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ARTICLE

Comprehensive analysis of the *LRRK2* gene in sixty families with Parkinson's disease

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Mutations in the gene *leucine-rich repeat kinase 2* (*LRRK2*) have been recently identified in families with Parkinson's disease (PD). However, the prevalence and nature of *LRRK2* mutations, the polymorphism content of the gene, and the associated phenotypes remain poorly understood. We performed a comprehensive study of this gene in a large sample of families with Parkinson's disease compatible with autosomal dominant inheritance (ADPD). The full-length open reading frame and splice sites of the *LRRK2* gene (51 exons) were studied by genomic sequencing in 60 probands with ADPD (83% Italian). Pathogenic mutations were identified in six probands (10%): the heterozygous p.G2019S mutation in four (6.6%), and the heterozygous p.R1441C mutation in two (3.4%) probands. A further proband carried the heterozygous

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p.I1371 V mutation, for which a pathogenic role could not be established with certainty. In total, 13 novel disease-unrelated variants and three intronic changes of uncertain significance were also characterized. The phenotype associated with *LRRK2* pathogenic mutations is the one of typical PD, but with a broad range of onset ages (mean 55.2, range 38–68 years) and, in some cases, slow disease progression. On the basis of the comprehensive study in a large sample, we conclude that pathogenic *LRRK2* mutations are frequent in ADPD, and they cluster in the C-terminal half of the encoded protein. These data have implications both for understanding the molecular mechanisms of PD, and for directing the genetic screening in clinical practice.

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Introduction

In most patients Parkinson's disease (PD) (MIM #168600) is a sporadic condition of unknown causes. However, in some cases the disease is inherited as a highly penetrant Mendelian trait, and the identification of families with monogenic forms of PD has been determinant for the recent progress in the understanding of the molecular mechanisms.[1,2] Mutations in five genes have been firmly implicated in the aetiology of PD. Mutations in the *SNCA*[3,4] gene, encoding the α-synuclein protein, cause autosomal dominant forms, whereas mutations in the *PARK2*,[5], *PARK7*,[6] and *PINK1*,[7] gene, encoding the parkin, DJ-1, and PINK1 protein, respectively, cause autosomal recessive forms. Additional loci for mendelian and more complex forms have been mapped, but the defective genes have not been identified yet.[1]

A different locus, PARK8 (MIM #607 060), was first mapped to chromosome 12 in a Japanese family with dominantly inherited parkinsonism.[8] Recently, mutations in the gene *leucine-rich repeat kinase 2* (*LRRK2*) (MIM *609 007) have been identified in PARK8-linked families.[9,10] The *LRRK2* gene encodes a predicted protein of 2527 amino acids, which has an unknown function. The LRRK2 protein, also termed dardarin, belongs to the ROCO group within the Ras/GTPase superfamily, characterized by the presence of several conserved domains: a Roc (Ras in complex proteins) and a COR (C-terminal of Roc) domain, together with a leucine-rich repeat region, a WD40 domain, and a protein kinase catalytic domain.[11]

To date, five *LRRK2* missense mutations associated with autosomal dominant PD (p.R1441C, p.R1441G, p.Y1699C, p.G2019S, and p.I2020T)[9,10,12–15] are considered definitely pathogenic on the basis of clear cosegregation with disease in large pedigrees and absence in controls. The evidence for cosegregation with PD is limited for another two mutations found in small families (p.L1114L and p.I1122 V),[9,16] whereas it is lacking for four additional mutations because DNA from relatives was unavailable (p.I1371 V and p.R1441 H),[17,18] or because the mutation was identified in single sporadic PD cases (IVS31 + 3A > G and p.M1869 T);[16,17] the pathogenic role of these last six mutations remains therefore uncertain.

With the exception of 34 ADPD families included in one of the original cloning papers,[9] in all the previous reports small numbers of families (from 2 to 23) were studied for all the 51 *LRRK2* exons;[12–15,18] most studies have instead screened large PD samples for only single or few mutations.[10,12,14–24]. Therefore, the prevalence and nature of *LRRK2* mutations, and the polymorphism content of this large gene remain poorly understood. Furthermore, since dardarin is a large protein with multiple functional domains, mutations in specific regions might result in different phenotypes. Genotype–phenotype correlation analyses are therefore warranted. We report here a comprehensive analysis of the *LRRK2* gene and its associated phenotypes in a large sample of ADPD families.

Materials and methods

We studied 60 PD families compatible with autosomal dominant inheritance (two or more PD cases in at least two consecutive generations, ADPD), consecutively collected at several PD clinical referral centers. Of the families, 50 were from Italy, nine from Brazil, and one from Portugal.

