

## ARTICLE

# A wide variety of mutations in the *parkin* gene are responsible for autosomal recessive parkinsonism in Europe

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**Autosomal recessive juvenile parkinsonism (AR-JP, PARK2; OMIM 602544), one of the monogenic forms of Parkinson's disease (PD), was initially described in Japan. It is characterized by early onset (before age 40), marked response to levodopa treatment and levodopa-induced dyskinesias. The gene responsible for AR-JP was recently identified and designated *parkin*. We have analysed the 12 coding exons of the *parkin* gene in 35 mostly European families with early onset autosomal recessive parkinsonism. In one family, a homozygous deletion of exon 4 could be demonstrated. By direct sequencing of the exons in the index patients of the remaining 34 families, eight previously undescribed point mutations (homozygous or heterozygous) were detected in eight families that included 20 patients. The mutations segregated with the disease in the families and were not detected on 110–166 control chromosomes. Four mutations caused truncation of the parkin protein. Three were frameshifts (202–203delAG, 255delA and 321–322insGT) and one a nonsense mutation (Trp453Stop). The other four were missense mutations (Lys161Asn, Arg256Cys, Arg275Trp and Thr415Asn) that probably affect amino acids that are important for the function of the parkin protein, since they result in the same phenotype as truncating mutations or homozygous exon deletions. Mean age at onset was  $38 \pm 12$  years, but onset up to age**

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58 was observed. Mutations in the *parkin* gene are therefore not invariably associated with early onset parkinsonism. In many patients, the phenotype is indistinguishable from that of idiopathic PD. This study has shown that a wide variety of different mutations in the *parkin* gene are a common cause of autosomal recessive parkinsonism in Europe and that different types of point mutations seem to be more frequently responsible for the disease phenotype than are deletions.

## INTRODUCTION

Parkinson's disease (PD) is a frequent neurodegenerative disorder with a prevalence close to 2% after age 65 (1). The major signs of the disease are tremor, rigidity and bradykinesia associated with a good response to treatment with levodopa. The disorder is caused by a massive loss of dopaminergic neurons in the pars compacta of the substantia nigra. The aetiology of the disease is still unknown, but the existence of genetic susceptibility factors is strongly suspected (2). Several familial forms of PD with autosomal dominant transmission have been reported. Mutations in the  $\alpha$ -synuclein gene on chromosome 4q21–q23 were described in several PD families with early onset and rapid disease progression (3,4). However,  $\alpha$ -synuclein is a minor locus, found only in a subset of families with dominant transmission (5–7). A second susceptibility locus was localized on chromosome 2p13 in German families with autosomal dominant PD (8). Autosomal recessive juvenile parkinsonism (AR-JP, PARK2; OMIM 602544) was first described in Japan (9,10). AR-JP patients show the typical signs of PD, but they are associated with: (i) early onset, typically before the age of 40; (ii) dystonia at onset; (iii) diurnal fluctuations; (iv) slow disease progression; and (v) early and severe levodopa-induced dyskinesias. In a few cases, neuropathological examination has shown a massive loss of dopaminergic neurons in the pars compacta of the substantia nigra, but the absence of Lewy bodies, the histopathological hallmark of PD (9,11,12). Recently, the AR-JP locus, designated PARK2, was mapped to chromosome 6q25.2–27 in consanguineous Japanese families (13). Subsequently, linkage analyses, our own (14) and others' (15), have demonstrated the existence of non-Japanese PARK2 families in Europe, the USA and the Middle East. Homozygous deletions of one or more microsatellite markers in three AR-JP families greatly reduced the initial 17 cM candidate interval (14–16). Very recently, Kitada *et al.* identified a novel gene, designated *parkin*, in which homozygous deletions of either exon 4 or exons 3–7 were detected in four Japanese families with AR-JP (17). The *parkin* gene has an estimated genomic size of 500 kb and consists of 12 coding exons with an open reading frame of 1395 bp. The corresponding protein, parkin, composed of 465 amino acids, shows moderate homology to ubiquitin at the N-terminus and contains a RING-finger motif at the C-terminus (17). Subsequently, homozygous deletions of exon 3 were found in two European families and of exons 8–9 in one Algerian family (18). To date, two point mutations in the *parkin* gene have been reported in two Turkish AR-JP families (19).

In order to determine the frequency and the diversity of mutations in the *parkin* gene as a cause for the AR-JP phenotype in Europe, we amplified the 12 coding exons of the *parkin* gene in 35 families with autosomal recessive early onset parkinsonism and sequenced the exons in the patients that did not show homozygous exon deletions.



**Figure 1.** Homozygous exon 4 deletion in the patients of the Italian family IT-005. Bands (PCR products of the indicated exons) correspond to the numbered individuals. Black squares (men) and circles (women) represent affected individuals. Age at onset (in years) is given above the patient's symbol. Slashed symbols indicate deceased individuals. Ex, exon; i, inner; o, outer.

