The role of LRRK2 in Parkinson’s Disease

Alessio Di Fonzo

Cover: "*Madame Me*", engraving on zinc, © Patrizia Pecorella, 2008

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The Role of *LRRK2*

in Parkinson's Disease

De rol van *LRRK2*

bij de ziekte van Parkinson

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Chapter 1 - Introduction

Parkinson's disease (PD) was first described by James Parkinson in 1817 in the monograph entitled 'An Essay on the Shaking Palsy'. PD is the most common neurodegenerative movement disorder, and the second most common neurodegenerative disease after Alzheimer's disease (AD), with more than 1% of people over 65 year old affected[1]. PD is clinically defined by adult-onset, progressive parkinsonism (the combination of akinesia, resting tremor, and muscular rigidity), and displays a beneficial response to dopamine-replacement therapy. This clinical syndrome correlates with neuronal loss and gliosis in the substantia nigra pars compacta and other brain areas, and in most patients with the formation of cytoplasmic inclusions called Lewy bodies (LB) and Lewy neurites in the surviving neurons[2, 3].

Enormous steps have been taken over the past two decades in understanding the neurobiology of PD, but the exact etiology of this devastating disease is still largely unknown.

Currently, several mechanisms are believed to be implicated in the selective degeneration of dopaminergic neurons in PD. Although a common, unifying pathway at the cellular level has not been identified, the culprits might include the formation of protein aggregates and dysfunction of the proteasomal and lysosomal system, oxidative stress leading to mitochondrial dysfunction and apoptosis[2, 3].

The Mendelian forms of PD

Until the end of last century hereditary influences in PD were thought to play a minor role. This led the scientific community to emphasize the role of environmental factors in the etiology of the disease. Certainly, ageing plays also an important role, as the incidence of PD increases dramatically with age. Epidemiological studies reveal that 10–15% of PD has a positive familial history for the disease while the majority of cases are sporadic. Through linkage analysis and positional cloning approaches five genes have been definitely implicated in the etiology of PD. Mutations in the *α-synuclein*[4, 5] and leucine-rich repeat kinase 2 (*LRRK2*)[6, 7] gene cause autosomal dominant forms whereas mutations in the *parkin*[8], *DJ-1*[9] and *PINK1*[10] gene cause

autosomal recessive forms of PD. Furthermore, mutations in the PARK9/*ATP13A2*[11], PARK14/*PLA2G6*[12], and PARK15/*FBXO7*[13, 14], have been reported as rare causes of early onset parkinsonism with atypical clinical features which might be mechanistically distinct from classical PD. Finally, mutations in the genes for the nuclear receptor 4A2 (*NR4A2, Nurr-1*)[15], ubiquitin C-terminal hydrolase L1 (*UCH-L1*)[16], *Omi/HtrA2*[17] and GRB10 interacting GYF protein 2 (*GIGYF2*)[18] gene have also been described in PD cases, but their role in the disease remeins uncertain. Another three PD loci have also been mapped (PARK3, PARK10, PARK12)[19-21], but the defective genes remain unknown.

In addition to the Mendelian forms of PD, genetic risk factors for the disease have been investigated in several candidate genes and, more recently, in genome wide association studies[22-25]. With the exceptions of *α-synuclein* and microtubule associated protein tau (*MAPT*)[26-31], none of the loci reported has so far been convincingly replicated in independent studies. Another exception is represented by the the gene involved in a recessive neurometabolic disease, the glucocerebrosidase gene (*GBA*). Mutations in the *GBA* gene have a conspicuously high prevalence of PD. Screening of PD patients for *GBA* mutations then found a higher number of heterozygous mutations carriers as compared to healthy controls. Mutations have been found in about 2 to 4% of Caucasian PD patients, and less than 1% of controls.

Table 1

Locus	**Gene**	**Chromosome**	**Inheritance**	**Clinical Presentation**
PARK1-4	***α-Synuclein***	4q21	AD	PD, Lewy body dementia
PARK2	***parkin***	6q25.2-27	AR	Early onset PD
PARK3	Unknown	2p13	AD	Typical PD
PARK5	*UCH-L1* (?)	4p14	AD	Typical PD
PARK6	***PINK1***	1p36	AR	Early-onset PD
PARK7	***DJ-1***	1p36	AR	Early-onset PD
PARK8	***LRRK2***	12q12	AD	Typical PD
PARK9	*ATP13A2*	1p36	AR	Early-onset parkinsonian-pyramidal syndrome with dementia
PARK10	Unknown	1p32	Unknown	Typical PD
PARK11	*GIGYF2* (?)	2q36-37	AD	Typical PD
PARK12	Unknown	Xq21-q25	Unknown	Typical PD
PARK13	*Omi/HTRA2* (?)	2p12	Unknown	Typical PD
PARK14	*PLA2G6* (?)	22q	AR	Adult-onset dystonia-parkinsonism
PARK15	*FBXO7*	22q	AR	Early-onset parkinsonian-pyramidal syndrome
(?)	*GBA*	1q21	Unknown	Typical PD

Table 1. Known loci and genes for PD

The *LRRK2* gene – mapping and cloning

Although the discovery of mutations in the *α-synuclein*, *parkin*, *PINK1* and *DJ-1* genes clearly contributed to our understanding of the pathogenesis of PD, they were identified in a limited number of PD cases, often with early-onset or pathologically atypical features.

In contrast, mutations in the *LRRK2* gene turned out to be a frequent determinant of typical PD, in both early and late onset forms of the disease. Moreover the identification for the first time of *LRRK2* mutations in sporadic cases has dramatically changed the previous opinion on the contribution of genetic factors in the development of PD in general.

Introduction

A new locus for PD, termed PARK8, was identified in a large family with autosomal dominant PD, known as the "Sagamihara family" from the region in Japan where the family originated from[32]. The clinical features in affected individuals of the kindred were reported to resemble very closely classical PD, with an average of symptoms onset at 51± 6 years. Initially, a pattern of "pure nigral degeneration" without Lewy bodies was found at autopsy in four PD patients examined, while another carrier of the disease haplotype developed pathologically-confirmed nigral degeneration, an atypical form of parkinsonism which is known to be associated with a synuclein-positive brain pathology. However, a pathological follow-up study on this kindred revealed two additional cases with pure nigral degeneration and one with classical LB pathology[33]. In this family a genome wide linkage scan yielded significant evidence for linkage of PD to the centromeric region of chromosome 12 (12p11.2-q13.1). The haplotype analysis suggested an incomplete penetrance of the mutation[32, 33]. Linkage to the same region was found in a genome scan for Alzheimer disease, the locus being termed AD5, but this was mostly supported by the subset of families containing at least one affected individual with LB pathology[34]. In 2004 the linkage to PARK8 was confirmed in two Caucasian families, "family A" (a German–Canadian kindred) and "family D" (from Western Nebraska) with dominantly inherited neurodegeneration[35], and thereafter in several Basque PD[36] families suggesting PARK8 to be a relatively common locus and refining the critical region. A wide clinical-pathological spectrum was shown in these families, including typical PD but also dementia and amyotrophy, diffuse Lewy body and tau pathology, nigral degeneration without inclusions and atypical, ubiquitin-positive inclusions[37].

In 2004 two independent groups, by positional cloning, identified mutations in a gene at that time annotated as DKFZp434H2111, which cosegregated with PD in several PARK8-linked pedigrees[6, 7]. The gene was renamed *LRRK2* (leucine-rich repeat kinase 2) and the encoded protein LRRK2 or Dardarin (from the Basque term *dardara*, meaning tremor, since resting tremor was a consistent clinical feature of the Basque patients who carried *LRRK2* mutations). The protein has an unknown function and the pathogenic mutations replace highly conserved residues among LRRK2 orthologues.

Subsequently, early in 2005, different groups identified a single *LRRK2* mutation (c.G6055A) leading to a G2019S substitution in the encoded protein, which was present in familial and sporadic PD with unprecedented high frequency[38-41]. The following years have seen an explosion of research into the *LRRK2* gene in PD and related disorders.

Due to the large size of the open reading frame (more than 7.5 kb across 51 exons), most of the published studies only screened for the a very few recurrent mutations, especially those reported in the cloning papers (R1441C, R1441G, Y1699C and I2020T) and the common G2019S, which are so far considered as definitely pathogenic on the basis of clear cosegregation with disease in families and absence in ethnically matched healthy controls. The I2020T mutation was detected as well as the cause of disease in the original "Sagamihara family"[42].

The G2019S mutation

Prevalence of G2019S across populations

G2019S is particularly important among the PD-causing mutations in *LRRK2*. This mutation was identified by several groups as a common cause of the disease, being detected initially in ~5–6% of large cohorts of familial PD in Europe and US[38, 40], and in ~1–2% of sporadic PD from UK[39]. Due to the unprecedented high frequency in familial and late-onset classical parkinsonism, which in the past would have been identified as "idiopathic PD", this specific mutation has been extensively studied worldwide. However, reviewing the literature on this specific mutation is difficult. The results from the different studies are often not directly comparable because of large differences in sample size. If the ethnic background of the population studied is not mentioned, especially in countries with highly mixed populations like U.S. and Brazil, the frequency of the mutation could be not representative of a given ethnic group. Moreover, the different definitions of ''familial'' versus ''sporadic'' disease can lead to an over representation of the mutation among (pseudo)sporadic cases who do have distant relatives with PD but lack affected first-degrees family members.

Taking all these concepts into account, so far large screenings revealed that the frequency of G2019S is population specific.

The G2019S mutation has been reported at the highest frequency (up to 37%) among familial PD cases of Arab descent and in 23% of familial Ashkenazi Jewish patients[43, 44]. Similar frequencies were replicated in independent studies on PD cases from Tunisia[45-47] and in Ashkenazi Jews[47-50]. Remarkably, the frequency of this mutation was considerably high among sporadic cases (41% Arabs and 13% Ashkenazi Jews), and also rarely identified in healthy controls (3% Arabs and 1.3% Ashkenazi Jews). Other studies reported the presence of the G2019S among 1-2% of healthy North Africans, Ashkenazi and Sephardic Jewish subjects[48, 49, 51, 52]. Little is known about the prevalence in specific Middle Eastern, North African, and sub Saharan populations. A study suggests that the G2019S might be frequent in Turkey, having

been found in 1/26 (3.8%) of sporadic PD cases[53], although this needs to be confirmed in a larger cohort. The mutation has not been identified so far in sub Saharan Africans (50 PD and 51 healthy Nigerian)[54] , Yemenite Jews [50] and in Iran (205 PD)[55].

In Western Europe there is a South-North gradient of frequency. The G2019S is found in 9-16% of familial and 3-4% of sporadic PD patients in Portugal[56, 57]; it accounts for 6-16% of familial and 2-6% of sporadic PD in different region of Spain: Catalunia[58], Cantabria[59, 60], Asturias[61], Galicia[59] and Basque regions (patients without Basque ancestry)[62]; while it is less common in patients of Basque origin (1-2%)[62].

In Italy the G2019S mutation has been reported in 6-7% of familial and ~1-2% of sporadic cases[38, 63-67]. Similarly, in France the mutation accounts for ~3.5% of familial and ~1.9% of sporadic cases[43, 68-71]. Two independent screening in Sardinians, an isolated population, reported a frequency of ~1.5% in both familial and sporadic cases[72, 73]. Interestingly, the mutation that appeared to be common in the western Mediterranean basin is instead very rare in Greece and Crete[74-77].

A slightly lower frequency was reported in U.K. screenings of PD patients of Caucasian ethnicity (2.5% familial and 0.3-1.6% sporadic)[39, 78, 79]; and also in populations of Celtic and Baltic origin (Ireland 1.1% of familial PD[41, 80]; Norway ~1.5% of familial PD[81] and Sweden 1.4% of sporadic cases[82]). Mutation analyses in more than 300 familial and 1200 sporadic PD in Germany suggested a very low frequency of this mutation (0.8% of familial cases,[83, 84] 0.2-0.9% of sporadic)[83-85], as well as in Belgium[86], The Netherlands[87] and Austria[88]; however, an independent study reported a higher frequency (6.4%) in familial PD from Germany, although the small sample size (30 families) limits the value of this finding[89].

Except from Poland and Serbia, where the G2019S appeared to be rare[83, 90], frequency data in Eastern Europe are still lacking. On the contrary four studies have been performed in Russia, where the mutation accounts for 4-7% of familial and 1% of sporadic cases[91-94]. However, subjects were included from a mixed ethnic background, since at least two PD families and one sporadic case reported their ethnic origin as Ashkenazi Jews[93].

An analogous observation can be done when analyzing patients from the U.S., where the frequency of the G2019S in Caucasian PD reaches 2-3.5% in familial and 0.5-1.6% in sporadic cases[45, 48, 95-101], it seems to be rare among American Indians and Afro-Americans (but the sample size for these two ethnic groups is still insufficient to make firm conclusions) [100, 101], whereas it was reported to be higher when patients of Ashkenazi Jewish ancestry were included[48]. In Canadian PD patients the G2019S is rare/absent[102, 103].

Four different populations of South America, where the Spanish, Portuguese and Italian ethnic backgrounds are strong, have been studied for this mutation. In Uruguay[104] and Chile[105] G2019S accounts for 3.5% of familial and 2,9-4.2% of sporadic cases. Controversial results came from large screening in Brazil (from 3 to 6.8% in familial and 0 to 1.7% in sporadic cases), probably due to the high degree of ethnic heterogeneity within the study cohorts[106-108].

G2019S is rare/absent among Chinese patients with familial and sporadic PD[109-112], as well as in Korea[113, 114] and in India [112, 115]. So far, only three patients have been reported with this mutation in Japan (0.7% of sporadic cases)[47, 116].

Finally, the mutation is present in Australia, among PD patients with European ancestry (2-6% of familial, 0.4% of sporadic PD)[117, 118], while it has not been identified in Australian Aboriginal.

Taken together, these data show that a single *LRRK2* mutation represents the most frequent known genetic determinant of PD. The frequency of the G2019S mutation varies widely across populations, indicating that ethnicity is an important factor. For some populations, independent studies on the prevalence of the mutation are already available and often, the reported results are consistent. This should influence and speed the diagnostic genetic testing, especially in those countries where the prevalence of the mutation among PD is considerably high (20-40% of patients in North Africa and in Ashkenazi Jews). Nevertheless in most European countries and in U.S. at least 1% of sporadic and 2-6% of familial PD carry this mutation. These observations imply that most neurologists who treat patients with movement

disorders will see patients with *LRRK2*-related PD. This estimation could be even higher if we include the other *LRRK2* definitely pathogenic mutations.

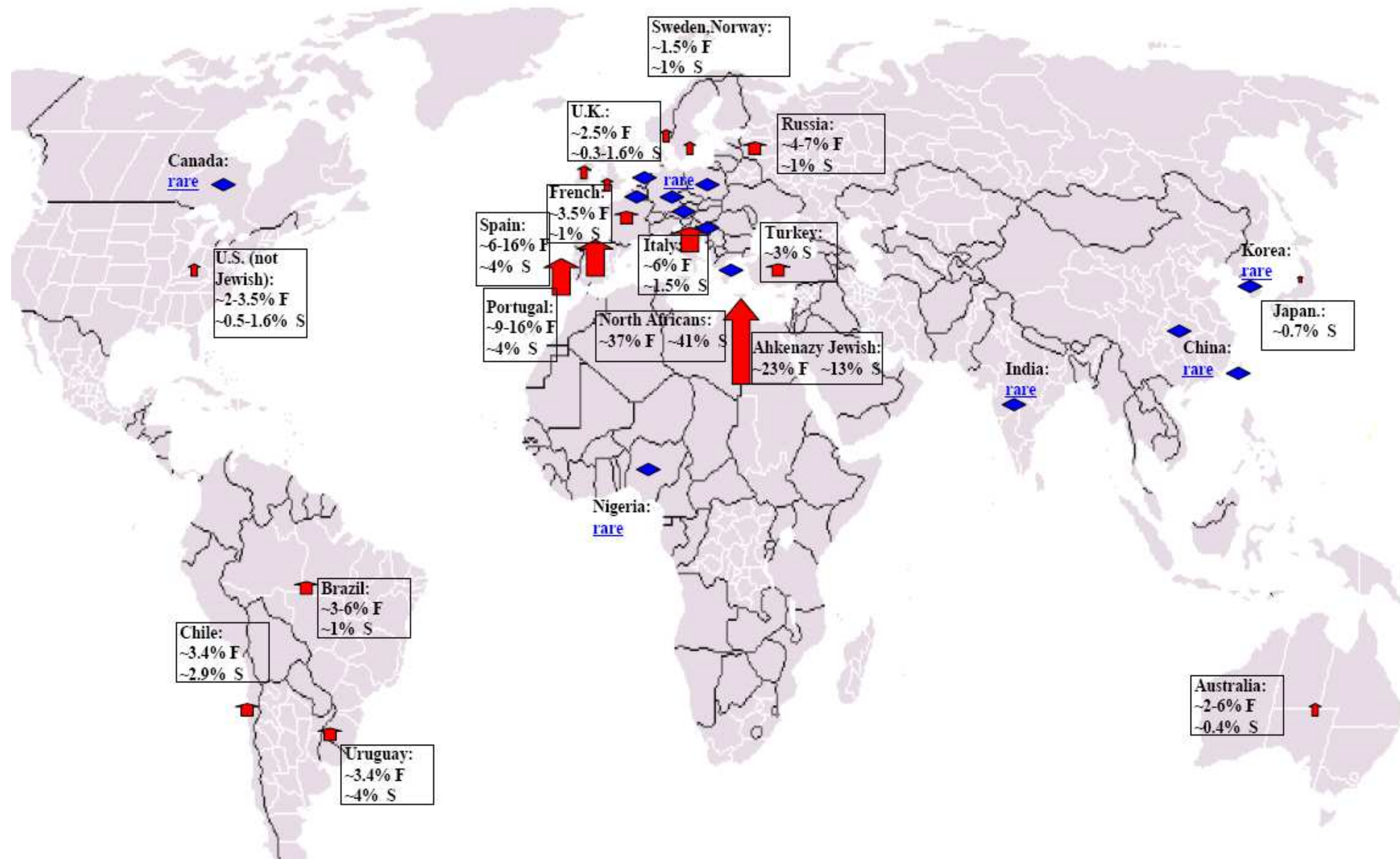

Figure 1

Worldwide distribution of the G2019S mutation.

