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PINK1, *Parkin*, and *DJ-1* mutations in Italian patients with early-onset parkinsonism

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Recessively inherited early-onset parkinsonism (EOP) has been associated with mutations in the *Parkin*, *DJ-1*, and *PINK1* genes. We studied the prevalence of mutations in all three genes in 65 Italian patients (mean age of onset: 43.2 ± 5.4 years, 62 sporadic, three familial), selected by age at onset equal or younger than 51 years. Clinical features were compatible with idiopathic Parkinson's disease in all cases. To detect small sequence alterations in *Parkin*, *DJ-1*, and *PINK1*, we performed a conventional mutational analysis (SSCP/dHPLC/sequencing) of all coding exons of these genes. To test for the presence of exon rearrangements in *PINK1*, we established a new quantitative duplex PCR assay. Gene dosage alterations in *Parkin* and *DJ-1* were excluded using previously reported protocols. Five patients (8%; one woman/four men; mean age at onset: 38.2 ± 9.7 (range 25–49) years) carried mutations in one of the genes studied: three cases had novel *PINK1* mutations, one of which occurred twice (homozygous c.1602_1603insCAA; heterozygous c.1602_1603insCAA; heterozygous c.836G>A), and two patients had known *Parkin* mutations (heterozygous c.734A>T and c.924C>T; heterozygous c.924C>T). Family history was negative for all mutation carriers, but one with a history of tremor. Additionally, we detected one novel polymorphism (c.344A>T) and four novel *PINK1* changes of unknown pathogenic significance (–21G/A; IVS1 + 97A/G; IVS3 + 38_40delTTT; c.852C>T), but no exon rearrangements. No mutations were found in the *DJ-1* gene. The number of mutation carriers in both the *Parkin* and the *PINK1* gene in our cohort is low but comparable, suggesting that *PINK1* has to be considered in EOP.

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Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder and the most frequent cause of parkinsonism. Parkinsonism has a significant genetic component, particularly in cases with an early onset of the disease (before the age of 51 years). To date, three different genes have been reported to be associated with early-onset parkinson-

ism (EOP): *Parkin* at *PARK2*¹ (OMIM*602544), *PINK1* at *PARK6*² (OMIM*608309), and *DJ-1* at *PARK7*³ (OMIM#606324) (for a review see Shen and Cookson⁴).

For the first two known genes (*Parkin* and *DJ-1*), different types of mutations – point mutations, small deletions, and deletions of whole exons – have been described.^{3,5–9} The latter type of mutations is only detectable upon gene dosage analysis.⁶ For the recently identified *PINK1* gene, only point mutations and one four-nucleotide insertion have been reported to date.^{2,10–14}

While the frequency of *Parkin* mutations in EOP patients has been estimated at 10–25%,⁵ and *DJ-1* mutations account for 1%^{15,16} to 2%^{7,8} of the tested EOP cases, the frequency of mutations in *PINK1* still remains to be accurately determined. Although a first study suggested a frequency of 7%, including patients with heterozygous mutations,¹⁰ recent findings revealed that the *PINK1* mutation rate is much lower in an Irish PD population.¹³

Factors associated with mutation frequencies in EOP have been studied most extensively in *Parkin*-associated PD and appear to be highly correlated with lower age of onset and positive family history.⁵ Ethnic background of the patients also affects the mutation rate with frequencies of *Parkin* mutations ranging from 66% in patients from Japan¹⁷ to less than 4% in a US or Serbian EOP population.^{8,18} However, such diverse findings may be the result of referral bias at specialty centers, attracting a higher number of patients with a positive family history, suggestive of a genetic cause. Finally, the rate of detected mutations is dependent on the extent of the mutational analysis performed.¹⁹

Thus, we investigated, for the first time, the prevalence of mutations in all three known EOP genes in an ethnically homogeneous group of (mostly) sporadic Italian patients by employing a comprehensive screening protocol.

