{123}\text{Ioflupaneuptake}>}{<\text{ROI}_{123}\text{Ioflupaneuptake}>}
\]

‘< ’ symbol denotes averaged value for three consecutive transverse slices in the different regions of interest (ROI) (whole striatum, putamen and caudate nucleus of each hemisphere) and frontal cortex. Right/left asymmetry ratio for different regions of interest (Asym) was calculated using the formula:

\[
\text{AsymROI} = \frac{\text{ROI}_{123}\text{Ioflupaneuptake} - \text{Frontal cortex}_{123}\text{Ioflupaneuptake}}{\text{Frontal cortex}_{123}\text{Ioflupaneuptake}}
\]

Normal values for Asym were set at 1.0 ± 0.05 (Kessler et al., 2005). Putamen-caudate uptake ratio (PC) was calculated using the formula (Isaias et al., 2006):

\[
\text{PC} = \frac{\text{ROI}_{123}\text{Ioflupaneuptake} - \text{Frontal cortex}_{123}\text{Ioflupaneuptake}}{\text{Frontal cortex}_{123}\text{Ioflupaneuptake}}
\]

**Neuropathological analysis**

The neuropathological study was carried out on selected brain sections, cerebellum and brainstem. Tissue samples were embedded in paraffin. Dewaxed sections, 5 μm thick, were stained with haematoxylin and eosin, and with Klüver-Barrera, or processed for immunohistochemistry following the EnVision + system peroxidase procedure (Dako, Barcelona, Spain). After incubation with methanol and normal serum, the sections were incubated with one of the primary antibodies at 4°C overnight. Antibodies to gial fibrillary acidic protein (Dako), β-amyloid (Boehringer, Barcelona, Spain) and ubiquitin (Dako) were used at dilutions of 1:250, 1:50, and 1:200, respectively. CD68 (Dako), as a marker of microglia, was diluted at 1:100. AT8 antibody (Innogenetics, Gent, Belgium) was used at a dilution of 1:50. Rabbit polyclonal anti-α-synuclein antibody (Chemicon, Barcelona, Spain) was used at a dilution of 1:3000 and mouse monoclonal anti-phosphorylated α-synuclein Ser129 antibody (Wako, Rafer, Barcelona, Spain) at a dilution of 1:2000. Rabbit polyclonal antibodies to TAR DNA-binding protein-43 C-terminal (Abcam, Cambridge, UK) and monoclonal TAR DNA-binding protein-43 antibodies (Abnova, Heidelberg, Germany) were used at dilutions of 1:1000 and 1:500, respectively. Sections processed for β-amyloid and α-synuclein immunohistochemistry were first pre-treated with formic acid, and incubated with methanol and hydrogen peroxide in phosphate buffered saline and normal serum. Following incubation with the primary antibody, sections were incubated with EnVision + system peroxidase for 15 min at room temperature. The peroxidase reaction was visualized with diaminobenzidine and hydrogen peroxide. Control of the immunostaining included omission of the primary antibody; no signal was obtained following incubation with only the secondary antibody. Sections were lightly counterstained with haematoxylin.

**Results**

A simplified pedigree of Family 476 is depicted in Fig. 1.

**Case reports**

**Individual III:2**

This patient was 68-year-old female who started showing rest tremor in both arms at age 34, which was more prominent in the left arm. At the age of 42 she developed postural instability and recurrent falls. She was given levodopa and selegiline with excellent motor response. Over the following years, she developed slowly progressive clumsiness in her arms and legs. When she was 59 she started to experience motor fluctuations and morning stiffness. At 60 she showed moderate asymmetric coreform peak-dose dyskinesias in her left leg and head 1 hour after having medication. Moreover, she experienced a ‘wearing off’ phenomenon 6 h after medication intake, described as leg clumsiness and slow gait. She reported difficulties in turning around while lying in bed. Currently, she is on levodopa (600 mg/day), rasagiline (1 mg/day) and amantadine (200 mg/day) with good clinical response and independence for daily activities.

**Individual III:3**

The 67-year-old sister of Individual III:2. At the age of 45, her walking became gradually slow and shuffling with a tendency to fall. One year later, she underwent brain surgery to remove a right frontal lobe meningioma. After surgery she showed transient mild left hemiparesis. At present, she wakes up with slight body stiffness that lasts for 1 h after she takes her first levodopa dose. She reports a slight ‘wearing off’ phenomenon that begins 3 h after medication intake, described as leg clumsiness and slow gait. She reported difficulties in turning around while lying in bed. Currently, she is on levodopa (400 mg/day) and amantadine (200 mg/day) with very good motor response.

