PINK1 Mutations Are Associated with Sporadic Early-Onset Parkinsonism

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We have recently reported homozygous mutations in the *PINK1* gene in three consanguineous families with early-onset parkinsonism (EOP) linked to the PARK6 locus. To further evaluate the pathogenic role of PINK1 in EOP and to draw genotype–phenotype correlates, we performed PINK1 mutation analysis in a cohort of Italian EOP patients, mostly sporadic, with onset younger than 50 years of age. Seven of 100 patients carried missense mutations in PINK1. Two patients had two PINK1 mutations, whereas in five patients only one mutation was identified. Age at onset was in the fourth-fifth decade (range, 37–47 years). The clinical picture was characterized by a typical parkinsonian phenotype with asymmetric onset and rare occurrence of atypical features. Slow progression and excellent response to levodopa were observed in all subject. Two of 200 healthy control individuals also carried one heterozygous missense mutation. The identification of a higher number of patients (5%) than controls (1%) carrying a single heterozygous mutation, along with previous positron emission tomography studies demonstrating a preclinical nigrostriatal dysfunction in PARK6 carriers, supports the hypothesis that haploinsufficiency of PINK1, as well as of other EOP genes, may represent a susceptibility factor toward parkinsonism. However, the pathogenetic significance of heterozygous PINK1 mutations still remains to be clarified.

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Parkinson's disease (PD) is one of the commonest neurodegenerative diseases, with age-related prevalence reaching 2% in subjects older than 65 years of age. The clinical phenotypic core (resting tremor, rigidity, bradykinesia, and postural instability) is caused by striatal denervation consequent to massive loss of dopaminergic neurons in the pars compacta of the substantia nigra. The identification of several genes responsible for mendelian forms of PD has brought important insights into the molecular mechanisms leading to neurodegeneration. Whereas dominant forms of PD are rare, autosomal recessive inheritance is relatively frequent especially among patients with early-onset parkinsonism (EOP).[1] The phenotype of EOP can be indistinguishable from sporadic, late-onset classic PD, yet peculiar features can be present, such as dystonia at onset, hyperreflexia, psychiatric disturbances, and long-lasting excellent response to L-dopa with early occurrence of L-dopa–induced dyskinesias.[2]

The Parkin gene is estimated to be responsible for up to 50% of familial cases and 10 to 15% of sporadic cases of EOP.[3] Many distinct mutations have been reported in diverse ethnic groups, with a clear negative correlation between mutation frequency and age of onset.[3–9] A second autosomal recessive gene, DJ-1, is less important than Parkin in terms of prevalence, because only eight mutations were identified in more than 500 patients with familial and sporadic EOP, with mutation frequency not exceeding 1%.[10–15]

In a considerable number of Parkin-positive and DJ-1–positive cases, only a single mutation in heterozygous state could be found despite extensive mutation screening. Although the existence of a second undetected mutation cannot be confidently ruled out, these findings suggest that haploinsufficiency of one of these genes could represent a risk factor to develop the disease, possibly in conjunction with other, as yet unidentified genetic or environmental factors.

We have recently identified *PINK1*, a third gene responsible for autosomal recessive EOP in three consanguineous families.[16] The clinical presentation in affected family members was closely overlapping that of

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late-onset idiopathic PD, with low incidence of peculiar features.[17] To further evaluate the pathogenic role of PINK1 in EOP and to draw genotype–phenotype correlations, we have performed PINK1 mutation analysis in a cohort of Italian patients with sporadic and familiar parkinsonism with onset at younger than 50 years of age.

Patients and Methods

Patients and Controls

One hundred consecutive Italian patients with EOP (age of onset, 18–49 years) were selected from the clinical database of the Movement Disorder Centre of the Catholic University, Rome. All patients were examined by a senior neurologist (A.A., A.R.B.), and the diagnosis of clinically definite parkinsonism was based on published criteria.[18] Patients with evidence of secondary parkinsonism or with atypical features such as early dementia, ophthalmoplegia, early autonomic failure, and pyramidal signs were not included in the study. All subjects signed a written informed consent. Two hundred unrelated healthy controls of Italian origin (age range, 20–70 years) were fully sequenced to assess the frequency of PINK1 nucleotide changes in the Italian population.