In all, 35 families contained each three or more members affected by PD, while the remaining 25 families had two individuals with PD. The mean age at disease onset in the probands was 49.2 years (range 28–75). Pathological studies could not be performed.

The clinical diagnosis of definite PD required: the presence of bradykinesia and at least one of the following: resting tremor, rigidity and postural instability; a positive response to dopaminergic therapy; the absence of atypical features or other causes of parkinsonism.[25] Neurological examination included the Unified Parkinson's Disease Rating Scale (UPDRS, motor part)[26] and Hoehn-Yahr staging. The project was approved from the relevant ethical authorities, and written informed consent was obtained from all subjects.

Genomic DNA was isolated from peripheral blood using standard protocols. In the probands from the 60 ADPD families, the whole coding sequence and exon–intron boundaries of the *LRRK2* gene were studied by polymerase chain reaction (PCR) using previously described primers and PCR conditions.[12] For exons 6, 22, 31, 38 and 49, we designed new primers (Supplementary Table S1). Direct sequencing of both strands was performed using Big Dye Terminator chemistry ver.3.1 (Applied Biosystems). Fragments were loaded on an ABI3100 and analysed with DNA Sequencing Analysis (ver.3.7) and SeqScape (ver.2.1) software (Applied Biosystems). The consequences of mutations at the protein level were predicted according to the *LRRK2* cDNA sequence deposited in Genbank (accession number AY792511). Novel variants identified in patients were tested by direct sequencing in a panel of at least 100 chromosomes from healthy Italian subjects aged more than 50 years.

For haplotype analysis in carriers of one of the *LRRK2* mutations (p.R1441C), we typed intragenic and flanking markers (microsatellites and single nucleotide polymorphisms, SNPs). Microsatellites were amplified by PCR using fluorescently labelled F-primers according to standard methods; fragments were loaded on an ABI3100 and analysed using the GeneMapper ver.3.0 software (Applied Biosystems). Exonic and intronic *LRRK2* SNPs were typed by direct sequencing using the primers and PCR conditions described above. Haplotypes were constructed manually. We included in the haplotype analysis the two families with the p.R1441C mutation detected in this study, a further PD family carrying this mutation detected by us in a different sample set,[27] as well as family 'D' (from Western Nebraska) and family '469', in which the p.R1441C mutation was initially identified.[9] The phase could be assigned unambiguously in family 'D' by typing a trio of parents/child.

For *in silico* analysis of dardarin, the closest homologues of the human protein were identified using the program BLASTP, and aligned using the ClustalW program.

Results

Genetic studies

The results of the genetic studies are summarized in the Figures 1–2 and in the Tables 1–2.

We identified four heterozygous carriers of an exon 41 mutation, c.6055G>A (p.G2019S), two heterozygous carriers of a exon 31 mutation, c.4321C>T (p.R1441C), and one heterozygous carrier of a exon 29 mutation, c.4111A>G (p.I1371V). Two families carrying the p.G2019S mutation originated from Italy, one from Brazil and one from Portugal; the two families with the p.R1441C and the family with the p.I1371V mutation were from Italy.

Initial results concerning the four families with the p.G2019S mutation have been previously published by us,[12] whereas the other three families with *LRRK2* mutations as well as the results of the comprehensive analysis of the *LRRK2* gene in the entire sample of 60 ADPD probands are reported here for the first time.

The three *LRRK2* mutations detected in this study replace amino acids, which have been highly conserved among species (Figure 2d for the p.I1371V mutation). The p.G2019S and p.R1441C mutations were previously shown to be absent in more than 800 and 500 Italian control chromosomes, respectively.[27] On the contrary, one heterozygous carrier of the p.I1371V mutation was detected in this study among 416 Italian control chromosomes (allelic frequency 0.002).

The p.R1441C mutation was present in the proband of family PV-12 and PV-78 (Figure 2a and Supplementary Figure S1). Cosegregation with PD could be studied in family PV-12, while DNA was not available from relatives in family PV-78. The results of the haplotype analysis in patients with the p.R1441C mutation are reported in the Figure 2b (see discussion).

The proband of family MI-007 was heterozygous carrier of the p.I1371V mutation (Figure 2c and Supplementary Figure S1). The parents were both affected by PD, and the presence of the p.I1371V mutation was confirmed in the mother.