**Individual IV:3**

This patient was a 40-year-old left-handed female who, at 31 years of age and pregnant, noticed slight, slowly progressive gait impairment. She stumbled because of left leg clumsiness. At the age of 34 she felt weakness in her left arm and had writing difficulties with micrographia. At that time she was given cabergoline (3 mg/day in two doses) with a good motor response. Four years later she noticed worsening of her walking. She felt clumsier and had more difficulties in moving both arms and legs. She had...
difficulty in turning when resting on the bed. She reported anxiety with obsessive trends. Cabergoline was replaced by ropinirole (9 mg/day in three doses) and rasagiline (1 mg/day), and maintained a good response. She showed end of dose fluctuations that manifested as tiredness. Neurological examination showed slight cogwheel rigidity in both arms and legs, which was more accentuated in the right limbs. She had no rest tremor but action tremor in her right arm was noticed. Moreover, she showed a diminished left side swing while walking. At present, she is on ropinirole (16 mg once a day) and rasagiline (1 mg/day) with maintained good motor response.

Individual IV:5
This patient was a 38-year-old male with no neurological complaints. Nevertheless, neurological examination showed slight impairment of tapping test in both hands and mild rigidity in both arms, which was more prominent in the right-hand side.

Individual IV:8
This patient was 31 years old when he complained of pain and rigidity in his left shoulder and arm. He was addicted to cocaine. Three years later he developed rigidity in his left leg. Neurological examination showed noteworthy right-left asymmetry with clumsiness in his left limbs, predominantly in his left leg. He also showed gait impairment and postural instability. He had an anxious personality trend. He did not tolerate treatment with selegiline and ropinirole. At 34 years of age he was treated with cabergoline (up to 8 mg/day), which improved his motor symptoms and anxiety but his gait problems remained. Moreover, he reported insomnia that was aggravated by cabergoline. At the age of 37, cabergoline treatment was replaced with levodopa (200 mg/day) and rasagiline (1 mg/day) with excellent motor response. However, he showed increasingly strange behaviour and began to isolate himself from his family and friends. After six years of illness he developed a severe psychosis with paranoid delirium against his own person. Two years later he died, at the age of 39, from non-natural causes.

Individual IV:11
A 36-year-old male whose first symptoms started at 27, complained of painless cramps in his left toes for 20 min, while he was walking. He had been addicted to cocaine in the past but had undergone successful detoxification therapy. Over the years he developed mild gait impairment due to slowness and shuffling in his legs, with greater involvement of the left leg. His gait and mood were markedly improved after he was given ropinirole (8 mg/day).

Individual V:2
This is the youngest person affected in Family 476. He is a 17-year-old male who started to experience gait disturbance when he was 14. He felt weakness and clumsiness in his left leg making him limp with a dystonic leg posture. He complained of nocturnal cervical rigidity. He was given 300 mg of levodopa daily, distributed in three doses, which resulted in an excellent response. Neurological examination on levodopa treatment showed impairment in finger tapping and in heel tapping on ground. His left hand and foot were clumsier than his right ones. Moreover, he showed bradykinesia in his left limbs with slight gait impairment. He had noteworthy arm swinging asymmetry and his right limbs showed a dystonic posture (right hand in flexion and extension of his right toes).

**Mutation analysis**

Sequencing of PINK1 in affected individuals revealed that 6 of them were carriers of both a novel splice-site mutation in the boundary of exon 7 (g.16378G > A; c.1488 + 1G > A; Fig. 2) and a deletion in exon 7 which extended from intron 6 to 7 (g.16089_16383del293; c.1252_1488del; Fig. 2). Two individuals were homozygous for the c.1488 + 1G > A mutation: Individual IV:3 who was affected and IV:6 who has no symptoms of disease to date (Fig. 1). No homozygous subjects for exon 7 deletion (c.1252_1488del) were found. We also found 18 asymptomatic heterozygous carriers for either exon 7 deletion (c.1252_1488del) (n = 10) or for the c.1488 + 1G > A mutation (n = 8).

The c.1488 + 1G > A variant modifies the +1U1-dependent 5’ splice site in exon 7 and disrupts the conserved consensus-site donor sequence for constitutive splicing at position g.16378 (Fig. 3). Bioinformatics analysis showed that the main effects of c.1488 + 1G > A on splicing were, first, that it decreases splicing likelihood from 95.95 to 69.12% at position g.16378. Second, c.1488 + 1G > A could activate two cryptic 5’ splice donor sites in exon 7 at position g.16093 (AGgig) and g.16344 (TGgig), with a likelihood of 91.69 and 90.18%, respectively (Fig. 3). As g.16093 cryptic 5’ splice donor site is located upstream from native exon 7 acceptor splice site (g.16141; gGT; likelihood = 86.91%), g.16083 (gGA) can be activated instead with a likelihood of 82.18% (Supplementary Fig. S1).

