DOI: 10.1093/brain/awg136 Advanced Access publication April 8, 2003 *Brain* (2003), **126,** 1271–1278

Parkin mutations are frequent in patients with isolated early-onset parkinsonism

Magali Periquet,[1] Morwena Latouche,[1] Ebba Lohmann,[1] Nina Rawal,[1] Giuseppe De Michele,[7] Sylvain Ricard,[6] Hélio Teive,[8] Valérie Fraix,[5] Marie Vidailhet,[1,4] David Nicholl,[10] Paolo Barone,[7] Nick W. Wood,[11] Salmo Raskin,[9] Jean-François Deleuze,[6] Yves Agid,[1,3] Alexandra Dürr,[1,2,3] Alexis Brice,[1,2,3] the French Parkinson's Disease Genetics Study Group* and the European Consortium on Genetic Susceptibility in Parkinson's Disease**

[1]*INSERM U289,* [2]*Département de Génétique, Cytogénétique et Embryologie and* [3]*Fédération de Neurologie, Hôpital de la Salpêtrière and* [4]*Service de Neurologie, Hôpital Saint Antoine, Paris,* [5]*Service de Neurologie, CHU de Grenoble, Grenoble, and* [6]*Biotechnology Department, Aventis Pharma, Vitry sur Seine, France,* [7]*Dipartimento di Scienze Neurologiche, Università Federico II, Napoli, Italy,* [8]*Departamento de Neurologia, Hospital de Clinicas and* [9]*Laboratorio Genetika, Curitiba Parana, Brazil,* [10]*Department of Clinical Neurology, Queen Elizabeth Hospital, Birmingham, and* [11]*Institute of Neurology, London, UK*

Correspondence to: Alexis Brice, INSERM U289, Hôpital de la Salpêtrière, 47, Boulevard de l'Hôpital, 75651 Paris cedex 13, France
E-mail: brice@ccr.jussieu.fr

**Y. Agid, A.-M. Bonnet, M. Borg, A. Brice, E. Broussolle, Ph. Damier, A. Destée, A. Dürr, F. Durif, J. Feingold, G. Fénelon, E. Lohmann, M. Martinez, C. Penet, P. Pollak, O. Rascol, F. Tison, C. Tranchant, M. Vérin, F. Viallet, M. Vidailhet and J.-M. Warter*
***N.W. Wood and D. Nicholl (UK); A. Brice, A. Dürr, M. Martinez and Y. Agid (France); T. Gasser and B. Müller-Myhsok (Germany); M. Breteler, S. Harhangi and B. Oostra (The Netherlands); V. Bonifati, N. Vanacore, G. De Michele, E. Fabrizio, A. Filla and G. Meco (Italy)*

Summary

***Parkin* gene mutations are reported to be a major cause of early-onset parkinsonism (age at onset ⩽45 years) in families with autosomal recessive inheritance and in isolated juvenile-onset parkinsonism (age at onset <20 years). However, the precise frequency of *parkin* mutations in isolated cases is not known. In order to evaluate the frequency of *parkin* mutations in patients with isolated early-onset parkinsonism according to their age at onset, we studied 146 patients of various geographical origin with an age at onset ⩽45 years. All were screened for mutations in the *parkin* gene using semi-quantitative polymerase chain reaction combined with sequencing of the entire coding region. We identified *parkin* mutations in 20 patients including three new exon rearrangements and two new missense mutations. These results, taken in conjunction with those of our previous study (Lücking *et al.,* 2000) show that *parkin* mutations account for at least 15% (38 out of 246) of our early-onset cases without family history, but that the proportion decreases significantly with increasing age at onset. There were no clinical group differences between *parkin* cases and other patients with early-onset parkinsonism. However, a single case presenting with cerebellar ataxia several years before typical parkinsonism extends the spectrum of *parkin* related-disease.**

Keywords: parkin; mutation frequency; isolated early-onset parkinsonism

Abbreviations: PCR = polymerase chain reaction

Introduction

Parkinson's disease is a common neurodegenerative disorder, with a prevalence of nearly 2% after age 65 years (Elbaz *et al.*, 1999). Clinically, it is characterized by resting tremor, rigidity and bradykinesia—a triad of neurological symptoms caused by the progressive and selective degeneration of dopaminergic neurons in the substantia nigra pars compacta

(Lang and Lozano, 1998). Lewy bodies—neuronal cytoplasmic inclusions containing aggregated ubiquitinated proteins—are a pathological hallmark of the disease.