## RESULTS

### A new family with an exon 4 deletion

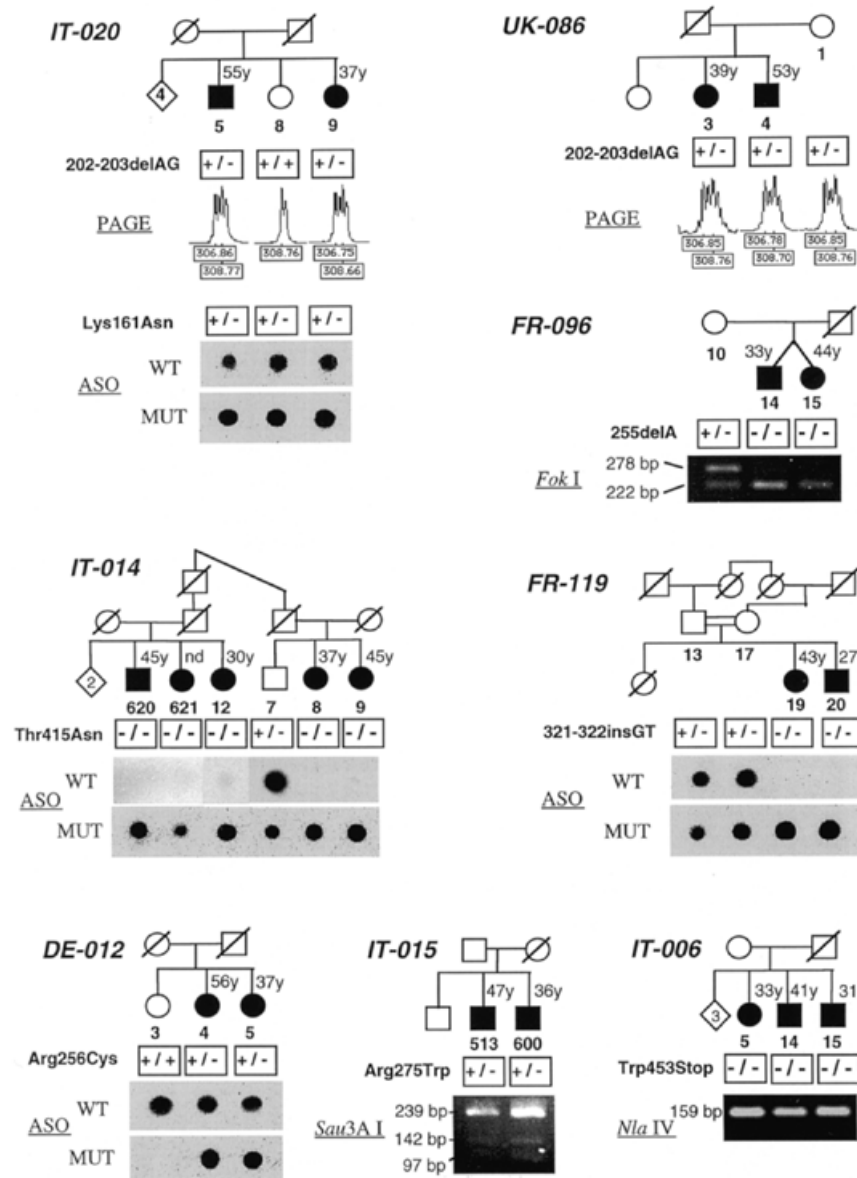
A homozygous deletion of exon 4 was detected in all three patients of an Italian family (IT-005), previously shown to be homozygous for four markers at the disease locus (14; Fig. 1).

### Point mutations in the *parkin* gene

In the index patients from the 34 families that did not show homozygous exon deletions, sequence analysis of all coding exons, including the exon–intron boundaries, revealed 11 sequence variations in exons and three in introns (Table 1). Eight of the exonic variations co-segregated with the disorder in the families (Fig. 2) and were not detected in 110–166 control chromosomes (Table 1). They are therefore most probably causative mutations (Fig. 3).

Four mutations resulted in truncated proteins: 202–203delAG in exon 2 of families IT-020 and UK-086 (from the UK); 255delA in exon 2 of family FR-096 (from France); 321–322insGT in exon 3 of family FR-119; 1459G→A (Trp453Stop) in exon 12 of family IT-006. These mutations were homozygous in patients, except for the 202–203delAG mutation in families IT-020 and UK-086.