In order to demonstrate c.1488 + 1G > A and c.1252_1488del effects on splicing machinery, PINK1 mRNA from IV:3, IV:6, IV:11, IV:12, V:1 and V:2 individuals was analysed (Fig. 1). Forward primers for cDNA amplification of exons 6–8 and 7–8 located ahead of cryptic 5’ splice sites virtually activated by the c.1488 + 1G > A mutation were designed (Supplementary Table S1). cDNA analysis of a heterozygous c.1252_1488del carrier showed a transcript (r.1252_1488del) resulting from deletion of both the entire exon 7 sequence as well as its native acceptor (g.16141) and donor (g.16378) splice sites, resulting in a predicted in-frame protein lacking exon 7 amino acid (p.E417_R497del; Supplementary Fig. S2).

cDNA polymerase chain reaction amplification and sequencing of exons 7–8 in c.1488 + 1G > A homozygous carriers, confirmed the native splice-site inhibition and cryptic 5’ splice-site activation located at g.16344 position by generating a PINK1 mRNA with a 33 bp deletion (r.1456_1488del) as shown by the new 117 bp cDNA fragment instead of the expected wild-type 150 bp cDNA fragment (Fig. 3B and C). The resulting r.1456_1488del transcript maintains the reading frame with a predicted protein lacking 11 amino acids encoded by exon 7 (p.L485_R497del; Supplementary Fig. S2). Interestingly, cDNA analysis of exons 6–8 of c.1488 + 1G > A homozygous lead to an unexpected major transcript (r.1252_1488del) corresponding to a new 262 bp cDNA.
band lacking exon 7 sequence (Fig. 3B and C). Sequencing of the 262 bp band confirmed an in-frame transcript lacking exon 7 amino acids identical to the one generated by the c.1252_1488del mutation (p.E417_R497del; Supplementary Fig. S2). However, according to bioinformatics analysis a cDNA band expected from the cryptic 5' splice-site activation at g.16093 position was not observed (Supplementary Fig. S1).

### SPECT analysis

SPECT images of Individuals III:3, IV:3, IV:6, IV:9, IV:11 and V:2 showed a statistically significant reduction in striatal $^{123}$I-Ioflupane uptake (Fig. 4 and Table 2). $^{123}$I-Ioflupane uptake reduction and asymmetry indices for striatum, putamen and caudate varied among individuals. Putamen–caudate uptake ratios were reduced bilaterally in all subjects ranging from 0.35 to 0.70, indicating that the bulk tracer binding loss involved mainly the putamen. Individual V:2, with earliest age at onset, showed lower striatum $^{123}$I-Ioflupane uptake than Individuals IV:3 and IV:11 with later disease onset, suggesting greater striatum impairment in young subjects. Global dopaminergic dysfunction at basal ganglia measured by IUBR striatum did not correlate with disease duration (Table 2 and Fig. 5). However, taking into account disease duration of the individuals examined, a temporal gradient in areas with dopamine transporter binding reduction was identified (Table 2). The asymptomatic subject (IV:6) or subjects with short disease duration such as Individuals IV:5 and V:2 showed mild asymmetrical putaminal impairment whereas Individuals IV:3 and IV:11, with 9 years of disease duration, revealed a symmetrical pattern consisting on severe reduction of putaminal $^{123}$I-Ioflupane and relative caudate integrity signal. The subject with the longest disease duration (Individual III:3) showed severe reduction of putaminal uptake and asymmetrical caudate $^{123}$I-Ioflupane binding (Fig. 4 and Table 2).