The aetiology of idiopathic Parkinson's disease remains obscure, but new insights into the molecular mechanisms of its pathogenesis have recently been provided by the discovery of several families with clearly established monogenic inheritance (Lansbury and Brice, 2002). Autosomal dominant forms of the disease are extremely rare. Missense mutations in the α-synuclein gene (A30P, A53T) have been described in a subset of Parkinson's disease families with autosomal dominant transmission (Polymeropoulos *et al*., 1997; Krüger *et al*., 1998). Despite the relative rarity of this gene in familial Parkinson's disease, the observation that normal α-synuclein is a major component of Lewy bodies indicates a more general role of this protein in the pathogenesis of sporadic Parkinson's disease (Spillantini *et al*., 1998). A point mutation (I93M) in the ubiquitin carboxyterminal hydrolase L1 (UCHL-1) has been found in a family with autosomal dominant Parkinson's disease (Leroy *et al*., 1998), but its role in the pathogenesis of the disease remains uncertain as it has not been found in other familial or sporadic cases with Parkinson's disease. Autosomal dominant parkinsonism has also been linked to three other loci: PARK3 on chromosome 2p13 (Gasser *et al*., 1998); PARK 4 on chromosome 4p14–16.3 (Farrer *et al*., 1999); and PARK8 on chromosome 12p11.2-q13.1 (Funayama *et al*., 2002). In families with autosomal recessive inheritance, one locus, PARK6, and one gene PARK7 or DJ-1, have recently been identified both on chromosome 1p35–36 (Valente *et al*., 2001; van Duijn *et al*., 2001; Bonifati *et al*., 2002)—but to date, the majority of such cases are caused by mutations in the *parkin* gene (PARK2), which result in autosomal recessive early-onset parkinsonism (Kitada *et al*., 1998; Hattori et al., 1998*a*; Lücking *et al*., 1998, 2000; Abbas *et al*., 1999). The phenotype associated with *parkin* gene mutations is variable, but is usually characterized by early-onset parkinsonism (mean age at onset around 30 years) and slow disease progression (Ishikawa and Tsuji, 1996; Lücking *et al*., 2000). Several *post mortem* brain studies have shown that *parkin* patients do not have Lewy bodies (Mori *et al*., 1998; van de Warrenburg *et al*., 2001), except for one case with a particular *parkin* mutation (Farrer *et al*., 2001). Lewy bodies are ubiquitinated cytoplasmic inclusions consisting of aggregates containing a number of proteins, including α-synuclein. Parkin is an E3 ubiquitin-protein ligase (Shimura *et al*., 2000) that ubiquitinates specific substrates that are targeted for their degradation through the ubiquitin–proteasome pathway (Joazeiro and Weissman, 2000). Interestingly, one of its recently identified substrates is a glycosylated form of α-synuclein, establishing a direct link between parkin and idiopathic Parkinson's disease (Shimura *et al*., 2001), although the role of this minor form of α-synuclein has not been clarified.

The clinical presentation of *parkin* patients is highly variable with an age at onset that ranges from 7 to 72 years (Klein *et al*., 2000; Lücking *et al*., 2000; Nichols *et al*., 2002). The mutations in the *parkin* gene are also extremely varied and include many different point mutations and exon rearrangements affecting all 12 of the coding exons (Kitada *et al*., 1998; Abbas *et al*., 1999; Lücking *et al*., 2000; Maruyama *et al*., 2000; Klein *et al*., 2000; Periquet *et al*., 2001; Hedrich *et al*., 2002; West *et al*., 2002). The frequency of these mutations in Europe was estimated at 50% in families with early onset parkinsonism with potentially recessive inheritance and 18% in patients with isolated early-onset parkinsonism with onset prior to 45 years (Lücking *et al*., 2000).