We also detected 16 novel sequence variants, 14 intronic and two exonic, and several known polymorphisms (Figure 1 and Tables 1–2). In all, 13 of the novel variants (including the two exonic variants p.P1542S and p.G2385G) were considered as neutral, disease unrelated changes, as they were observed with similar frequency in cases and controls, or they did not cosegregate with disease (Table 2). On the contrary, the allelic frequency of the novel intronic variant IVS30+12delT was higher in patients than in controls ($P<0.05$, Fisher Exact test), and another two intronic variants (IVS4-38A>G and IVS5+33T>C) were rarely observed in cases but absent in 200 control chromosomes; these variants could not be tested for cosegregation (Table 2), and their pathogenic role remains uncertain.

Clinical studies

The clinical features in the four families with the p.G2019S mutation have been published previously by us.[12] In the carriers of p.R1441C, age at disease onset ranged between 63 and 65 years, while the two patients with the p.I1371V mutation had onset at 33 and 61 years.

All treated patients responded well to levodopa. Asymmetric onset and complications typically associated with long-term treatment with levodopa (motor fluctuations and dyskinesias) were noted in some. Severe cognitive disturbances were observed only in one patient (carrying the p.I1371V mutation).

A rather slow progression of the parkinsonism was also noted in some cases, as also shown by the low UPDRS

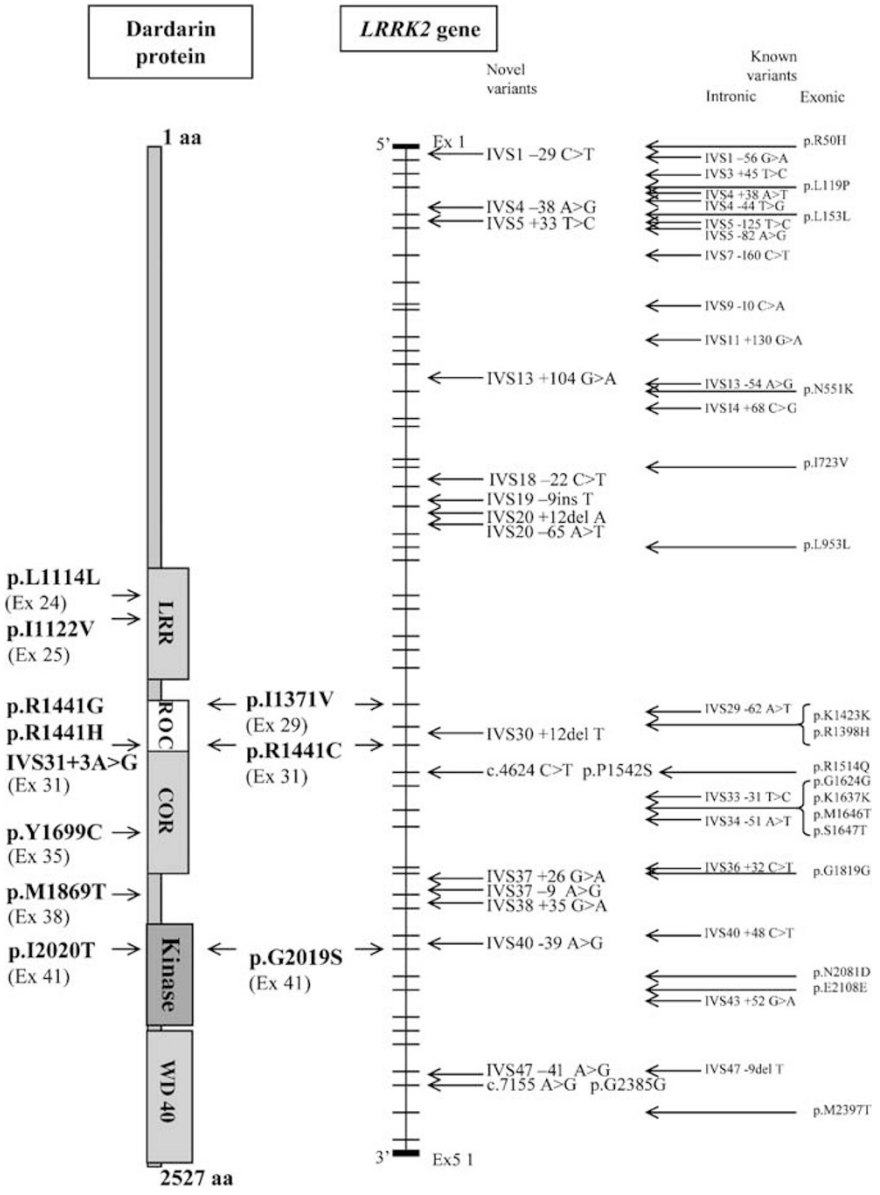


Figure 1 Schematic representation of the *LRRK2* gene, the dardarin protein and its known functional domains. Known and novel *LRRK2* polymorphisms are indicated on the right side of the gene. Mutations are indicated, those identified by us and by others, on the left and right side of the protein, respectively.