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**Figure 2** PINK1 analysis of Family 476. (A) Left: fluorescent DNA chromatogram showing the c.1488 + 1G>A PINK1 mutation: AA = homozygous for the mutant allele, AG = heterozygous for the mutant allele, GG = homozygous for the wild-type allele. Right: Simplified PINK1 genomic sequence. U1 small nuclear ribonucleoprotein recognizes at 5'-AAG/(g/a)tggg-3' sequence between -3 and +6 positions. Exon 7 sequence is shown in capital letters and intron 7 sequence in lower letters. Asterisk denotes the intronic single substitution in PINK1 (c.1488 + 1G>A). Fw/Rv and Fw' /Rv' denote the position of the primers used for exon 7 and cDNA exon 7–8 polymerase chain reaction amplification, respectively. (B) Left: simplified PINK1 genomic sequence showing exon 7 deletion (c.1252_1488del; g.16089_16383del293). Fw/Rv and Fw'/Rv' denote the location of primers used for genomic exon 7 and cDNA exon 6–8 polymerase chain reaction amplification, respectively. Right: agarose gel electrophoresis showing a 123 bp DNA band in PINK1 exon 7 deletion heterozygous carriers (g.16089_16383del293; c.1252_1488del) and the 416-bp fragment observed in the wild-type individuals (GG) were obtained after polymerase chain reaction of genomic DNA with primers Fw/Rv.
The neuropathological examination of Individual IV:9 revealed neuronal loss of the substantia nigra pars compacta accompanied with astrocytic gliosis and moderate microgliosis, together with presence of neuromelanin in the neuropil and scattered macrophages. Semi-quantitative studies based on the neuronal counts of five non-consecutive sections of the substantia nigra at the level of the third nerve revealed an approximate loss of 50% of neurons in the lateral and medial tier of the substantia nigra pars compacta. A few remaining neurons contained Lewy bodies, as revealed with anti-ubiquitin and anti-α-synuclein antibodies. In contrast, no apparent cell loss was seen in the locus ceruleus and Lewy bodies and abnormal neurites were also absent in this nuclei. Lewy bodies and neurites were present, albeit in small numbers, in the reticular formation of the medulla oblongata and pons, covering the medialis, lateralis and gigantocellularis nuclei. Lewy bodies and neurites were also present in the basal nucleus of Meynert. The amygdala and the hippocampus, as well as the neocortex, were spared (Fig. 6). Parallel sections immunostained with anti-phosphorylated synuclein antibodies Ser129 disclosed a strong α-synuclein phosphorylation in Lewy bodies and aberrant neurites (Fig. 7).
Figure 4: $^{123}$I-Ioflupane SPECT transversal slices ordered feet to head from six family members showing the (A) left putamen hypointensity (Individual IV:6, 36 years old), (B) left putamen hypointensity (Individual IV:5, 38 years old), (C) right putamen hypointensity (Individual V:2, 18 years old), (D) bilateral putamen hypointensity and light reduction of $^{123}$I-Ioflupane uptake in caudate nucleus (Individual IV:9, 37 years old), (E) bilateral uptake reduction in putamen (Individual IV:3, 39 years old), (F) absence of signal in putamen and significant reduction of $^{123}$I-Ioflupane uptake in caudate (Individual IV:11, 36 years old) and (G) absence of signal in putamen and asymmetric uptake in caudate with greater impairment on right caudate (Individual III:3, 67 years old).
improvement and falls owing to lower limb awkwardness in most of the affected individuals (Table 1). Parkinsonism was characterized by predominant asymmetric rigid-akinetic syndrome with very slow progression over decades, as shown by lower motor Unified Parkinson’s Disease Rating Scale scores despite long disease duration (Table 1). Presence of gait disorder in idiopathic Parkinson’s disease is usually a late disease event often associated with motor deterioration and cognitive decline (Burn et al., 2006). However, the most frequent clinical characteristic of Family 476 was initial gait impairment without cognitive decline over decades. Predominant disease onset in lower limbs and greater gait impairment has been described to be more prevalent in PINK1 homozygous carriers than in both heterozygous PINK1 carriers and in idiopathic Parkinson’s disease (Marongiu et al., 2008), suggesting that early gait impairment could be very specific to PINK1-linked parkinsonism (Leutenegger et al., 2006; Marongiu et al., 2008). A high frequency of psychiatric disorders such as drug addiction (IV:9 and IV:11), psychosis (IV:9), anxiety and obsessive behaviour (IV:3) was observed in Family 476 (Table 1). Other studies have reported the presence of affective or schizophrenic disorders in 40–72% of PINK1 mutation carriers (Steinlechner et al., 2007; Marongiu et al., 2008), suggesting that psychiatric features are more frequently associated with PINK1 than with idiopathic Parkinson’s disease. Some individuals in Family 476 complained of mild rest and/or postural tremor, but often rest tremor was only observed by the physician. All affected individuals showed very good response to low doses of levodopa or to dopamine agonist treatment. A previous study showed that individuals with PINK1-linked parkinsonism more frequently develop dyskinesias (90%; n = 10) than PINK1 heterozygous Parkinson’s disease carriers (55%; n = 11) as well as non-mutated PINK1 Parkinson’s disease patients (47.2%; n = 320) (Marongiu et al., 2008). Similarly, Individuals III:2 and III:3 showed moderate levodopa-induced dyskinesias after 10 years of levodopa treatment that improved substantially with amantadine treatment. Whether the presence of late levodopa-induced dyskinesias responsive to amantadine is a phenotype specific to PINK1 should be investigated in other PINK1 families.