In order to evaluate more accurately the frequency of *parkin* mutations in patients with isolated early onset parkinsonism, we studied 146 patients selected with an age at onset ⩽45 years. All subjects were screened for mutations in the *parkin* gene with the use of semi-quantitative polymerase chain reaction (PCR) combined with the sequencing of the entire coding region.

Subjects and methods

Patients and families

One hundred and forty-six isolated patients with early-onset parkinsonism were selected according to the following criteria: (i) parkinsonism, defined by at least two of the following signs: akinesia, rigidity or rest tremor, and a good response to levodopa treatment (>30% of improvement); (ii) no first degree family history of Parkinson's disease; and (iii) age at onset ⩽45 years. There were eight cases with known consanguinity. The majority of patients were from France ($n = 92$). The reminder were from Italy ($n = 13$), Brazil ($n = 15$), Algeria ($n = 13$), United Kingdom ($n = 6$), Spain ($n = 3$), Turkey ($n = 2$), Portugal ($n = 1$) and Israel ($n = 1$).

Molecular analysis

Blood samples were taken with written informed consent from all the 146 patients and genomic DNA was extracted from peripheral blood leukocytes using standard procedures. The patients were screened for *parkin* mutations with the use of the semi-quantitative PCR assay established in our laboratory (Lücking *et al*., 2000). Briefly, exons 2 to 12 are amplified by PCR simultaneously, associated in groups defined by the amplification conditions: group 1 includes exons 4, 7, 8 and 11; group 2 includes exons 5, 6, 8 and 10; and group 3 includes exons 2, 3, 9 and 12, and an external control, the transthyretin gene. PCR conditions were set up so that amplification was exponential for all of the exons that are co-amplified. The DNA from a patient known to have a heterozygous deletion of exons 8 and 9 was always processed in parallel as an internal control. To identify the mutations, the PCR products were analysed on denaturing polyacrylamide gels on a ABI 377 automated sequencer with GENESCAN 3.1 and GENOTYPER 1.1.1 softwares (all from Applied Biosystems, Foster City, CA, USA). The ratios

Table 1 *Mutations detected in isolated patients with early-onset parkinsonism*

Patient	Age at onset (years)	Country of origin	Dosage exon 2–12	Sequence exon 1–12
SPD-145-012	12	France	Ex 3 het del	Arg275Trp het
SPD-134-10[a]	30	France	**Ex 2-4het dupl**/Ex3 het del	ND
SPD-146-005	35	France	Ex 3-4 het del	Arg275Trp het
SPD-164-10	27	France	Ex 6 het del	Normal
SPD-169-003[b]	29	France	Ex 2 het del/Ex3 het del	Normal
SPD-188-008	39	France	Normal	Arg256Cys het
JMP28	28	Italy	Ex 3 hom del	ND
BHAM 18[c]	18	UK	Ex 3,4,5 het del	Normal
SPD-099-003	28	Italy	Ex 6 het dupl	Normal
SPD-100-003	40	France	Normal	Arg275Trp het
SPD-110-001[a]	34	Algeria	Ex 3-4 het del	Normal
SPD-112-001	36	France	**Ex 10 het del**	Normal
SPD-123-007	30	France	Ex 3 het dupl	Normal
SPD-166-010[b]	45	France	Ex 2 het del/Ex 3-4 het del	Normal
SPD-181-009	27	France	Ex 4 het del	Arg275Trp het
SPD-187-003[c]	29	France	Ex 3,4 het dupl	Normal
SPD-225-008	38	France	Normal	Arg275Trp het
SPD-229-003	45	Algeria	Normal	**Ala398Thr het**
SPD-245-001[c]	43	Brazil	**Ex 2,**3 **het dupl**	Normal
JMP 37	7	Italy	Ex 2-4 het del	**Thr240Met het**

Bold characters indicate new mutations. [a]The phase of transmission was deduced by co-segregation analysis. [b]The phase of transmission was deduced by RT–PCR analysis. [c]The phase of transmission is not known for these patients who may be compound heterozygotes. het = heterozygous; hom = homozygous; del = deletion; dupl = duplication; ND = not determined.

between the heights of the peaks corresponding to each of the exons amplified in a given reaction were calculated and then compared with the ratios measured with the non-rearranged exons in the control sample from a normal subject. For a detailed description of this method, see Lücking and Brice (2002).