Ninety patients were sporadic, whereas 10 probands had at least one first-degree relative affected. Among these, five had a family history compatible with autosomal recessive inheritance, and the remaining five had one affected parent. Only two patients were aware of parental consanguinity.

Conventional Mutational Analysis

DNA was extracted from peripheral blood using standard protocols. The eight exons of PINK1 and exon–intron junctions were polymerase chain reaction (PCR)–amplified as previously described.[16] PCR fragments then were sequenced in both forward and reverse directions using the Big Dye Terminator chemistry and an ABI 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Gene Dosage Studies

To evaluate the presence of heterozygous exonic deletions or multiplications in the five patients and two controls in which only one heterozygous mutation could be detected (see Results), we developed a quantitative PCR-based exon dosage assay for each of the eight exons of PINK1, using the TaqMan chemistry and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Gene dosage analysis also was performed in an additional sample of 15 EOP patients with clinical presentation similar to PINK1-positive cases (age of onset between 37 and 47 years, slow progression with excellent response to L-dopa therapy, absence of atypical features), in which direct sequencing already had excluded point mutations, small deletions and homozygous exon deletions, as well as sequence mismatches within the annealing sites of the primers and probes used for quantitative studies.

We used the comparative C_t method ($\Delta\Delta C_t$) to obtain a quantitative measure of the target product.[19,20] A control gene (RNAse P) was coamplified with each PINK1 exon and served as internal standard. Exonic primers and a fluorescently labeled probe for each PINK1 exon were obtained ready-made as "Assays by Design" (Applied Biosystems) and are available upon request. RNAse P primers and probe and TaqMan PCR core reagent kit were from Applied Biosystems. A DNA from a healthy individual was fully sequenced to exclude sequence changes within the primers and probe annealing sites of each exon and used as a negative control. All experiments were performed in triplicate and then averaged. The use of an internal control within each reaction provides a relative ratio of starting copy numbers of PINK1/starting copy numbers of RNAse P ($2^{-\Delta\Delta Ct}$), which normally is expected to be around 1 (0.80–1.30). A ratio of 0.45 to 0.65 suggests an heterozygous exonic deletion, whereas a ratio higher than 1.4 is found in exon multiplications. Because no positive controls were available, we tested the sensitivity of the technique to detect mismatches even of a single base pair within the primers or probe annealing site, resulting in loss of signal and therefore in a ratio indicating a false-positive deletion.[4,14] We could identify one heterozygous mutation (203_204GC→CT) and one heterozygous polymorphism (G1018A) falling within the forward primer annealing site of exons 1 and 5, respectively. Quantitative real-time PCR in DNA samples carrying these mismatches produced false-positive ratios of 0.53 and 0.59 (Fig, A, B).

Results

Two of 100 patients carried two mutations in the *PINK1* gene. Both cases were sporadic. None of the 200 control individuals carried two PINK1 mutations.

Patient GE910 was a 53-year-old man carrying a homozygous G502C mutation (Ala168Pro). He denied any parental consanguinity; still both parents originated from the same village in central Italy. His parkinsonian syndrome started at the age of 39 years with gait impairment due to akinesia. On the latest examination, after 14 years of disease, he presented a typical parkinsonian picture with excellent response to dopaminergic treatment. In the *on* state, parkinsonian signs were mild (United Parkinson's Disease Rating Scale III motor section, 6), with appreciable right-hand side prevalence. A bilateral (albeit asymmetrical) severe parkinsonian phenotype with freezing became evident after treatment withdrawal. In the last 6 months, the patient reported the occurrence of mild dystonic dyskinesias. Urinary urgency and mild orthostatic hypotension were reported in absence of other disautonomic features. Peculiar features of EOP such as dystonia at onset, early or severe L-dopa–induced dyskinesias, and deep tendon hyperreflexia were not present.

