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# *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 (^{123}I-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

neuropathological report of the brain from an early-onset phosphatase and tensin homolog-induced putative kinase 1-linked parkinsonism showing that mutated *phosphatase and tensin homolog-induced putative kinase 1* protein induces Lewy body pathology. Unbalanced preservation of the locus ceruleus may well play a role in the slow evolution of motor symptoms and, probably, in the psychiatric symptoms often encountered in Parkinson's disease associated with *phosphatase and tensin homolog-induced putative kinase 1* mutation.

# 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.*, 2004*a*). *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 exonic 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.*, 2004*a*). 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.*, 2004*a*).

An ^{18}F-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, ^{123}I-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 ^{123}I-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.
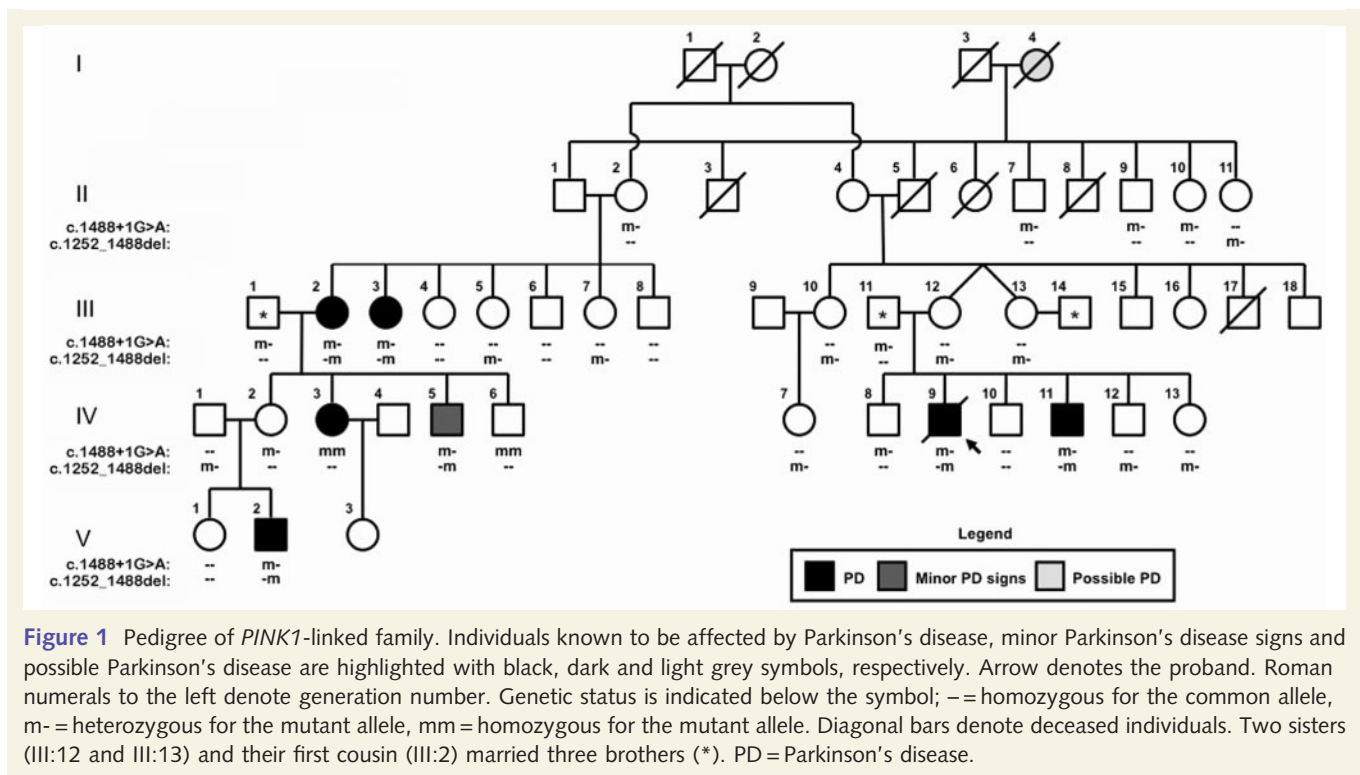
# Materials and methods