In the patients in whom the dosage technique detected only one or no mutations, the entire coding sequence was analysed by sequencing as described previously (Abbas *et al*., 1999). Briefly, the 12 exons and the intron–exon boundaries of the *parkin* gene were amplified and sequenced on both strands using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), according to the manufacturer's recommendations, on an ABI 377 automated sequencer with Sequence Analysis 3.0 software (Applied Biosytems).

In addition, 162 European and 68 North African control chromosomes were analysed by restriction assay for the new point mutation Ala398Thr. Primers were chosen to create a restriction site for *Bal*I enzyme on the mutant allele giving a product of 207 bp instead of 228 bp in the wild-type control. The restriction products were analysed by electrophoresis on 2.5% agarose gel. For the new Thr240Met mutation, 106 European control chromosomes were analysed by direct sequencing of exon 6.

For two patients (SPD-169–03 and SPD-166–10), total RNA was isolated from lymphoblastoid cell lines and cDNA was prepared using the Super script II reverse transcriptase–polymerase chain reaction (RT–PCR) system (Invitrogen, Glasgow, UK) in order to determine the phase of transmission. A first-round PCR was performed with the forward and reverse primers Ex1iFor (5′-CGCGCATGGGCCTGTTCCT-3′) and Ex9iRev (5′-CCATACTGCTGGTACCGGTTG-3). A second-round PCR was performed with primers Ex1iForNes (5′-CAGCCGCCACCTACCCAGT-3′) and Ex5iRev (5′-GATTGGCATTCACCACTCATCC-3′). The PCR products were sequenced directly using the second-round PCR primers and a Big Dye Terminator Cycle Sequencing Ready Reaction DNA Sequencing Kit (Applied Biosystems).

The five patients with the Arg275Trp mutation were genotyped for marker D6S305. The genotyping was performed by PCR using the primers specified in the Genome Database (GDB). The primers were labelled fluorescently and PCR products were analysed on an ABI377 automated sequencer with GENESCAN 3.1 and GENOTYPER 1.1.1 software.

Statistical analysis

Raw data for means and for proportions were compared with Student's *t*-test and the χ^2 test or the Fisher exact test, respectively.

Results

Frequency of mutations in the parkin *gene*

Among the 146 patients with early-onset parkinsonism but without family history, 20 (14%) had mutations in the *parkin* gene (Table 1). None of the eight patients with consanguinity carried a *parkin* mutation. Two mutations of the *parkin* gene

Table 2 *Frequency of parkin gene mutations in patients with isolated early-onset parkinsonism, by age at onset*

Age at onset (years)	Patients with *parkin* mutations	Patients without *parkin* mutation	Total*	Frequency (%)	95% CI
<20	10	5	15	67	38–88
20–24	4	11	15	27	8–55
25–29	9	29	38	24	11–40
30–34	4	49	53	8	2–18
35–39	4	67	71	6	2–14
40–45	5	46	51	9	3–21
Total	38[a]	208[b]	246[c]	15	11–20

[a, b, c]Age at onset was not known for 2, 1 and 3 patients, respectively, who were younger than 45 years when examined. *Including 100 cases from Lücking *et al*. (2000). CI = confidence interval.

were identified in eight cases, but in nine, a single mutation was detected. However, in three patients, the mutation was a rearrangement involving consecutive exons and it was not possible to determine if there were rearrangements of different consecutive exons on the two alleles or a single rearrangement on the same allele. Unfortunately, the unaffected relatives or the cell lines for RT-PCR experiments were unavailable for these ambiguous cases. In this study, all patients with one mutation were considered to have *parkin*-related disease, based on the assumption that the second mutation could not be detected by the methods.