Patient GE735 was a 60-year-old woman, compound heterozygote for missense mutations G275T (Cys92Phe) and G1391A (Arg464His). The presenting sign was resting tremor of the right upper limb at age 37 years. Progression was slow and response to L-dopa sustained. L-Dopa–induced dyskinesias occurred after 10 years of treatment. On the latest clinical examination, 23 years after onset, she presented a typical, fully

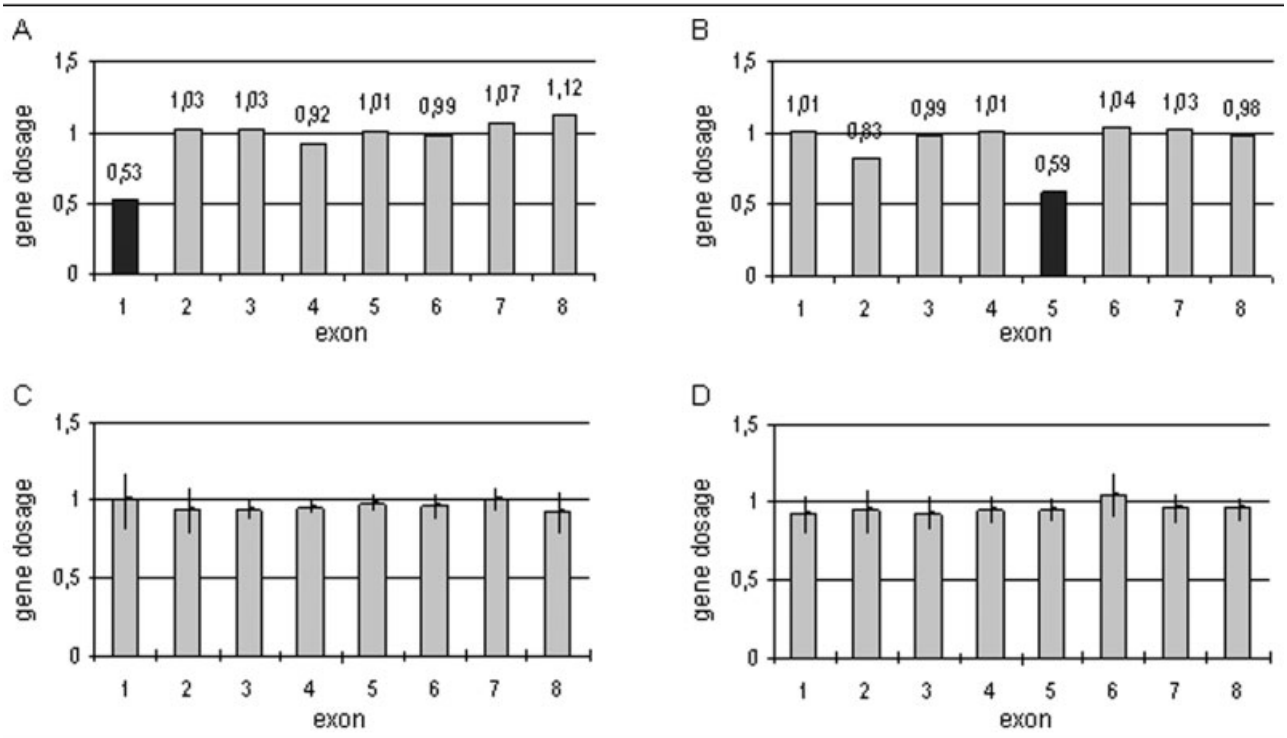

Fig. Gene dosage analysis of PINK1. (A, B) Results for Patient GE1111, carrying the 203_204GC→CT heterozygous mutation in exon 1, and for Patient GE469, carrying the G1018A heterozygous polymorphism in exon 5. These two nucleotidic changes fall within the forward primers used for the quantitative assay of exons 1 and 5, respectively, generating false-positive ratios below 0.65 (black bars). *(C) Mean dosage analysis of five patients and two controls carrying one heterozygous PINK1 missense mutation. For exon 1, GE1111 was excluded from mean calculation (see A). (D) Mean dosage analysis of 15 EOP patients (see Patients and Methods). Error bars indicate standard deviation.*

symptomatic phenotype and mild orthostatic hypotension. United Parkinson's Disease Rating Scale III motor section in *on* state was 37.

Five sporadic cases of 100 patients and 2 of 200 controls (aged 50 and 65 years) carried heterozygous missense changes in the PINK1 gene (Table 1). Quantitative real-time PCR assay in these seven individuals failed to show heterozygous exon rearrangements. These also were excluded in a sample of 15 EOP subjects who had tested negative by direct sequencing (see Fig, C, D).