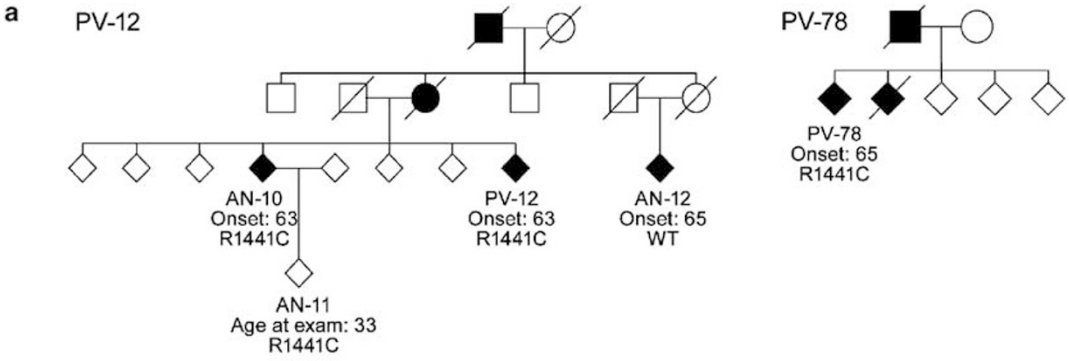
motor scores after many years of disease course. In the PV-78 proband, brain computerized tomography (CT) showed symmetric frontal atrophy. Additional clinical details are reported in Table 3.

Discussion

Frequency and nature of *LRRK2* mutations

To our knowledge, this is the first study which comprehensively analysed all the 51 exons and the exon–intron boundaries of the *LRRK2* gene in a large sample of 60 ADPD probands (mostly from Italy), revealing the presence of two recurrent pathogenic mutations, p. G2019S and p.R1441C, in six families (10% of the whole sample, 8% of the Italian sample), and a third mutation, p.I1371V, in another family. These frequencies are in substantial agreement with those reported in the only two previous studies of comparable size, which comprehensively screened the *LRRK2* gene, and found mutations in 3/23 and 6/34 families, respectively (13% and 17%).[9,18] ADPD represents a relevant fraction of the whole population of PD. According to the results of this

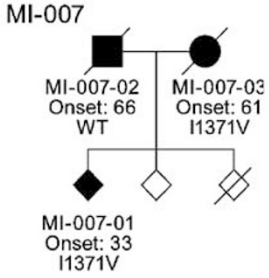
a



b

	Country	USA	USA	Italy	Italy	Italy
	Family	**D**	**469**	**PV-12**	**PV-78**	**PD-768**
Marker	Physical position					
D12S2514	38874 K	**308**	314/314	314/317	314/308	314/314
D12S2515	38974 K	**251**	235/239	235/243	235/239	235/235
D12S2516	38989 K	277/275	277/277	277/277	277/277	277/277
R1441C	38990 K	**t**	t	t	t	t
rs1896252	39000 K	**t**	c/c	c/c	c/c	c/t
rs1427263	39000 K	c/a	a/a	a/a	a/a	a/a
rs11176013	39000 K	**a**	g/g	g/g	g/g	g/a
rs11564148	39000 K	**t**	t/a	t/a	t/a	t/a
D12S2518	39034 K	**189**	176/176	176/176	176/198	176/176
D12S2519	39116 K	**156**	156/156	156/158	156/162	156/156
D12S2520	39120 K	**278**	278/278	278/278	278/278	278/281
D12S2521	39128 K	**394**	394/377	394/401	394/394	394/390
D12S2522	39132 K	**321**	321/321	321/323	321/317	321/321
D12S2523	39147 K	**342**	342/342	342/327	342/336	342/342
D12S2517	39282 K	**206**	206/210	206/212	206/229	206/204