Materials and methods

Patients

After obtaining informed consent, all patients underwent a standardized neurological examination by a movement disorder specialist (GA, PM, PPP). The diagnosis of PD was based on the UK Brain Bank diagnostic criteria (with the exception that positive family history was not regarded an exclusion criterion).²⁰ All patients represent a consecutive series, collected at three Northern Italian Movement Disorder Centers, and did not originate in a geographic isolate. None of them were included in previous *Parkin* or *DJ-1* studies.

Family history was regarded positive if parkinsonism was reported by a first- or second-degree relative. PD was rated using the Unified Parkinson's Disease Rating Scale (UPDRS) III²¹ and Hoehn and Yahr Scales²² in the off state; major cognitive dysfunction was excluded by applying the Mini-Mental State Examination.²³

Mutational analysis

We screened all 12 exons for *Parkin* mutations by SSCP analysis and gene dosage studies as reported previously.⁶ To test for *DJ-1* mutations, we carried out a dHPLC analysis, and performed gene dosage studies of all six coding *DJ-1* exons by quantitative duplex PCR.⁷ Exons 2–8 of *PINK1* were analyzed by dHPLC analysis. Exon 1 was tested in two overlapping parts (1a and 1b) based on the large size (480 bp + exon/intron boundary). Since the PCR for both parts of exon 1 needed additives that can damage the column of the dHPLC system (see On-line Supplementary Information 1), we used SSCP analysis to detect mutations in this exon. For samples that showed a conspicuous band/elution pattern (SSCP/dHPLC) for any exon in any of the three analyzed genes, the entire coding region of the gene in question was sequenced. Quantitative duplex PCR of all *PINK1* exons was adapted from that of the *Parkin* gene⁶ and was carried out for 55 patients. The amount and quality of the DNA for the remaining 10 samples were not sufficient for quantitative PCR analysis. Exons that carried a heterozygous polymorphism were not included in the quantitative analysis since heterozygous deletions had already been excluded. Primers and assay conditions are available on-line (see On-line Supplementary Information 1 and 2).

Positive controls with known mutations or polymorphisms were available for all exons of *Parkin*, except for exon 5, for all amplicons of *PINK1*, and for exon 5 of *DJ-1* for the conventional mutational analysis. Regarding the quantitative analysis, the *Beta globin* gene was coamplified with each individual *Parkin*, *DJ-1*, and *PINK1* exon, and served as

Table 1 Haplotype analysis of samples with recurrent mutations in *PINK1* (a) and *Parkin* (b)

			Case 1	Case 2		
(a) <i>PINK1</i>						
D1S552	37.30 cM	248	252	256	252	
D1S2732	39.04 cM	258	268	264	268	
<i>c.189C/T</i>	<i>PINK1</i>	C	C	T	C	
<i>IVS1-7A/G</i>	<i>PINK1</i>	G	G	G	G	
<i>IVS4-5G/A</i>	<i>PINK1</i>	A	A	A	A	
<i>*37A/T</i>	<i>PINK1</i>	T	T	T	T	
D1S2828	40.64 cM	251	251	251	251	
D1S2864	41.17 cM	141	159	141	159	
D1S2734	42.89 cM	118	120	112	120	
D1S1622	48.69 cM	255	258	270	258	
Case 4			Case 5			
(b) <i>Parkin</i>						
D6S1599	133	133	131	155		
D6S955	149	153	149	153		
D6S305	220	224	220	202		
D6S411	155	155	155	155		
D6S1550	128	128	128	128		

Intragenic *PINK1* SNP markers are italicized; potentially shared alleles are bolded. The marker order in *PINK1* region is based on the deCODE genetic map.³⁸