Origins of the G2019S mutation

So far three different haplotypes have been identified in patients carrying the G2019S mutation.

Haplotype 1

The first studies on unrelated carriers of the G2019S of European (Italy, Norway, Poland, Ireland) or Middle Eastern–North African origin revealed that all shared the same haplotype, consistent with a common founder[41, 43, 44, 66, 119, 120]. Initially, the centromeric border of this haplotype was defined at the marker D12S2515, which led to a minimum ~60 kb shared region[41, 121]. Using a likelihood based haplotype approach the mutation has been estimated to have arisen 725 years ago[121]. However, we found that a sample of G2019S-related haplotypes (mostly Italians) contained a very rare (0.1% in controls) allele at marker rs28903073, located upstream from D12S2515, which was in strong linkage disequilibrium with G2019S[66]. This suggested that the allelic differences observed among G2019S haplotypes at D12S2515 were likely due to recurrent mutation in an unstable microsatellite rather than recombination. Excluding the D15S2515 marker, the minimum shared region appeared larger, spanning ~160 kb which includes the promoter and most of the *LRRK2* gene. The identification of a larger (at least at the 5' end) shared haplotype suggests that variation at the promoter or other cis-acting regulatory elements are not important determinants of the phenotypic variation observed among G2019S carriers.
Subsequently, the same haplotype has been identified among subjects carrying the G2019S mutation from Italy (independent subset)[67], French[46], Germany[83], Russia[91] Sardinia[72, 73], Spain[61], Portugal[57, 66], Brazil[66], Chile,[105] Uruguay and Peru[104], and Australia[117].
According to a general rule in population genetics, the geographic center of the origin of a mutation corresponds to the area where the same mutation is most frequent[122]. The highest prevalence of the G2019S mutation has been reported in Berbers[51], followed by North African Arabs, Ashkenazi and Sephardic Jews. The frequency data combined with the identification of a common haplotype among these populations support the hypothesis that the mutation originated in North Africa or in the Middle East and than spread to other countries following the patterns of migration.

Three further studies provided important insights on the estimated age of the common founder for the haplotype 1 carriers. Analyzing the haplotypes of European and Ashkenazi Jews [120], and Tunisian G2019S carriers[123], the age estimated of the common ancestor (using the 30-year intergeneration interval) was 2250 (95% CI 1650-3120) and 3120 (95% CI 2340-4620) years ago, respectively (the results form the two dataset do not differ significantly since the 95% CI overlap). A third study, on Ashkenazi Jews only, estimated a more recent founder approximately 1525-1830 years ago (150-450 A.D)[124]. The latter estimation would fit with the absence of the G2019S in Yemenite Jews [50]. The Yemenite Jews evolved completely separate from all of the other Jewish populations. Most of them arrived in Yemen in the early second century A.D. (~160 A.D.). Instead the Ashkenazi Jews migrated through Italy and southern Germany (Ashkenazi means ‘‘German’’ in medieval Hebrew) in the seventh/eighth century A.D., and spread from there to northern, central and eastern Europe. If the mutation occurred in a Jewish founder after the separation of the Yemenite, that would explain the absence of the G2019S only in this specific subgroup. Alternatively it is possible that the mutation was absent among the founders of the Yemenite subgroup simply by chance (genetic drift). In another subgroup of Jews, the Sephardim (Sephard means ‘‘Spanish’’ in Hebrew), which migrated to the Iberian Peninsula, the prevalence of the G2019S (in the context of haplotype 1) is similar to that of the Ashkenazi[51]. The precise origins of the Jewish communities of the Iberian Peninsula are unclear. Although the spread of Jews into Europe is most commonly associated with the Diaspora, it is thought that substantial Jewish immigration in Spanish regions probably occurred during the period of Roman occupation of Spain (after the Second Punic War, 218-202 B.C.), which would be much earlier than the Yemenite separation.

In this picture it is not easy to speculate on the precise region of G2019S origin. It must be considered that patterns of migration, especially of Jews, often occurred in more separate waves, making their relation with the estimated age of the mutation complex.

To explain the relative younger age of the Jewish haplotype compared to that estimated in North Africans, a possible hypothesis is that an ancient common founder (~3000 years ago , ~1000 B.C.) led to a descendant who served as a Jewish (Ashkenazi and Sephardic, but not Yemenite) subfounder [120].

Further studies on the prevalence of the G2019S and haplotype analysis in genetically homogeneous North African populations (Arabs from Saudi Arabia, populations with presumed Phoenician ancestry [Syrians/Lebanese?], Berbers and Jewish subgroups) might provide important insight into the origin of the mutation[125].

Haplotype 2

A different G2019S haplotype was identified in three families from Western Europe, but the geographic origin of this haplotype is less certain[120].

Haplotype 3

The third haplotype has been found in Japanese patients carrying the G2019S[116]. This haplotype differs even across the markers closest to the mutation, which would suggest an independent origin of the mutation in Japanese and European populations rather than a single ancient founder. Interestingly, the haplotype 3 has also been observed in a single sporadic Turkish patient[53]. This may be the result of a common ancestry (plausibly explained by the large centuries-long migration of the Turkic people across Central Asia) or coincidental presence of Japanese ancestors. Further screening and haplotype analysis in this country are warranted.

Incomplete penetrance of G2019S

Incomplete penetrance was already suspected for the mutations underlying the PARK8 locus at the time of the linkage studies. Since G2019S represents the most frequent *LRRK2* mutation, most of the penetrance analyses have been performed on this specific mutation.

Initially, a high penetrance for the G2019S mutation was reported: 17% at age 50 years and 85% at 70 years[41], and 33% at 55 years and 100% at 75 years[46]. However, the patients included in the analysis were from selected, large autosomal dominant familial PD, and probands were not excluded, leading to a marked overestimation.

More recent analyses performed on Ashkenazi Jews revealed a lifetime penetrance of 31.8% [44]. A slighter lower penetrance (24% at 80 years) was estimated in an independent

group of U.S. and Ashkenazi Jews[48] (without significant differences between the two groups).
Moreover, a family-based study on 36 G2019S carriers, mainly from Italy, estimated a penetrance of 30% at 80 years[126]. The latter result was consistent with those found in the Ashkenazi Jews[44]. The International *LRRK2* Consortium performed a penetrance study on the largest dataset of G2019S carriers. By analyzing a large sample PD patients and controls with and without the G2019S mutation, Healy et al. calculated a 28% risk of PD at 59 years, 51% at 69 years, and 74% at 79 years for *LRRK2* G2019S carriers. There was no difference in penetrance by sex or ethnic group[127]. It is worth underlining that, as in the Italian study[126], also in this multicentric study the selection bias was minimized by excluding the probands and including the families of sporadic cases, when data were available for both parents. A second consortium also enrolled a large sample of familial PD and controls. G2019S was present in 29 families and the calculated penetrance (~67% at 85 years) was substantially higher then that reported by the first consortium[128].
Finally, a penetrance study in Tunisian G2019S PD cases, after stratifying by homozygous (n=23) and heterozygous carriers, reported a penetrance consistently higher in homozygotes in each age group. Considering possible biases in estimating penetrance only from families, this finding, if true, would indicate a gene dosage effect, although the age of onset was not dissimilar between the two groups. This study needs replication in a larger independent dataset[129].

The reduced penetrance of this frequent mutation is in keeping with the *LRRK2* G2019S being the most important genetic determinant, known so far, of sporadic PD. In populations like the Ashkenazi Jews, several studies consistently reported a lifetime penetrance of ~30%. This can also be expressed in terms of risk (calculated as odd ratio) to develop the disease. For a Ashkenazi Jew who carries the G2019S the risk of developing PD increases of ~18 fold[44]. By analyzing the G2019S in North Africans, a lifetime odds ratio for developing PD of 48.6 (CI 11.2–211.0)[43] has been calculated.
Nevertheless, additional studies in different populations are warranted before G2019S genetic counseling can be implemented, since the precise estimation of the penetrance in some countries is still controversial.

It is possible that dissimilar results across the above-mentioned studies are not yet due to different methodological approaches, but actually revealed an intrinsic variability of the mutation penetrance among different subjects/families.

The reduced penetrance suggests that the G2019S mutation itself is needed, but is somehow not sufficient for developing the disease. In this picture it is tempting to speculate that the presence of other genetic factors or simply stochastic events can act as modifiers of this (and may be other) *LRRK2* mutation, thereby influencing the penetrance.

The analysis of a few candidate genes involved in neurodegeneration as potential genetic modifiers of *LRRK2* has been reported. The first to be explored was *parkin*, since patients who simultaneously harbored *parkin* mutations and *LRRK2* G2019S have been mentioned in several studies[57, 69, 91, 130-132]. However, the clinical and cosegregation analysis of patients carrying heterozygous *parkin* mutations and the G2019S reveled that the combination of the two does not influence the symptoms or the age at disease onset.

Recently, polymorphic variations in the microtubule associated protein tau (MAPT) have been proposed to be significantly associated with age of disease onset in individuals with *LRRK2* mutations[133]. Further analyses, especially on large samples and families carrying the G2019S, are warranted to identify genetic factors that can act as modifiers of *LRRK2* mutations.

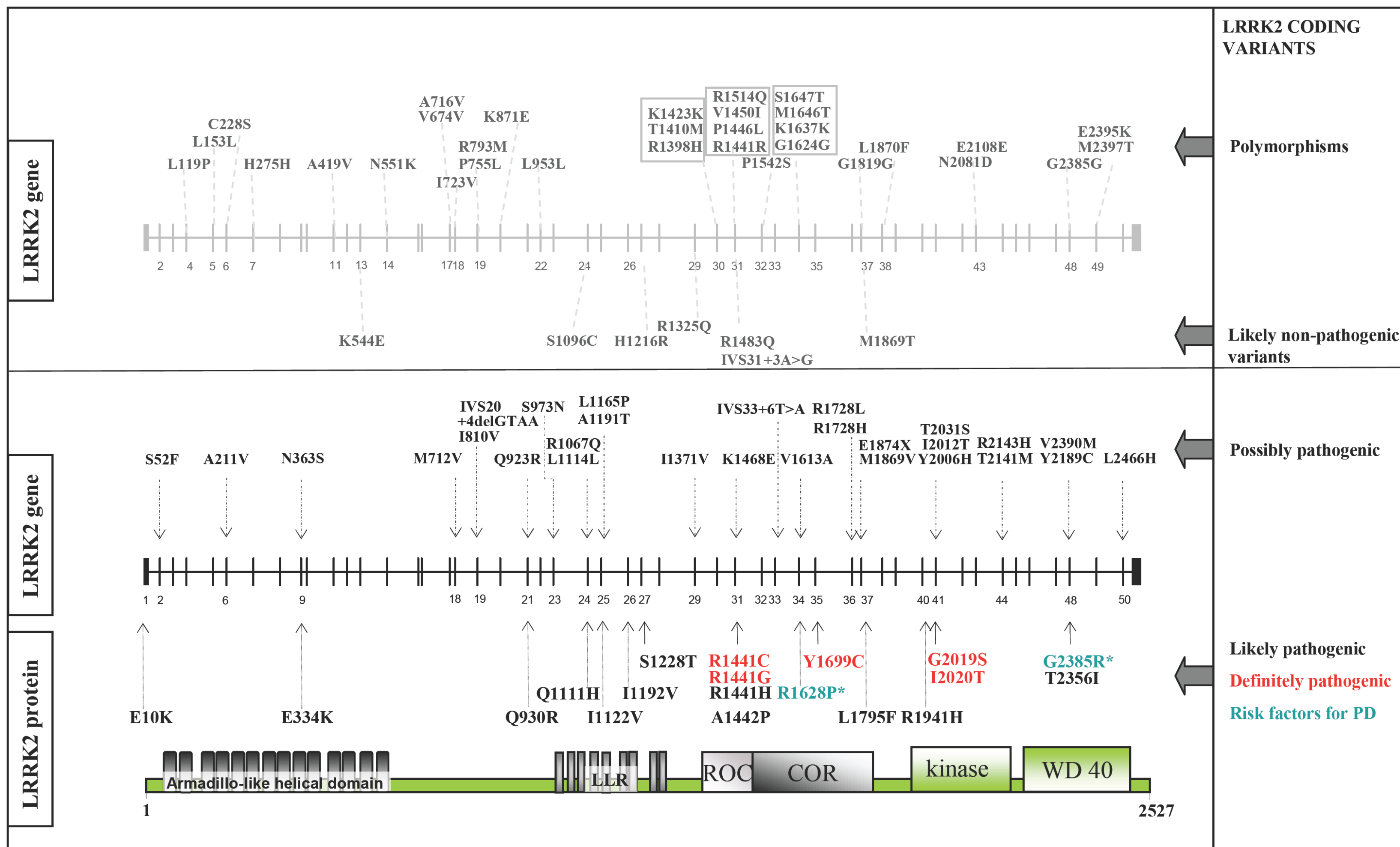

Figure 2

Schematic representation of the *LRRK2* gene, the dardarin protein and its known functional domains. *LRRK2* 83 coding variants and three putative splice variants are grouped according to evidence of pathogenicity (see main text).

The R1441 mutational hot spot

The LRRK2 R1441 residue is the second most common spot of pathogenic *LRRK2* mutations, after G2019S. Three non-synonymous substitutions (R1441C, R1441G and R1441H), and the synonymous R1441R have been reported in several patients.

R1441C – the second most frequent pathogenic LRRK2 mutation

This mutation (c. 4321C>T) represents the second known most common mutation of the *LRRK2* gene. Its pathogenic role was clear when it was firstly shown to cosegregate with PD in the PARK8-linked "Family D" (Western Nebraska)[7]. Cosegregation was consistent also in smaller PD families from Germany[7, 83], Italy[65], Belgium[86], US[41, 128] and Iran[55]. The mutation has also been reported in a few other families, but additional affected relatives were not available for cosegregation analysis[58, 65, 86, 134]. The R1441C is also found among sporadic cases and has been reported in patients from Italy[66], Sardinia[73], Russia (Slavic origin)[92], China[135] and Belgium[86]. The variant was absent in large cohorts of ethnically matched controls (>1000 German, 530 Italian, 208 Sardinian, 400 Chinese, 178 Belgian and 300 U.S.). Being the nucleotide mutated close to the splice acceptor (+4 from the AG), cDNA analysis was performed in order to exclude deleterious consequence at the transcript level[65].

Haplotype analysis of *LRRK2* R1441C carriers from 20 families of different geographical areas revealed in total four classes of haplotypes. Only for the two major haplotypes could the phase be established[136]. A first haplotype was identified in all Italian carriers, as well as in German, Spanish, and American patients.

A second haplotype was present in the American Family D (Western Nebraska) and in all Belgian R1441C families. A German and an Irish patient shared a third haplotype for which phase could not be unambiguously determined. Finally, a Chinese proband carried alleles that could not be assigned to any of the previous three haplotype classes.

The phenotype associated with this mutation is similar to that of classic PD[136]. The mutation exhibits incomplete penetrance, which could explain its presence in sporadic cases, but calculations performed so far must be interpreted with caution as only a small number of R1441C mutation carriers have been identified until now.

Intriguingly, the neuropathology associated with the R1441C mutation in "Family D" (Western Nebraska) revealed α-synucleinopathy (presence of Lewy bodies) in one member, a tauopathy with neurofibrillary tangles and Progressive Supranuclear Palsy (PSP)-like distribution in a second affected member, and loss of neurons with no intracytoplasmic neuronal inclusions in a third[37].

R1441G – a founder pathogenic mutation in the Basques

The *LRRK2* R1441G (c. 4321C>G) was initially described in patients with autosomal dominant late-onset PD in PARK8-linked families of Basque ethnicity[6]. The Basques are a homogeneous ethnic group who historically were isolated by linguistic and geographical barriers. The first report on the frequency of this mutation in Basque PD (~8% of familial cases)[6] and the absence in other large populations screened (except for a U.S. patient reported to be of Hispanic descent[49]) suggested that this variant was population specific. Further studies investigated the prevalence of this mutation in Basque. One group detected the R1441G in 16.4% and 4.0% of familial and sporadic Basque PD, respectively[137]; while a more recent study reported a prevalence of 46% in familial Basque patients and 2.5% of sporadic cases[62]. It has also been identified at lower frequencies in patients from nearby provinces in Spain who did not report Basque ancestry (6% of non-Basques living in the Basque countries[62], 2.7% in Asturias[138], 0.7% in Catalonia[58], two families from the neighbouring region of Navarre, and one from la Rioja[59]), while is rare in Cantabria[60]. Haplotype analysis on R1441G carriers from Basque and neighborhood regions[59, 137, 139] indicates that this mutation occurred in a single common ancestor, which in one study was estimated to have lived 1,350 (95% CI, 1,020–1,740) years ago[139]. Since the Basque population has a history of emigration to Europe, and North, Central and South Americas, it would not be surprising to find isolated cases in those countries. However, recently a

single case from Uruguay carrying the R1441G, who denied her ancestry as European, has been reported with a different haplotype than the Basque, suggesting in this case an independent mutational event[104].

The clinical phenotype of the patients with the R1441G mutation is similar to that of classical late onset PD[36, 58, 59, 62, 137, 138]. A poor response to bilateral deep brain subtalamic stimulation in patients with the R1441G compared to idiopathic PD has been reported[140], but confirmation in a larger number of carriers treated with this approach is warranted.

The only post-mortem material from a patient with the R1441G (Basque) mutation has briefly been described. Loss of dopaminergic neurons in the substantia nigra, free neuromelanin in the neuropil and absence of α-synuclein-, hyperphosphorylated tau- and ubiquitin-immunoreactive inclusions were mentioned[141].

R1441H and R1441R – uncommon, but also likely pathogenic

This variant, c.G4322A on *LRRK2* cDNA, occurs immediately adjacent to the two previously reported pathogenic mutations, c.C4321T (R1441C) and c.C4321G (R1441G), resulting in a different substitution of the same amino acid residue (R1441H).

R1441H has been described in a U.S. PD family but only the proband and an unaffected sibling were available for testing[134]. Is was also reported in PD families from Crete[77], Portugal[57] and Taiwan[80], all not large enough to demonstrate definitive cosegregation with the disease.