## 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.*, 2004*a*) and sequenced in both directions in affected and non-affected individuals on an ABI3130*xl* 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*[TM] vacuette blood

**Figure 1** Pedigree of *PINK1*-linked family. Individuals known to be affected by Parkinson's disease, minor Parkinson's disease signs and possible Parkinson's disease are highlighted with black, dark and light grey symbols, respectively. Arrow denotes the proband. Roman numerals to the left denote generation number. Genetic status is indicated below the symbol; − = homozygous for the common allele, m- = heterozygous for the mutant allele, mm = homozygous for the mutant allele. Diagonal bars denote deceased individuals. Two sisters (III:12 and III:13) and their first cousin (III:2) married three brothers (*). PD = Parkinson's disease.

**Table 1 Clinical features of affected family relatives with *PINK1* mutations**

| Individual identification | III:2 | III:3 | IV:3 | IV:5 | IV:6 | IV:9 | IV:11 | V:2 |
|---|---|---|---|---|---|---|---|---|
| Clinical status | Parkinson's disease | Parkinson's disease | Parkinson's disease | Minor Parkinson's disease signs | Asymptomatic | Parkinson's disease | Parkinson's disease | Parkinson's disease |
| Gender | Female | Female | Female | Male | Male | Male | Male | Male |
| Age, years | 68 | 67 | 40 | 38 | 36 | 39 | 36 | 17 |
| Disease onset, years | 34 | 45 | 31 | 38 | NA | 31 | 27 | 14 |
| Duration, years | 34 | 22 | 9 | 1 | NA | 8 | 9 | 3 |
| c.1488 + 1G > A | +/− | +/− | +/+ | +/− | +/+ | +/− | +/− | +/− |
| c.1252_1488del | −/+ | −/+ | −/− | −/+ | −/− | −/+ | −/+ | −/+ |
| Progression | Slow | Slow | Slow | Slow | NA | Moderate | Slow | Slow |
| Hoehn-Yahr Stage | 2.5 | 2 | 2 | 1 | – | 2.5 | 1 | 1 |
| UPDRS-III 'ON' scores | 17 | 18 | 19 | 5 | NA | 16 | NA | 6 |
| Rest tremor | + | − | − | − | − | + | NA | − |
| Postural tremor | + | − | + | − | − | + | NA | − |
| Bradykinesia | − | + | + | − | − | + | + | − |
| Rigidity | + | + | + | + | − | + | + | + |
| Postural instability | + | + | + | − | − | + | − | + |
| Dystonia at onset | − | − | − | − | − | − | + | + |
| Motor fluctuations | + | + | − | − | − | − | − | − |
| Dyskinesias | + | + | − | − | − | + | − | − |
| Psychosis | − | − | − | − | − | + | − | − |
| Anxiety | − | − | + | − | − | + | − | − |
| Motor phenotype at onset | Symmetrical | Asymmetrical | Asymmetrical | Asymmetrical | – | Asymmetrical | Asymmetrical | Asymmetrical |
| Levodopa response | Good | Good | Good | NA[a] | NA[a] | Good | NA | Good |
| Response to dopaminergic agonists | NA[b] | NA | Good | NA[a] | NA[a] | Good motor response | Good | NA |
| Upper limb reflexes | ↓ | ↓ | NA | ↓ | ↓ | ↓ | Normal | Normal |
| Lower limb reflexes | Abolished | ↓ | NA | ↓ | ↓ | ↓ | ↑ | Normal |

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*$^{TM}$ reagents according to manufacturer's protocol in an ABI Prism$^{TM}$ 6100 Nucleic Acid PrepStation$^{TM}$ (Applied Biosystems, Foster City, CA, USA). Leukocyte-derived *PINK1* cDNA was amplified using *Superscript*$^{TM}$ *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 $^{123}$I-Ioflupane SPECT examination. SPECT acquisition protocol is displayed in the online supplementary data. Tracer-specific $^{123}$I-Ioflupane uptake binding ratio ($^{123}$IUBR) 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):

$$^{123}\text{IUBR}_{ROI} = \frac{<\text{ROI}_{123}\text{Ioflupane uptake}> - <\text{Frontal cortex}_{123}\text{Ioflupane uptake}>}{<\text{Frontal cortex}_{123}\text{Ioflupane uptake}>}$$