Four missense mutations were observed. In family IT-020, a heterozygous 584A→T transversion (Lys161Asn) in exon 4 was detected in addition to the 202–203delAG mutation described above. Segregation analysis showed that the two mutations were located on different alleles and that all patients were compound heterozygotes (Fig. 2). Two other heterozygous, non-conservative amino acid changes were observed in exon 7 of patients from families DE-012 (from Germany) and IT-015: 867C→T (Arg256Cys) and 924C→T (Arg275Trp), respectively. Finally, a



**Figure 2.** Families with point mutations in the *parkin* gene. Complete co-segregation of the mutations with the disease is shown. Black squares (men) and circles (women) represent affected individuals with age at onset (in years) indicated above the patient's symbol. Slashed symbols indicate deceased individuals. The numbers of unaffected sibs not analysed are given in diamonds. For each mutation the genotype of the family member is indicated (+/+, homozygous wild-type; +/-, heterozygous for the mutation; --, homozygous for the mutation). Under each genotype, the detection data are shown. PAGE: electropherograms with allele sizes in bp; ASO: autoradiograms of mutated and wild-type allele; restriction assay: PCR products after digestion with the appropriate restriction enzyme. Fragment lengths in bp are indicated. Mut, mutated; nd, age at onset not determined, as patient is unaware of her clinical signs.

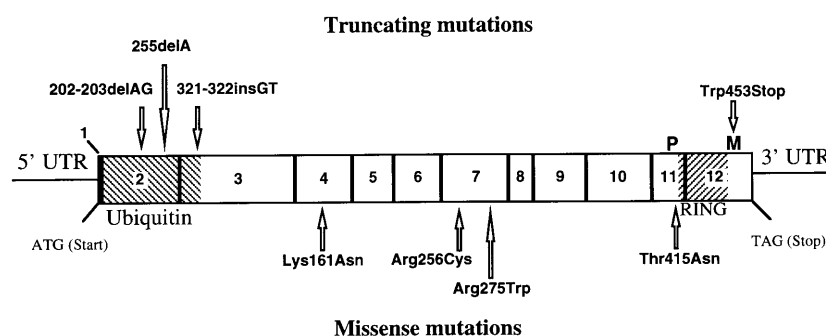
homozygous 1345C→A transversion in exon 11 of all patients from family IT-014 resulted in a conservative (Thr415Asn) amino acid change.

In three of the families with heterozygous mutations (UK-086, DE-012 and IT-015), no complementary heterozygous mutation could be detected by sequencing.

### Polymorphisms

Three of the 11 exonic sequence variations were detected in control chromosomes and were thus classified as polymorphisms (Table 1). They resulted in two conservative and one non-conservative amino acid change. A 601G→A transition (Ser167Asn) was heterozygous

in only one of the two affected members of family FR-730 and on 1% of the control chromosomes (2/166). In addition, it was found in all sibs, including an unaffected, of family IT-020, in which the disease is already explained by the compound heterozygosity of two causative mutations (see above). A 1239G→C transversion (Val380Leu) in exon 10 was either heterozygous or homozygous in 11 families (IT-014, IT-020, IT-058, FR-017, FR-029, FR-038, FR-096, FR-431, UK-006, UK-086 and DE-022). It did not segregate with the disease and was found on 16% of control chromosomes. A 1281G→A transition (causing the non-conservative Asp394Asn change) in exon 11 was heterozygous in the index patient of family UK-046 and heterozygous or homozygous on 7% of control chromosomes.



**Figure 3.** Point mutations in the *parkin* gene. The coding sequence of the gene with its 12 exons is represented as a bar. Exons are numbered 1–12. The eight causative point mutations are positioned according to their effect on the protein (truncating versus missense). The ubiquitin-like domain and the RING-finger motif are hatched. For the mutations Thr415Asn and Trp453Stop the putative phosphorylation sites (P) and the *N*-myristoylation site (M), respectively, are indicated. UTR, untranslated region.