Table 2 Case reports of patients with mutations

	Case 1	Case 2	Case 3	Case 4	Case 5
Gene and exon with mutation	<i>PINK1</i> Exon 8	<i>PINK1</i> Exon 8	<i>PINK1</i> Exon 4	<i>Parkin</i> Exons 6 and 7	<i>Parkin</i> Exon 7
Mutation type	Homozygous c.1602_1603 ins CAA	Heterozygous c.1602_1603 ins CAA	Heterozygous c.836G>A	Heterozygous c.734A>T and c.924C>T	Heterozygous c.924C>T
Protein change	p.534_535insQ	p.534_535insQ	p.R279H	p.K211N and p.R275W	p.R275W
Gender	Female	Male	Male	Male	Male
Age at onset	32 years	25 years	49 years	40 years	45 years
Disease duration	25 years	23 years	6 years	8 years	9 years
Family history of parkinsonism	Negative, no evidence for consanguinity	Negative	Negative (bilateral hand tremor in his father aged 70 years)	Negative	Negative
Initial symptoms	Dystonia of left foot	Slowing of movements, clumsiness and stiffness in left hand	Clumsiness in right hand	Muscle stiffness	Slowing of movements, intermittent resting tremor in left hand, reduction of libido, and depression
Most recent neurologic examination	Resting tremor, slowness, and clumsiness of movements in left hand, left-sided muscle stiffness, spread of symptoms to the contralateral side after 2 years. Motor symptoms improved after sleep	Resting tremor of left hand, followed by tremor in left leg, and later in both right extremities, dyskinesias 5 years after initiation of treatment	Resting tremor of both hands, more pronounced on the right-hand side; head tremor; and mild postural and kinetic tremor in upper limbs (right>left). General slowing of movements; change in posture (anteflexion) in combination with micrographia, and hypophonia. Mild hyper-reflexia	Resting tremor and left foot dystonia 2 years after initial symptoms	Resting tremor of left hand, bradykinesia; sleep benefit for motor symptoms, mild wearing-off, and no dyskinesias
UPDRS III score at last exam (off state)	34	56	15	12	29
Hoehn and Yahr stage at last exam (off state)	3	4	2	1.5	1
Cognitive signs	None	None	None	None	None
Psychiatric symptoms	None	Depression and anxiety	Severe depression and claustrophobia	None	Feeling of depression and anxiety
Autonomic symptoms	None	Sexual dysfunction	Tendency towards orthostatic hypotension	None	Sexual dysfunction
Levodopa responsiveness	Good to a low dose (200 mg)	Good to a low dose (200 mg)	Excellent to a low dose (100 mg)	Not tested Good responsiveness to dopamine agonist	Excellent to a low dose (100 mg)
Neuroimaging	Brain MRI scan normal; [¹²³ I]FP-CIT SPECT scan revealed a reduction of tracer uptake of the putamen, more evident on the right-hand side, contralateral to more severely affected body side	Brain MRI unremarkable; [¹²³ I]FP-CIT SPECT scan disclosed a severe reduction of tracer uptake in this caudate nucleus and putamen	Not performed because of claustrophobia	Brain MRI unremarkable	Brain MRI normal, except for a moderate cortical-subcortical atrophy

an internal standard. A relative ratio (concentration of each *Parkin*, *DJ-1*, or *PINK1* exon to concentration of *Beta globin*) between 0.8 and 1.2 was considered as normal, a heterozygous deletion was expected at the ratio between 0.4 and 0.6, and a heterozygous duplication between 1.3 and 1.7.⁶ In all, 100 chromosomes of Italian and 100 chromosomes of German control individuals were screened for the presence of the novel mutations and the novel polymorphism. The mean age of the controls at the time of the blood collection was 52.2 ± 14.3 years.

To investigate whether the carriers of recurrent *Parkin* and *PINK1* mutations shared the same haplotype, a set of microsatellite markers in the *PARK2* and *PARK6* region was used (Table 1). To determine the phase of one of the *PINK1* mutation carriers with two base pair substitutions in exon 4, we separated the alleles by cloning the PCR product of exon 4 in the pCRII-Topo vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. We selected four insert-bearing colonies, amplified the insert, and sequenced it using vector-specific primers.