'$<>$' 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:

$$\text{Asym}_{ROI} = \frac{\text{Right}\_^{123}\text{IUBR}_{ROI}}{\text{Left}\_^{123}\text{IUBR}_{ROI}}$$

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

$$\text{PC} = \frac{^{123}\text{IUBR}_{putamen}}{^{123}\text{IUBR}_{caudate}}$$

## 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 glial 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 coreiform 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 with clumsiness in her legs. Moreover, she has moderate peak-dose dyskinesias. At present, she takes 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:9

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).
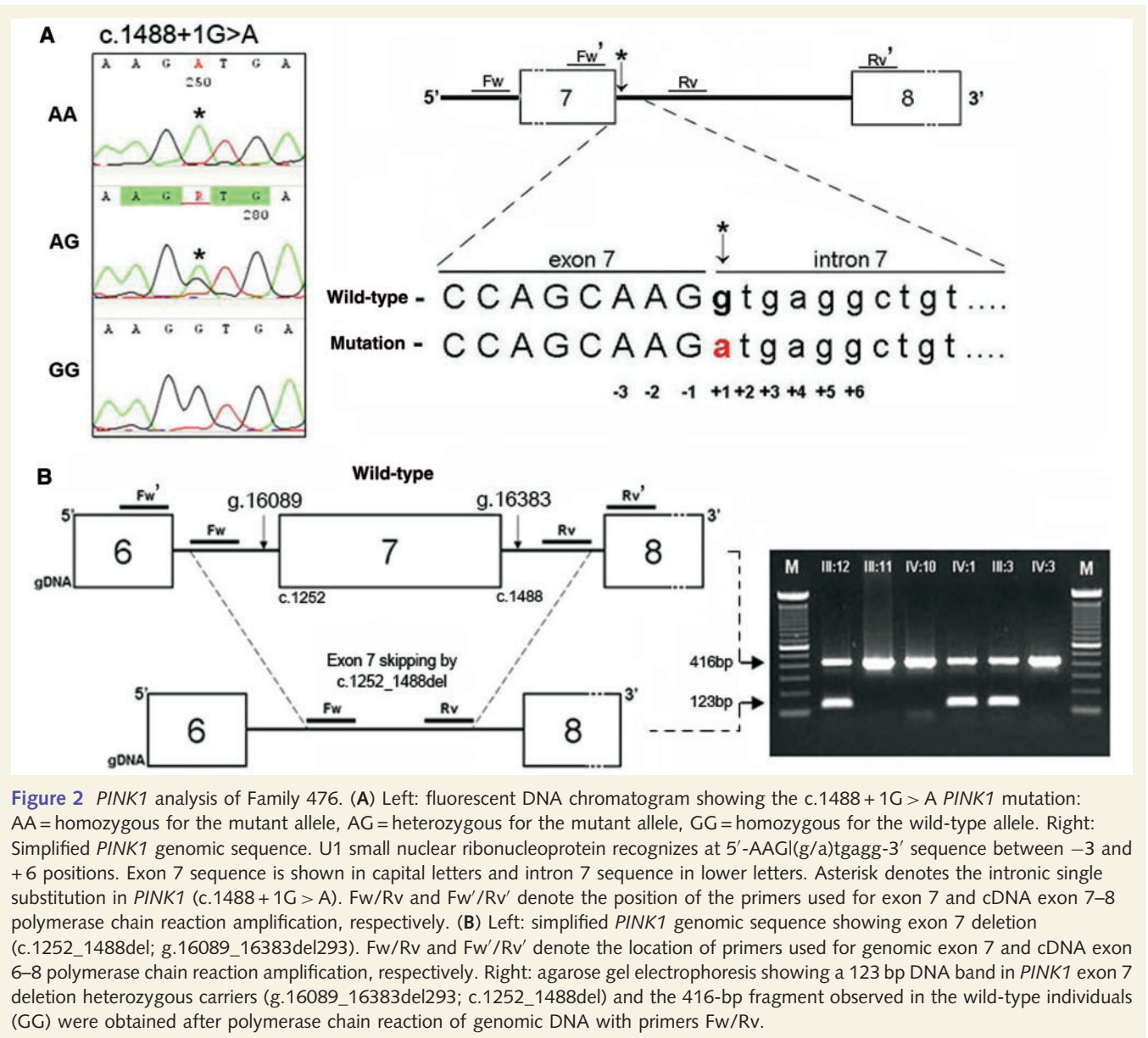
## Mutation analysis