**Table 1.** Variants of the *parkin* gene

	Nucleotide change <sup>a</sup>	Amino acid change/ stop codon position <sup>a</sup>	Mutation type	Detection technique	Expected fragment length (bp)	No. of controls	Variant frequency (%)
Exonic							
Ex2	<i>202–203delAG</i>	<i>Gln34/Stop37</i>	Frameshift	PAGE	WT: 308 V: 306	55	0
Ex2	<i>255delA</i>	<i>Asn52/Stop81</i>	Frameshift	<i>FokI</i> Creation of site	WT: 278+30 V: 222+57+30	83	0
Ex3	<i>321–322insGT</i>	<i>Trp74/Stop81</i>	Frameshift	ASO		83	0
Ex4	<i>584A→T</i>	<i>Lys161Asn</i>	Missense	ASO		83	0
Ex4	<i>601G→A</i>	<i>Ser167Asn</i>	Missense	<i>AlwNI</i> Loss of site	WT: 164+97 V: 261	83	1
Ex7	<i>867C→T</i>	<i>Arg256Cys</i>	Missense	ASO		83	0
Ex7	<i>924C→T</i>	<i>Arg275Trp</i>	Missense	<i>Sau3AI</i> Loss of site	WT: 142+97 V: 239	83	0
Ex10	<i>1239G→C</i>	<i>Val380Leu</i>	Missense	ASO		45	16
Ex11	<i>1281G→A</i>	<i>Asp394Asn</i>	Missense	<i>TaqI</i> Loss of site	WT: 221+84 V: 303	90	7
Ex11	<i>1345C→A</i>	<i>Thr415Asn</i>	Missense	ASO		83	0
Ex12	<i>1459G→A</i>	<i>Trp453Stop</i>	Nonsense	<i>NlaIV</i> Loss of site	WT: 142+17+35+61 V: 159+35+61	83	0
Intronic							
Intron2	IVS2+25T→C			<i>BstNI</i> Creation of site	WT: 308 V: 264+44	45	19
Intron3	IVS3–20C→T			<i>MnII</i> Loss of site	WT: 201+60 V: 261	45	10
Intron7	IVS7–35A→G			<i>MaeIII</i> Creation of site	WT: 206 V: 159+47	36	27

The nucleotide numbers are given according to the cDNA sequence published in the DNA Data Bank of Japan (DDBJ accession no. AB009973).

Ex, exon; PAGE, polyacrylamide gel electrophoresis; ASO, allele-specific oligonucleotide.

<sup>a</sup>Causative mutations are given in italic.

Variants in introns 2, 3 and 7 (IVS2+25T→C, IVS3–20C→T, IVS7–35A→G) were located at a distance from the splice sites. These polymorphisms were detected on control chromosomes at a frequency of 19, 10 and 27%, respectively (Table 1). Finally, a homozygous 768C→T transition (Pro223Ser) was found in all individuals sequenced, suggesting that 768C was an error in the initial cDNA sequence (17).

### Functional domains of the parkin protein

According to the PROSITE program, the conservative amino acid change Thr415Asn is located within the consensus sequence for putative phosphorylation sites of cAMP- and cGMP-dependent protein kinases (KKTT) and protein kinase C (TTK). The truncating nonsense mutation Trp453Stop is located within a putative *N*-myristoylation site (GCEWNR).

**Table 2.** Clinical characteristics of 31 patients from 12 families according to the type of mutation in the *parkin* gene

Clinical characteristics	Mutation type			
	Deletion	Truncating	Missense	Total
No. of families (patients)	4 (11)	4 (9)	4 (11)	12 (31)
Mean age at onset in years (range)	33.9 ± 16.3 (7–58)	38.2 ± 8.0 (27–53)	42.5 ± 8.5 (30–56)	38.1 ± 12.1 (7–58)
Mean disease duration in years (range)	14.8 ± 6.5 (3–26)	16.3 ± 9.4 (4–29)	16.3 ± 8.9 (0.5–31)	15.8 ± 8.0 (0.5–31)
Hoehn and Yahr score	3.4 ± 1.1	2.2 ± 0.9	2.8 ± 0.9	2.8 ± 1.0
Bradykinesia	11/11	8/9	11/11	97%
Rigidity	10/11	9/9	11/11	97%
Tremor	6/11	8/9	7/11	68%
Dystonia at onset	6/11	1/7	0/5	30%
Good response to levodopa ( <i>de novo</i> cases)	10/10 (1)	9/9	9/9 (2)	100%
Dyskinesia	6/10	4/9	8/9	71%
Fluctuations	5/10	3/6	ND	50%
Brisk reflexes lower limbs	4/11	0/6	3/4	33%

### Genotype–phenotype correlations

Point mutations were present in eight families, that included 20 patients (Table 2). The patients of family IT-020 with a truncating mutation on one allele and a missense mutation on the other allele were classified in the missense mutation group, assuming that missense mutations may be less deleterious if they result in only partial loss of function of the parkin protein, thereby determining the severity of the illness. Accordingly, the patients of the families for whom only one heterozygous mutation could be detected were included among the truncating (UK-086) or missense mutations (DE-012 and IT-015). The 11 patients with homozygous exon deletions of the *parkin* gene [three previously published families (18) and family IT-005], the 11 patients with missense mutations (four families) and the nine patients with truncating mutations (four families) showed an overall phenotype of early onset parkinsonism (Table 2). Mean age at onset was  $38 \pm 12$ , ranging from 7 to 58. Parkinsonian rigidity (97%) and bradykinesia (97%) were almost always present, whereas tremor was observed in only 68%. These features were associated with dystonia at onset in 30% and with brisk reflexes in the lower limbs without a Babinski sign in 33%. After a mean disease duration of  $16 \pm 8$  years, dyskinesias and fluctuations were present in 71 and 50%, respectively. The effect of the mutations on age at onset, severity or frequency of signs did not differ significantly among the patients carrying different types of mutations.