Haplotype analysis of the above mentioned R1441H carriers showed diversity suggesting a number of independent founders[142]. Subsequently, the R1441H mutation has been identified in two cases from Australia, both of British origin and with a possible common haplotype, although in these cases the phase was not assessed[117]. A further proof in favor of a pathogenic role of this variant came from the identification of R1441H in two slightly larger French families [68].

R1441H was not found in 281 U.S., 300 Cretans, 200 Portugueses, 174 Europeans and a set of 1,000 control samples (600 North Americans, 200 Taiwaneses, 200 Norwegians, 200 Irish, and 200 Spanish). Moreover several studies screened by sequence the *LRRK2* exon

31 in a large sample of healthy controls (>3000 Caucasian[6, 7, 65, 86]) in order to check for the R1441C and G, and none reported mutation in the adjacent nucleotide.

The clinical presentation of affected R1441H carriers appears to be similar to typical Parkinson's disease with an age at onset range of 32–66 years. All display levodopa responsive parkinsonism, however, the disease in one of the siblings from the Greek R1441H family appeared to transition into a progressive supranuclear palsy-like disorder[77].

To further highlight the nature of codon 1441 as a mutational hotspot, two groups reported a R1441R (c.C4323T) in a sporadic PD patient[93] from Russia and a PD patient with ascertained LB pathology who additionally developed dementia and dysautonomia (PDD)[143]. As for the R1441H we can indirectly assume that the variant is rare in the Caucasian population, since sequencing controls for the other mutations at the same codon did not reveal any R1441R carriers. This variant is predicted to lead to a synonymous substitution, which would suggest a non-pathogenic role. Moreover, being the nucleotide change close to the splice site, cDNA analysis from brain of the PDD patient was performed and did not reveal any aberrations on the *LRRK2* transcript[143]. Taken together these results suggest that R1441R is likely to represent a rare but non-pathogenic polymorphism.

The other *LRRK2* variants: which are pathogenic?

Besides the most recurrent G2019S and R1441 C/G/H, more than 50 different *LRRK2* sequence variants have been reported in familial and sporadic PD cases so far; moreover few novel *LRRK2* substitutions have been found in healthy control subjects only (Figure 2).
The Y1699C and I2020T mutations are considered as definitely pathogenic. The Y1669C was identified in two independent large families, the Lincolnshire kindred[6, 78] (family PL) of European ancestry, and "Family A" (German-Canadian)[7, 37]. Interestingly, the detailed analysis of these families revealed not only an interfamilial but, surprisingly, an intrafamilal variability in clinical and pathological features (see paragraph on Neuropathology). The I2020T was identified in "family 32"[7] and "T10738"[85], both of German ancestry, and absent in more than 1000 German control subjects. Additionally the same mutation was identified segregating in the large PARK8-linked Sagamihara kindred[42] and in two smaller Japanese families coming from the neighborhood of the Sagamihara region[47].
The role of several other variants remains unclear, since often no family members were available to assess cosegregation and a limited number of ethnically matched controls was screened.
Some of these substitutions are recurrent, like the L1114L in "family 38"[7] (two affected siblings were carriers) and in a Irish sporadic patient[144]; the same variant has been detected in an unaffected sibling but not in the correspondent proband, making its pathogenic role uncertain[144]. The L1114L (c.3342A>G) affects a nucleotide close to the splice donor of exon 24, but so far no analysis on cDNA has been reported. The I1371V has been reported in four independent probands[65, 68, 132], but convincing evidence of cosegregation is still missing. The I1371V is absent in large number of French[68] and US controls, and was not tested in Indians from where one patient carrier comes[132], and it was present in one (out of 416) Italian control subject sampled at the age of 55 years who might be still at risk of developing PD[65]. Similarly, the R1067Q mutation, has been reported in one sporadic Chinese patient[145], two sporadic Japanese patients[146] and a single Japanese-American control who at his last assessment at 83 years of age displayed no signs or symptoms of parkinsonism and reported no family history of PD.

While the role of the last three variants remains controversial, a fourth *LRRK2* mutation, T2356I, identified in a small family from UK[78] with at least two affected carriers, in a single sporadic case from US[96] and in a sporadic Dutch patient[87], and absent in more than 1300 ethnic matched controls, is likely to be pathogenic. Interestingly, the threonine at the position 2356 is not completely conserved among LRRK2 orthologues (see paragraph on LRRK2 Protein)

Of the remaining private variants, ten display some evidence of cosegregation with PD, although in small families (E10K[147], E334K[147], Q930R[85], Q1111H[147], I1122V[7], I1192V[147], S1228T[85], A1442P[117], L1795F[147], R1941H[78]).

Twenty-three had no cosegregation data available (S52F[68], A211V[148], N363S[68], M712V[98], I810V[68], Q923H[106], S973N[88], A1151T[89], L1165P[95], K1468E[86] V1613A[92], R1728L[98], R1728H[98],M1869V[149], E1874X[149], Y2006H[69], I2012T[47], T2031S[69], T2141M[98], R2143H[98], Y2189C[86], V2390M[150], L2466H[98]).

Three intronic variants, proposed to be splice mutations (IVS20+4delGTAA[96], IVS31+3A>G[55, 134], IVS33+6T>A[145]) have been reported in patients, but never in controls. Although expression of the IVS20+4delGTAA variant in the pSPES splicing vector yielded a cDNA fragment lacking the exon 19, the precise effect of all these variants on patients' cDNA is still unclear. Recently the IVS31+3A>G[55, 134] has been studied in an Iranian family, where the variant did not segregate with PD, suggesting that it likely represents a non-pathogenic variant. The IVS33+6T>A was found in one patient who developed typical parkinsonian signs after an 8-year course of isolated essential tremor, which rather differs from the classical PD course associated with *LRRK2* mutations. The role of all these intronic variants remains to be elucidated.

K544E[148], S1096C[85], H1216R[68], R1483Q[86] and M1869T[80] (the last reported also in a sporadic case from US[144]) represent variants which are absent in large number of controls but with an incomplete cosegregation with PD, which would suggest either no association with PD or the presence of phenocopies in those families.

The role of the R1325Q also remains unclear. It was identified in a Belgian family where the mother of the proband was affected by initial tremor and is a carrier for the variant in a homozygous state[86]; the same change has been reported in a French familial PD case, but no family members were available for cosegregation[68].

Finally, several variants, initially not present in SNP databases, have been reported in PD cases and controls with similar frequencies (L119P[7, 65], A419V[149], P755L[149, 151-153], R793M[7, 81, 85, 95, 144], T1410M[68], P1446L[146], V1450I[146], R1514Q[7, 65, 154, 155], P1542S[7, 65, 80], M1646T[7, 65]) and some only in healthy subjects (C228S, A716V, K871E, L1870F, E2395K)[98]. These most likely represent non-pathogenic polymorphisms.

In conclusion, the *LRRK2* gene displays a high polymorphic content in terms of single nucleotide substitutions. No deletions or duplications have been identified until now. Variants identified in patients are located in almost all exons. However, most of them still lack a definite proof of pathogenicity. This has direct practical consequences for the genetic studies. *LRRK2* is a large gene containing 51 exons. A time/cost-saving strategy to perform the mutational analysis could be to first screen for the frequent G2019S mutation. If negative, other validated mutations (R1441G/C/H, I2020T and Y1699C) can be tested next. Where a considerable number of affected family members are available for testing, an option is to screen the entire *LRRK2* gene, which raises the possibility of discovering one of the above reported doubtful variants, or even a novel mutation that could be tested for cosegregation in order to verify its pathogenic role.
Concerning the significance of these data for the genetic counseling, it is worth to consider that screening the whole coding region or single variants of uncertain role in unselected cases is still a matter of debate, since the identification of any variant would result in more questions than answers for both clinicians and patients.

The clinical phenotype associated with *LRRK2* mutations

Mutations in PD-causing genes such as *parkin*, *PINK1* and *DJ-1* were mostly associated with some clinical features that differ from classical PD, like an early age of symptoms onset and a slow disease course. Differently, mutations in *α-synuclein* have been associated with both, classical and aggressive forms of the disease and some mutation carriers developed symptoms more typical of diffuse LB disease, rather than PD[156]. In this scenario, the *LRRK2* associated phenotype represents an exception.

Reviewing the core features of 356 patients with *LRRK2* G2019S one can summarize that the associated phenotype is characterized by asymmetrical, tremor-predominant parkinsonism with bradykinesia and rigidity that responded to dopamine replacement and functional neurosurgery. Tremor in this study was reported to be more common in patients with *LRRK2* G2019S-associated PD than in idiopathic PD. The age of PD onset for all *LRRK2* mutation carriers is broad, with an average of ~58·1 years ± 14·0, which does not differ significantly between patients with *LRRK2* G2019S (57·5 years ± 13·9 years) and carriers of all *LRRK2* non-G2019S mutations (59·9 years ±13·8 years). Moreover, there is no difference in the frequency of the G2019S mutation in probands with early vs late onset disease[66]. The disease duration, the rate of disease progression in *LRRK2* mutation carriers (measured by the time to progression through each point on the Hoehn and Yahr scale), and the clinical response to L-Dopa is similar to those in patients with idiopathic PD[127]. Several PET studies documented in *LRRK2* G2019S patients a reduced uptake of tracers for the membrane dopamine transporter (DAT) and vesicular dopamine transporter (VMAT2), as well as a reduction of ^{18}F/dopa uptake in the striatum with a classic pattern of asymmetry with the putamen more severely affected than the caudate[78, 119, 157].

At least 29 homozygous carriers of the G2019S have been described so far, and they display a mean age of disease onset and phenotype features similar to the heterozygous patients. [129] [40, 43, 46, 48, 95, 100, 127]

From the currently available data, one can conclude that there are not clinical features that distinguish *LRRK2* associated PD from idiopathic Parkinson's disease.

The neuropathology associated with *LRRK2* mutations

Mutations in *LRRK2* are associated with a pleomorphic range of pathologies, although the LB-positive pathology is the most common pattern, particularly for the G2019S mutation.[37, 78, 158-160]

The presence of LB positive pathology was briefly mentioned in three cases included in one of the first papers on this mutation[39]. Interestingly one patient showed signs of pathological aging with neocortical plaques and occasional neurofibrillary tangles.

In a large screen of 405 LB-positive brains, 8 (~2%) have been found to be carriers of the G2019S mutation, including 4 with brainstem-type, three with transitional-type, and 1 with diffuse LB pathology[160]. In two G2019S positive brains, Alzheimer-type pathology was also present and it was of enough severity to make a concomitant pathological diagnosis of Alzheimer's disease.

A further study on 80 brains with PD or LB dementia screened for the G2019S mutation, and 3 were found to be carriers[158]. Typical brainstem-type LB-positive pathology was found in one, while the Lewy body variant of Alzheimer's disease was diagnosed in the second. The third brain showed only cell loss in the substantia nigra and locus coeruleus, but no α-synuclein inclusions were detected. There were only rare tau-positive tangles and occasional plaques. No other ubiquitin positive inclusions were present either.

Finally, we described a neuropathological study in an Italian PD case carrying a different *LRRK2* mutation, I1371V, who showed typical ubiquitin- and α-synuclein–positive Lewy body pathology[159].

The atypical phenotypes

Even though most of the patients with *LRRK2* pathogenic mutations have clinically and pathologically typical PD, some exceptions with different clinical and/or neuropathological features exist in the literature.

One member of the original Sagamihara family, later found to carry the I2020T mutation, developed pathologically-confirmed striatonigral degeneration, a variant of the multiple

system atrophy (MSA) which is known to be associated with α-synuclein-positive brain pathology[32]. A pattern of pure nigral degeneration was reported in other family members[32]. Recently, the authors conducted a clinical and neuropathologic follow up of the family, and reported the finding of pure nigral degeneration in two additional affected family members and classical Lewy body pathology in a third case, all carrying the I2020T mutation[161].

Furthermore, the "family A" (German-Canadian), associated with the *LRRK2* Y1699C mutation, included two members who manifested primarily with dementia and two individuals who developed parkinsonism associated with a distal limb muscle weakness, atrophy and fasciculation[7]. Neuropathological examination of the latter showed evidence of nigral neuronal loss with gliosis and spinal cord anterior horn neurodegeneration with ubiquitin-immunoreactive axonal spheroids, reminiscent of amyotrophic lateral sclerosis (ALS)[37]. Interestingly, an autopsy performed in the British family with PD and the same Y1699C mutation, the Lincolnshire kindred, revealed LB-positive pathology in the brain and olfactory bulb, as observed in classical PD[78].

Two carriers of G2019S were identified among 1,179 LB-negative brains, including 326 progressive supranuclear palsy, 43 multiple-system atrophy, 654 Alzheimer's disease, and 156 healthy control brains. One G2019S carrier was a sporadic case with both clinical and neuropathological confirmed Alzheimer disease[160] .

G2019S has been described in a Brazilian sporadic patient who developed both Parkinson's disease and Alzheimer disease[162]. Another patient carrying the G2019S had a clinical diagnosis of corticobasal syndrome (CBS), with some extrapyramidal symptoms and no response to levodopa[95]. A 77-year-old patient with a 14 year history of PD disclosed at the pathological examination mild neuronal loss in the substantia nigra without α-synuclein, tau or ubiquitin cytoplasmic inclusions[163]. Again, a patient with the G2019S developed no symptoms of parkinsonism and presented neuropathology features typical for FTD with ubiquitin-immunoreactive inclusions. This patient had a family history of essential tremor, but not of parkinsonism or dementia[143].

Last, a family from U.S. with parkinsonism, with poor response to levodopa (Family SK) and dementia, has been reported to carry the G2019S mutation[164]. Neuropathological analysis of the proband's brain revealed PSP-like tau pathology similar in type, quantity, and distribution to that observed in one member of "Family D" with *LRRK2* R1441C[37, 164].

The occasional association of *LRRK2* mutations with MSA-like pathology, dementia and ALS-like phenotype prompted several groups to determine the frequency of *LRRK2* mutations in neurodegenerative disorders other than PD. The most studied mutation was the G2019S, although also the Y1699C, R1441C and I202T have been screened in several studies.

The *LRRK2* G2019S was absent in hundred of cases with Alzheimer disease from U.S.[165], Norway[166], Italy[167] and Ashkenazi Jews[52].

Moreover, the mutation has not been identified in large cohorts of Caucasian and Asian patients, mostly with diagnosis of dementia, but also with MSA, PSP, FTD, CBD, vascular dementia and Lewy body dementia[84, 88, 135, 168-171].

The G2019S, I2020T and I2012T were also absent in 272 patients with the diagnosis of essential tremor[172].

In conclusion, all the above reported data strongly suggest that *LRRK2* mutations are specific for PD, but the associated pathology might differ considerably, even between cases with the same mutation belonging to the same family, suggesting an important role of still unknown modifiers. Although most of the patients had the classical findings of nigral cell loss, gliosis and Lewy bodies, some had no Lewy bodies, suggesting that the LB pathology might not be the unique pathological hallmark of the disease. Moreover few *LRRK2* mutation carriers displayed a spectrum of features more reminiscent of tauopathy.

These observations suggest that the classical definition and boundaries of the disease should be reconsidered.

Association studies on *LRRK2*

In the past few years many groups put special effort in search of common risk factors for complex diseases. Among these, Parkinson’s disease and other neurodegenerative disorders have been extensively studied. However, even using high throughput techniques allowing to genotype hundred of thousand of SNPs and covering the whole genome in cases and controls (genome wide association studies, GWA), no reproducible risk loci have been reported so far.

One caveat is that the GWA approach can be problematic because the massive number of statistical tests performed presents an unprecedented potential for false-positive results.
After the discovering of mutations in the *LRRK2* gene, several studies aimed to explore whether common variant of this gene could represent a risk factor for PD.
Two association studies on *LRRK2* have been performed in Caucasians. The first enrolled 340 PD patients and 608 controls from Germany. 121 SNPs (81 tagging SNPs) were genotyped attempting to represent the complete DNA variation of the *LRRK2* gene[173]. The second study analyzed four common coding SNPs (L953L, H1398R, G1624G and T2397M) in 250 controls and 121 unrelated PD, mostly with early onset and positive family history[132]. Neither of these studies revealed any evidences of association between PD and the *LRRK2* SNPs at both allelic and genotypic levels.
In 2005, one study performed in Singapore yielded a significant association. A set of 21 tagging SNPs covering the *LRRK2* gene were genotyped in 466 sporadic PD and 374 control individuals all of Chinese ancestry. The authors identified a common haplotype that was highly overrepresented within cases ($p = 0.005$) and, when present in two copies, significantly increased the risk of PD (OR = 5.5, 95% C.I. = 2.1–14.0, $P = 0.0001$)[174]. However, no *LRRK2* variants within the risk haplotype were reported as the biologically relevant factors.

The G2385R variant

The *LRRK2* G2385R represents the fist common genetic risk factor for PD in the Asian population. This variant was first reported in a small PD family from Taiwan[80]. Evidence for cosegregation with PD in that family was limited due to the small pedigree size; however, the mutation was reported to be absent in 200 ethnically matched controls, and therefore, interpreted as putatively pathogenic. At that time very limited data were available on the nature and frequency of *LRRK2* mutations and on the polymorphism content of the gene in patients from Asia.

We and other groups first conducted a mutational screening of three known PD-causing mutations (I2012T, G2019S, and I2020T) which appeared to be very rare or absent in Asian PD patients[42, 109, 111, 112].

In order to identify other *LRRK2* variants which could play a role in PD in Asian populations we screened by sequencing the whole *LRRK2* coding region and splice sites in 15 Chinese Han patients with sporadic PD. Four of the identified coding variant (A419V, P755L, M1869V which were novel substitutions, and the G2385R) were tested for association with PD in our entire sample of 608 Chinese Han cases and 373 ethnically matched controls.

The heterozygosity for the G2385R variant was significantly higher among PD cases (61 heterozygous carriers out of 608 tested cases) than controls (18 heterozygous carriers out of 373 tested controls [nominal p value=0.004, corrected for multiple comparisons=0.012; permutation test, p=0.02 (5,000 replicates); gender- and age-adjusted odds ratio 2.24, 95% C.I.: 1.29–3.88]. This suggested that the G2385R variant, or another variant in linkage disequilibrium, is associated with PD in the Taiwanese population.