Results

Patients

We included 65 unrelated Italian patients (34 women, 31 men). The mean age of onset was 43.2 ± 5.4 (range 25–51) years. Three patients (5%) had a positive family history (an affected parent), compatible with a dominant mode of inheritance. In total, we found five (8%) mutation carriers in our patient group (one woman, four men; average age of onset: 38.2 ± 9.7 (range 25–49) years (Table 2)). None of these patients had a positive family history for parkinsonism, with the possible exception of one case with late-onset

bilateral arm tremor in his father (mutational status unknown).

Molecular findings

Three patients carried *PINK1* mutations, one of which occurred twice (homozygous c.1602_1603insCAA in exon 8 [p.534_535insQ]; heterozygous c.1602_1603insCAA; heterozygous c.836G>A in exon 4 [p.R279H]; (Figure 1). In addition, two patients showed *Parkin* mutations (c.734A>T in exon 6 [p.K211N] and c.924C>T in exon 7 [p.R275W]; heterozygous c.924C>T).

Genotyping analysis revealed that the two patients with the homozygous and heterozygous *PINK1* insertion, respectively, shared at least one allele at each of the six tested microsatellite and the four single-nucleotide polymorphism (SNP) markers at the *PARK6* locus. The carrier of the homozygous insertion (Case 1) was heterozygous at five microsatellite markers (Table 1a). The two carriers of the recurrent *Parkin* mutation (c.924C>T) had alleles in common at four of the five tested markers situated in intronic regions of the *Parkin* gene, including the rare '220 bp' allele at the marker D6S305 (Table 1b).

None of the patients carried mutations in two different genes, and we did not find any mutations in *DJ-1* in our patient sample. Also, there was no gene dosage alteration in any of the genes tested in our patient cohort. The novel *PINK1* mutations were not present in 200 control chromosomes.

We detected one as yet undescribed *PINK1* polymorphism (c.344A/T), which results in an amino-acid change (p.Q115L) and is present in six out of 100 (6%) screened control individuals. We also found four novel changes of unknown pathogenic significance (–21G/A; IVS1 + 97A/G;

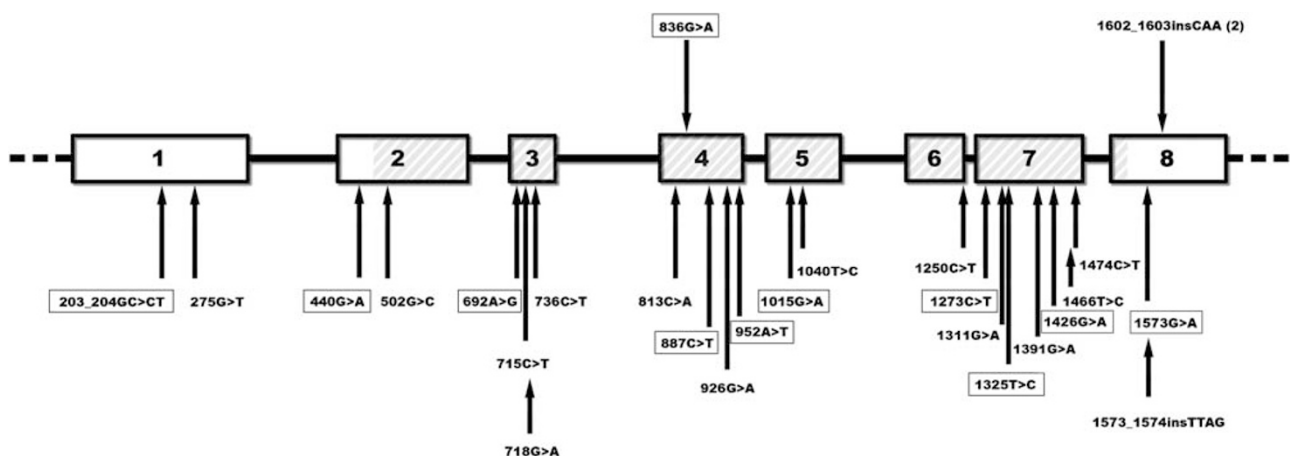


Figure 1 Summary of mutations in the *PINK1* gene. A schematic drawing of the eight exons of the *PINK1* gene and introns is shown and parts encoding the Ser/Thr protein kinase domain (amino acid 156–509) are hatched. The novel mutations that we found in our sample are indicated above the scheme. The 1602_1603insCAA insertion was found in two patients (once in the homozygous and once in the heterozygous state). The positions of all presently known mutations are marked with black arrows below the scheme of the gene. Mutations detected in the (single) heterozygous state only are boxed.