### DISCUSSION

The initial report of Kitada *et al.* describing homozygous deletions of exon 4 or exons 3–7 in four Japanese families (17), our own observation of homozygous deletions of exon 3 or exons 8–9 in two European and one Algerian families (18) and the newly identified exon 4 deletion in an Italian family show that a variety of deletions in the *parkin* gene can cause autosomal recessive parkinsonism, with age at onset as late as 58, as observed in the Italian family IT-005 (Fig. 1). However, it was suspected that other types of mutations (e.g. point mutations) might be a more common cause of the disease (18). The only point mutations reported to date were two homozygous single

base pair substitutions in two consanguineous Turkish families, which resulted in a Thr240Arg missense mutation in exon 6 and an Ala311Stop nonsense mutation in exon 8. Until now, however, it could not be determined whether point mutations in the *parkin* gene account for a significant proportion of autosomal recessive early onset parkinsonism.

Taken together, our three families with previously reported exon deletions (18) and the nine families with mutations detected in the present study demonstrate that mutations in the *parkin* gene are the cause of the disease in ~30% of the families with autosomal recessive parkinsonism analysed (12/38), including 11 of 35 from Europe. Point mutations were detected in two-thirds of our families (eight of 12 families) and thus seem to be more frequent than homozygous exon deletions (four of 12 families).

The fact that a second mutation could not be detected in families DE-012, IT-015 and UK-086 (which were heterozygous for only one mutation) suggests the presence of a complementary mutation that could not be detected by the techniques used in this study. This second mutation might be located in regions of the gene that were not sequenced (e.g. the promotor region) or might be present as a heterozygous exon deletion that cannot be analysed by simple PCR amplification. However, in order to estimate the frequency of compound heterozygotes resulting from the combination of a point mutation and an exon deletion or of two different exon deletions, we compared the frequencies of the homozygous exon deletions ( $n = 4$ ) and the homozygous point mutations ( $n = 4$ ) in eight families with known or suspected consanguinity. The frequency for the two types of homozygous mutations was 50% each in this sample. The frequency of compound heterozygotes for each of the two combinations (deletion + point mutation or deletion + deletion) could therefore reach up to 25%. However, the small sample size precludes accurate estimation of these frequencies.

Only one of the point mutations, the 202–203delAG frameshift, was found in two families of different origins (Italy and UK), suggesting that the mutation occurred independently. Although the patients from these families shared two frequent alleles for the PARK2 markers D6S411 (allele 2 = 59%) and D6S1550 (allele 2 = 68%), the alleles for two other tightly linked markers, D6S305

and D6S1579, were different (data not shown), indicating that a recent common founder effect is unlikely.

All the point mutations are novel and show that a wide variety of different mutations in the *parkin* gene can account for the disease. The pathogenic role of the point mutations was shown by: (i) co-segregation of the mutations with the disorder in the families; and (ii) their absence in a large number of control chromosomes (110–166 depending on the mutation). All the point mutations identified are likely to have major functional consequences. The four truncating mutations (202–203delAG, 255delA, 321–322insGT and Trp453Stop), which were detected in the homozygous state in three of five families, clearly cause a loss of function of the parkin protein, compatible with a recessive mode of inheritance. Three of the missense mutations result in non-conservative amino acid changes (Lys161Asn, Arg256Cys and Arg275Trp). In family IT-020, the 202–203delAG frameshift mutation on one allele, which results in a loss of function, is associated with an apparently deleterious Lys161Asn missense mutation on the other allele. The conservative Thr415Asn amino acid change, which involves neutral amino acids with different polar side chains, homozygous in all five patients of family IT-014, is located within two consensus sequences of different protein kinases (cAMP- and cGMP-dependent protein kinases and protein kinase C) and might alter post-translational modifications. In addition, codon 415 is located very close to the first cysteine of the RING-finger motif (codon 418) and could affect its structure.

The mutations, which had different effects on the parkin protein, were distributed over six exons, excluding a mutational hotspot. It is interesting to note that, in contrast to the reported Gln311Stop mutation (19), the truncating point mutations identified in this study correspond to the N- and C-terminal regions of the parkin protein (ubiquitin-like and RING-finger motif, respectively), whereas the missense mutations affect the more central regions of the protein, as does the Thr240Arg mutation (19). The previously described ubiquitin-like and RING-finger domains were not affected by the missense mutations. The C-terminal region appears to be extremely important since a homozygous Trp453Stop nonsense mutation, which only removes the last 12 C-terminal residues, causes the disease, perhaps by altering an *N*-myristoylation site between codons 450 and 455, which prevents a necessary post-translational modification of the protein. The absence of significant clinical differences in this study among the patients with different types of mutations suggests that the modified amino acids are all of functional importance and that their replacement, like the truncating mutations, causes loss of function. The location of the mutations may therefore point to unknown functional domains.