The five patients carrying a heterozygous missense PINK1 mutation had a typical parkinsonian phenotype with asymmetric onset in the upper or lower limb. Mean age at onset was 44.0 ± 4.2 years (range, 37–47). The presenting sign was bradykinesia in three cases and resting tremor in the others. Only one patient reported dystonia at onset. Progression was slow in all cases with good to excellent response to L-dopa, and all patients developed L-dopa–induced dyskinesias. Hyperreflexia was present in two cases. Three patients presented mild mood disturbances (depression and anxiety). One patient, who underwent two surgical procedures for implanting thalamic and then subthalamic deep brain stimulation, developed imbalance, gait impairment, dysarthria, and behavioral changes at the age of 54 years. Mental deterioration was documented a few years later.

We also identified several novel exonic and intronic polymorphic variants in PINK1 (Table 2). Three novel exonic substitutions resulted in silent changes (Leu63Leu, Gln355Gln, and Ser365Ser). Each of these variants was found only in one patient (with the exception of Ser365Ser, found in two cases) and was not present in any of the control chromosomes. It is still unclear whether these changes represent rare polymorphisms or alternatively could bear a pathogenic role, for instance, altering mRNA processing or splicing. Unfortunately, neither RNA from these patients nor parents were available for further studies.

Discussion

We recently observed that PINK1 is responsible for autosomal recessive EOP in three large consanguineous families linked to the PARK6 locus.[16] Here, we demonstrate that mutations in PINK1 are also found in sporadic EOP. We identified PINK1 mutations in 7 sporadic cases of 100 patients with onset below 50 years of age. Two patients (2%) carried two PINK1 mutations, whereas in five cases (5%) only one het-

Table 1. Heterozygous Missense Mutations Found in the PINK1 Gene in Patients and Controls

exon	nucleotide change	aa/codon	patients (n = 100)	controls (n = 200)
1	203_204GC>CT	Arg68Pro	1	0
4	C887T	Pro296Leu	0	1
7	T1325C	Ile442Thr	2	0
7	G1426A	Glu476Lys	1	0
8	G1573A	Asp525Asn	1	1
total			5 (5%)	2 (1%)

erozygous mutation could be identified. A single heterozygous mutations was also found in 2 of 200 healthy controls (1%). In these seven individuals, extensive sequencing of the coding region and splice sites of the gene and exon dosage analysis failed to detect a second mutation, although the presence of a second undetected intronic or promoter mutation cannot be ruled out with certainty.

The pathogenic significance of a single heterozygous PINK1 mutation remains to be clarified. Although statistical comparisons are constrained by the limited number of patients and controls so far analyzed, the frequency of heterozygous mutations appears to be higher in EOP patients than in healthy controls, raising the intriguing hypothesis that heterozygous PINK1 mutations could represent a risk factor to develop parkinsonism, in the frame of a certain genetic and environmental background. This hypothesis is supported by several lines of evidence. First, in the two Italian families with PINK1 mutations, several healthy individuals were heterozygous carriers of the Trp437OPA mutation. However, the youngest sister of the two affected siblings of Family IT-GR (IV:9, Figure 2 in reference 17), who carries the Trp437OPA mutation in heterozygous state, developed at the age of 38 years a clinical picture of mild parkinsonian phenotype consisting in depression of mood, asymmetric arm rigidity, bradykinesia, and gait impairment. It is unlikely that this subject was carrier of a second unidentified mutation in PINK1, given the family history and close parental consanguinity. Second, several large studies have reported mutations in a single Parkin allele in up to 60% of Parkin-positive sporadic EOP cases, despite extensive mutation analysis of the gene, including gene dosage analysis and sequencing of the promoter region and of the cDNA.[5,6,8,9] Single heterozygote mutations in the DJ-1 gene have been found in four of the six patients so far described (66%), excluding the original consanguineous families reported by Bonifati and colleagues.[11,12,14] Third, positron emission tomography studies in Parkin and PINK1 patients with two mutated alleles and heterozygous carriers demonstrated for both genes a reduction of striatal ^{18}F-dopa uptake proportional to the number of mutated alleles, with heterozygous healthy carriers always showing a significant decrement of ^{18}F-dopa uptake compared with controls.[21–23] Taken together, these data suggest that, while the presence of two mutations in one EOP gene invariably leads to parkinsonism, a single mutant allele is likely to produce a subclinical dopaminergic dysfunction which other additional genetic or environmental factors may push over a threshold of clinical disease. This pathogenetic mechanism should always be taken into account when giving genetic counseling to EOP patients and their families.