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 (AG|g) and g.16344 (TG|g), 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; g|GT; likelihood = 86.91%), g.16083 (g|GA) 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

**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′-AAGI(g/a)tgagg-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.

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:5, 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).
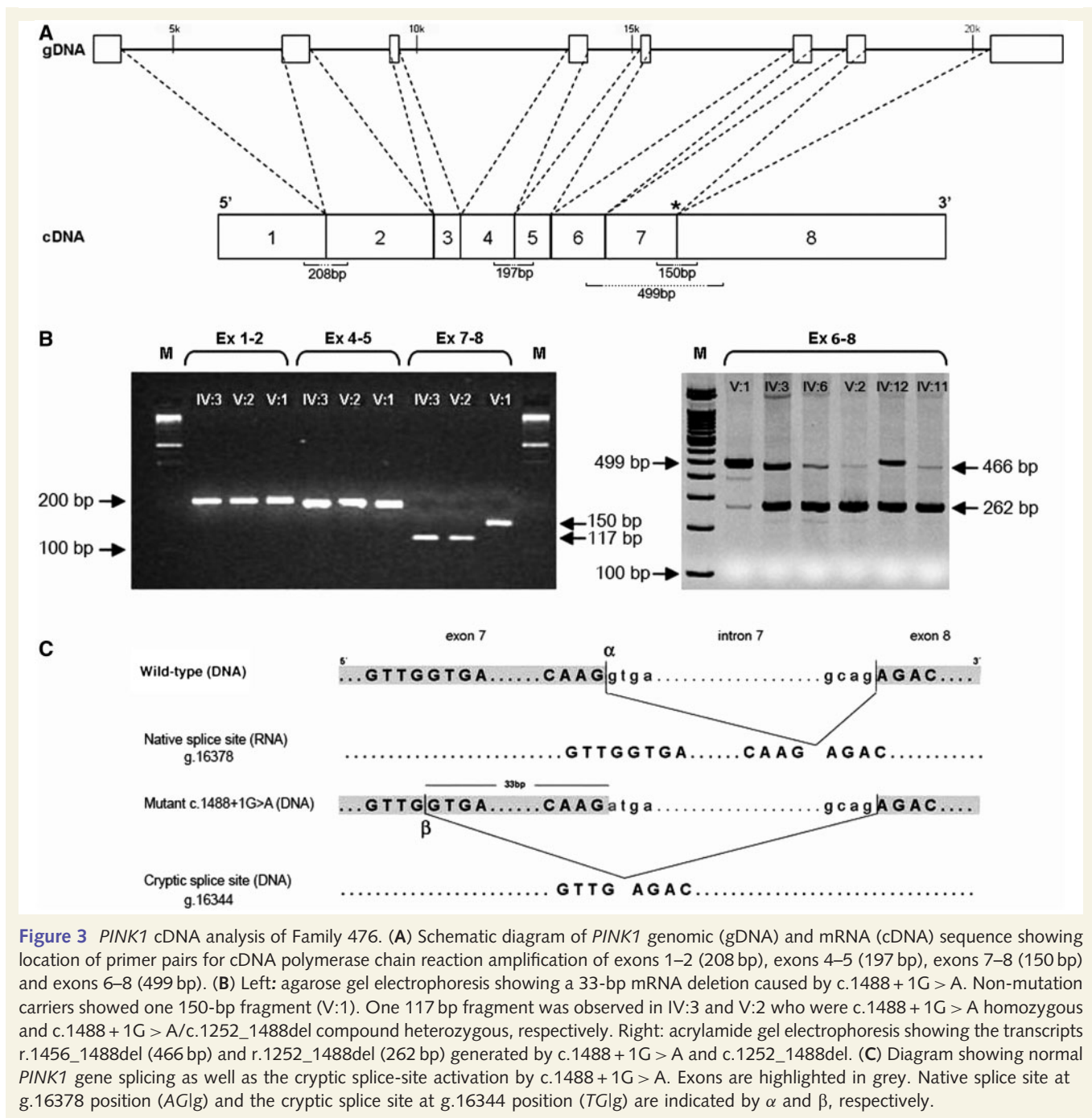
**Figure 3** *PINK1* cDNA analysis of Family 476. (**A**) Schematic diagram of *PINK1* genomic (gDNA) and mRNA (cDNA) sequence showing location of primer pairs for cDNA polymerase chain reaction amplification of exons 1–2 (208 bp), exons 4–5 (197 bp), exons 7–8 (150 bp) and exons 6–8 (499 bp). (**B**) Left**:** agarose gel electrophoresis showing a 33-bp mRNA deletion caused by c.1488 + 1G > A. Non-mutation carriers showed one 150-bp fragment (V:1). One 117 bp fragment was observed in IV:3 and V:2 who were c.1488 + 1G > A homozygous and c.1488 + 1G > A/c.1252_1488del compound heterozygous, respectively. Right: acrylamide gel electrophoresis showing the transcripts r.1456_1488del (466 bp) and r.1252_1488del (262 bp) generated by c.1488 + 1G > A and c.1252_1488del. (**C**) Diagram showing normal *PINK1* gene splicing as well as the cryptic splice-site activation by c.1488 + 1G > A. Exons are highlighted in grey. Native splice site at g.16378 position (AG|g) and the cryptic splice site at g.16344 position (TG|g) are indicated by α and β, respectively.