The frequency of mutations in the patients of our series decreased with increasing age at onset. Three out of seven patients with onset before 20 years of age had *parkin* mutation compared with only 7 out of 75 with onset after 29 years. When our results are combined with those of our previous study (Lücking *et al*., 2000), which used the same inclusion criteria, the frequency of *parkin* gene mutations in isolated cases with onset ⩽45 years of age is 15% (38 out of 246) (Table 2). Taken together, these two studies demonstrate a significant negative correlation between age at onset and the presence of mutations in the *parkin* gene: mutations were detected in 67% with onset before age 20 years, but only in 7% with onset after 29 years ($P < 0.001$, Table 2).

Type of mutations in the parkin *gene*

Twelve different homozygous and heterozygous exon rearrangements were found in 16 patients, including eight deletions and four duplications of one or more exons (Fig. 1 and Table 1). Three of these mutations were detected for the first time—the deletion of exon 10, the duplication of exon 2 and the duplication of exons 2 to 4. Duplication of exon 2 and exons 2–4 would predict a frameshift and a stop codon at position 98 and 226, respectively, whereas exon 10 deletion would predict an in-frame deletion.

Sequencing of the entire coding region of the *parkin* gene revealed four different exonic point mutations in eight patients. The causative nature of mutations Arg256Cys and Arg275Trp has already been established. The Ala398Thr mutation was detected for the first time in an Algerian patient and was not found in 162 European and 68 North African control chromosomes. The Thr240Met mutation was found for the first time in an Italian patient and was not found in 106 European control chromosomes. The Arg275Trp mutation, located in exon 7, was found repeatedly in five unrelated French patients. The genotyping of these patients revealed that four of them carry a very rare allele (220 bp) at the marker D6S305, which is located in intron 7.

Clinical studies

Patients were identified not only in Europe, but also in Algeria and Brazil, indicating the widespread distribution of *parkin* mutations. The relative frequency of *parkin* cases was similar in French (18 out of 114, 16%), Italian (4 out of 31, 13%), North African (3 out of 14, 21%) and Brazilian (1 out of 13, 8%) cases. There were 88 males and 58 females (Table 3). Age at onset was not significantly different between the 20 *parkin* patients and those without mutation (31 ± 10 versus 34 ± 7 years). There were no significant group differences in clinical features between *parkin* cases and other patients (Table 3). In addition, at the individual level, there was no clinical feature that distinguished the two groups.

However, clinical presentation was highly atypical in one patient (JMP28) (Table 1). This 35-year-old Italian woman presented the initial signs of the disease at 28 years (immediately after a second pregnancy), with unsteadiness, inconstant hand tremor, motor slowing and difficulty with fine movements at the left side, distal numbness in all limbs. Symptoms were absent at awakening, worsened during the day and culminated in severity in the evening. At age 30 years, examination showed unsteadiness and retropulsion**,** nystagmus and slight left limb dysmetria. Other investigations were normal except cerebral MRI, which showed bilateral sickle-shaped areas of abnormal signal (decreased in T_1-weighted images, increased in T_2-weighted images) in the cerebellum. She was diagnosed as having idiopathic cerebellar ataxia**.** At age 33 years, neurological examination revealed left limb increased tone and left foot dystonia. Levodopa treatment (150 mg daily) improved the symptoms and she was

Table 3 *Clinical characteristics of early-onset autosomal recessive parkinsonism patients with or without parkin mutations*

	With (n = 20)	Without (n = 126)
Female/male	9/11	49/77
Age in years (range)	46 ± 11 (16–56)	44 ± 11 (9–68)
Age at onset in years (range)	31 ± 10 (7–45)	34 ± 7 (12–45)
Duration in years (range)	14 ± 8 (4–35)	11 ± 8 (0.5–36)
Clinical signs at onset		
Bradykinesia	13 out of 20 (65%)	71 out of 106 (67%)
Tremor	10 out of 20 (50%)	67 out of 105 (64%)
Dystonia	3 out of 19 (16%)	13 out of 104 (13%)
Asymmetry of clinical signs at onset	19 out of 20 (95%)	103 out of 106 (97%)
Clinical signs at examination		
Bradykinesia	19 out of 20 (95%)	102 out of 105 (97%)
Rigidity	19 out of 20 (95%)	100 out of 106 (94%)
Tremor	11 out of 20 (55%)	81 out of 107 (76%)
Micrography	6 out of 20 (30%)	23 out of 104 (22%)
Postural tremor	3 out of 17 (18%)	19 out of 84 (23%)
Hyperreflexia	4 out of 16 (20%)	18 out of 95 (19%)
UPDRS without treatment (range)	39 ± 21 (8–70) (n = 11)	35 ± 21 (2–89) (n = 45)
Hoehn and Yahr without treatment (range)	3.4 ± 1.3 (2–5) (n = 8)	2.7 ± 1.3 (1–5) (n = 76)
Characteristics of treatment		
Daily dose of levodopa in mg (range)	475 ± 340 (150–1500)	540 ± 400 (50–3000)
Duration of treatment in years (range)	11 ± 8 (0.2–28) (n = 19)	9 ± 10 (0.1–37) (n = 74)
Levodopa-induced dyskinesia	17 out of 20 (85%)	65 out of 79 (82%)
Levodopa-induced fluctuations	18 out of 20 (90%)	65 out of 82 (79%)
Levodopa-induced dystonia	12 out of 20 (60%)	46 out of 81 (57%)