c



d

homo sapiens	1358-	K K S D L G M Q S A T V G	I D V K D W P I Q I R D K R K - 1385
bos taurus	107-	K K S D L G M Q G A T V G	I D V K D W P I Q I R G K G K - 134
rattus norvegicus	1370-	K K S E L G M Q G A T V G	I D V R D W P I Q I R G K R K - 1397
mus musculus	1358-	K K P E L G M Q G A T V G	I D V R D W S I Q I R G K R R - 1385
gallus gallus	893-	K R T E L G C P K A T V G	I D V K D W I I Q G K G K M K - 910
tetraodon nigroviridans	1350-	K R S Q L K S K S T S V G	I D V Q D W T I K D W D R K K - 1376
trichodesmium erythraeum	439-	N - Y Q L K D E D T T K G	I E V H Q Y K F Q T K N Q - - - 463

and the previous studies,[9,18] *LRRK2* mutations are clearly the most frequent cause of PD known so far. None of the genes previously implicated in PD showed such a high frequency of involvement.[1,2] Yet, the frequency of *LRRK2* involvement may be still underestimated, since neither in this nor in any of the previous studies were the gene promoter or the UTR regions explored, or was the presence of genomic rearrangements investigated. In addition, some of the unclassified intronic variants may prove to be pathogenic. It will also be important to investigate whether *LRRK2* mutations show similar or different prevalence in different populations, because this has implications for the genetic counselling. For example, the p.G2019S mutation seems rare in Asian populations.[22]

The pathogenic role of the p.G2019S and p.R1441C mutations is well established on the basis of the absence in a large number of control chromosomes, cosegregation with disease, conservation and crucial structural position of the amino acids involved.[9,12–14,16–18]

The p.G2019S mutation was identified previously by us and other groups in ~3–6% of samples with familial PD (autosomal dominant families, and sib-pairs) from several European and North American countries, and even in ~1% of sporadic PD cases from the United Kingdom and Italy, while it was absent in more than 4000 control individuals.[12–14,16–20,27]. The presence of a shared haplotype in all the p.G2019S carriers from many populations strongly suggests that this mutation originated from an ancient founder.[14,27,28]

The p.R1441C mutation, present in two families in this study (3.4% of our ADPD panel), has been initially reported in one of the original *LRRK2* cloning papers in family 'D' and in the smaller family '469',[9] and later in two sporadic PD cases.[17,27] The results of our haplotype analysis (Figure 2b) are compatible with the presence of a founder effect in the Italian p.R1441C carriers and in family '469'. In family 'D', however, the disease haplotype differs for most markers (Figure 2b), and only a very short region might be shared with the other p.R1441C families.

Taken together, these findings suggest an independent origin of this mutation, or a very ancient founder. The occurrence of another two different mutations at the same codon (c.4321C>G, p.R1441G in Basque families,[10,21], and c.4322G>A, p.R1441H in a sporadic PD case[17]), is also in keeping with the presence of a mutational hot spot at this position.

Interestingly, one first cousin in family PV-12 was also affected by PD but did not carry the p.R1441C mutation. Phenocopies have previously been detected in other families with *LRRK2* mutations, including the p.R1441C and the p.G2019S mutation.[9,13,20] The frequent occurrence of phenocopies illustrates the complexity of genetic studies in aetiologically heterogeneous, highly prevalent diseases such as PD.

The pI1371V mutation was recently identified in one proband with familial PD from Eastern India.[18] However, cosegregation with PD in that family, and occurrence in ethnically matched controls, were not assessed in that study. We report here this mutation in two affected members of an Italian family, but also in one of 208 Italian controls. This control individual was 55 years old at the time of sampling, and he might be still at risk of developing PD. Further work, including case–control studies and functional analyses, might help clarifying whether the p.I1371V mutation is pathogenic.

All the *LRRK2* pathogenic mutations previously reported in PD are located between exon 24 and 41.[9,10,12–18] The results of this study confirm this pattern (mutations in exon 29, 31 and 41), suggesting that most of the pathogenic mutations cluster in a discrete, albeit large region of the gene, which encodes the ROC, COR, leucine-rich repeat and the kinase catalytic domains (Figure 1). This region plays therefore likely a critical role in the mechanism of *LRRK2*-related neurodegeneration.