# Neuropathological study

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 abnormal 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).
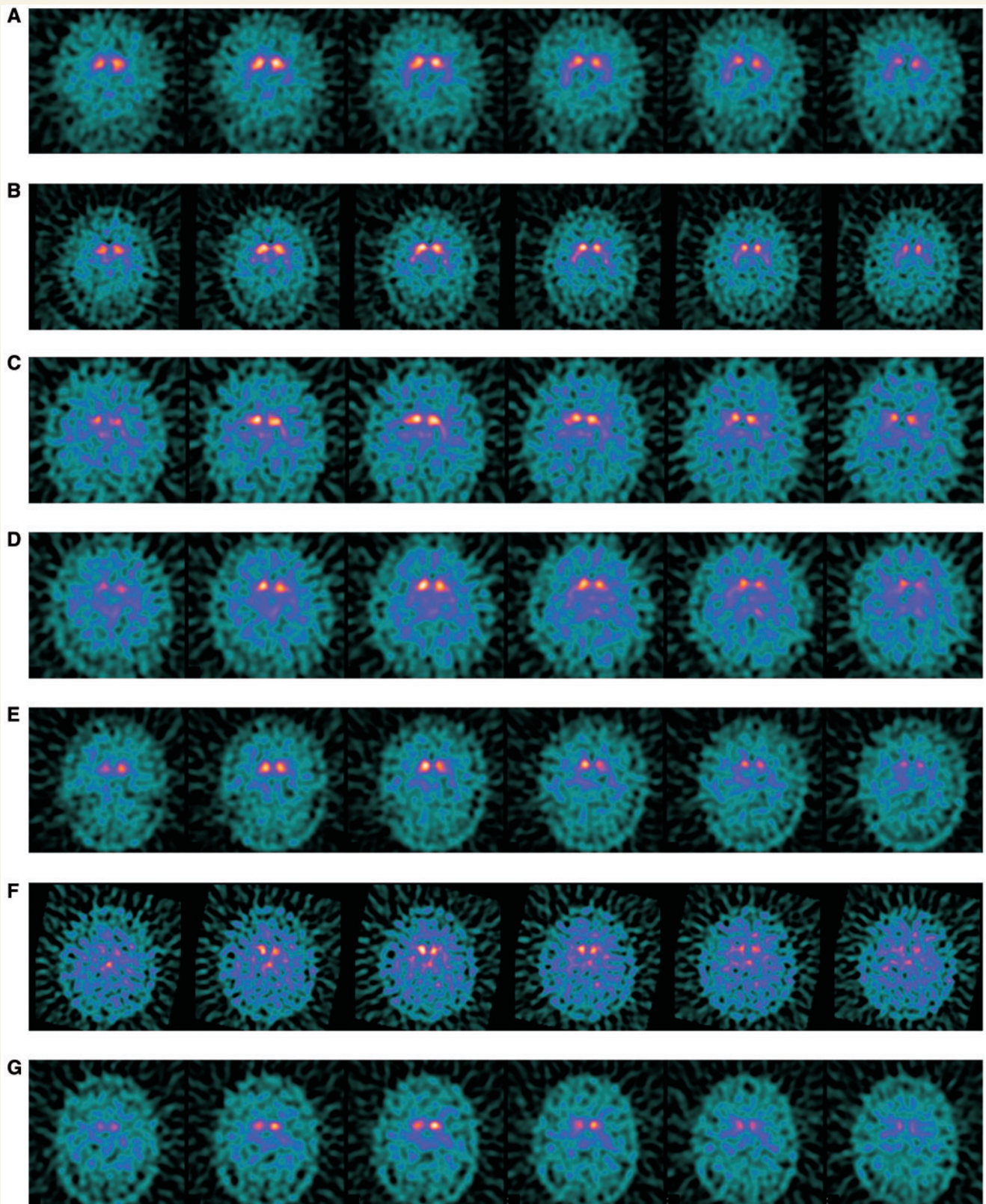
**Figure 4** $^{123}$I-Ioflupane SPECT transversal slices ordered feet to head from six family members showing the (**A**) left putamen hypointensity (Individual IV:6, 36 years old), (**B**) left putamen hypointensity (Individual IV:5, 38 years old), (**C**) right putamen hypointensity (Individual V:2, 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).