The phenotype of the families with point mutations in the *parkin* gene and those with exon deletions covered a clinical spectrum that was broader than in the Japanese families originally described (17,19) and is often very close to that of idiopathic PD (Table 2). Mean age at onset (38 years) was that of early onset PD. In 13 patients, however, onset occurred after age 40. Dystonia or brisk reflexes were less frequent than previously reported (10,19). Overall, the phenotypes of patients classified according to the effect of the mutations on the parkin protein were similar, although earlier ages at onset (7–18 years) were observed in the Algerian family with deletion of exons 8 and 9 (18). Similar early onset was also observed in the Japanese family with deletion of exons 3–7 (17) as well as in the patient with the Gln311Stop

mutation (19), raising the question of the functional consequences of exon deletions and truncating events in specific regions of the parkin protein, especially as onset within our patients group with truncating mutations was later (between 27 and 53 years).

Finally, three exonic variants (Ser167Asn, Val380Leu and Asp394Asn) were classified as polymorphisms, since they were detected at different frequencies (between 1 and 16%) on control chromosomes. In some families, they did not segregate with the disease. Furthermore, they were found in a family in which the disease was explained by other mutations (the polymorphism Ser167Asn in family IT-020) or were homozygous in healthy controls (Val380Leu and Asp394Asn). They are therefore insufficient to cause the disease by themselves. They might, however, alter the function of the parkin protein and contribute to the pathogenesis of idiopathic PD. Association studies will help to clarify this question.

In conclusion, this study shows that point mutations in the *parkin* gene are a common cause of autosomal recessive parkinsonism in Europe and seem to be more frequent than the exon deletions so far described. Furthermore, the mutations in the *parkin* gene are associated with a wide range of ages at onset as well as of clinical signs and can result in familial parkinsonism clinically indistinguishable from idiopathic PD. The mutations detected are diverse in their effects on the parkin protein as well as in their localization within the gene. The diversity of the mutations and the absence of a mutational hotspot will complicate molecular diagnosis, but the evident importance of the amino acids affected will help to analyse the function, still unknown, of the parkin protein.

## MATERIALS AND METHODS

### Patients and statistical analysis

Thirty-eight families were selected according to the following criteria: (i) presence of parkinsonism with good response to levodopa ( $\geq 30\%$  improvement) in at least two siblings and absence of excluding criteria such as extensor plantar reflexes, ophthalmoplegia, early (after 2 years of disease evolution) dementia or autonomic failure; (ii) onset at  $\leq 45$  years in at least one of the siblings; and (iii) inheritance compatible with autosomal recessive transmission (several patients in a single generation with or without known consanguinity). The families originated from France ( $n = 12$ ), Italy ( $n = 10$ ), Germany ( $n = 7$ ), Great Britain ( $n = 4$ ), Algeria ( $n = 1$ ), Morocco ( $n = 1$ ), The Netherlands ( $n = 1$ ), Portugal ( $n = 1$ ) and Vietnam ( $n = 1$ ). Four families from Algeria, France, Italy and Portugal were excluded from sequence analysis because they were found to carry homozygous deletions of either exon 3 or exons 8–9 (18) or exon 4 (family IT-005).

The patients and unaffected relatives were examined by us in one of the movement disorder clinics of the European Consortium on Genetic Susceptibility in Parkinson's Disease or the French Parkinson's Disease Genetics Study Group, according to a standardized protocol using the same inclusion and exclusion criteria. All patients were videotaped and the clinical data were centralized. The Kruskal–Wallis test was used for comparisons of means and the  $\chi^2$  test for comparison of frequencies (Yates corrected). Blood samples were taken with informed consent from the patients and their first degree relatives.

## PCR amplification and sequence analysis

The 12 coding exons of the *parkin* gene from 35 index patients were amplified from genomic DNA by PCR as described by Kitada *et al.* (17). In family IT-005, an additional pair of exonic primers was used for exon 3 (Ex3iFor, 5'-AATTGTGAC-CTGGATCAGC-3'; Ex3iRev, 5'-CTGGACTTCCAGCTGG-TGGTGAG-3'). For exons 4 and 7 only the intronic primer pairs were used. The same primers were used for the sequencing of the PCR products of the 12 exons on both strands using the Big Dye Terminator Cycle Sequencing Ready Reaction DNA Sequencing kit (Applied Biosystems, Foster City, CA), according to the manufacturer's recommendations, on an ABI 377 automated sequencer with the Sequence Analysis v.3.0 (Applied Biosystems) software.