LRRK2 polymorphisms

We excluded the pathogenic role of 13 novel exonic and intronic variants on the basis of a similar frequency in cases and controls, or of absence of cosegregation with disease (Tables 1-2). On the contrary, the allelic frequency of the intronic variant IVS30 + 12delT was higher in patients than in controls ($P<0.05$, Fisher Exact test), and two other intronic substitutions (IVS4 −38A>G, IVS5 +33T>C) were detected in patients but not in controls. These variants could not be studied for cosegregation with disease, and their significance in disease causation remains unclear. They might be in LD with other pathogenic variants located in other regions of the *LRRK2* gene, which were not screened in this study. *In silico* analysis (http://l25.itba.mi.cnr.it/~webgene/www.spliceview.html) showed that none of the intronic variants appear to significantly modify the recognition of the natural splice site. The IVS30 + 12delT variant, as well as other

Figure 2 (**a**) Simplified pedigrees of families carrying the p.R1441C mutation. Black symbols denote individuals affected by PD. Age at PD onset or age at examination is shown (years). To protect confidentiality, sex of individuals in the youngest generation has been disguised. WT: wild type genotype. (**b**) Haplotype analysis in families with the p.R1441C mutation. The minimum shared region is highlighted in gray. Clinical and genealogical data have been published previously about the PD-768 family,[27] and the ''D'' and ''469'' families[9,32]. (**c**) Simplified pedigree of family MI-007. (**d**) Conservation of the Isoleucine1371 residue (asterisk) in the dardarin homologues.

Table 1 *LRRK2* gene variants-detected in this study

Position	Ref. No.	Nucleotide change	Protein change	Frequency
Exon1	rs2256408	c.149G>A	p.R50H	A 1.00
Intron1		**IVS1-29C>T**		**T 0.008**
Intron1	rs2723273	IVS1-56G>A		A 1.00
Intron3	rs1352879	IVS3+45T>C		C 1.00
Exon4		c.356T>C	p.L119P*	C 0.016
Intron4	rs2131088	IVS4+38A>T		T 0.075
Intron4	rs2723270	IVS4-44T>G		G 0.042
Intron4		**IVS4-38A>G**		**G 0.008**
Exon5	rs10878245	c.578T>C	p.L153L	C 0.6
Intron5		**IVS5+33T>C**		**C 0.008**
Intron5	rs6581622	IVS5-125T>C		C 0.24
Intron5	rs11564187	IVS5-82A>G		G 0.05
Intron7	rs732374	IVS7-160C>T		T 0.325
Intron9	rs7955902	IVS9-10C>A		A 0.35
Intron11	rs7969677	IVS11+130G>A		A 0.183
Intron13	**ss#37042808**	**IVS13+104G>A**		**A 0.034**
Intron13	rs10784461	IVS13-54A>G		G 0.3
Exon14	rs7308720	c.1653C>G	p.N551K	G 0.025
Intron14	rs10784462	IVS14+68C>G		G 0.417
Exon18	rs10878307	c.2167A>G	p.I723V	G 0.1
Intron18		**IVS18-22C>T**		**T 0.058**
Intron19		**IVS19-9ins T**		**insT 0.45**
Intron20		**IVS20+12delA**		**delA 0.017**
Intron20		**IVS20-65A>T**		**T 0.008**
Exon22	rs7966550	c.2857T>C	p.L953L	C 0.134
Exon29		*c.4111A>G*	*p.I1371V*	*G 0.008*
Intron29	rs7305344	IVS29-62A>T		T 0.55
Exon30	rs7133914	c.4193G>A	p.R1398H	A 0.025
Exon30	rs11175964	c.4269G>A	p.K1423K	A 0.025
Intron30		**IVS30+12delT**		**delT 0.059**
Exon31		*c.4321C>T*	*p.R1441C*	*T 0.017*
Exon32		c.4541G>A	p.R1514Q*	A 0.008
Exon32		**c.4624C>T**	**p.P1542S**	**T 0.017**
Intron33	rs1896252	IVS33-31T>C		C 0.483
Exon34	rs1427263	c.4872C>A	p.G1624G	A 0.62
Exon34	rs11176013	c.4911A>G	p.K1637K	G 0.541
Exon34		c.4937T>C	p.M1646T*	C 0.025
Exon34	rs11564148	c.4939T>A	p.S1647T	A 0.241
Intron34	rs10878368	IVS34-51A>T		T 0.51
Intron36	rs7137665	IVS36+32C>T		T 0.6
Exon37	rs10878371	c.5457T>C	p.G1819G	C 0.508
Intron37		**IVS37+26G>A**		**A 0.008**
Intron37		**IVS37-9A>G**		**G 0.008**
Intron38		**IVS38+35G>A**		**A 0.059**
Intron40	rs2404834	IVS40+48C>T		T 0.1
Intron40		**IVS40-39A>G**		**G 0.008**
Exon41		*c.6055G>A*	*p.G2019S*	*A 0.034*
Exon42		c.6241A>G	p.N2081D*	G 0.059
Exon43	rs10878405	c.6324G>A	p.E2108E	A 0.317
Intron43	rs11176143	IVS43+52G>A		A 0.092
Intron47		**IVS47-41A>G**		**G 0.008**
Intron47	rs11317573	IVS47-9delT		delT 0.408
Exon48		**c.7155A>G**	**p.G2385G**	**G 0.108**
Exon49	rs3761863	c.7190T>C	p.M2397T	C 0.55