diagnosed as having dopa-responsive dystonia. At age 34 years, she had mild nystagmus on lateral gaze, slight bradykinesia and rigidity on the left side and left foot dystonia. Tendon reflexes were moderately increased; eye movements, speech, motor coordination, sensation and plantar responses were normal. The Unified Parkinson's Disease Rating Scale Motor Section score was 16 and the Hoehn and Yahr stage was 1, while she was taking levodopa. Extrapyramidal symptoms fluctuated during the day and mild limb dyskinesias were present. She was diagnosed as having juvenile onset parkinsonism, confirmed by the presence of a homozygous deletion of exon 3 in the *parkin* gene.

Discussion

Combining our results with those obtained by Lücking *et al*. (2000) results in a frequency of *parkin* gene mutations of 15% (38 out of 246) in patients with early-onset parkinsonism with an age at onset ⩽45 years. Since our population was selected for patients with juvenile and early onset parkinsonism, *parkin* cases might be over-represented compared with the general population of patients with early-onset parkinsonism. However, our calculation of the relative frequency of *parkin* cases in 5-year classes excludes this potential bias. In addition, the frequency might also be underestimated since current techniques might not detect all mutations. To our knowledge, this is the first study in which the frequency of *parkin* gene mutation is evaluated in such large series of patients.

The frequency of mutations in the patients decreased with increasing age at onset. Mutations were detected in 67% with onset before 20 years, but only in 7% with onset after 29 years ($P < 0.001$, Table 2). This raises the question of the frequency of *parkin* mutations in late-onset cases, which is considered to be rare (Oliveri *et al*., 2001). However, ages at onset ⩽72 years have been described in *parkin* cases (Klein *et al*., 2001; Nichols *et al*., 2002) and no systematic study of a large group of late-onset cases has been performed with the appropriate molecular tools (e.g. combining sequence analysis and exon dosage). Nevertheless, although the frequency of *parkin* mutations might be underestimated because of undetected mutations, our results demonstrate that *parkin* is the most important known aetiological factor for early-onset parkinsonism, which represents at least 10% of cases with Parkinson's disease (Lang and Lozano, 1998).

Nine out of the 20 patients with *parkin* mutations unambiguously carry a single mutation. This raises two questions: (i) do other mutations remain to be discovered in other regions of the *parkin* gene or (ii) are single mutations in *parkin* sufficient to cause the phenotype? The observation of patients with both normal and mutant alleles may reflect that haploinsufficiency is a risk factor for disease or that certain mutations are dominant, conferring dominant-negative or toxic gain of function. A kindred has been reported recently with a novel mutation in the *parkin* gene and autosomal dominant inheritance of Parkinson's disease with Lewy bodies (Farrer *et al*., 2001). Since none of our patients had