Since then, five association studies on Chinese Han population from Taiwan, Singapore and mainland of China replicated this finding with a similar size effect (Table 2). An additional study on Chinese recruited in Hong Kong reported a prevalence of G2385R in early and late onset PD of ~8.5%, and not in controls[175]. However, this study was not suitable to test association due to the small number of subjects.

Study	Geographical location	Ethnicity	PD	CTR	OR (95% CI)	p value
Di Fonzo et al. (2006)	Taiwan	Chinese Han	61/608 (10%)	18/373 (4.8%)	2.20 (1.28-3.78)	0.012
Tan et al. (2006)	Singapore	Chinese	37/495 (7.5%)	18/494 (3.6%)	2.14 (1.20-3.81)	0.002
Fung et al. (2006)	Taiwan	Chinese Han	27/305 (9%)	1/176 (0.5%)	17.00 (2.29-126.20)	0.0002
Farrer et al. (2007)	Taiwan	Chinese	34/410 (9.3%)	13/335 (3.9%)	2.24 (1.16-4.32)	0.014
Funayama et al. (2007)	Japan	Japanese	52/448 (11.6%)	22/457 (4.8%)	2.60 (1.55-4.35)	0.0002
Tan et al. (2007)	Singapore	Malay	2/98 (2%)	2/173 (1.2%)	1.75 (0.25-12.85)	0.6
		Indian	0/66 (0%)	0/133 (0%)		
Li et al. (2007)	Shanghai	Chinese Han	14/235 (6%)	0/214 (0%)	28.08 (1.66-473.72)	<0.001
An et al. (2008)	Mainland of China	Chinese Han	71/600 (11.8%)	11/334 (3.3%)	3.94 (2.06-7.55)	<0.001
Zabetian et al. (2009)	Japan and U.S.	Japanese	69/601 (11.5%)	101/1628 (6.2%)	1.96 (1.42-2.70)	<0.001
Total			**367/3800 (9.6%)**	**168/4184 (4.4%)**	**2.55 (2.10-3.10)**	**<0.001**

Table 2. Overview of results of *LRRK2* G2385R association studies in PD. Number of subjects homozygous or heterozygous for the G2385R and total number of individuals studied are reported. OR: odds ratio with 95% confidence interval (CI)

Interestingly the association was also reported in Japanese PD patients and controls, giving a risk of developing PD increased of ~2-fold[116, 176]. The clinical presentations of all G2385R carriers showed no difference from typical idiopathic PD with definite asymmetric onset, bradykinesia, resting tremor and rigidity. All carriers showed good response to dopamine replacement therapy[149, 176-179]. A meta-analysis on five studies suggested that the G2385R has a small but significant effect in lowering the age at onset of PD[180]. In one study a slightly higher prevalence in females with early onset was reported[178].
Two groups performed a haplotype analysis of G2385R carriers in a cohort of Chinese Han from Taiwan[177, 178]. A single common haplotype shared by all the carriers has been identified, likely originated from a single ancestor who lived approximately 4800 years ago. Also all Japanese G2385R carriers shared the same haplotype, with a set of markers (D12S2516, D12S2519 and D12S2521) which overlapped with the Chinese haplotype.

This might suggest that the G2385R of Chinese Han and Japanese ancestry has arisen from a common ancestor[116].

The R1628P variant

The *LRRK2* R1628P has been identified in a multicentric study which combined 1986 Chinese individuals from 3 independent centers in Taiwan and Singapore, and so far represents the second more frequent genetic risk factor for PD in Asia[181]. This variant was approximately twice as frequent in affected individuals as control subjects [~6% of PD and ~3.5% of controls, odds ratio 1.84, 95% C.I.: 1.20–2.83, nominal p value= 0.006] [181].
This finding was replicated in two independent Chinese Han cohorts from Singapore[182] and Taiwan[183] [8.4% vs 3.4% odds ratio 2.5, 95% C.I.: 1.1-5.6 p=0.046 and 7.4% vs 3.7% odds ratio 2.13, 95% C.I.: 1.29-3.52 p= 0.004, respectively]. On the contrary the R1628P is rare in Japan and in non-Chinese Asians[146, 181, 184].
Haplotype analysis strongly indicates that carriers of the R1628P variant share an extended haplotype, indicative of a founder effect[181]. The mutation has been estimated to arise ~2500 years ago and, in contrast to the older G2385R has remained confined to subjects of Chinese Han ethnicity.
Like for the G2385R, the clinical phenotype of the affected R1628P carriers is that of typical late onset L-dopa responsive PD[181, 183, 184].
Taken together, these studies indicate for the first time that common population specific genetic risk factors for PD exist. The association of both *LRRK2* variants with PD in Asia has been extensively confirmed in independent dataset of patients. These findings open several opportunities of studies for researchers and clinicians. Discovering how those variants can increase the risk of death of dopaminergic neurons might provide important insight into the pathogenesis of the disease. Other interesting prospects can be provided in clinical practice, for example studying the effect of neuroprotective drugs in large cohorts of asymptomatic carriers of these two *LRRK2* variants, in order to explore whether the risk of developing PD would decrease in the treated subjects.

Functional Studies on LRRK2

The LRRK2 protein

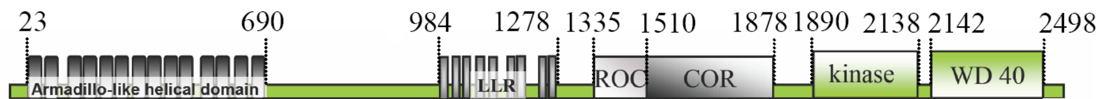

Figure 3

Schematic representation of the LRRK2 protein and its known functional domains. The domain boundaries are indicated by the residue numbers. Abbreviations: LRR, leucine-rich repeat domain; Roc, Ras of complex (GTPase).,COR, C terminal of Roc.

LRRK2 (also named dardarin) is a 2,527-amino acid protein which belongs to a newly identified protein family termed ROCO. LRRK2 has a complex structure, which includes several types of repeats and three large domains, called Roc (Ras in complex protein) , COR (C-terminal of Roc), and a mixed lineage kinase domain[185]. Two known protein-protein-interaction domains are present, the leucine-rich repeats (LRR) before the Roc domain, and the WD40 in C-terminus.

ROCO family genes are present in both prokaryotes and eukaryotes, but the function of the encoded proteins is still unknown. The paralogue LRRK1 is the closest relative of LRRK2; however, it largely differs from LRRK2 at its N-terminus. Recently a phylogenetic analysis on LRRK2 revealed an ancient orthologue of LRRK2 in N.*vectensis*[186]. By aligning the LRRK2 orthologue it was possible to recognize a specific conserved N-terminus repeat structure which can be considered as Armadillo-like helical domain. This kind of structure is considered to be a protein-protein interaction surface.

The newly identified LRRK2 conserved N-terminus region is absent in LRRK1, and interestingly also indicate that no true orthologue of LRRK2 exists in protostomes like

Drosophila melanogaster and *Caenorhabditis elegans*[186], that could be better to considered as LRRK1 orthologues.

In silico analysis

The LRRK2 residues replaced by the definitely pathogenic mutations are highly conserved among LRRK2 orthologues suggesting important roles on those residues for the LRRK2 function and possible loss of function as effect of the mutations (figure 4). Regarding the substitutions that are still of uncertain pathogenicity the conservation during evolution is variable. However, especially for mutations which would result in a gain of protein function, the conservation of the residues is not an absolute requisite for pathogenicity. If a definitely pathogenic role of those less conserved variant will be established, this would support a hypothesis of a gain of function effect.

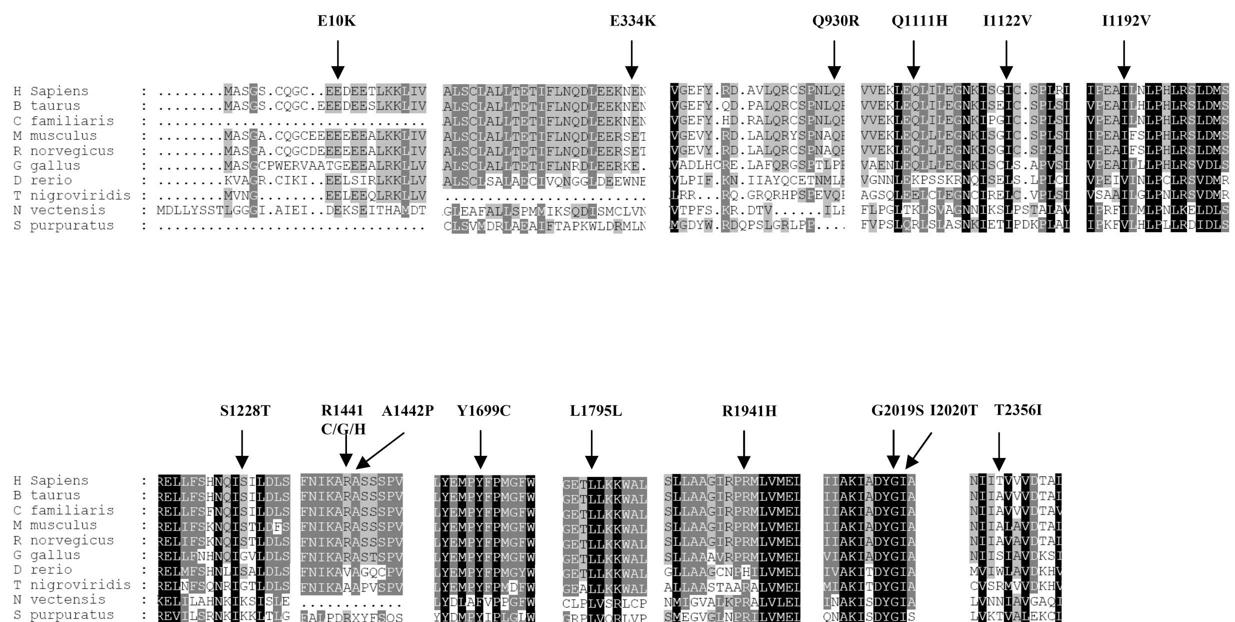

Figure 4

Multiple alignment of LRRK2 protein with orthologues. The most likely and definite pathogenic mutations are indicated.

mRNA expression patterns

The human *LRRK2* transcript is ubiquitously expressed, with highest levels in lung and kidney[187]. The expression of *LRRK2* mRNA has been examined in neurologically normal human brains, where it was found to be expressed in both dopaminergic and non dopaminergic areas, which would suggest an important role in basic cellular functions (reviewed in [188]).

In vitro studies

Several studies on the expression of the LRRK2 protein in brain areas and sub-cellular compartments have been published (reviewed in [141]). The major caveat is the specificity of LRRK2 antibodies, most of which have so far been unable to detect on western blot analysis a single band corresponding to the endogenous LRRK2 protein[141]. This technical limit has a major impact on the results of immunohistochemistry and sub-cellular localization studies as well. Additionally, the dissimilar specificity of the various LRRK2-antibodies used so far (together with the different experimental procedures) could also explain the different results in detecting LRRK2 in Lewy bodies[158, 189-193], and tau-immunoreactive inclusions[160, 190, 194].
In vitro studies on human LRRK2 revealed that the Roc domain, which exists as a dimer[195], has a GTPase activity and, by binding GTP[196-200], it activates the kinase domain[200].
The purified LRRK2 protein, through its kinase domain, is able to autophosphorylate or to phosphorylate a generic substrate[201, 202]. The G2019S mutation lies within the activation segment of the kinase domain of LRRK2. Interestingly, the activity of a large variety of protein kinases is controlled by activation segment conformation, whereby phosphorylation of this segment leads to a conformational switch of the kinase from an ‘off’- to an ‘on’-state[203]. Both overexpressed and purified LRRK2-G2019S increase the kinase activity in terms of autophosphorylation [189, 201, 202]. Initial studies reported that R1441C and R1441G mutations, which lie within the GTPase domain, decrease GTPase activity[196, 197], and, interestingly, lead to an increased kinase activity[197] , although the latter observation was not

confirmed in a further study[204]. An increased kinase activity has been found by overexpressing LRRK2 Y1699C and I2020T mutants as well[199, 205]; however, the effect of the Y1699C was not confirmed elsewhere[204].

The results of cell culture experiments are derived by using overexpressed LRRK2 and therefore should be considered with caution. Mutations in LRRK2 which lead to increased kinase activity are also associated with formation of toxic aggregates [189] and increased apoptotic cell death in dopaminergic cell lines and primary neurons[198]. In addition, LRRK2 kinase-dead constructs reduced neuronal toxicity[198].

Mutations affecting the kinase domain (G2019S and I2020T) might play a crucial role also in neurite outgrowth, leading to dramatic reductions in neurite length and branching of axons and dendrites[206]; on the contrary, overexpressing the kinase-dead K1906M construct or LRRK2 short hairpin RNA leads to an increased neurite length[206].

In summary, these results, though considering the limits of the current experimental procedures, suggest that the mutations leading to a reduced GTPase or an increased kinase activitiy would result in apoptotic cell death. Whether this effect has to be considered a gain or a loss of protein function is still controversial, since the final outcome of the LRRK2 activity is still unknown. Moreover, only the effect of the proved pathogenic mutations has been studied so far. It would be interesting to explore the functional consequence of other variants, at the moment classified as possible pathogenic, like the IVS20+4delGTAA, IVS33+6T>A and E1874X, which at least *in silico* are predicted to result in a truncated protein. Most of the observations still need further confirmation in more physiological models to clarify the normal function and the consequence of the mutations of the LRRK2 protein.

Animal models

- C. elegans

The *C. elegans* knockout for LRK-1 gene (which was considered to be a LRRK2 homologue) displays an aberrant sorting of synaptic vesicle proteins to axons[207] and a hypersensitivity to endoplasmic reticulum stressor tunycamicin[208]. In addition, overexpressing human LRRK2 strongly protects C. *elegans* against the mitochondrial toxin rotenone. The G2019S LRRK2 construct also protected against rotenone toxicity, but to a lesser degree than wild-type LRRK2, while knocking down LRK-1 potentiated rotenone toxicity. These data suggest a role of LRRK genes in mitochondrial physiology[209].

- D. melanogaster

The first transgenic Drosophila was generated using FLAG-tagged transgenic alleles for wild type LRRK and a mutant containing R1069C mutation corresponding to the pathogenic human LRRK2 R1441C mutation. No deleterious effect of the mutation was detected.
On the contrary, loss-of-function dLRRK mutations induced locomotive impairment and a reduction in tyrosine hydroxylase (TH) immunostaining within dopaminergic neurons, as well as loss of fertility in females[210].
A second study described a transgenic Drosophila with a P-element insertion in the dLRRK gene which results in a truncated protein lacking the C-terminal kinase domain. Experimental data demonstrated that dLRRK kinase activity is not required for normal development and growth[211].
Finally transgenic Drosophila overexpressing either wild-type human LRRK2 or LRRK2-G2019S were generated. Neuropathological analysis revealed that, at 1 day after eclosion, there were no differences in anti-TH-positive staining between of control and LRRK2 or LRRK2-G2019S flies. However, at 5 weeks of age, anti-TH staining decreased

significantly in flies expressing either wild-type or mutant LRRK2-G2019S. There was statistically significant TH-positive neuronal loss in all of the DA clusters examined. In addition, mutant LRRK2-G2019S caused more TH-positive neuronal loss than wild-type LRRK2 at equivalent expression levels. The expression of the human WT and mutant LRRK2 in neurons produced also locomotor dysfunction and early mortality. Expression of mutant G2019S-LRRK2 caused a more severe parkinsonism-like phenotype than expression of equivalent levels of wild-type LRRK2[212].

- <u>Rodents</u>

LRRK2-knockout mice[213, 214], and bacterial artificial chromosome (BAC) transgenic mice expressing murine FLAG-tagged WT Lrrk2[197] have been briefly mentioned in several papers, but their detailed descriptions are still lacking. A conditional Lrrk2 G2019S mouse model has been created and it was useful to observe LRRK2 interacting with Hsp90 *in vivo*[215]. However, the human LRRK2 G2019S mice did not shown obvious neuropathological or motor abnormalities at 12 months of age, although primary hippocampal neurons derived from the mice displayed retarded axon outgrowth during neuronal morphogenesis[215].

Electroporating Lrrk2 WT and mutant cDNA expression constructs in neural progenitors of the periventricular region of rat embryos resulted in dramatic reductions in neurite process length and branchpoint number in G2019S and I2020T mutants. Furthermore, embryos that were electroporated with lentiviral *LRRK2* shRNA had increased branchpoint number and a tendency towards increased process length[206]. In adult rodents, adeno-associated viral expression of the kinase domain WT or G2019S in the substantia nigra results in striatal abnormalities, including inclusions containing phosphorylated tau[206].

Recently a BAC transgenic mouse model expressing the human LRRK2 R1441G has been described[216]. An age-dependent levodopa-responsive motor-activity deficit was observed. Moreover, neuropathological analyses showed diminished dopamine release and axonal pathology of nigrostriatal dopaminergic projection. This model seems to successfully

recapitulate the phenotypical, neurochemical and histopathological features of the human disease.

Considering all these data, it seems that it is still too early to draw any conclusion on the physiologic role of LRRK2. The observation of retarded axon outgrowth in primary neurons of transgenic G2019S mice and the clinical and pathological features of the LRRK2-R1441G transgenic mice seems to represent an exquisite model to study the disease, but how and why the mutated LRRK2 would affect only dopaminergic neurons and which interactors play a role in this mechanism remains to be explored.

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Chapter 2 - A frequent LRRK2 gene mutation associated with autosomal dominant Parkinson's disease

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A frequent *LRRK2* gene mutation associated with autosomal dominant Parkinson's disease

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Mutations in the *LRRK2* gene have been identified in families with autosomal dominant parkinsonism. We amplified and sequenced the coding region of *LRRK2* from genomic DNA by PCR, and identified a heterozygous mutation (Gly2019Ser) present in four of 61 (6·6%) unrelated families with Parkinson's disease and autosomal dominant inheritance. The families originated from Italy, Portugal, and Brazil, indicating the presence of the mutation in different populations. The associated phenotype was broad, including early and late disease onset. These findings confirm the association of *LRRK2* with neurodegeneration, and identify a common mutation associated with dominantly inherited Parkinson's disease.