Each time a nucleotide variant was identified in an index case, its co-segregation with the disease was established in the patient's family and its frequency was determined in 36–90 controls (with or without other neurological diseases). Three different techniques were used to detect mutations in the PCR products corresponding to the different exons: (i) allele-specific oligonucleotides (ASOs) to detect the wild-type and the variant sequence; (ii) digestion with the appropriate restriction endonuclease; or (iii) PAGE (Table 1). Nucleotide positions were determined according to the cDNA sequence published in the DNA Data Bank of Japan (DDBJ accession no. AB009973).

## ASOs

Ten microlitres of the PCR product were blotted onto Hybond N<sup>+</sup> nylon membranes (Amersham Life Science, Little Chalfont, UK) after denaturation at 95°C for 5 min and fixed in a microwave oven at 600 W for 2 min. For exon 3, the primers Ex3iFor and Ex3iRev were used. The oligonucleotides (Table 3), labeled with [<sup>32</sup>P]CTP using the Terminal Transferase kit (Boehringer Mannheim, Mannheim, Germany), were hybridized to the membrane at 44°C overnight in a buffer consisting of 5× SSPE, 5× Denhardt's and 0.1% SDS. The membranes were washed twice for 30 min in 2× SSC at 59°C and exposed to MP film (Amersham) for 3–6 h.

**Table 3.** Oligonucleotides used for the ASO technique

Location	Nucleotide change	Oligonucleotide sequence
Ex3	321–322insGT	WT: 5'-TGCAGAGACC–GTGGAGAAAA-3' V: 5'-GCAGAGACCGTGTGGAGAAA-3'
Ex4	584A→T	WT: 5'-GCCGGGAAA <u>A</u> CTCAGGGTA-3' V: 5'-GCCGGGAAATCTCAGGGTA-3'
Ex7	867C→T	WT: 5'-TGCAACTCC <u>C</u> GCCACGTGA-3' V: 5'-TGCAACTCC <u>T</u> GCCACGTGA-3'
Ex10	1239G→C	WT: 5'-TGCAAGTCCGTAATTGAAG-3' V: 5'-TGCAAGTCCGTAATTGAAG-3'
Ex11	1345C→A	WT: 5'-AGAAAACCA <u>C</u> CAAGCCCTG-3' V: 5'-AGAAAACCA <u>A</u> CAAGCCCTG-3'

The nucleotide change in the oligonucleotides is underlined.  
WT, wild-type; V, variant.

## Restriction assay

Fifteen microlitres of PCR product were digested with restriction enzymes according to the manufacturer's recommendations. The expected fragment lengths are given in Table 1.

## Polyacrylamide gel electrophoresis

A 5'-fluorescent (Hex) forward primer (17) was used to amplify exon 2. The presence of the 202–203delAG variant, resulting in a shorter PCR product (306 versus 308 bp), was established by fragment size measurement using an ABI 377 automated sequencer with the Genescan v.2.0.2 and Genotyper v.1.1.1 software (Applied Biosystems).

## Determination of additional functional domains in the parkin protein

The Internet web site PROSITE (<http://expasy.hcuge.ch/cgi-bin/scanprosite?1>) was used to determine additional functional domains of the parkin protein.

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## ABBREVIATIONS

AR-JP, autosomal recessive juvenile parkinsonism; ASO, allele-specific oligonucleotide; DE, German family; FR, French family; IT, Italian family; PD, Parkinson's disease; UK, British family; V, variant; WT, wild-type.

## REFERENCES

- de Rijk, M.C., Tzourio, C., Breteler, M.M., Dartigues, J.F., Amaducci, L., Lopez-Pousa, S., Manubens-Bertran, J.M., Alperovitch, A. and Rocca, W.A. (1997) Prevalence of parkinsonism and Parkinson's disease in Europe: the EUROPARKINSON Collaborative Study. European Community Concerted Action on the Epidemiology of Parkinson's disease. *J. Neurol. Neurosurg. Psychiatr.*, **62**, 10–15.
- Wood, N. (1997) Genes and parkinsonism [editorial]. *J. Neurol. Neurosurg. Psychiatr.*, **62**, 305–309.
- Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I. and Nussbaum, R.L. (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*, **276**, 2045–2047.
- Kröger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., Przuntek, H., Epplen, J.T., Schöls, L. and Riess, O. (1998) Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nature Genet.*, **18**, 106–108.
- Vaughan, J.R., Farrer, M.J., Wszolek, Z.K., Gasser, T., Durr, A., Agid, Y., Bonifati, V., DeMichele, G., Volpe, G., Lincoln, S., Breteler, M., Meco, G., Brice, A., Marsden, C.D., Hardy, J. and Wood, N.W. (1998) Sequencing of the alpha-synuclein gene in a large series of cases of familial Parkinson's disease fails to reveal any further mutations. The European Consortium on Genetic Susceptibility in Parkinson's Disease (GSPD). *Hum. Mol. Genet.*, **7**, 751–753.
- The French Parkinson's Disease Study Group (1998)  $\alpha$ -Synuclein gene and Parkinson's disease. *Science*, **279**, 1116–1117.