Although recent studies reported that PINK1-linked parkinsonism is associated with brisk or normal tendon reflexes, similar to Parkin carriers (Albanese et al., 2005; Ibanez et al., 2006), we observed that 62% of the affected members of Family 476 showed a reduction or abolition of tendon reflexes. However,

**Table 2** 

<table>
<thead>
<tr>
<th>Individual ID</th>
<th>IV:6</th>
<th>IV:5</th>
<th>V:2</th>
<th>IV:9</th>
<th>IV:3</th>
<th>IV:11</th>
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<td>6</td>
<td>9</td>
<td>9</td>
<td>22</td>
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<td>0.96</td>
<td>1.93</td>
<td>1.82</td>
<td>1.80</td>
</tr>
<tr>
<td>123IUBR, left striatum</td>
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<td>2.93</td>
<td>1.46</td>
<td>0.88</td>
<td>1.88</td>
<td>1.77</td>
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<td>1.04</td>
<td>1.11</td>
<td>0.92</td>
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<tr>
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<tr>
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<tr>
<td>PC, right</td>
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<td>0.59</td>
<td>0.36</td>
<td>0.38</td>
<td>0.49</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Asym = right/left asymmetry ratio; PC = putamen–caudate ratio; 123IUBR = 123I-Ioflupane binding ratio calculated in reference to frontal cortex. 

**Figure 5** 123I-Ioflupane uptake binding ratio (123IUBR) in striatum of Family 476. Individuals are ordered from left to right according to disease duration (in brackets); AAO = age at onset; y = years.

**Discussion**

We analysed a large Spanish family with levodopa-responsive early-onset parkinsonism characterized by predominant early gait impairment due to PINK1 c.1488 + 1G > A splicing mutation and exon 7 deletion. These two segregating PINK1 mutations were either in homozygous or heterozygous compound state in all of the affected family members. Neuropathology of one of the affected members (IV:9) showed neuronal loss, Lewy bodies and aberrant neurites in the substantia nigra pars compacta, and other nuclei, but the locus ceruleus and the amygdala were spared.

**Clinical features of Family 476**

The main clinical features observed in Family 476 were variable age at onset, between the second and fifth decade, with gait impairment and falls owing to lower limb awkwardness in most of the affected individuals (Table 1). Parkinsonism was characterized by predominant asymmetric rigid-akinetic syndrome with very slow progression over decades, as shown by lower motor Unified Parkinson’s Disease Rating Scale scores despite long disease duration (Table 1). Presence of gait disorder in idiopathic Parkinson’s disease is usually a late disease event often associated with motor deterioration and cognitive decline (Burn et al., 2006). However, the most frequent clinical characteristic of Family 476 was initial gait impairment without cognitive decline over decades. Predominant disease onset in lower limbs and greater gait impairment has been described to be more prevalent in PINK1 homozygous carriers than in both heterozygous PINK1 carriers and in idiopathic Parkinson’s disease (Marongiu et al., 2008), suggesting that early gait impairment could be very specific to PINK1-linked parkinsonism (Leutenegger et al., 2006; Marongiu et al., 2008). A high frequency of psychiatric disorders such as drug addiction (IV:9 and IV:11), psychosis (IV:9), anxiety and obsessive behaviour (IV:3) was observed in Family 476 (Table 1). Other studies have reported the presence of affective or schizophrenic disorders in 40–72% of PINK1 mutation carriers (Steinlechner et al., 2007; Marongiu et al., 2008), suggesting that psychiatric features are more frequently associated with PINK1 than with idiopathic Parkinson’s disease. Some individuals in Family 476 complained of mild rest and/or postural tremor, but often rest tremor was only observed by the physician. All affected individuals showed very good response to low doses of levodopa or to dopamine agonist treatment. A previous study showed that individuals with PINK1-linked parkinsonism more frequently develop dyskinesias (90%; n = 10) than PINK1 heterozygous Parkinson’s disease carriers (55%; n = 11) as well as non-mutated PINK1 Parkinson’s disease patients (47.2%; n = 320) (Marongiu et al., 2008). Similarly, Individuals III:2 and III:3 showed moderate levodopa-induced dyskinesias after 10 years of levodopa treatment that improved substantially with amantadine treatment. Whether the presence of late levodopa-induced dyskinesias responsive to amantadine is a phenotype specific to PINK1 should be investigated in other PINK1 families.

Although recent studies reported that PINK1-linked parkinsonism is associated with brisk or normal tendon reflexes, similar to Parkin carriers (Albanese et al., 2005; Ibanez et al., 2006), we observed that 62% of the affected members of Family 476 showed a reduction or abolition of tendon reflexes. However,
**Figure 6** α-Synuclein immunohistochemistry in Individual IV:9 showing (A) Lewy bodies and abnormal neurites in lateral reticular nucleus, (B and C) substantia nigra pars compacta, (D–F) Meynert nucleus, (G) reticular formation, nucleus gigantocellularis, (H) reticular formation, nucleus centralis and (I) reticular formation, nucleus lateralis. Magnification: ×400 (A–I).

**Figure 7** α-Synuclein-PSer129 immunohistochemistry in Individual IV:9 showing phosphorylated α-synuclein in (A) Lewy bodies and neuritis, (B) substantia nigra pars compacta, (C) lateral reticular nucleus, (D) medial reticular formation and (E and F) Meynert nucleus. Magnification: ×400 (A–F).
nerve conduction and H-reflex studies performed on Individuals IV:6, IV:11 and IV:12 were normal (data not shown), suggesting that further studies are needed to interpret the presence of hyporreflexia in some of the subjects with PINK1-linked parkinsonism.