Similar to Parkin-related EOP, the age of onset of PINK1-related parkinsonism is variable. Considering

Table 2. Polymorphisms Found in the PINK1 Gene

			EOP (n = 100)		controls (n = 200)	
exon	nucleotide change	aa/codon	het %	hom %	het %	hom %
1	**C189T**	**Leu63Leu**	**20**	**4**	**32.5**	**5**
2	IVS1 −7 A>G	—	18	7	15.5	6.5
5	IVS4 −5 G>A	—	21	4	18	3
5	G1018A	Ala340Thr	5	0	5	0
5	**A1065G**	**Gln355Gln**	**1**	**0**	**0**	**0**
5	**C1095T**	**Ser365Ser**	**2**	**0**	**0**	**0**
6	**IVS6 +9 T>C**	**—**	**1**	**0**	**0**	**0**
6	IVS6 +43 C>T	—	4	0	4.5	0
7	**IVS7 +14 C>G**	**—**	**1**	**0**	**0**	**0**
8	A1562C	Asn521Thr	29	3	34.5	4

Novel polymorphisms are in bold.

the three families that we already have described (n = 9)[16] and an additional six PINK1 families identified by Hatano and colleagues (n = 10),[24] the age of onset of patients with two PINK1 mutations (n = 21) is mostly in the third-fourth decade (mean ± standard deviation [SD], 36 ± 6.9), with only two patients with onset in the second decade (18 and 19 years) and two with onset in the fifth decade (45 and 48 years), respectively. The age of onset of patients with only one PINK1 mutation seems on average to be higher (range, 37–47 years; mean ± SD, 44 ± 4.2), although the few cases so far described do not allow statistical correlates to be drawn. Conversely, at difference from Parkin, the clinical presentation of PINK1-mutated cases seems to be fairly homogeneous resembling that of late-onset classic PD, with rare occurrence of atypical features such as dystonia at onset, diurnal fluctuations of symptoms, and hyperreflexia. A very slow progression of the disease with good and sustained response to L-dopa appears to be consistently related to the PINK1 phenotype, and L-dopa–induced dyskinesias are frequent particularly in those patients with higher on-state United Parkinson's Disease Rating Scale motor score. Screening of larger cohorts of EOP patients are required to fully establish the PINK1 phenotypic spectrum.

All PINK1 mutations identified in this study were missense changes affecting conserved residues of the protein. We could not find heterozygous exonic deletions or multiplications in any of the five patients or two controls carrying one heterozygous missense mutation, despite quantitative dosage assay of each exon. Moreover, no exon dosage alterations were found in an additional sample of 15 patients with a phenotype closely resembling that of PINK1-mutated patients, but in which direct sequencing failed to show pathogenic mutations. Our findings suggest that exon deletions or multiplications are not a frequent mechanism of PINK1 mutations, in contrast with what was observed for Parkin and DJ-1. However, these data need further confirmation in larger series of EOP patients.

The *PINK1* gene encodes a mitochondrial protein with putative kinase activity. The predicted kinase domain spans 354 highly conserved amino acids in eukaryotic homologs (amino acids 156–509) and shows high degree of homology with the serine-threonine kinases of the Ca^{2+}/calmodulin family. Preliminary data show that PINK1 could play a major role in protecting cells against stress conditions and mitochondrial dysfunction.[16] Most mutations identified by us and by Hatano and colleagues[24] fall within the kinase domain of PINK1, likely affecting either kinase activity or substrate recognition. These findings support the hypothesis that altered phosphorylation of target proteins could represent a key pathogenetic mechanism leading to abnormal stress response and neurodegeneration in PINK1-mutated patients. The identification of PINK1 substrates and the effect of PINK1 mutations on its physiological activity undoubtedly will give important insights into the molecular mechanisms leading to cell death in PD.

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