7. Farrer, M., Wavrant-De Vrieze, F., Crook, R., Boles, L., Perez-Tur, J., Hardy, J., Johnson, W.G., Steele, J., Maraganore, D., Gwinn, K. and Lynch, T. (1998) Low frequency of alpha-synuclein mutations in familial Parkinson's disease. *Ann. Neurol.*, **43**, 394–397.
8. Gasser, T., Müller-Mysok, B., Wszolek, Z.K., Oehlmann, R., Calne, D.B., Bonifati, V., Bereznoi, B., Fabrizio, E., Vieregge, P. and Horstmann, R.D. (1998) A susceptibility locus for Parkinson's disease maps to chromosome 2p13. *Nature Genet.*, **18**, 262–265.
9. Yamamura, Y., Sobue, I., Ando, K., Iida, M. and Yanagi, T. (1973) Paralysis agitans of early onset with marked diurnal fluctuation of symptoms. *Neurology*, **23**, 239–244.
10. Ishikawa, A. and Tsuji, S. (1996) Clinical analysis of 17 patients in 12 Japanese families with autosomal-recessive type juvenile parkinsonism. *Neurology*, **47**, 160–166.
11. Takahashi, H., Ohama, E., Suzuki, S., Horikawa, Y., Ishikawa, A., Morita, T., Tsuji, S. and Ikuta, F. (1994) Familial juvenile parkinsonism: clinical and pathologic study in a family. *Neurology*, **44**, 437–441.
12. Mori, H., Kondo, T., Yokochi, M., Matsumine, H., Nakagawa-Hattori, Y., Miyake, T., Suda, K. and Mizuno, Y. (1998) Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. *Neurology*, **51**, 890–892.
13. Matsumine, H., Saito, M., Shimoda Matsubayashi, S., Tanaka, H., Ishikawa, A., Nakagawa Hattori, Y., Yokochi, M., Kobayashi, T., Igarashi, S., Takano, H., Sanpei, K., Koike, R., Mori, H., Kondo, T., Mizutani, Y., Schaffer, A.A., Yamamura, Y., Nakamura, S., Kuzuhara, S., Tsuji, S. and Mizuno, Y. (1997) Localization of a gene for an autosomal recessive form of juvenile Parkinsonism to chromosome 6q25.2–27. *Am. J. Hum. Genet.*, **60**, 588–596.
14. Tassin, J., Dürr, A., de Broucker, T., Abbas, N., Bonifati, V., De Michele, G., Bonnet, A.M., Broussolle, E., Pollak, P., Vidailhet, M., De Mari, M., Marconi, R., Medjbeur, S., Filla, A., Meco, G., Agid, Y. and Brice, A. (1998) Chromosome 6-linked autosomal recessive early-onset parkinsonism: linkage in European and Algerian families, extension of the clinical spectrum and evidence of a small homozygous deletion in one family. *Am. J. Hum. Genet.*, **63**, 88–94.
15. Jones, A.C., Yamamura, Y., Almasy, L., Bohlega, S., Elibol, B., Hubble, J., Kuzuhara, S., Uchida, M., Yanagi, T., Weeks, D.E. and Nygaard, T.G. (1998) Autosomal recessive juvenile parkinsonism maps to 6q25.2–q27 in four ethnic groups: detailed genetic mapping of the linked region. *Am. J. Hum. Genet.*, **63**, 80–87.
16. Matsumine, H., Yamamura, Y., Hattori, N., Kobayashi, T., Kitada, T., Yoritaka, A. and Mizuno, Y. (1998) A microdeletion of D6S305 in a family of autosomal recessive juvenile parkinsonism (PARK2). *Genomics*, **49**, 143–146.
17. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. and Shimizu, N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*, **392**, 605–608.
18. Lücking, C.B., Abbas, N., Dürr, A., Bonifati, V., Bonnet, A.M., de Broucker, T., De Michele, G., Wood, N.W., Agid, Y., Brice, A. for the European Consortium on Genetic Susceptibility in Parkinson's Disease and the French Parkinson's Disease Genetics Study Group (1998) Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism. *Lancet*, **352**, 1355–1356.
19. Hattori, N., Matsumine, H., Asakawa, S., Kitada, T., Yoshino, H., Elibol, B., Brookes, A.J., Yamamura, Y., Kobayashi, T., Wang, M., Yoritaka, A., Minoshima, S., Shimizu, N. and Mizuno, Y. (1998) Point mutations (Thr240Arg and Ala311Stop) in the Parkin gene. *Biochem. Biophys. Res. Commun.*, **249**, 754–758.