Additionally, we also observed variable clinical expression in some affected individuals in Family 476. In fact, age at onset ranged from 14 to 45 years and the youngest affected started with levodopa-responsive leg dystonia. Intra-familial variability in age at onset has been previously reported in other familial early-onset Parkinson’s disease due to PINK1 and Parkin mutations (Lohmann et al., 2003; Ibanez et al., 2006). We found no clinical differences between PINK1 compound heterozygous individuals and the individual who was homozygous for c.1488 + 1G > A mutation (Table 1), suggesting a similar effect on PINK1 function for both mutations.

Currently, there is a discussion about how PINK1 heterozygous mutations could lead to a greater risk of developing Parkinson’s disease. It has been suggested that PINK1 haploinsufficiency may represent a factor of susceptibility toward parkinsonism (Valente et al., 2004b). Although SPECT data from heterozygous PINK1 carriers of the Family 476 were not available, neurological examination showed no clinical signs of Parkinson’s disease, suggesting that one PINK1 mutation is not sufficient to develop Parkinson’s disease. This fact may be due to different reasons: (i) these mutations would lead to phenotypic expression only when other genetic or environmental factors are present; (ii) the clinical manifestations that they cause are so slight that they are subclinical and can only be detected by functional neuroimaging techniques (Khan et al., 2002); or (iii) simply, they are not risk factors for Parkinson’s disease in heterozygous status and the presence of Parkinson’s disease in heterozygous PINK1 mutation carriers is owed to other genetic or environmental causes. The latter observation is in concordance with the normal neurological status of PINK1 heterozygous carriers reported by others (Ibanez et al., 2006; Ishihara-Paul et al., 2008; Savettieri et al., 2008). In fact, a study in which PINK1 gene was analysed in a series of 1126 patients with Parkinson’s disease and 400 controls, showed that PINK1 heterozygotes were slightly more frequent among the Parkinson’s disease group than controls (1.7% versus 1.0%; odds ratio = 1.62) suggesting that PINK1 heterozygous rare variants play only a minor role in Parkinson’s disease genetic susceptibility (Marongiu et al., 2008).

**PINK1 mutations associated with early-onset Parkinson’s disease**

c.1488 + 1G > A is the first PINK1 mutation located in the +1U1-dependent 5’ splice-site sequence of exon 7. Conserved sequence at the 5’ splice donor site and an AG sequence at the receptor 3’ splice site in mammalians are necessary for an efficient splicing process. Cryptic splice sites are hidden throughout the DNA sequence and silenced under normal conditions, but nucleotide substitutions at native 5’ splice site may decrease U1 small nuclear ribonucleoprotein affinity for DNA and thus activate cryptic splice sites (Nelson and Green, 1990). In Family 476, c.1488 + 1G > A disrupts native 5’ donor sequence of intron 7 of PINK1 gene and activates a cryptic 5’ donor sequence leading to an aberrant PINK1 transcript, as demonstrated in leukocyte-derived mRNA analysis from mutated individuals (Fig. 3). The subsequent 33 bp deletion at mRNA level (r.1456_1488del) disrupts part of the C-terminal PINK1 region where a crucial PINK1 serine–threonine kinase domain is located. Recently, a 23 bp micro deletion in exon 7 of PINK1 has been reported to induce multiple abnormal splicing transcripts as well as disruption of the native exon 7 splice acceptor site (Marongiu et al., 2007). The other major c.1488 + 1G > A transcript (r.1252_1488del) lacking exon 7 sequence was probably caused by steric inhibition of spliceosome owing to the short distance left between the cryptic 5’ splice acceptor (g.16083) and the cryptic 5’ splice donor site (g.16093). c.1488 + 1G > A could also lead to exon 7 skipping by an inefficient interaction of U1 small nuclear ribonucleoprotein by reducing base-pairing affinity of stem-loop at g.16378 and g.16093 donor sites thus promoting U1 small nuclear ribonucleoprotein recognition of the g.15789 site. In addition, g.16083 and g.16093 cryptic sites could still be functional, generating an out-of-frame transcript containing eleven intronic base pairs with a premature STOP codon (r.1251_1252ins1251 + 304_1252-47; p.V418DfsX427; Supplementary Fig. S2). The latter’s transcript could undergo a nonsense-mediated mRNA decay (Kuzmiak and Maquat, 2006).

Interestingly, we observed different intensity for the bands corresponding to r.1456_1488del transcript in c.1488 + 1G > A homozygous (Fig. 2), suggesting that other factors can influence the activation of certain cryptic splice sites in c.1488 + 1G > A mutation carriers. It would be a matter for further research to elucidate the causes favouring the differential expression of PINK1 mutated transcripts.