IVS3+38_40delTTT; c.852C/T) (Table 3). Further, 11 known *PINK1* polymorphisms were found.

The patient with the heterozygous c.836G>A mutation in exon 4 of *PINK1* also carried the novel c.852C/T substitution (Table 3) that was situated on the same chromosome, 16 bp downstream of the c.836G>A mutation (Figure 2).

Discussion

This is the first investigation that comprehensively tested for mutations in all three known genes associated with recessive EOP by employing both conventional methods (SSCP/dHPLC/sequencing) and gene dosage analysis of all coding exons.

Inclusion of PD patients was solely based on the age of onset ≤ 51 years, regardless of family history, that was negative in the vast majority of our cases. Only one of the five mutation carriers (heterozygous mutation in *PINK1*) reported that his father had had late-onset, bilateral arm tremor starting at the age of 70 years. No other cases of (possible) parkinsonism were reported in any of the families of our five mutation carriers. In particular, the parents of the carrier of the homozygous *PINK1* insertion died at ages 68 and 80 years. Since they had not undergone a detailed neurological examination, it remains unknown whether they had had any (mild) parkinsonian signs. Even if they had been unaffected, this could have been explained by reduced penetrance.

Ages of onset of the heterozygous *PINK1* mutation carriers included the youngest (25 years) and one of the oldest ages of onset (49 years) of our patient cohort. It is curious that the heterozygous carrier of the trinucleotide insertion in *PINK1* has an earlier age of onset (25 years) than the homozygous carrier of the same mutation (32 years). Thus, in our three *PINK1* mutation carriers, we could not confirm a potential correlation of the number of mutations and age of onset, as suggested in a previous study.¹⁰ Nevertheless, it is possible that additional genetic and/or environmental factors contribute to the early age of onset. The homozygous carrier first presented with foot

dystonia, not supporting the notion that lack of dystonia may be a distinct finding in patients with *PINK1* mutations.¹¹ As described previously,^{10–14} disease progression was relatively slow in both *PINK1* cases with long-term follow-up. Two of the three patients developed depression and autonomic dysfunction. Mild hyperreflexia was present in one case, and sleep benefit in another patient. The two patients with *Parkin* mutations did not obviously differ clinically from the *PINK1* mutation carriers; dystonia was present in one of the *Parkin* mutation carriers, whereas the other developed depression and sexual dysfunction in addition to the parkinsonian motor signs. All five mutation carriers identified in our study were not clinically different from the remainder of the study cohort. Our findings and the first case descriptions from the literature suggest that patients with *Parkin*, *PINK1*, and also *DJ-1* mutations may be clinically indistinguishable not only from one another but also from age-matched patients not carrying mutations in any of the three genes.

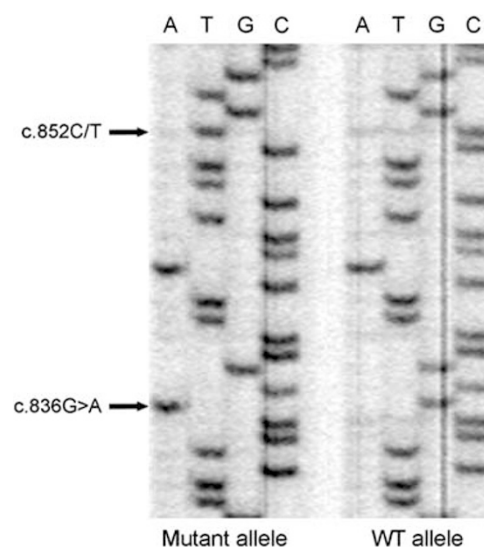


Figure 2 Sequence analysis of clones containing PCR fragments of the mutated (left) and of the wild-type allele (right) of Case 3 demonstrating that both sequence changes (arrows) are localized on the same chromosome.