Novel variants detected in our study are in **bold**. The p.I1371V, p.R1441C, and p.G2019S mutations are highlighted in italic.
Accession number (rs or ss) is given for each known *LRRK2* polymorphism. The nucleotide numbers are according to the *LRRK2* cDNA sequence deposited in Genbank (accession number AY792511).
For each polymorphism, the variant allele is reported after the >symbol, and its allelic frequency in our sample of autosomal dominant PD patients is also given.
*Polymorphisms, which are not present in the database but have been reported previously (Zimprich *et al*. *Neuron* 2004).

polymorphisms in the gene, deserve further consideration in larger case–control studies for a possible role as risk factor for PD.

One of the novel variants, the IVS13 + 104 G>A, was found in all PD cases carrying the p.G2019S mutation, and in 3% of controls (not carrying p.G2019S). Our haplotype

Table 2 16 novel LRRK2 variants-frequency in patients and controls, and cosegregation studies

Position	Nucleotide change	No. of patients carriers	Allelic frequency in PD cases	Cosegregation with PD	Allelic frequency in controls (at least 100 chrom.)
Intron1	IVS1-29C>T	1/60	0.8%	NO	0%
Intron4	**IVS4-38A>G**	**1/60**	**0.8%**	**NA**	**0%****
Intron5	**IVS5+33T>C**	**1/60**	**0.8%**	**NA**	**0%****
Intron13	IVS13+104G>A	4/60	3.3%	YES*	1.5%
Intron18	IVS18-22C>T	5/60	5.8%	NA	6%
Intron19	IVS19-9insT	45/60	45%	NO	64%
Intron20	IVS20+12delA	2/60	1.6%	NA	4%
Intron20	IVS20-65A>T	1/60	0.8%	NO	0%
Intron30	**IVS30+12delT**	**5/60**	**5.8%#**	**NA**	1.5%
Exon32	c.4624C>T (p.P1542S)	2/60	1.6%	NA	1.14%
Intron37	IVS37+26G>A	1/60	0.8%	NO	0%
Intron37	IVS37-9A>G	1/60	0.8%	NO	0%
Intron38	IVS38+35G>A	6/60	6.6%	NO	2%
Intron40	IVS40-39A>G	1/60	0.8%	NO	0%
Exon48	c.7155A>G (p.G2385G)	12/60	10.8%	NO	11%
Intron47	IVS47-41A>G	1/60	0.8%	NO	0%

When possible, cosegregation of variant with disease was tested. Three intronic substitutions, for which a pathogenic role remains unknown, are highlighted in **bold**.
NA: cosegregation data not available. *Variant in LD with the p.G2019S mutation.
**200 control chromosomes tested.
#$P<0.05$ *vs* controls, Fisher Exact test.

Table 3 Clinical features in three novel families with *LRRK2* mutations

Family (*country*)	**PV-12** (*Italy*)	**PV-78** (*Italy*)	**MI-007** (*Italy*)
Mutation	**p.R1441C**	**p.R1441C**	**p.I1371V**
N. generations with PD	3	2	2
N. mutation carriers with PD	2	1	2
PD onset age in mutation carriers (years)	63/63	65	33/61
Mean age at PD onset	63	65	47
Disease duration (years)	13/2	9	17/12
UPDRS motor score	11/11	13	NA/NA
Dementia	−/−	−	−/+
Dysautonomia	−/−	−	−/−
Levodopa response	+/NA*	+	+/+
N. unaffected mutation carriers	1	0	0
Age at examination of unaffected mutation carriers	33	NA	NA

NA: not available or not applicable; +: present; −: absent; *untreated with levodopa.

analysis in a large panel of patients with the p.G2019S mutation[27] suggested that IVS13 + 104 G>A is in strong LD with this mutation.

The allelic frequencies of all *LRRK2* known and novel polymorphic variants detected in our sample are reported in the Tables 1-2. It will be interesting to resolve the haplotype-block structure of the *LRRK2* gene in Italians and in other populations, and to identify haplotype-tagging SNPs, in order to investigate whether *LRRK2* variants act as susceptibility factors for the common forms of PD.