Thus, c.1488 + 1G > A PINK1 mutation behaves as the first pure 5’ splicing PINK1 mutation, since one of its main mutation effects is the activation of a cryptic 5’ donor site and exon 7 skipping (Fig. 3C). c.1252_1488del leads to exon 7 skipping as observed for the c.1488 + 1G > A mutation. The r.1456_1488del and r.1252_1488del transcripts generated by the c.1252_1488del and c.1488 + 1G > A mutations lead to in-frame shorter protein products that appear to be stable, suggesting that they do not undergo nonsense-mediated mRNA decay (Fig. 3; He et al., 1993).

PINK1 exon 7 deletion has recently also been described in early-onset Parkinson’s disease families (Camargos et al., 2009). Exon 7 codifies for the C-terminal region of PINK1 and also for the two last amino acids of the highly conserved serine–threonine kinase activation loop, which is not only involved in protein folding but also protects cells from stress-induced mitochondrial dysfunction (Valente et al., 2004a). At the protein level, c.1252_1488del and c.1488 + 1G > A mutations partly destroy the PINK1 C-terminus. Although, PINK1-deficient mice showed a Parkinson’s disease-like phenotype and reduction of preprotein mitochondrial transport correlating with defects in core mitochondrial functions like ATP-generation and respiration, no cytoplasmic aggregates of α-synuclein or nigrostriatal degeneration were observed (Gispert et al., 2009).
123I-Ioflupane SPECT scan of PINK1 mutation carriers

This is the second report on 123I-Ioflupane SPECT analysing dopaminergic terminal integrity in PINK1-linked parkinsonism. Major involvement of the dorsal striatum, compared with the caudate, was observed, as previously reported in another PINK1 family (Kessler et al., 2005). These results support the hypothesis that striatal degeneration in PINK1-linked parkinsonism follows a posterior-anterior gradient similar to idiopathic Parkinson’s disease. Considering disease duration, there was initial asymmetrical reduction of putaminal uptake, followed by absence of bilateral putaminal 123I-Ioflupane binding and, finally, asymmetrical impairment of the caudate nuclei. 123I-Ioflupane SPECT of Individual IV:6, who was a heterozygous compound PINK1 mutation carrier without Parkinson’s disease symptoms, showed an asymmetrical reduction of putaminal uptake, as seen in 123I-Ioflupane SPECT studies performed in early idiopathic Parkinson’s disease, suggesting an early impairment of nigrostriatal pathways projecting to the putamen (Tissingh et al., 1998; Booi and Knol, 2007). Similar 123I-Ioflupane binding ratios were found in Individuals IV:11 and III:3 after 9 and 22 years of disease, respectively, suggesting lack of correlation between striatal denervation and disease duration. Interestingly, Individual IV:9 showed a disproportionate striatal impairment after six years of disease, indicating that other environmental or genetic factors can modify intrafamilial expressivity of mutated PINK1 protein. Although it has been hypothesized that different PINK1 mutations can lead to different 123I-Ioflupane SPECT patterns (Kessler et al., 2005), we found no differences in patterns of dopaminergic activity between homozygous and compound heterozygous carriers, suggesting a similar biological effect of both mutations.

Neuropathology of PINK1-linked parkinsonism

No neuropathological reports of autosomal recessive PINK1-linked Parkinson’s disease have been reported so far. The presence of Lewy bodies in brainstem and neocortex and nigral neuronal cell loss was described in four brains from Parkinson’s disease with only one heterozygous PINK1 mutation (Gandhi et al., 2006). The authors found no neuropathological differences between heterozygous PINK1 mutation carriers and non-carriers. Additionally, available genetic studies of PINK1 families, including ours, do not support the notion that one mutated copy of PINK1 causes Parkinson’s disease. Therefore, it is likely that the heterozygous PINK1 mutations in those Parkinson’s disease brains (Gandhi et al., 2006) are not the sole cause of Parkinson’s disease and that the presence of heterozygous PINK1 mutations was coincidental. Yet the present case bearing a novel splice-site mutation due to a substitution in the +1U1-dependent 5’ splice site in exon 7 (c.1488+1G>A), as well as an exon 7 deletion (c.1252_1488del), showed loss of neurons in the substantia nigra pars compacta and α-synuclein inclusions (Lewy bodies and aberrant neurites) in selected nuclei of the brainstem, substantia nigra pars compacta and nucleus basalis of Meynert, thus supporting the idea that PINK1 mutations are causative of α-synucleinopathy. The distribution of Lewy body pathology was reminiscent of that seen in Braak stage 4 (Braak et al., 2002), but the lack of involvement of the locus ceruleus makes the neuropathology of this case ‘atypical’ regarding Braak staging of Parkinson’s disease-related pathology. However, atypical cases are not uncommon in cases with sporadic Parkinson’s disease (Jellinger, 2009). The neuropathology in the present case differs from that encountered in several inherited and apparently sporadic cases of Parkinson’s disease due to Parkin or LRRK2 mutations. All neuropathological studies of brains from individuals with Parkin-linked parkinsonism consistently report neuronal loss in substantia nigra and locus ceruleus whereas Lewy bodies have been found only in two individuals (Farrer et al., 2001; Pramstaller et al., 2005), suggesting that the presence of Lewy bodies is an extremely rare finding in Parkin-linked parkinsonism (Mori et al., 1998; Hayashi et al., 2000; Farrer et al., 2001; van de Warrenburg et al., 2001; Gouder-Khouja et al., 2003; Pramstaller et al., 2005). Additionally, some Parkinson’s disease cases bearing LRRK2 mutations in which Lewy body pathology is absent have been reported (Mizuno et al., 2001; Gouder-Khouja et al., 2003; Santperere and Ferrer, 2009). Whether those cases should be considered α-synucleinopathies is a matter for further discussion. In any case, the neuropathological study of Individual IV:9 provides an anatomical substrate that may sustain the presence of cardinal neurological deficits linked with long-lasting parkinsonism.