Table 3 Novel changes detected in the *PINK1* gene

Nucleic acid level	Position	Protein level	Number (%) of homozygous carriers among 65 patients	Number (%) of heterozygous carriers among 65 patients
–21G/A	5'UTR	—	0 (0)	1 (1.5)
c.344A/T	Exon 1	p.Q115L	0 (0)	3 (4.6)
IVS1+97G/A	Intron 1	—	0 (0)	2 (3.1)
IVS3+38_40delTTT	Intron 3	—	0 (0)	1 (1.5)
c.836G>A	Exon 4	p.R279H	0 (0)	1 (1.5)
c.852C/T	Exon 4	p.S284S	0 (0)	1 (1.5)
c.1602_1603insCAA	Exon 8	p.Q534_S535insQ	1 (1.5)	1 (1.5)

Potentially pathogenic mutations are bolded

Both *PINK1* mutations (the point mutation and the 3 bp insertion) were novel, whereas both *Parkin* mutations had been reported before. There was no genealogical evidence that the patients with recurrent mutations were related. However, although chromosomal phase could not be set with certainty, haplotype analysis of the two carriers of the c.1602_1603insCAA mutation would be compatible with a shared allele. Interestingly, however, the homozygous mutation carrier appeared to have two different alleles, suggesting that this mutation has arisen at least twice independently. Alternatively, the insertion may be ancient enough for two recombination events to have occurred delineating the 2.13-cM shared *PINK1* and flanking region (Table 1a). A common founder was also conceivable for both carriers of the recurrent *Parkin* mutation since they shared a rare allele suggestive of a European founder haplotype associated with this mutation.²⁴

Both *PINK1* mutations are likely to have a pathogenic role. The arginine to histidine substitution at position 279 in *PINK1* is located in the functional Ser/Thr protein kinase domain of the protein. Out of the 24 previously described mutations, 19 are localized in this domain (Figure 1). In addition, the arginine is conserved throughout all species for which data are available in public databases. The role of the silent change (c.852C/T; p.S284S) detected on the same chromosome and only 16 nucleotides away from the c.836G>A (p.R279H) mutation currently remains elusive. It is possible that it is nonpathogenic or that the change leads to abnormalities in splicing. Unfortunately, no RNA was available to test for this possibility. One may speculate that the occurrence of these two changes, so close to one another, may be somehow related.

The p.534_535insQ mutation is located very close to the C-terminus of *PINK1* and causes the incorporation of an additional glutamine after two glutamines encoded by another two CAA triplets. This change was not found in any of the control chromosomes. Interestingly, a homozygous mutation in the C-terminus (c.1573_1574insTTAG) has recently been described in a familial EOP case with a similar age of onset at 28 years.¹² Another example of a disease-causing, in-frame mutation close to the end of the coding sequence is the GAG deletion in the *DYT1* gene that is associated with early-onset torsion dystonia.²⁵

Both detected *Parkin* mutations (p.L211N and p.R275W) have been previously described in several EOP patients and were thus considered pathogenic.²⁴ The latter point mutation within RING-finger 1 was shown to be the most prevalent mutation among Europeans and probably results from a founder effect.²⁴ It was reported to produce an unusual distribution of the Parkin protein, with large cytoplasmic and nuclear inclusions.²⁶

The rate of *PINK1* mutations in EOP is currently largely unknown, but estimated to be almost as high as the frequency of *Parkin* mutations based on a single study that reported seven *PINK1* mutation carriers among 100 EOP

patients, including five with heterozygous mutations.¹⁰ Taking a different approach, a second investigation evaluated the role of *PINK1* mutations in a set of 39 families with autosomal recessively inherited EOP and detected homozygous *PINK1* mutations in six out of the eight EOP families that were shown to be linked to the *PINK1* chromosomal region.¹¹ One of the most recent studies identified only one heterozygous carrier among 290 unrelated PD patients in Ireland.¹³ This last result could be explained by the low number of EOP cases (approximately one-third) included in this study, but could also indicate that the *PINK1* mutational rate is ethnically dependent. Furthermore, the analysis of a large cohort of patients from North America (consisting of more than 55% EOP patients) revealed a much larger number of mutation carriers.¹⁴ Our study design and the results are compatible with those from the recently published study on Italian EOP patients¹⁰ and suggest that the frequency of *PINK1* mutations may be comparable to that of *Parkin* mutations in patients with mainly sporadic EOP, at least in some populations.