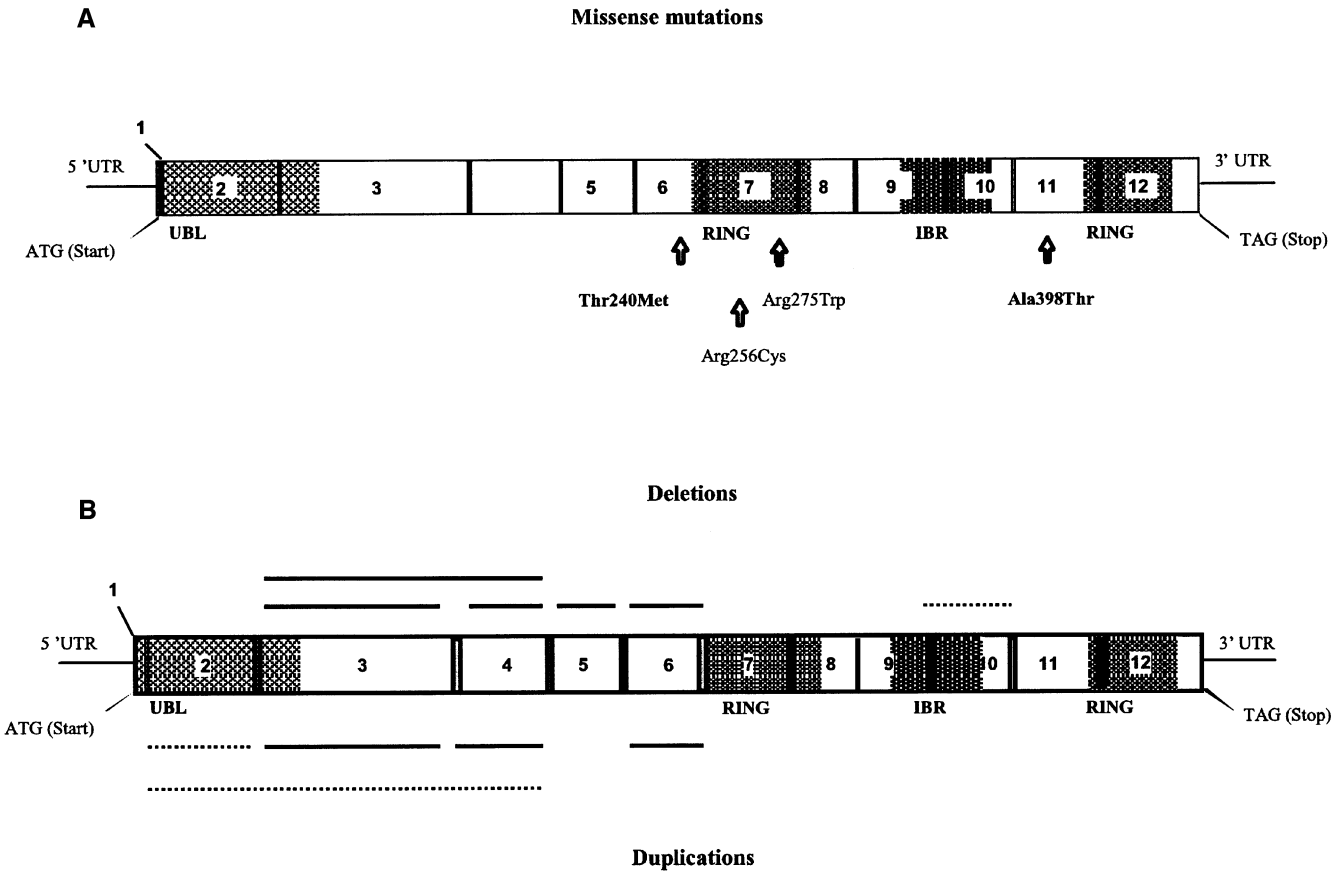

Fig. 1 Point mutations and exon rearrangements in the *parkin* gene found in this study. (**A**) The two new point mutations are indicated in bold characters. (**B**) The deletions and duplications are represented by lines indicating their sizes and positions. Dashed lines represent new deletions or duplications. The functional domains of *parkin* are indicated below the schematic representation: UBL = ubiquitin-like domain; RING-IBR-RING = RING-in between RING-RING; UTR = untranslated region.

family histories of Parkinson's disease, the hypothesis of autosomal dominant transmission is unlikely, except in the case of reduced penetrance or *de novo* mutations. Furthermore, the nature and putative consequences of these mutations does not appear to differ from those detected in cases with mutations on both alleles. However, mutations in unexplored regions of the *parkin* gene, including the promotor, cannot be excluded, although a recent study exploring a large portion of the promotor region found no disease causing mutations (West *et al*., 2002).