Parkinson's disease is the second most common neuro-degenerative disease after Alzheimer's disease, with a prevalence of more than 1% after the age of 65 years. The condition is defined clinically by resting tremor, bradykinesia, and muscular rigidity, and pathologically by brain dopaminergic neuronal loss, with inclusion formation (Lewy bodies) in surviving neurons. The cause of the disease remains unknown in most cases. About 15–20% of patients have a positive family history of Parkinson's disease in first-degree relatives, suggesting that genes have a role. However, until recently, causative mutations had been identified only in rare cases of Parkinson's disease, usually of early-onset, and sometimes with atypical clinical or pathological features.[1]

Linkage of an autosomal dominant form of parkinsonism (PARK8) to chromosome 12 was shown in a Japanese family,[2] and later confirmed in two white families. Recently, mutations in a gene termed *LRRK2* (leucine-rich repeat kinase 2) were identified in families with PARK8.[3,4] The ranges of clinical and pathological characteristics associated with *LRRK2* mutations are broad, and include typical late-onset Parkinson's disease with Lewy-body pathology, showing that mendelian mutations are associated with the classic form of Parkinson's disease. In other cases, Lewy bodies are absent, and unusual inclusions or pathological findings usually associated with different neurodegenerative diseases are present.[4]

The *LRRK2* gene encodes a large protein of 2527 amino acids and unknown function. The protein, dardarin,[3] belongs to a group within the Ras/GTPase superfamily, termed ROCO, characterised by the presence of two conserved domains named Roc (Ras in complex proteins) and COR (C-terminal of Roc), together with other domains including a leucine-rich repeat region, a WD40 domain, and a tyrosine kinase catalytic domain.[4]

We recruited a consecutive series of 61 families with Parkinson's disease and a family history compatible with autosomal dominant inheritance. 51 families were from Italy, nine from Brazil, and one from Portugal. The clinical diagnosis of definite Parkinson's disease was established according to widely accepted criteria.[5] Pathological studies were not done. The project was approved by the local ethics authorities. Written informed consent was obtained from all participants.

We isolated genomic DNA from peripheral blood from patients with Parkinson's disease and unaffected relatives by standard methods. The 51 exons of *LRRK2* were amplified from genomic DNA using PCR and directly sequenced in both strands. PCR reactions were

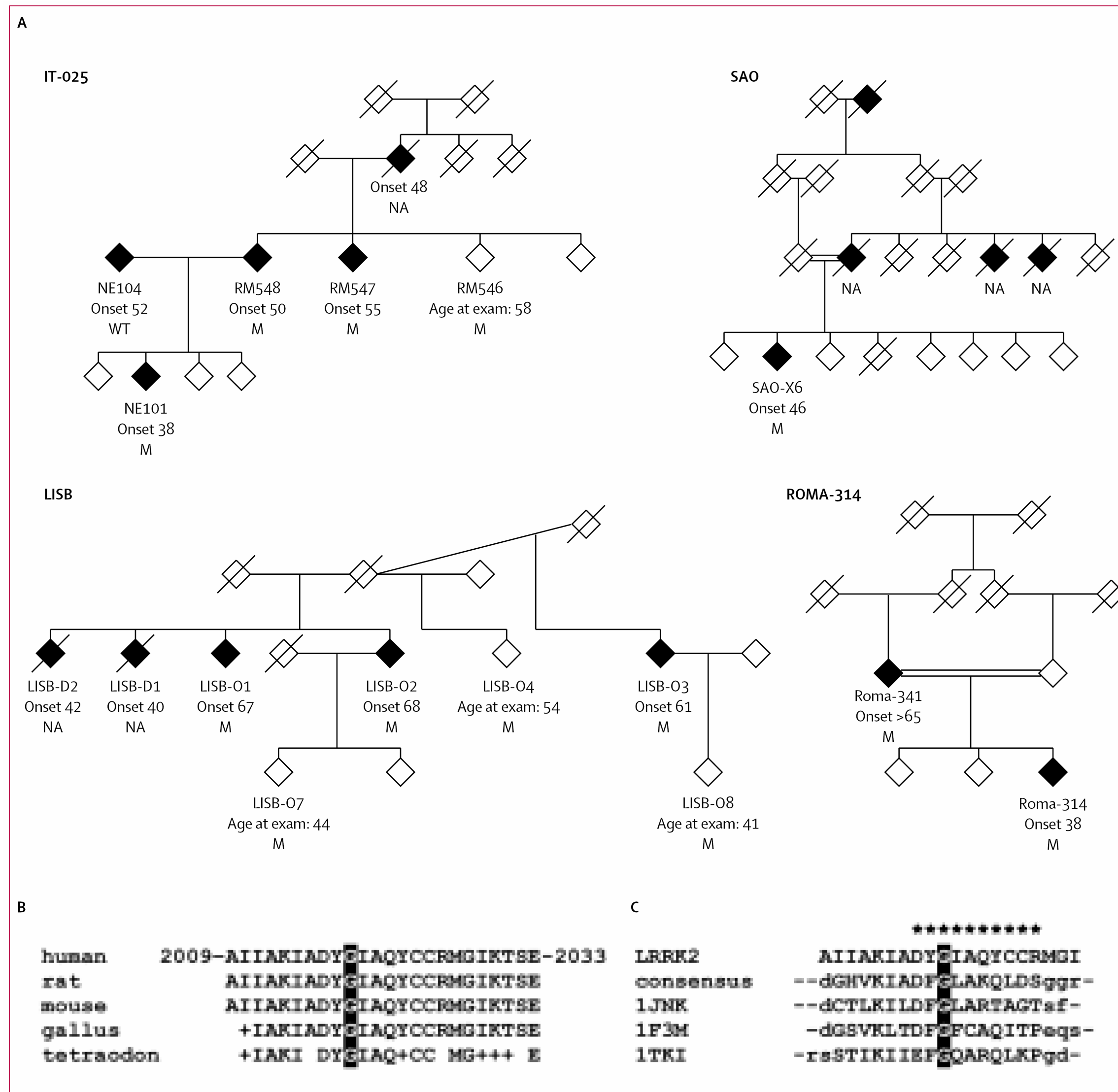

***Figure:* Clinical and molecular findings**

Simplified pedigrees of families with *LRRK2* mutations. Black symbols denote individuals affected by Parkinson's disease. Age at onset of disease or at examination shown in years. To protect confidentiality, sex of individuals is disguised and mutation carriers among youngest relatives are not indicated. One spouse (NE104) was also affected by Parkinson's disease (sporadic form), and did not carry the Gly2019Ser mutation. M=carrier of heterozygous Gly2019Ser mutation. WT=wild type genotype. NA=DNA not available. (B) Alignment of human dardarin protein and closest homologues: *Rattus norvegicus* (GenBank accession number XP_235581), *Mus musculus* (AAH34074), *Gallus gallus* (XP_425418), and *Tetraodon nigroviridis* (CAG05593). The mutated residue G2019 is highlighted. (C) Alignment of catalytic domains of human protein kinases. Asterisks indicate part of the activation segment. 1JNK=C-JUN N-terminal kinase. 1F3M=human serine-threonine kinase PAK1. 1TKI=serine kinase domain of the giant muscle protein titin. Consensus: consensus sequence for human protein kinase catalytic domain.

done in 25 μL containing 1× Invitrogen PCR buffer, 1·5 mmol/L $MgCl_2$, 0.01% W1 detergent, 25 μmol/L of each dNTP, 0·4 μmol/L forward primer, 0·4 μmol/L reverse primer, 2·5 units of Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA, USA), and 50 ng genomic DNA. Cycle conditions were: 5 min at 94°C; 30 cycles of 30 s denaturation at 94°C; 30 s annealing; and 90 s extension at 72°C; final extension 5 min at 72°C (primers and annealing temperatures reported in the webtable, http://image.thelancet.com/extras/04let12084webtable.pdf). Direct sequencing of both strands was done with Big Dye Terminator chemistry version 3.1 (Applied Biosystems, Foster City, CA, USA). Fragments were loaded on an ABI3100 automated sequencer and analysed with DNA Sequencing Analysis (version 3.7) and SeqScape (version 2.1) software (Applied Biosystems).

We predicted the consequences of mutations at the protein level according to the *LRRK2* cDNA sequence deposited in Genbank (accession number AY792511).

Novel variants that co-segregated with disease were tested in a panel of 250 chromosomes from healthy Italian people aged older than 60 years, by use of allelic specific oligohybridisation. For the Gly2019Ser mutation, PCR products containing *LRRK2* exon 41 were blotted into Hybond-N+ membranes (Amersham Biosciences, Amersham Biosciences, Buckinghamshire, UK). The blots were hybridised for 1 h at 37°C in 5× sodium chloride/sodium phosphate/EDTA (SSPE), 1% sodium dodecyl sulphate, and 0·05 g/L single-strand salmon sperm DNA with either the normal or mutated sequence oligonucleotides (wild-type allele: tgactacggcattg; mutant allele: gactacagcattgc). Filters were washed in buffer containing 0·045 mol/L sodium chloride, 0·0045 mol/L sodium citrate, and 0·1% sodium dodecyl sulphate, at 37°C.

By sequencing the whole *LRRK2* coding region in the probands from 15 families, we identified two heterozygous carriers of an exon 41 mutation, 6055G→A (numbered from the A of the ATG-translation initiation codon), predicted to replace the glycine at position 2019 of the dardarin protein with serine (Gly2019Ser; electropherogram available at http://image.thelancet.com/extras/04let12084webfigure.pdf). The mutation co-segregated with Parkinson's disease in the families (figure 1A), and was absent in the 250 control chromosomes. In these two probands, we detected several polymorphisms but no further variants that co-segregated with Parkinson's disease and were absent in control chromosomes.

Direct sequencing of exon 41 in the remaining 46 probands identified another two heterozygous carriers, bringing the prevalence of the Gly2019Ser mutation to four of 61 autosomal dominant families (6·6%, 95% CI 0·4–12·8).

16 individuals in these four families had Parkinson's disease, but accurate clinical information was available for only ten of them (table). These individuals had a broad range of age of disease onset (table; average 50·5 years, range 38–68, n=10), including two patients with onset before age 40 years. All patients responded well to levodopa. Dementia and additional neurological signs were not present. Asymmetric onset and complications typically associated with long-term treatment with levodopa (motor fluctuations and choreic dyskinesias) were noted in some patients, lending support to the accuracy of the clinical diagnosis of typical Parkinson's disease.[5] The broad range of ages of onset suggests that factors other than the mutation identified have a role in modifying the disease. Clinical features in patients who carried the Gly2019Ser mutation were similar to those of patients who did not (data not shown).

Several unaffected family members carried the mutation, but were younger than the latest age of onset observed in these families (figure 1A). These individuals are still at risk of developing Parkinson's disease. This finding indicates an age-dependent (perhaps incomplete) penetrance for this mutation, as reported for other *LRRK2* mutations.[3,4] The families carrying the Gly2019Ser allele lived in Italy (two families), Portugal, and Brazil, suggesting that this mutation is present in different populations.

Further evidence for the pathogenic role of the mutation is provided by the observation that the Gly2019 residue is not only conserved among the dardarin protein homologues, but is also part of a motif of three amino acids (AspTyrGly or AspPheGly) that is required by all human kinase proteins (figure 1B and C).

Our data provide independent confirmation that *LRRK2* mutations cause human neurodegeneration, and identify a single common mutation associated with autosomal dominant Parkinson's disease. Precise information about the penetrance of this mutation will be important for clinical practice. Since penetrance is age-dependent, this mutation might be found in patients with negative family history. These findings have implications for the diagnosis and counselling of patients with Parkinson's disease.

	Patient number									
	1	2	3	4	5	6	7	8	9	10
Onset age (years)	67	68	61	40	42	50	55	38	38	46
Duration (years)	5	1	3	30	31	16	6	5	8	15
UPDRS	12	14	14	NA	NA	17	20	12	15	26
Rest tremor	+	+	+	NA	+	+	+	-	-	+
Bradykinesia	+	+	+	NA	+	+	+	+	+	+
Rigidity	-	+	+	NA	+	+	+	+	+	+
Asymmetric onset	+	+	+	NA	+	+	+	NA	+	+
Levodopa response	+	+	+	+	+	+	+	+	+	+
Motor fluctuations	-	-	-	NA	+	+	+	-	+	+
Dyskinesias	-	-	-	NA	+	+	+	-	+	+
Dementia	-	-	-	-	-	-	-	-	-	-
Dysautonomia	+	-	-	-	-	-	-	-	-	-
Others	S, D	S	S, D	-	-	D	-	-	D	-

UPDRS=unified Parkinson's disease rating scale, motor score under the effect of medication (maximum 108). S=sleep disturbance. D=early morning dystonia. NA=not available. Patient codes: 1=LISB-01, 2=LISB-02, 3=LISB-03, 4=LISB-D1, 5=LISB-D2, 6=RM-548, 7=RM-547, 8=NE-101, 9=ROMA-314, 10=SAO-X6

***Table:* Clinical features of the ten individuals with Parkinson's disease in families with the mutation**

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Conflict of interest statement
We declare that we have no conflict of interest.

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Chapter 3 - The G6055A (G2019S) mutation in LRRK2 is frequent in both early and late onset Parkinson's disease and originates from a common ancestor

S Goldwurm, A Di Fonzo, E J Simons, C F Rohé, M Zini, M Canesi, S Tesei, A Zecchinelli, A Antonini, C Mariani, N Meucci, G Sacilotto, F Sironi, G Salani, J Ferreira, H F Chien, E Fabrizio, N Vanacore, A Dalla Libera, F Stocchi, C Diroma, P Lamberti, C Sampaio, G Meco, E Barbosa, A M Bertoli-Avella, G J Breedveld, B A Oostra, G Pezzoli and V Bonifati

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ELECTRONIC LETTER

The G6055A (G2019S) mutation in *LRRK2* is frequent in both early and late onset Parkinson's disease and originates from a common ancestor

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Background: Mutations in the gene *Leucine-Rich Repeat Kinase 2* (*LRRK2*) were recently identified as the cause of PARK8 linked autosomal dominant Parkinson's disease.
Objective: To study recurrent *LRRK2* mutations in a large sample of patients from Italy, including early (<50 years) and late onset familial and sporadic Parkinson's disease.
Results: Among 629 probands, 13 (2.1%) were heterozygous carriers of the G2019S mutation. The mutation frequency was higher among familial (5.1%, 9/177) than among sporadic probands (0.9%, 4/452) ($p<0.002$), and highest among probands with one affected parent (8.7%, 6/69) ($p<0.001$). There was no difference in the frequency of the G2019S mutation in probands with early *v* late onset disease. Among 600 probands, one heterozygous R1441C but no R1441G or Y1699C mutations were detected. None of the four mutations was found in Italian controls. Haplotype analysis in families from five countries suggested that the G2019S mutation originated from a single ancient founder. The G2019S mutation was associated with the classical Parkinson's disease phenotype and a broad range of onset age (34 to 73 years).
Conclusions: G2019S is the most common genetic determinant of Parkinson's disease identified so far. It is especially frequent among cases with familial Parkinson's disease of both early and late onset, but less common among sporadic cases. These findings have important implications for diagnosis and genetic counselling in Parkinson's disease.

Parkinson's disease affects more than 1% of people after the age of 65 years, and is the second most common neurodegenerative disorder after Alzheimer's disease.[1] The disease is defined clinically by the association of bradykinesia, resting tremor, muscular rigidity, and postural instability, and pathologically by loss of dopaminergic neurones in the substantia nigra-pars compacta and other brain sites, with formation of ubiquitin containing inclusions (Lewy bodies) in the surviving neurones.[1]

The cause of the disease remains unknown in most patients, but a positive family history of Parkinson's disease is found in ~15–25% of cases, and mutations in five genes have been firmly implicated in the aetiology of rare inherited forms of the disease.[2 3]

An autosomal dominant form of Parkinson's disease (PARK8) was first mapped to chromosome 12 in a Japanese family[4]; this linkage was later confirmed in white families.[5 6] Recently, mutations in the gene *Leucine-Rich Repeat Kinase 2(LRRK2)* (MIM *609007) were identified in PARK8 linked families.[7 8] The *LRRK2* gene encodes a predicted protein of 2527 amino acids, which has unknown function. This protein, termed dardarin, belongs to the ROCO group within the Ras/GTPase superfamily, and contains several conserved domains: an Roc (Ras in complex proteins) and a COR (C-terminal of Roc) domain, together with a leucine-rich repeat, a WD40 domain, and a tyrosine kinase catalytic domain.[9]

To date, seven *LRRK2* pathogenic mutations have been reported in autosomal dominant Parkinson's disease. Four of these mutations recurred in at least two unrelated families: Y1699C (present in two large kindreds, family "A" of German-Canadian ancestry, and one British kindred)[7 8]; R1441C (found in family "D" of Western Nebraska origin, and another family)[8]; R1441G (found in several families and a few sporadic cases in the Basque population)[7]; and G2019S, which we and other groups have recently identified.[10–14]

Mutations in the *LRRK2* gene, particularly G2019S, appear to be relevant for Parkinson's disease, but the frequency of these mutations according to clinical features of the probands—such as onset age and pattern of presentation (familial or sporadic)—has not been assessed in large consecutive series of probands from homogeneous well defined populations. The frequency of known or novel *LRRK2* mutations might be different in different populations; moreover, the previous studies have targeted mainly late onset Parkinson's disease series. Therefore the frequency of mutations remains unknown among early onset patients.

The penetrance of *LRRK2* mutations appears strongly age related, and is probably incomplete[4 7 8 10 12 14]; these mutations might therefore also be expected in patients with the sporadic presentation (the vast majority of cases of Parkinson's disease). It is therefore urgent to assess the prevalence and associated phenotype of the G2019S and other *LRRK2* mutations in clinically and ethnically well defined series of familial and sporadic Parkinson's disease cases, including early and late onset patients.

Here, we report the first study of all four so far known recurrent *LRRK2* mutations in a large sample of 629 probands with Parkinson's disease ascertained at a single centre in Italy. We also analyse the haplotypes and the clinical phenotypes associated with the G2019S mutation.