**Table 2** $^{123}$I-Ioflupane binding ratios in striatum in seven *PINK1*–linked parkinsonism subjects

| Individual ID | IV:6 | IV:5 | V:2 | IV:9 | IV:3 | IV:11 | III:3 |
|---|---|---|---|---|---|---|---|
| Disease duration[a] | 0 | <1 | 3 | 6 | 9 | 9 | 22 |
| $^{123}$IUBR, right striatum | 3.01 | 3.23 | 1.12 | 0.96 | 1.93 | 1.82 | 1.80 |
| $^{123}$IUBR, left striatum | 3.19 | 2.93 | 1.46 | 0.88 | 1.88 | 1.77 | 2.10 |
| Asym, putamen | 1.03 | 1.18 | 0.75 | 1.04 | 1.11 | 0.92 | 1.03 |
| Asym, caudate | 0.98 | 1.00 | 0.88 | 1.08 | 1.05 | 1.03 | 0.80 |
| Asym, whole striatum | 1.01 | 1.10 | 0.77 | 1.09 | 1.03 | 1.03 | 0.86 |
| PC, right | 0.64 | 0.70 | 0.51 | 0.35 | 0.37 | 0.43 | 0.58 |
| PC, left | 0.60 | 0.60 | 0.59 | 0.36 | 0.38 | 0.49 | 0.45 |

Asym = right/left asymmetry ratio; PC = putamen–caudate ratio; $^{123}$IUBR = $^{123}$I-Ioflupane binding ratio calculated in reference to frontal cortex.
a Related to the moment at SPECT acquisition expressed in years.
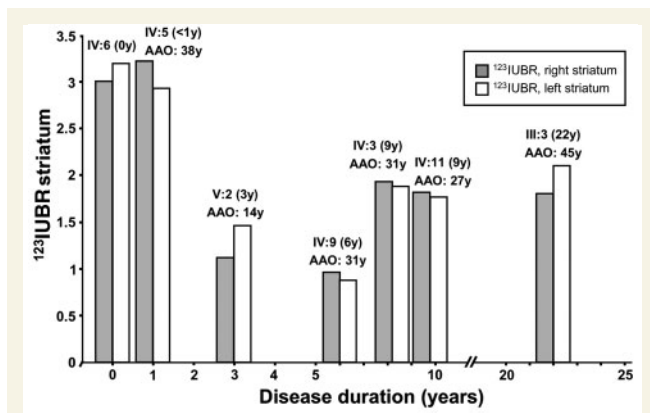