This screening yielded three new exon rearrangements (duplications of exons 2, duplication of exons 2–4 and deletion of exon 10) and two point mutations (Ala398Thr and Thr240Met) (Fig. 1) to be added to the growing list of known mutations in the *parkin* gene which now includes 45 different point mutations and 34 exon rearrangements (Kitada *et al*., 1998; Abbas *et al*., 1999; Lücking *et al*., 2000; Klein *et al*., 2000; Maruyama *et al*., 2000; Periquet *et al*., 2000; Hedrich *et al*., 2001; West *et al*., 2002). The Ala398Thr mutation concerns an amino acid located in exon 11, which is conserved among species (human, rat and mouse). The Thr240Met mutation, located in exon 6, is a variant of the previously reported Thr240Arg mutation (Hattori *et al*., 1998*b*). Four out of five patients with the Arg275Trp mutation carried the very rare 220 bp allele at marker D6S305 located in intron 7 close to the mutation. This mutation is the most common point mutation of the *parkin* gene in Europe, where it probably results from a founder effect (Periquet *et al*., 2001), and was already detected in 8 out of 8 patients previously analysed (Periquet *et al*., 2001). The present results confirm the existence of this founder effect.

There were no significant group differences in clinical features of *parkin* cases and other patients (Table 3). In addition, at the individual level, no clinical features distinguished the two groups. These results demonstrate that *parkin* cases do not represent a phenotypically distinct group, as was reported in Japanese families (Ishikawa and Tsuji, 1996). This could have an impact on molecular analyses for *parkin* mutations which are useful for genetic counselling. Since there is no obvious genotype–phenotype correlation, the decision to perform *parkin* analysis can only be based on the frequency of *parkin* cases as a function of age at onset that sharply decreases after 30 years of age.

Clinical analysis of patient JMP28 indicates that a *parkin* analysis should also be considered in patients with a highly atypical presentation. This patient was diagnosed on clinical grounds as having cerebellar ataxia, levodopa-responsive dystonia and, finally, levodopa-responsive parkinsonism. The difficulty in distinguishing patients with levodopa-responsive dystonia from those with *parkin* mutations has been reported previously (Tassin *et al*., 2000) and is confirmed by this case.

This study demonstrates that a monogenic form of parkinsonism caused by *parkin* mutations represents an important cause of early-onset parkinsonism without family history, especially before the age of 30 years. However, even when onset occurs between age 30 and 45 years, *parkin* cases still account for 8% of isolated parkinsonism. The *parkin* phenotype is variable, but cannot be distinguished from non-*parkin* cases. Therefore, molecular analysis is necessary to identify *parkin* cases, whose presentations are sometimes highly atypical. However, even with the combination of gene dosage and sequencing, a significant proportion of mutations might remain undetected due to the size and the complexity of the *parkin* gene and negative results should be interpreted with caution.

Electronic database information

Accession numbers and URLs for data in this article are as follows: The Genome Database (GDB) (for the primer sequences of marker D6S305), http://www.gdb.org; The DNA Databank of Japan (DDBJ) (for the cDNA sequence of the *parkin* gene, accession no. AB009973), http://www.ddbj.nig.ac.jp.

Acknowledgements

We wish to thank Béatrice Debarges, Sophie Lainé and Wilson Dos Santos Bele for technical assistance. This work was supported by the Assistance Publique-Hôpitaux de Paris and the Association France-Parkinson, and grants from the European Community Biomed 2 (BMHCT960664), INSERM Ministère de la Recherche (4CH03G), AVENTIS-PHARMA and National Institutes of Health (grants NS41723–01A1). G.D.M. was supported by the Italian Ministry for University, Scientific and Technological Research (Italy) and N.W.W. was supported by the Brain Research Trust and UK Parkinson's Disease Society and the Doris Hillier Award (British Medical Association).

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Received August 28, 2002. Revised January 29, 2003.
Accepted January 29, 2003