Considerations on the dardarin protein

The mutations reported here are diverse in their predicted effect on the dardarin protein. The pathogenic role of the p.G2019S mutation is strongly supported by the observation that the Glycine2019 residue is extremely conserved in the human kinase domains, and in all dardarin homologues.[12,29] It is part of three residues (DYG, or DFG) which form the so-called 'anchor' of the activation segment of the kinase domain, necessary for the activation of the catalytic domain.[29,30] If the kinase activity of dardarin is required for the phosphorylation of target proteins, or if this activity plays an auto-regulatory role, is currently unknown. Mutations in the DYG/DFG residues are predicted to destabilize the anchor of the activation segment; a possible outcome is a loss-of-function of the kinase activity, suggesting haploinsufficiency as disease mechanism. However, it is also possible that the mutation renders the kinase domain more susceptible to activation, as shown for mutations in the activation segment of other kinases.[31] This mechanism would confer a gain of a toxic function for the dardarin protein. Haploinsufficiency and gain-of-function are both compatible with the dominant

pattern of inheritance seen in families with *LRRK2* mutations.

The p.R1441C substitution is also highly significant for the dardarin protein: arginine is a positively charged residue, whereas cysteine is polar and weakly acidic, and the sulphydryl group is often involved in protein folding by forming disulphide bonds. The Arginine1441 residue is located in the ROC domain and is highly conserved in various species.

The p.I1371V mutation is located in a Rab family motif within the ROC domain. Although Isoleucine and Valine are both aliphatic amino acids, Isoleucine1371 is highly conserved among the dardarin protein homologues (Figure 2d).

Genotype/phenotype correlations analysis

Overall, the phenotype in patients with the different mutations was similar and close to classical PD, despite the fact that the mutations are predicted to impact on different functional domains of the dardarin protein. Common features include asymmetric onset, good response to levodopa treatment and, in some cases, slow disease course. Severe cognitive disturbances occurred in only one case. Restless leg syndrome (RLS) was noted in other PD patients who carried the p.G2019S mutation (Z Wszolek, personal communication); however, in this study we did not look specifically for the presence of RLS.

A broad range of disease onset ages is observed (mean 55.2, range 38–68 years including all the three mutations found in our sample: p.G2019S, p.R1441C, and p.I1371V), suggesting that other genetic and/or non-genetic factors likely play a role as disease modifiers.

Among nine PD patients shown to carry the G2019S mutation, and for whom accurate clinical information is available (data from reference[12]), the mean age at symptoms onset was 54.2 years (range 38–68 years), while the age at last examination in unaffected p.G2019S carriers ($n=6$) was 49.3 years (range 41–58 years). In order to estimate the penetrance of the p.G2019S mutation, we calculated the ratio between the number of affected carriers and the total number of carriers of this mutation at a given age. The values range from 15% at 40 years, to 78% at age 65 years. These findings are in agreement with the reported p.G2019S penetrance in another study,[14] and have important implications for genetic counselling. However, analysis of larger series of families with the p.G2019S mutation is needed in order to define the penetrance of this frequent pathogenic mutation more accurately.

Neurological examination of three patients with the p.R1441C mutation revealed a classical PD phenotype and age at disease onset of 63–65 years. In the two previously published families with this mutation (family 'D' and family '469') the phenotypes and onset ages were similar, but a broader range of onset ages was evident (range 48–78 years).[9,32]

Onset age ranged from 33 to 61 years in our family with the p.I1371V mutation, and from 41 to 72 years in the other family with this mutation published previously[18] (though in that family the mutation status was only tested in the proband, with PD onset at age 41 years). In our family, it is possible that the inheritance of additional genetic factors from the father (also affected by PD and not carrying the p.I1371V mutation) contributed in the proband to the onset of PD at a younger age (Figure 2c).

Conclusion

Our comprehensive analysis of all the 51 exons of *LRRK2* in a large sample of families allowed for the first time a more accurate estimate of the frequency of *LRRK2* involvement in ADPD, delineating further the mutations in this gene as the most frequent cause of ADPD known so far, at least in the studied populations. Unraveling the mechanism of the disease caused by *LRRK2* mutations might therefore greatly promote the understanding of the pathogenesis of the common forms of PD. Owing to their frequency, *LRRK2* mutations should be considered in the diagnostic workup. *LRRK2* is a large gene and mutation analysis of the whole coding region is expensive and time consuming. We suggest that large-scale screening of this gene should begin by searching the most common, recurrent mutations for a given population, followed by the systematic scrutiny of the central region of *LRRK2*, where most of the mutations are located.

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Appendix

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