Abbreviations: LD, linkage disequilibrium; SNP, single nucleotide polymorphism

Table 1 Distribution of study sample according to 10 year onset age classes

	Early onset (years)			Late onset (years)			
	<30	30–39	40–49	50–59	60–69	⩾70	Total
All cases	7	83	140	224	142	33	629
G2019S heterozygous	–	3	4	4	–	2	13
Familial	1	21	46	65	33	11	177
G2019S heterozygous	–	2	3	3	–	1	9
Sporadic	6	62	94	159	109	22	452
G2019S heterozygous	–	1	1	1	–	1	4

METHODS

Subjects and clinical analyses

We studied 629 probands, representing two consecutive cohorts of Parkinson's disease cases with early onset disease (<50 years old at symptoms onset, n = 230) or late onset disease (⩾50 years old at onset, n = 399). The age at which the patient noticed the first symptom was considered to be the age of disease onset. Thirty three relatives affected by Parkinson's disease were also included, giving a total of 662 cases with the disease. All cases were examined and collected at the Parkinson Institute, Istituti Clinici di Perfezionamento, Milan, one of the largest referral centres for diagnosis and treatment of Parkinson's disease in Italy. Most cases were of Italian origin, but one case originated from each of the following countries: Argentina, Colombia, Ethiopia, France, Greece, Iceland, Ireland, Israel, and the United Kingdom.

The mean (SD) age at disease onset was 52.7 (10.9) years in the whole series of 629 probands, and 40.8 (5.6) years and 59.5 (6.6) years in the early onset and late onset groups, respectively. The clinical diagnosis of definite Parkinson's disease was established according to widely accepted criteria,[15] and required the presence of bradykinesia and at least one of the following: resting tremor, rigidity, and postural instability; a positive response to dopaminergic therapy; and the absence of atypical features or other causes of parkinsonism.

Patients were classified as "familial" if at least one relative was reported with a formal diagnosis of Parkinson's disease among the first, second, or third degree relatives. The other probands were classified as "sporadic".

The four mutations were tested—using the same method as for the Parkinson's disease cases—in 440 Italian controls, including 304 elderly individuals free from Parkinson's disease or dementia (spouses of Parkinson's disease cases, outpatients of general practices, and blood donors, average age 66.4 (9.3) years), and 136 inpatients with cerebrovascular disease (average age 64.9 (8.4) years). The whole sample of controls (880 chromosomes) was tested for the G2019S mutation. The remaining mutations were tested in a total of 530 chromosomes. The project was approved from the local ethics authorities, and written informed consent was obtained from all subjects.

Mutation analysis

Genomic DNA was isolated from peripheral blood using standard protocols.[16] The primers and polymerase chain reaction (PCR) protocol used to amplify the *LRRK2* exons (Nos 31, 35, and 41) containing the C4321T (R1441C), C4321G (R1441G), the A5096G (Y1699C), and the G6055A (G2019S) mutation have been reported previously.[10] The consequences of mutations at the protein level were predicted according to the *LRRK2* cDNA sequence (Genbank accession number AY792511).

About 20 ng of pooled PCR product (exons 31, 35, and 41) were purified using ExoSAP-IT (USB) and used in a primer extension reaction (SNaPshot) including the following primers: for the R1441C and the R1441G mutations in exon 31 (sense strand), 5′-agaatcacaggggaagaagaagcgc-3′, product size 26 base pairs (bp) (primer length plus one base); for the Y1699C mutation in exon 35 (antisense strand), 5′-taatc-gattgattaatcttgaccaaaatcccattggaaaa-3′, product size 41 bp; for the G2019S mutation in exon 41 (antisense strand), 5′-aatgctgccatcattgcaaagattgctgactac-3′, product size 34 bp.

Reactions were carried out in 10 µl containing 1 µl SNaPshot multiplex ready reaction mix (Applied Biosystems, Foster City, California, USA); 2.5 µM R1441C/R1441G, 7.5 µM Y1699C, 2.5 µM G2019S extension primer, and 1 µl ½ term buffer (200 mM TrisHCl; 5 mM $MgCl_2$, pH 9). Additional thermal cycling was undertaken for 40 cycles of 10 seconds at 95℃, five seconds at 50℃, and 30 seconds at 60℃. Removal of the 5′-phosphoryl groups was done using 1 unit of shrimp alkaline phosphatase (SAP) (Roche Diagnostics, Monza, Italy) for 30 minutes at 37℃.

One microlitre of SNaPshot product was diluted in 10 µl Hi-Di formamide (Applied Biosystems) containing GeneScan-120 LIZ size standard (Applied Biosystems), denatured for five minutes at 95℃, cooled on ice, and loaded on an ABI3100 Genetic Analyzer (Applied Biosystems). Fragments were analysed using GeneMapper V3.0 software (Applied Biosystems).

Negative and positive controls for the G2019S and R1441C mutations were included in all experiments. Positive controls were not available for the R1441G and the Y1699C mutation. All the mutations identified in the SNaPshot screening were confirmed by direct sequencing using a second DNA aliquot.

In one case carrying the G2019S mutation and one control, total RNA was isolated from blood cells and cDNA was prepared using standard protocols. A 251 bp fragment of the *LRRK2* cDNA spanning exons 41–42 was amplified using the following primers: forward 5′-cacgtagctgatggtttgagatacc-3′; reverse 5′-ccaaatgaataaacatcagcctgt-3′.

Haplotype analysis

Nineteen intragenic and flanking markers (13 microsatellites and six single nucleotide polymorphisms (SNP)) were typed, including both known exonic and a newly discovered *LRRK2* intronic SNP (IVS13+104G/A) in linkage disequilibrium (LD) with the G2019S mutation. Microsatellites were selected from the Marshfield integrated map and from Kachergus *et al*[14]; they were amplified by PCR using fluorescently labelled F-primers according to standard methods; fragments were loaded on an ABI3100 and analysed using the GeneMapper version 3.0 software (Applied Biosystems). Exonic and intronic *LRRK2* SNPs were typed by direct sequencing using the primers and PCR conditions reported previously.[10]

The frequency of the IVS13+104G/A SNP was assessed in 100 chromosomes from Italian Parkinson's disease cases and 200 chromosomes of Italian controls.

We included in the haplotype analysis 12 families with the G2019S mutation detected in this series, the four families reported by us previously,[10] and another two unpublished families (IT-023 and TH-08, from Italy and Morocco,

Table 2 Frequency of the G2019S mutation according to familial aggregation

Proband category	n (%)	Male/female	Onset (years) (mean (SD))	Range	G2019S	%
All probands (early and late onset)	629 (100%)	369/260	52.7 (10.9)	23 to 82	13	2.1%
All familial probands (1st, 2nd, 3rd degree affected relatives)	177 (28.1%)	103/74	52.6 (10.9)	23 to 82	9	5.1%*
All sporadic probands	452 (71.9%)	266/186	52.7 (11.0)	23 to 80	4	0.9%
All early onset probands	230 (100%)	149/81	40.8 (5.6)	23 to 49	7	3.0%
Familial early onset	68 (29.6%)	43/25	41.5 (5.5)	23 to 49	5	7.4%†
Sporadic early onset	162 (70.4%)	106/56	40.6 (5.7)	23 to 49	2	1.2%
All late onset probands	399 (100%)	220/179	59.5 (6.6)	50 to 82	6	1.5%
Familial late onset	109 (27.3%)	60/49	59.6 (6.9)	50 to 82	4	3.7%‡
Sporadic late onset	290 (72.7%)	160/130	59.4 (6.5)	50 to 80	2	0.7%
Probands with "dominant" PD (1st or 2nd degree affected relatives)	114	68/46	50.1 (10.7)	23 to 74	8	7.0%§
Probands with one affected parent	69	42/27	51.0 (9.7)	23 to 71	6	8.7%§
Probands with affected 2nd degree relative only	42	24/18	49.2 (11.7)	32 to 74	2	4.8%[NS]
Probands with affected siblings and 2nd degree relative	3	2/1	41.0 (15.6)	32 to 59	0	–
Probands with affected siblings only	49	27/22	56.9 (10.5)	36 to 82	1	2.0%[NS]
Probands with affected 3rd degree relative only	14	7/7	58.1 (6.9)	57 to 65	0	–

*$p<0.002$ *v* the frequency among the 452 sporadic probands (Fisher exact test).
†$p<0.025$: frequency in familial *v* sporadic early onset probands.
‡$p=0.05$: frequency in familial *v* sporadic late onset probands.
§$p<0.001$ *v* the frequency among the 452 sporadic probands.
[NS]No significant difference compared with the frequency among the 452 sporadic probands.
None of the differences between early onset and late onset groups (familial, sporadic, or all) was statistically significant.
PD, Parkinson's disease.

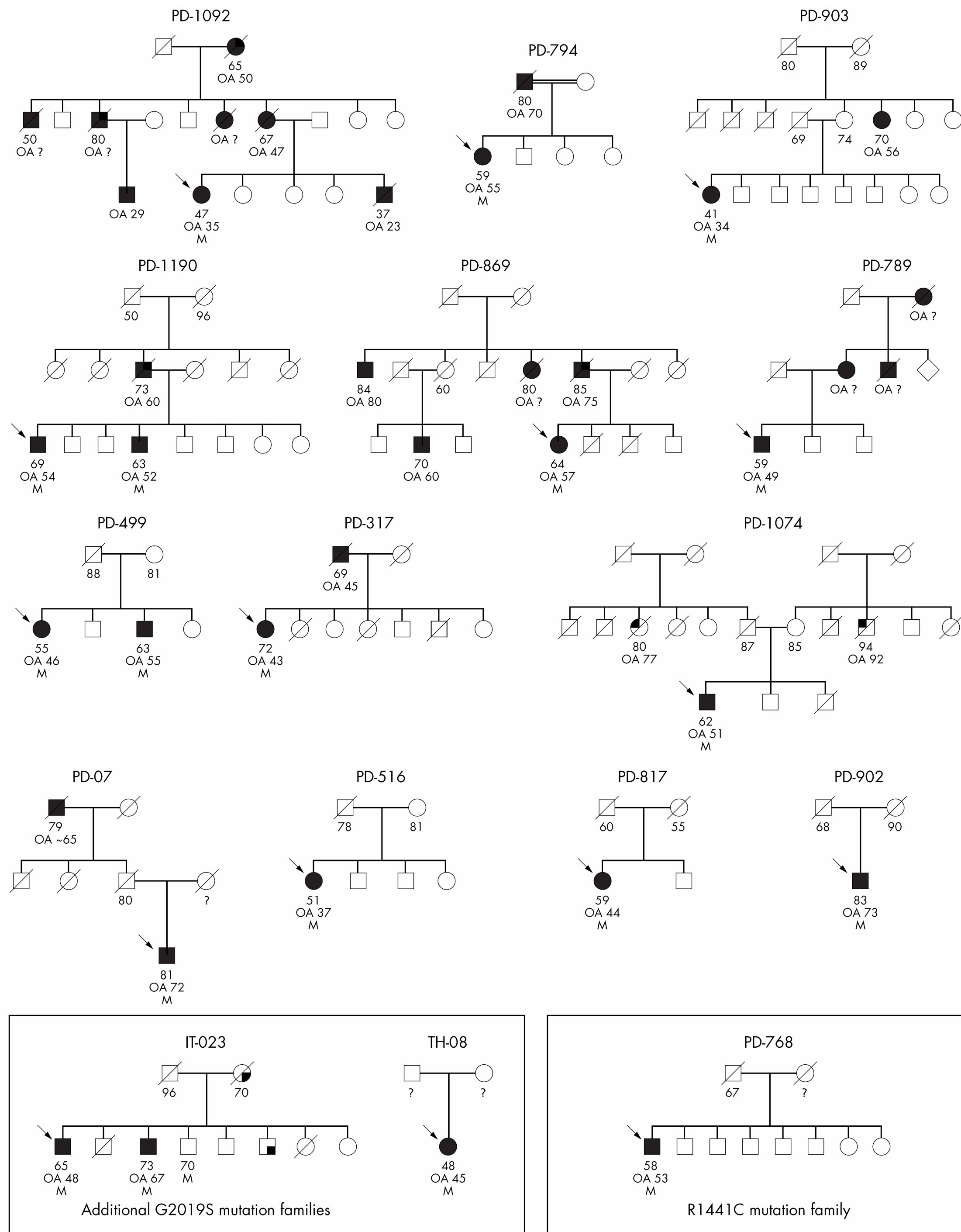


Figure 1 Simplified pedigrees of families with *LRRK2* mutations. Full black symbols: individuals affected by Parkinson's disease; symbols with black upper corner: individuals affected by senile dementia; symbols with black lower corner: individuals with tremor only. To protect confidentiality the order of individuals in sibships was altered. The first number below symbols indicates age at examination or age at death (years). OA, age at disease onset (years). Question mark indicates that information is not available (individuals who lost contacts with their family). M, carrier of heterozygous G2019S mutation. In family PD-768, M indicates the carrier of the R1441C mutation. No further individuals were known to be affected by Parkinson's disease among the more distant relatives, including the families of the sporadic Parkinson probands. Extended versions of these pedigrees are available on request.

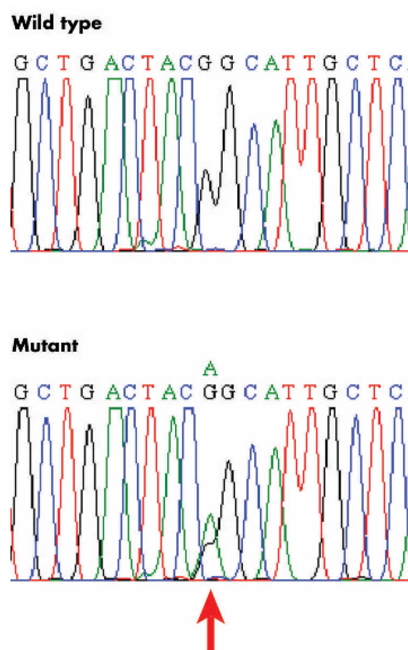


Figure 2 Electropherogram of part of *LRRK2* cDNA sequence from one Parkinson's disease patient and one control. The position of the heterozygous G6055A mutation (G2019S) is indicated (arrow).

respectively) identified by us from unrelated series of patients. Haplotypes were constructed manually. In four families phase could be assigned unambiguously for most markers by genotyping of trios of parents and child. In the remaining families, the phase was estimated using PHASE version 2.1.[17] Haplotypes with known phase were included to improve the performance of the program. Statistical analysis was undertaken using contingency tables and the Student's *t* test, as appropriate.

RESULTS

Frequency of mutations

The G2019S mutation was not detected in 880 control chromosomes, whereas it was identified in heterozygous state in 13 of the 629 probands (overall frequency 2.1%, $p<0.01$ *v* controls). The distribution of probands according to onset age classes and pattern of familial aggregation is presented in tables 1 and 2.

The carriers of the G2019S mutation included nine of 177 familial probands (5.1%) and four of 452 sporadic probands (0.9%) ($p<0.002$ familial *v* sporadic). The frequency of the G2019S mutation among the familial Parkinson's disease probands remained five times higher than among the sporadic probands when early onset or late onset groups were considered separately (table 2).

Considering together the familial and the sporadic sample, seven of 230 early onset probands (3.0%), and six of the 399 late onset probands (1.5%) carried the G2019S mutation (table 2). The frequency of carriers among early onset cases remained about twofold higher than among late onset case when either the whole sample or only familial or sporadic Parkinson's disease was considered; however, the differences between early and late onset groups did not reach statistical significance.

Among 600 probands tested, there was one heterozygous for the R1441C mutation but none carrying the R1441G or the Y1699C mutation. These mutations were not observed in controls.

The simplified pedigrees are shown in fig 1. These include the families of the 13 probands with the G2019S mutation and one with R1441C mutation identified in this study, and two unpublished families with the G2019S mutation (IT-023 and TH-08), identified from other Parkinson's disease cohorts, that were included in the haplotype study. Thirteen probands with the G2019S mutation were from Italy, one (PD-1092) was from Greece and another (TH-08) from Morocco.

In three families (PD-499, PD-1190, and IT-023), DNA was available from one affected relative; the G2019S mutation was found in heterozygous state in all these three secondary cases. The lack of DNA samples from other affected or unaffected relatives precludes further detailed analyses of co-segregation and penetrance of the mutation. The cDNA analysis from blood cells documented the expression of the mutant G2019S allele (fig 2).

Haplotype analysis

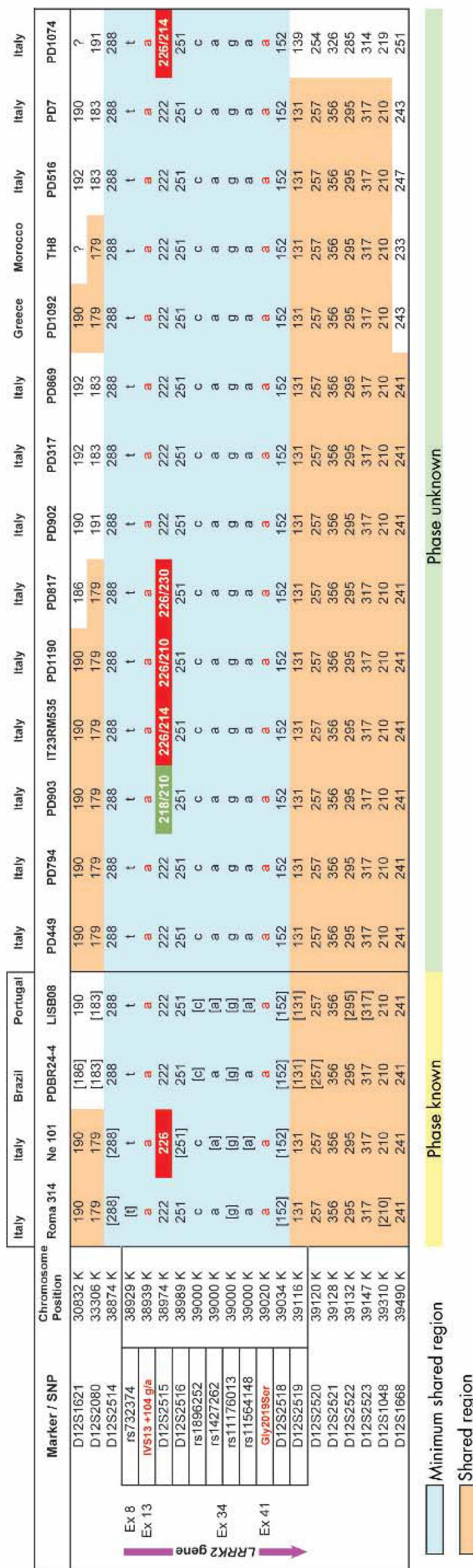
The results of the haplotype analysis are reported in fig 3. An extended shared region was present in the patients from all the families with phase assigned. For all patients with uncertain phase, the genotypes were compatible with the presence of the same haplotype (fig 3), as also predicted by the results of the PHASE program. These findings strongly suggest that the mutant G2019S allele was inherited from a common founder. The minimum size of the shared region is ~160 kb, defined by markers D12S2514 and D12S2518, while the maximum size is defined in our dataset by markers D12S2519 (~80kb from D12S2518) and D12S2080 (~570 kb from D12S2514).