There are several mechanisms by which PINK1 mutated protein could be related to Lewy body formation. Mitochondrial impairment could induce aggregation of ubiquitin and α-synuclein (Sherer et al., 2002). Recent studies have shown that PINK1, Parkin and DJ-1 proteins form an E3 ubiquitin ligase complex that promotes proteasomal degradation of unfolded proteins (Xiong et al., 2009). Therefore, loss of PINK1 function may have consequences in the ubiquitin proteasome system by losing its capability of degrading aggregated proteins. In support of this hypothesis, PINK1 defects can impair proteasomal function and induce α-synuclein aggregation in cell cultures (Liu et al., 2009). Recent studies suggest a protective role of PINK1 in autophagy cascade resulting from mitochondrial dysfunction induced by oxidative stress (Cherra et al., 2009; Dagda et al., 2009). It has been suggested that in PINK1-dependent recruitment of Parkin to the mitochondria, damaged mitochondria are delivered to the perinuclear area and then degraded by autophagy. Parkin or PINK1 mutations may alter turnover of dysfunctional mitochondria and lead to neurodegeneration (Vives-Bauza et al., 2009).

Additionally, overexpression of PINK1 can rescue α-synuclein-induced phenotype in Drosophila, suggesting that wild-type PINK1 plays a protective role against toxic proteins such as α-synuclein (Sherer et al., 2002; Todd and Staveley, 2008; Tan et al., 2009). In fact, in vitro experiments showed that PINK1W437X increases mitochondrial dysfunction promoted by mutant α-synucleinAS, and suggests that PINK1 mutations can reinforce α-synuclein pathology (Marongiu et al., 2009). It is clear that additional work is needed to understand the relationship between mutant PINK1 and α-synuclein pathology in
Parkinson’s disease. Unfortunately, lack of frozen tissue has not permitted further molecular studies in the present case. It is noteworthy that the locus ceruleus, which is early-impaired in idiopathic Parkinson’s disease, was well preserved in the present case. We suggest that this finding may be related to certain clinical symptoms. Experimentally-induced parkinsonism in rodents has shown that animals with locus ceruleus impairment have worse motor performance than those in which the locus ceruleus is spared (Marien et al., 1993; Bing et al., 1994; Fornai et al., 1995). We hypothesize that preservation of the locus ceruleus in Individual IV:9 could be related to the slowly progressive parkinsonism observed in most of the affected members of the Family 476.

On the other hand, we wondered whether some of the neuropathological features of Individual IV:9 could explain the high frequency of psychiatric manifestations associated to PINK1-linked parkinsonism. Imbalance between dopaminergic, noradrenergic and serotonergic innervation has been suggested as a common denominator of certain psychiatric manifestations, including depression-related changes in parkinsonism (van Dongen, 1981; Wolters, 2001; Aston-Jones and Cohen, 2005; Papapetropoulos and Mash, 2005; Weiss et al., 2005). Norepinephrine imbalance at the locus ceruleus has also been involved in the aetiology of affective disorders (Baumann et al., 1999). We argue that an in-adapted norepinephrine balance in the locus ceruleus could be related to the psychiatric manifestations of some PINK1 mutation carriers. However, this hypothesis cannot be ascertained on the basis of a single case.

In summary, our findings suggest that PINK1-linked parkinsonism is related to Lewy body pathology with unique distribution. Although these results are based on a single neuropathological examination, they can help our understanding of Parkinson’s disease aetiology and open the way to develop new therapies targeted at non-dopaminergic neuronal populations.

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Supplementary material

Supplementary material is available at Brain online.

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