**Figure 5** $^{123}$I-Ioflupane uptake binding ratio ($^{123}$IUBR) in striatum of Family 476. Individuals are ordered from left to right according to disease duration (in brackets); AAO = age at onset; y = years.

No other alterations were noticed. β-amyloid plaques and hyper-phosphorylated tau inclusions were absent. No abnormalities were seen with anti-TAR DNA-binding protein-43 antibodies in any region.

# Discussion

We analysed a large Spanish family with levodopa-responsive early-onset parkinsonism characterized by predominant early gait impartment 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,
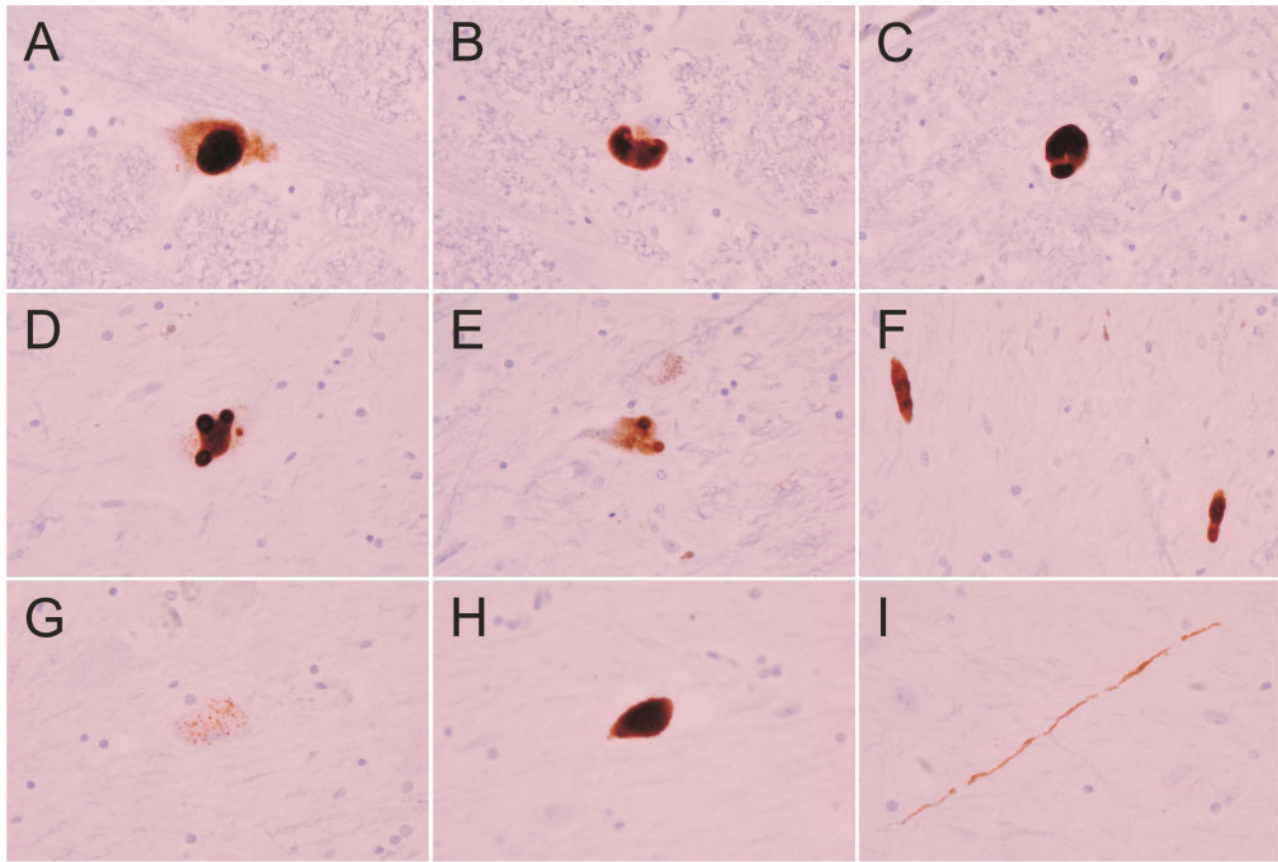
**Figure 6** α-Synuclein immunohistochemistry in Individual IV:9 showing (**A**) Lewy bodies and abnormal neurites in lateral reticular nucleus, (**B** and **C**) substantia nigra pars compacta, (**D–F**) Meynert nucleus, (**G**) reticular formation, nucleus gigantocellularis, (**H**) reticular formation, nucleus centralis and (**I**) reticular formation, nucleus lateralis. Magnification: ×400 (**A–I**).
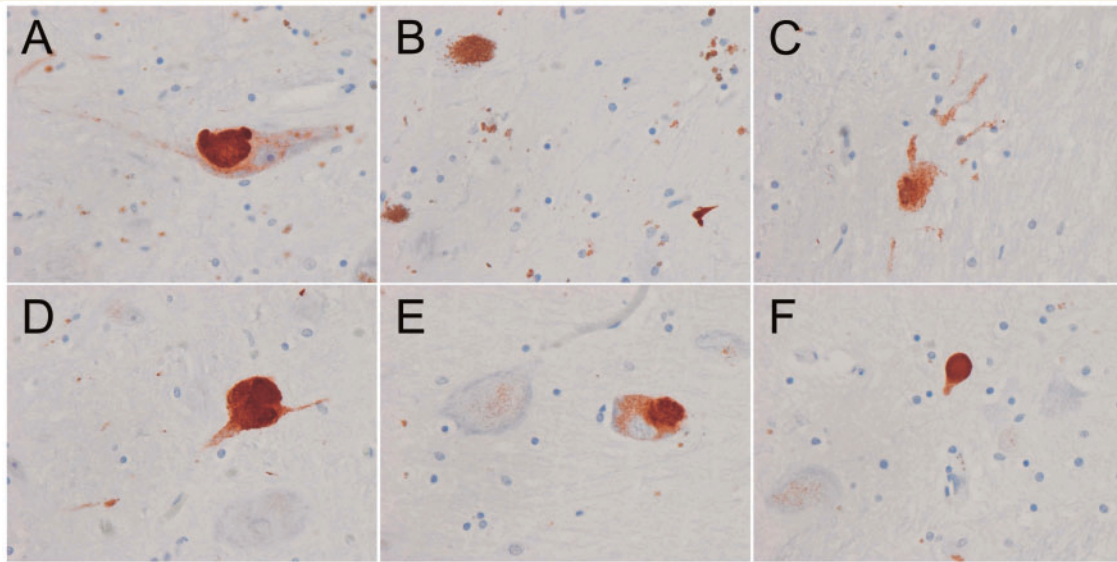


**Figure 7** α-Synuclein-PSer129 immunohistochemistry in Individual IV:9 showing phosphorylated α-synuclein in (**A**) Lewy bodies and neuritis, (**B**) substantia nigra pars compacta, (**C**) lateral reticular nucleus, (**D**) medial reticular formation and (**E** and **F**) Meynert nucleus. Magnification: ×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.*, 2004*b*). 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.V418D*fs*X427; 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.*, 2004*a*). 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).

## <sup>123</sup>I-Ioflupane SPECT scan of PINK1 mutation carriers

This is the second report on $^{123}$I-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 $^{123}$I-Ioflupane binding and, finally, asymmetrical impairment of the caudate nuclei. $^{123}$I-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 $^{123}$I-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; Booij and Knol, 2007). Similar $^{123}$I-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 $^{123}$I-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; Gouider-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; Gouider-Khouja *et al.*, 2003; Santpere 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 PINK1$^{W437X}$ increases mitochondrial dysfunction promoted by mutant α-synuclein$^{A53T}$ 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 serotoninergic 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 inadequate 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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