Clinical features

Clinical features were similar in patients who carried the G2019S mutation and those who did not (table 3). Among the 15 cases detected from the consecutive cohort in this study (13 probands and two affected relatives) the first symptom at onset was rest tremor in five cases, bradykinesia in nine, and rigidity in one. Body distribution of signs and symptoms at onset was asymmetrical in all but one case. Bradykinesia and rigidity were present in all 15 cases on examination, while in nine cases rest tremor was documented at some time during the disease course. Decreased postural reflexes were documented in 11 cases. Response to levodopa was good in all. Motor fluctuations were observed in 13 cases, and levodopa induced dyskinesias in 12 of these. Two cases showed dystonic features. Freezing of gait was noted in 12. Severe autonomic dysfunction was not observed. Psychiatric disturbances were common: four cases had psychotic phenomena (hallucinations, delusions); two had depression years after the onset of motor symptoms, another three cases had depression at the time of onset, and in one case depression occurred seven years before the onset of motor symptoms.

Dementia was present in only one case. Sleep disturbances were also common, present in nine cases. In one case, amelioration of symptoms after sleep was noted (sleep benefit). Three cases were treated with deep brain stimulation, and one with thalamolysis.

In the patient carrying the R1441C mutation, Parkinson's disease started with asymmetrical rest tremor, later followed by bradykinesia, rigidity, and postural instability. Freeezing of gait, levodopa induced motor fluctuations, and dyskinesias also developed. Depression occurred three years before the onset of motor symptoms.



	Marker / SNP	Chromosome Position	Roma 314	Ne 101	PDBR24-4	LISB08	PD449	PD794	PD903	IT23RM535	PD1190	PD817	PD902	PD317	PD869	PD1092	TH8	PD516	PD7	PD1074
			Italy	Italy	Brazil	Portugal	Italy	Italy	Italy	Italy	Italy	Italy	Italy	Italy	Italy	Greece	Morocco	Italy	Italy	Italy
	D12S1621	30832 K	190	190	[186]	190	190	190	190	190	190	186	190	192	192	190	?	192	190	?
	D12S2080	33306 K	179	179	[183]	[183]	179	179	179	179	179	179	191	183	183	179	179	183	183	191
	D12S2514	38874 K	[288]	[288]	288	288	288	288	288	288	288	288	288	288	288	288	288	288	288	288
Ex 8	rs732374	38929 K	[t]	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
Ex 13	IVS13 +104 g/a	38939 K	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
	D12S2515	38974 K	222	226	222	222	222	222	218/210	226/214	226/210	226/230	222	222	222	222	222	222	222	226/214
	D12S2516	38989 K	251	[251]	251	251	251	251	251	251	251	251	251	251	251	251	251	251	251	251
	rs1896252	39000 K	c	c	[c]	[c]	c	c	c	c	c	c	c	c	c	c	c	c	c	c
Ex 34	rs1427262	39000 K	a	[a]	a	[a]	a	a	a	a	a	a	a	a	a	a	a	a	a	a
	rs11176013	39000 K	[g]	[g]	[g]	[g]	g	g	g	g	g	g	g	g	g	g	g	g	g	g
	rs11564148	39000 K	a	[a]	a	[a]	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Ex 41	Gly2019Ser	39020 K	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
	D12S2518	39034 K	[152]	[152]	[152]	[152]	152	152	152	152	152	152	152	152	152	152	152	152	152	152
	D12S2519	39116 K	131	131	[131]	[131]	131	131	131	131	131	131	131	131	131	131	131	131	131	139
	D12S2520	39120 K	257	257	[257]	257	257	257	257	257	257	257	257	257	257	257	257	257	257	254
	D12S2521	39128 K	356	356	356	356	356	356	356	356	356	356	356	356	356	356	356	356	356	326
	D12S2522	39132 K	295	295	295	[295]	295	295	295	295	295	295	295	295	295	295	295	295	295	285
	D12S2523	39147 K	317	317	317	[317]	317	317	317	317	317	317	317	317	317	317	317	317	317	314
	D12S1048	39310 K	[210]	210	210	210	210	210	210	210	210	210	210	210	210	210	210	210	210	219
	D12S1668	39490 K	241	241	241	241	241	241	241	241	241	241	241	241	241	243	233	247	243	251

Figure 3 Haplotypes of the *LRRK2* locus in 18 cases carrying the G2019S mutation. The minimum ~160 kb of DNA shared by all patients is in blue. The G2019S mutation and the IVS13+104G/A SNP, in linkage disequilibrium (LD) with the mutation among Parkinson's disease cases, are reported in red. Variations in 4 bp observed at tetranucleotide marker D12S2515 are probably the consequence of mutations occurring in this microsatellite, thereby defining subclasses within the ancestral haplotype. Both alleles are shown for this marker, in individuals who are not carrying the consensus allele (222 bp) in LD with G2019S. The marker D12S2518 contributed to the haplotype build but was not polymorphic in our entire dataset. For some other markers, genotypes with phase unknown or not informative are indicated between square brackets.

DISCUSSION

Frequency of *LRRK2* mutations in Parkinson's disease

This is the first comprehensive study of *LRRK2* recurrent mutations targeting large groups of Italian cases with early onset and late onset Parkinson's disease, with familial as well as sporadic presentation. We found a frequent occurrence of the G2019S mutation. On the other hand, the R1441C, R1441G, and Y1699C mutation were rare, suggesting they are not a relevant cause of Parkinson's disease in the Italian population. In addition to Italy, Portugal, and Brazil, and the countries reported by others,[10–14] we expand the presence of the G2019S mutation to Parkinson's disease cases from Greece and Morocco.

In the initial studies, the G2019S mutation was found in ~3–6% of selected samples with familial Parkinson's disease (autosomal dominant families, and sibling pairs) from several European and North American countries, and in ~1% of sporadic Parkinson's disease cases from the United Kingdom, while it was absent in more than 4000 control individuals.[10–14] However, the frequency may vary considerably between populations—recent studies suggest a very high prevalence in North African and a very low prevalence in Asian populations.[18 19]

The pathogenic role of the G2019S mutation is further supported by the observation that the G2019 residue is extremely conserved in human kinase domains and in all dardarin homologues.[20]

Here we report the frequency of G2019S in a large sample of clinically and ethnically well defined patients, showing that G2019S is significantly more frequent among the cases with familial Parkinson's disease than among those with sporadic disease, further supporting the pathogenic role of this mutation in Parkinson's disease inheritance. The phenotype associated with the mutation encompasses early and late onset Parkinson's disease, and we show here for the first time that this mutation is also common among cases with onset before the age of 50 years. However, as late onset disease represents the vast majority of cases, it is anticipated that a larger number of patients with this mutation will be identified among the cases with late onset classical Parkinson's disease.

Origin of the G2019S mutation from a common founder

Our haplotype analysis strongly suggests that the G2019S mutation is transmitted from a single ancient founder. This confirms the results of a previous study,[14] and refines the size of the shared region on the 3′ end of the *LRRK2* gene, excluding markers D12S2519 and D12S2520. More importantly, in our data the ~160 kb minimum shared region spans the promoter and most of the *LRRK2* gene, suggesting that variation at the promoter or other *cis*-acting regulatory elements are not important determinants of the phenotypic variation observed among G2019S carriers. However, variants at regulatory elements in the other allele might play a modifier role. In the previous study the minimum shared region was reduced to 145 kb from marker D12S2515 to D12S2521, thereby excluding the promoter and the first 21 exons and 20 introns of *LRRK2*.[14] However, our data suggest that D12S2515 is a highly unstable microsatellite, and the observed data in this study and the previous study[14] are also compatible with mutations occurring in this

Table 3 Clinical features in carriers and non-carriers of the G2019S mutation

	Carriers	n	Non-carriers	n
Onset age (years)	50.5 (11.6)	15	52.7 (10.9)	615
Onset age, women (years)	43.9 (8.7)*	8	53.9 (10.7)	254
Onset age, men (years)	58.0 (10.1)	7	51.9 (11.0)	361
Disease duration† (years)	11.4 (5.8)	15	10.4 (6.3)	615
Disease duration, women (years)	12.1 (7.8)	8	10.3 (5.9)	254
Disease duration, men (years)	10.6 (2.2)	7	10.5 (6.6)	361

Values are mean (SD).
†Years from the age at onset of symptoms to the age at last examination.
*$p<0.02$ *v* G2019S het. male carriers (Student's *t* test).

polymorphic marker instead of recombination events. We propose that alleles at D12S2515 define a cluster of subhaplotypes in the context of the ancestral G2019S bearing haplotype. The presence of the newly discovered IVS13+104A variant in all carriers of the mutant haplotype supports the contention that the shared region extends beyond the D12S2515 marker. We did not observe the IVS13+104A variant in any Italian Parkinson's disease cases, which do not carry the G2019S mutation (50 cases tested), and we have observed it in only three of 100 Italian controls (allele frequency ~1.5%) (the three controls were also sequenced and confirmed to be non-carriers of G2019S). The low frequency of the haplotype carrying the IVS13+104A variant in the general population also strongly suggests that G2019S originated from a single ancestor. The evidence of a common founder for this mutation in cases from many populations suggests that the mutant allele is very ancient.

The clinical phenotype associated with G2019S

The phenotype associated with the G2019S mutation in this and other studies is broad, encompassing a wide range of onset ages (from 34 to 73 years in this study), and a wide spectrum of penetrance, resulting in pattern ranging from sporadic presentation to autosomal dominant, highly penetrant familial aggregation. Pedigree inspection in our sporadic mutant probands (five carrying G2019S and one carrying R1441C) reveals that four of the 12 parents died before the age of 73 years, the latest onset age known in our patients with these mutations, including both parents of proband PD-817; information was unavailable for three parents, including both parents of proband TH-08. For the remaining five parents (both parents for probands PD-1074 and PD-516) the age at death or at examination was later than 73, and these might represent examples of non-penetrance of the G2019S mutation. For two more probands (PD-07 and PD-903) with unaffected parents but affected second degree relatives, the ''transmitting'' parent also died or is still alive at an age greater than 73. These observations strongly suggest that the penetrance and phenotype associated with this mutation might be markedly modified by other genetic or non-genetic factors. Future studies must address this issue, which complicates the genetic counselling of Parkinson's disease patients with *LRRK2* mutations.

In this study, the average disease onset and duration showed no differences between the patients who carried the G2019S mutation and those who did not (table 3). However, female patients carrying the mutation ($n = 8$) had an age of onset that was almost 10 years earlier than male patients with the mutation ($n = 7$) ($p<0.02$, Student's *t* test) (table 3); if the other carriers of the same mutation detected in our previous study,[10] with accurate onset age data available, are considered together, the difference remains significant (women 47.1 (10.3) years, $n = 17$; men 56.5 (10.5) years, $n = 11$; $p<0.03$). Larger numbers of cases are needed to substantiate this observation; however, it is possible that the penetrance of the G2019S mutation is higher or the onset earlier in female carriers. Further studies are also needed to assess prospectively the rate of progression of the disease associated with this and other *LRRK2* mutations.

Dementia is within the phenotypical spectrum of *LRRK2* mutations.[8 21] The fact that dementia is rare in carriers of the G2019S mutation in this and previous studies suggests that the phenotype associated with this mutation is that of classical Parkinson's disease. However, our study targeted patients with the pure Parkinson's disease phenotype; the presence of the G2019S and other *LRRK2* mutations should be investigated among patients with Parkinson's disease-dementia, or dementia with Lewy bodies.

Conclusions

Our study delineates the G2019S mutation in *LRRK2* as the most important single genetic determinant of Parkinson's disease so far identified and provides sound evidence that this mutation originated from a common founder. G2019S is especially frequent among cases with familial Parkinson's disease of both early and late onset, but it also occurs—albeit more rarely—among patients with sporadic Parkinson's disease. Understanding the mechanisms of the disease caused by G2019S and other *LRRK2* mutations might provide important clues for the dissection of the Parkinson's disease pathogenesis and for designing novel therapeutic strategies. The identification of a first, frequent genetic determinant of Parkinson's disease also has important implications for the diagnosis and genetic counselling of this disease.

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Chapter 4 - Comprehensive analysis of the LRRK2 gene in sixty families with Parkinson's disease

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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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Keywords: Parkinson; PARK8; *LRRK2*; familial; autosomal dominant; mutation

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.I1371 V 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

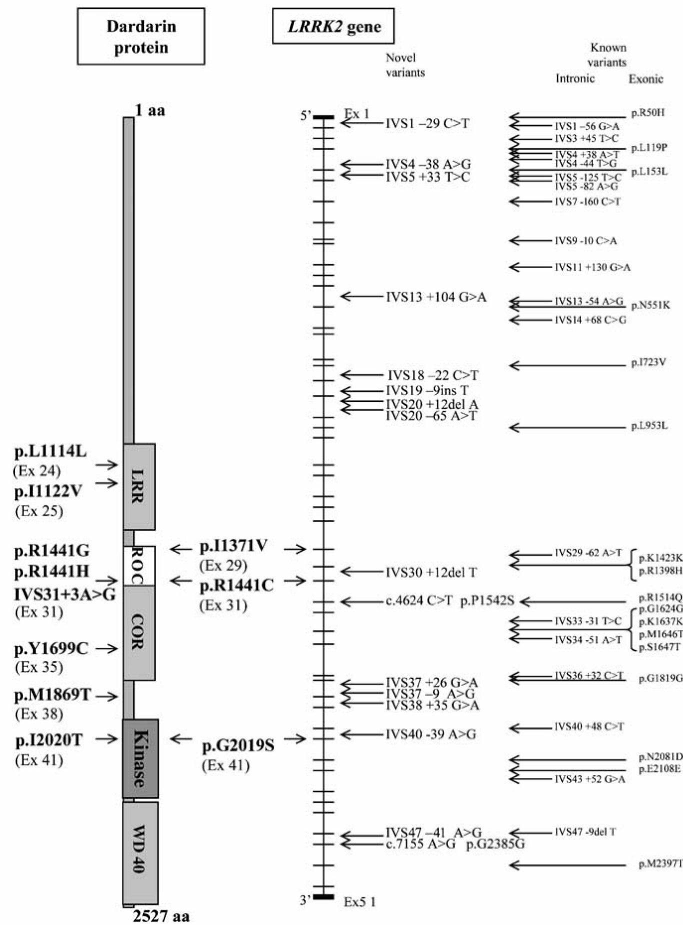


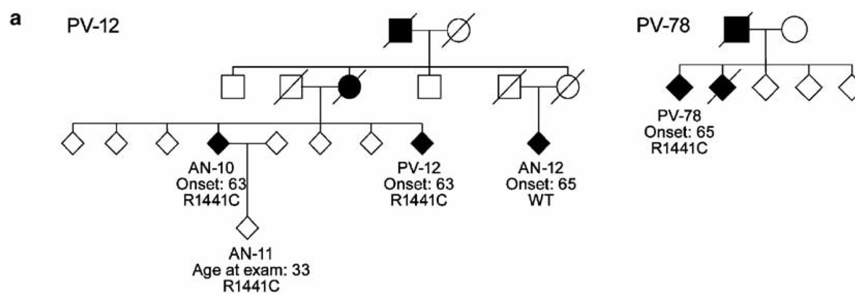
Figure 1 Schematic representation of the *LRRK2* gene, the dardarin protein and its known functional domains. Known and novel *LRRK2* polymorphisms are indicated on the right side of the gene. Mutations are indicated, those identified by us and by others, on the left and right side of the protein, respectively.

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



b

Marker	Physical position	Country: USA Family: D	Country: USA Family: 469	Country: Italy Family: PV-12	Country: Italy Family: PV-78	Country: Italy Family: PD-768
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

d

Species	Start	Sequence (* = position of I1371)	End
homo sapiens	1358-	KKSDLGMQSATVG I DVKDWPIQIRDKRK	- 1385
bos taurus	107-	KKSDLGMQGATVG I DVKDWPIQIRGKGK	- 134
rattus norvegicus	1370-	KKSELGMQGATVG I DVRDWPIQIRGKRK	- 1397
mus musculus	1358-	KKPELGMQGATVG I DVRDWSIQIRGKRR	- 1385
gallus gallus	893-	KRTELGCPKATVG I DVKDWIIQGKGKMK	- 910
tetraodon nigroviridans	1350-	KRSQLKSKSTSVG I DVQDWTIKDWDRKK	- 1376
trichodesmium erythraeum	439-	N-YQLKDEDTTKG I EVHQYKFQTKNQ---	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.I1371 V, 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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Chapter 5 - A common missense variant in the LRRK2 gene, Gly2385Arg, associated with Parkinson’s disease risk in Taiwan

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A common missense variant in the *LRRK2* gene, Gly2385Arg, associated with Parkinson's disease risk in Taiwan

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Abstract Mutations in the *LRRK2* gene are a cause of autosomal dominant Parkinson's disease (PD). Whether *LRRK2* variants influence susceptibility to the commoner, sporadic forms of PD remains largely unknown. Data are particularly limited concerning the Asian population. In search for novel, biologically relevant variants, we sequenced the *LRRK2* coding region in Taiwanese patients with PD. Four newly identified variants and another variant recently found in a Taiwanese PD family were tested for association with the disease in a sample of 608 PD cases and 373 ethnically matched controls. Heterozygosity for the Gly2385Arg variant was significantly more frequent among PD patients than controls (nominal p value=0.004, corrected for multiple comparisons=0.012, gender- and age-adjusted odds ratio=2.24, 95% C.I.: 1.29–3.88); this variant was uniformly distributed across genders and age strata. Two novel variants, Met1869Val and Glu1874Stop, were found in one PD case each; their pathogenic role remains, therefore, uncertain. The remaining two novel variants (Ala419Val and Pro755Leu) were present with similar frequency in cases and controls, and were therefore, interpreted as disease-unrelated polymorphisms. Our findings suggest that the *LRRK2* Gly2385Arg is the first identified, functionally relevant variant, which acts as common risk factor for sporadic PD in the population of Chinese ethnicity.