The prevalence of detected *Parkin* mutations was low but still in keeping with previous studies on ethnically homogeneous patient samples,^{8,18} particularly in sporadic patients. Technical reasons explaining the low mutation rate in the present sample are unlikely, since we employed a comprehensive genetic screening method. This technique was previously demonstrated to detect accurately *Parkin* mutations in a large number of patients at much higher frequencies.^{6,27,28} With regard to the *DJ-1* gene, recent studies demonstrated a mutation frequency between 1 and 2% among EOP patients.^{7,8,15,16} Only two of the published investigations included testing for gene dosage alterations and, as expected, revealed higher mutation rates (2 *versus* 1%).^{7,8} However, based on those previous investigations, one would only expect one *DJ-1* mutation carrier in our 65 study subjects, and the present study confirms the notion that *DJ-1* mutations are a rare cause of EOP.

Despite extensive mutational analysis, a considerable percentage of EOP cases have been shown to carry a single heterozygous mutation in the *Parkin*, *DJ-1*, and *PINK1* gene.^{10,14,16,29–31} In addition, preclinical changes have been detected in asymptomatic carriers of heterozygous *Parkin* mutations using positron emission tomography (PET)^{32,33} or transcranial ultrasound.³⁴ Interestingly, haploinsufficiency or a dominant-negative effect have been suggested for heterozygous *PINK1* mutations even prior to the identification of the *PARK6*-related gene: asymptomatic, heterozygous carriers of the *PARK6*-associated haplotype revealed a significant reduction in caudate (18)F-dopa uptake in a PET study in comparison with controls.³⁵ However, the potential pathogenic role of heterozygous mutations in 'recessive genes' remains a matter of vivid debate. There are several potential ways to address this important issue: (i) comparison of the

frequency of single heterozygous mutations among ethnically matched cases and controls as recently described for *Parkin*³⁶ and *PINK1*.^{10,14} *Parkin* mutation frequencies were similar between patients and controls,³⁶ whereas *PINK1* mutations were much rarer in controls than in patients.^{10,14} However, it may be argued that at least some of the controls may not have reached the age of onset yet and have not undergone the same rigorous neurological examination as the cases, so that mild parkinsonian signs may have been present in affected but asymptomatic controls. (ii) (Prospective) examination of heterozygous offspring of homozygous or compound heterozygous mutation carriers, in whom it is much more unlikely that a second mutation may have been overlooked. (iii) Functional studies of the protein. Haploinsufficiency or a dominant effect could be one explanation why we were unable to find a second mutation in two of our *PINK1* mutation carriers and in one of the two *Parkin* mutation carriers (assuming that the second case is compound heterozygous for his *Parkin* mutations). Alternatively, a second mutation may be localized in an area of the gene that was not screened, such as the promoter or intronic regions.

The finding of *PINK1* (and *Parkin*) mutations in apparently sporadic EOP patients and the relatively high rate of *PINK1* mutation carriers (Valente *et al*¹⁰ and present study) suggest that *PINK1* mutations are an important cause to be considered in EOP patients, even in cases with a negative family history. Although genotyping a set of tagging SNPs in a cohort of PD patients did not provide evidence for the existence of a common functional variant influencing PD risk in late-onset disease,³⁷ the frequency of *PINK1* mutations in 'idiopathic', late-onset PD remains to be established. Similarly, frequency and type of *PINK1* mutations need to be determined in PD patients of different ethnic background.

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