Keywords Parkinson's disease · *LRRK2* · Mutation · Polymorphism · Risk factor · Taiwan

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Introduction

Mutations in the gene *leucine-rich repeat kinase 2(LRRK2, PARK8)* are the most frequent known cause of familial autosomal dominant Parkinson's disease (PD) [1, 2]. Moreover, due to incomplete penetrance or missing genealogical information, *LRRK2* mutations are also found in apparently sporadic PD cases. The available data suggest that the prevalence of the *LRRK2* mutation varies markedly across populations. A first founder mutation, Arg1441Gly, is frequent but is limited to the Basque population, while a second founder mutation, Gly2019Ser, is common in countries of Southern Europe, being found in ~3–7% of familial PD and in ~1–2% of sporadic cases, and it might be much more frequent in populations from North Africa and the Middle East [3–9]. Very limited data are available on the nature and frequency of *LRRK2* mutations and on the polymorphism content of this large gene in patients of Asian and other ethnicities.

Three known PD-causing mutations (Ile2012Thr, Gly2019Ser, and Ile2020Thr) appear to be very rare or absent in Asian PD patients [10–12], while novel variants of uncertain pathogenic role were recently identified in single Taiwanese families with PD (Arg1441His and Gly2385Arg) [13]. Whether *LRRK2* variants influence the susceptibility to the commoner, sporadic forms of PD also remains largely unknown. No evidence for a major role of common *LRRK2* variants was found in the German population [14], while evidence for a PD-associated haplotype was identified in the population of Chinese ethnicity from Singapore [15]. However, the biologically relevant variant within this haplotype remains unknown. A third smaller study found no association between PD and four coding *LRRK2* variants in Caucasians [16]. In search for novel, biologically relevant variants in the Chinese population, we sequenced the *LRRK2* coding region in Taiwanese PD patients. Five variants were then studied for association with PD in a large sample of cases and ethnically matched controls.

Patients and methods

We studied 608 sporadic patients (246 females, 362 males) of Chinese ethnicity with a clinical diagnosis of idiopathic PD, ascertained at a single referral center in Taiwan. The average age at the last examination and at disease onset were 66±12 years (range 24–97) and 54.9±11.9 years (range 12–94), respectively. The clinical diagnosis of PD was established according to published criteria [17]. The whole PD sample was previously screened by us and found negative for three *LRRK2* mutations (Ile2012Thr, Gly2019Ser, and Ile2020Thr) [12]. In cases with disease onset before the age of 40 years [n=68], the *parkin* and *PINK1* genes were also screened by single-strand conformation polymorphism analysis (SSCP), and no mutations were found.

The control sample included 373 individuals free from PD, matched to the patients by ethnicity, collected at the same center, and originating from the same geographical areas (212 females and 161 males, mean age at sampling 54.7±18.2 years, range 11–87). More exactly, the controls were spouses of PD patients (67.5%), spouses of patients with unrelated diseases (16.5%), and healthy volunteers (16%). The project was approved from the local Ethical authorities and written informed consent was obtained from all subjects. Genomic DNA was isolated from peripheral blood using standard protocols.

In 15 randomly chosen PD patients, the whole *LRRK2* coding sequence (51 exons) and exon–intron boundaries were studied by polymerase chain reaction (PCR) using previously described primers and protocols [18]. Direct sequencing of both strands was performed using Big Dye Terminator chemistry ver.3.1 (Applied Biosystems). Fragments were loaded on an ABI3100 and analyzed with DNA Sequencing Analysis (ver.3.7) and SeqScape (ver.2.1) software (Applied Biosystems). Mutations were named according to the *LRRK2* cDNA sequence deposited in Genbank (accession n. AY792511). Novel sequence variants identified were submitted to Genbank.

Variants identified in these 15 patients were prioritized for further study and tested by direct sequencing in the whole sample of 608 sporadic PD patients and 373 controls. Positive and negative controls were included in each PCR plate. Statistical analyses included contingency tables, multiple logistic regression, and Student's t-test as appropriate, and were performed using the SPSS ver. 13.0 package. Permutation test was performed using the program Haploview ver. 3.2.

Table 1 Coding variants identified in 15 Taiwanese patients with PD

Exon	Accession number	Amino acid effect	MAF	Genotypes
1	rs2256408	Arg50His	1.00	15 hom
5	rs10878245	Leu153Leu	0.3	2 hom and 5 het
11	ss49853007	Ala419Val	0.03	1 het
14	rs7308720	Asn551Lys	0.1	3 het
19	ss49853008	Pro755Leu	0.03	1 het
22	rs7966550	Leu953Leu	0.23	1 hom and 5 het
30	rs7133914	Arg1398His	0.06	2 het
30	rs11175964	Lys1423Lys	0.06	2 het
34	rs1427263	Gly1624Gly	0.47	4 hom and 6 het
34	ss48398561	Arg1628Pro	0.06	2 het
34	rs11176013	Lys1637Lys	0.47	4 hom and 6 het
34	rs11564148	Ser1647Thr	0.4	3 hom and 6 het
37	rs10878371	Gly1819Gly	0.47	4 hom and 6 het
38	ss pending	Met1869Val	0.03	1 het
43	rs10878405	Glu2108Glu	0.4	3 hom and 6 het
48	ss48398568	Gly2385Arg	0.06	2 het
49	rs3761863	Met2397Thr	0.5	4 hom and 7 het

MAF Minor allele frequency, *hom* homozygous, *het* heterozygous

Results

By sequencing the whole *LRRK2* coding region and splice sites in 15 patients with sporadic PD, we identified 17 exonic (Table 1 and Fig. 1) and 22 intronic variants (not shown). Similar to what was previously found by us and others in Caucasians [13, 18], one variant (rs2256408, predicted amino acid effect Arg50His) was present in all samples in homozygous state. This is likely due to an error in the genomic reference sequence. Among the remaining 16 variants, we initially prioritized four for further analysis: the three novel missense variants (Ala419Val, Pro755Leu, and Met1869Val) and one additional variant (Gly2385Arg), which was recently identified in one Taiwanese family with PD and considered as a putative pathogenic mutation [13]. The prioritized variants were tested for association with PD in our entire sample of 608 cases and 373 ethnically matched controls. While sequencing the large sample, a further, novel heterozygous coding variant, Glu1874Stop, was found in one PD case.

The results of the association analyses are shown in Table 2. All variants were in Hardy–Weinberg equilibrium in controls and cases. The allelic or genotypic frequencies were not statistically different in cases and controls for two novel variants (Ala419Val, and Pro755Leu). The remaining novel variants, Met1869Val and Glu1874Stop were not observed in other PD or control individuals tested (Table 2). The heterozygosity for the Gly2385Arg variant was significantly higher among PD cases (61 heterozygous carriers out of 608 tested cases) than controls (18 heterozygous carriers out of 373 tested controls [nominal p value=0.004, corrected for multiple comparisons=0.012; permutation test, p=0.02 (5,000 replicates); gender- and age-adjusted odds ratio 2.24, 95% C.I.: 1.29–3.88]. The frequency of this variant was uniform across gender and age strata (Table 3).

The age at examination was similar in PD cases that carried the Gly2385Arg variant and those who did not carry it (carriers: 66.5±12 years, non-carriers: 66.2±11.6 years, p=0.84, Student's t-test). The average disease onset in the 61 carriers of the Gly2385Arg variant was 53.2±12.5 years, range 12–82, which is earlier though not significantly different from the average onset age in non-carriers (55±11.9 years, range 20–94, p=0.24). In the Gly2385Arg carrier with the earliest onset, foot dystonia started at the age of 12 years, followed by bradykinesia and rigidity. The clinical phenotype was otherwise typical of juvenile PD, including good levodopa response, slow course and levodopa-induced dyskinesias. *Parkin* mutations were not identified.

The average disease duration in carriers of the Gly2385Arg variant was slightly longer than that in non-carriers (average: 13.4±6.2 years in carriers and 11.2±6 in non-carriers, p=0.007, Student's t-test). Male patients accounted for much of the difference in disease duration [average: 15.1±5.9 years in male carriers (n=34) and 11.6±6.2 in non-carriers, p=0.002], while disease duration was not significantly different among the female patients [average 11.3±6 years in female carriers (n=27) and 10.5±5.8 in non-carriers, p=0.53]. Additional relatives of the cases carrying the novel variants were not available for study. Alignment of LRRK2 protein homologues at the level of the Met1869Val and Gly2385Arg variants is shown in Fig. 1.

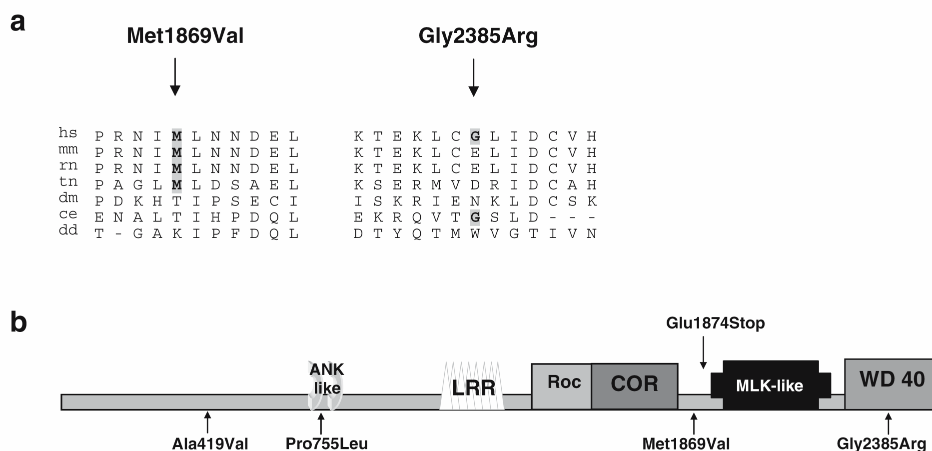

Fig. 1 **a** Aligment of LRRK2 protein homologues at positions of two missense mutations analysed in this study. The alignment was generated using T-COFFEE. The conserved amino acids are highlighted. *hs Homo sapiens* (accession number NP_940980), *mm Mus Musculus* (NP_080006.2), *rn Rattus norvegicus* (XP_235581.3), *tn Tetraodon nigroviridans* (Q4S189), *dm Drosophila melanogaster* (Q9VDJ9), *ce Caenorhabditis elegans* (NP_492839.3), *dd Dictyostelium discoideum* (AAO12857.1). **b** Protein domain structure of LRRK2. The position of the variants studied is indicated. *ANK-like* Ankyrin-like repeat region, *LRR* leucine-rich repeat region, *Roc* Ras in complex proteins, *COR* C-terminal of Roc, *WD40* WD40-like repeats domain, *MKL-like* mixed-lineage kinase-like consensus region

Table 2 Allelic and genotypic frequencies in cases and controls

Exon	Nucleotide change	Amino acid change	Cases Genotypes WT/WT	Cases Genotypes WT/Var	Cases Genotypes Var/Var	Cases Alleles WT	Cases Alleles Var	Controls Genotypes WT/WT	Controls Genotypes WT/Var	Controls Genotypes Var/Var	Controls Alleles WT	Controls Alleles Var
11	C1256T	Ala419Val	582 (0.98)	10 (0.02)	0 (0.00)	1174 (0.992)	10 (0.008)	341 (0.99)	3 (0.01)	0 (0.00)	685 (0.996)	3 (0.004)
19	C2264T	Pro755Leu	578 (0.99)	7 (0.01)	0 (0.00)	1163 (0.994)	7 (0.006)	339 (0.97)	10 (0.03)	0 (0.00)	688 (0.985)	10 (0.015)
38	A5605G	Met1869Val	592 (0.999)	1 (0.001)	0 (0.00)	1185 (0.999)	1 (0.001)	370 (1.00)	0 (0.00)	0 (0.00)	740 (1.00)	0 (0.00)
38	G5620T	Glu1874Stop	592 (0.999)	1 (0.001)	0 (0.00)	1185 (0.999)	1 (0.001)	370 (1.00)	0 (0.00)	0 (0.00)	740 (1.00)	0 (0.00)
48	G7153A	Gly2385Arg	547 (0.9)	61 (0.1)	0 (0.00)	1155 (0.950)	61 (0.05)	355 (0.95)	18 (0.05)	0 (0.00)	728 (0.975)	18 (0.025)

Nominal *p* value =0.004, corrected *p* value=0.012—comparison of Gly2385Arg genotypic and allelic frequency in PD cases vs controls (chi square test), permutation test: *p*=0.02 (5,000 replicates); Nominal *p* value=0.065, corrected *p* value=0.195—comparison of Pro755Leu genotypic and allelic frequency in PD cases vs controls (chi square test); differences between genotypic and allelic frequency of the Ala419Val polymorphism in PD cases vs controls are not significant

Discussion

In this association study, we focused on coding variants, which are more likely to be biologically relevant, identified by direct scanning of the *LRRK2* gene in the target population [19]. Four of the five variants investigated are reported here for the first time (Tables 1 and 2).

Two novel variants (Ala419Val, and Pro755Leu) were present with similar frequencies in cases and controls, and therefore, are likely to represent neutral, disease-unrelated

Table 3 Frequency of the Gly2385Arg variant in different age strata and genders

Age at PD onset	Total cases	Gly2385Arg carriers	Percentage (%)	Females Carrier	Males Carrier
Cases					
<50	215	23	0.107	9	14
50<60	152	15	0.099	7	8
60<70	184	20	0.109	9	11
70 and >70	57	3	0.053	2	1
<60	367	38	0.104		
60 and >60	241	23	0.095		
Total females	246	27	0.110		
Total males	362	34	0.094		
Total	608	61	0.100		
Controls					
Age at examination	Total controls	Gly2385Arg carriers	Percentage	Females carrier	Males carrier
<50	109	7	0.064	4	3
50<60	78	2	0.026	2	0
60<70	111	6	0.054	3	3
70 and >70	75	3	0.040	1	2
<60	187	9	0.048		
60 and >60	186	9	0.048		
Total females	212	10	0.047		
Total males	161	8	0.050		
Total	373	18	0.048		

changes. The remaining two novel variants, Met1869Val and Glu1874Stop, were found each in only one PD case and were not observed in other PD cases (n=592) or controls (n=370). Due to their rarity, these are, therefore, to be considered as mutations, rather than polymorphisms. However, their pathogenic significance remains uncertain. The patients carrying these mutations are sporadic, and relatives were dead or not available for testing. Hence, we cannot determine if these are "de novo" mutations. The fact that the same codon targeted by the *Met1869Val* mutation was also affected by another mutation found in PD cases, *Met1869Thr* [13, 20], supports the contention that these changes are pathogenic. The Met1869 residue is close to one of the known LRRK2 functional domains (Cor, C-terminal of Roc), but has not been highly conserved in evolution, and some species even have a threonine at this position (Fig. 1). However, the conservation of the residue is not an absolute requisite of pathogenic mutations.

In addition to be a novel variant, the Glu1874Stop is, to our knowledge, the first *LRRK2* nonsense mutation identified in a PD case and is predicted to encode a protein lacking the entire kinase and WD domains (Fig. 1). The identification of this allele could be a coincidental finding due to the large number of individuals sequenced for this exon. However, the Glu1874Stop could also represent a true disease-causing de novo mutation or an inherited mutation displaying reduced penetrance. In this patient, PD symptoms started at age 69. Inspection of the pedigree of this patient shows no other PD cases among first-degree relatives (parents, five siblings and two children). Both parents died at around 65 years of age during World War II. Only one brother of the patient remains alive and is, today, aged 90, but he could not be sampled for gene testing. This is potentially a very important finding for understanding the mechanisms of the disease caused by *LRRK2* mutations. Recent studies found evidence that some of the *LRRK2* mutations (Gly2019Ser, Ile2020Thr, and Arg1441Cys) increase the kinase activity of the encoded protein, thus, suggesting a gain-of-function mechanism [21, 22]. If carrying a heterozygous truncating allele, which lacks the whole kinase domain, was the cause of PD in our patient, we would have to postulate that an increased kinase activity is not the invariable mechanism of LRRK2-related neurodegeneration. Functional studies of the *Glu1874Stop* allele are now warranted.

The last variant studied (Gly2385Arg) was recently reported in a PD family from Taiwan [13]. Evidence for co-segregation with PD in that family was limited due to the small pedigree size; however, the mutation was reported to be absent in 200 ethnically matched controls, and therefore, interpreted as putatively pathogenic. We identified this variant in several PD patients and controls, but its frequency was significantly higher among PD cases, also after adjustment for possible confounders such as gender and age and after correction for multiple testing. This suggests that the Gly2385Arg variant, or another variant in linkage disequilibrium, is associated with PD in the Taiwanese population. The residue targeted (Gly2385) lies in the WD domain and is not conserved in evolution, but amino acids with a net positive charge (such as arginine) are not observed at this codon in the known LRRK2 protein homologues (Fig. 1). Functional studies are needed to investigate whether the presence of the arginine residue influences folding or stability of the LRRK2 protein, or the strength of binding to other, still unknown interactor(s).

The average disease onset in carriers of Gly2385Arg was 2 years earlier but was not significantly different than that of non-carriers. The longer disease duration in male carriers is likely to be a chance finding, in part, as a consequence of the earlier onset; however, it might also suggest, albeit indirectly, a slower disease course among the male carriers. Further, prospective clinical analyses are warranted to further characterize the clinical phenotype associated with the Gly2385Arg variant. It will also be interesting to study the prevalence of the Gly2385Arg variant in Chinese patients with familial forms of PD.

Genetic risk factors might be population specific, and therefore, it is not surprising that evidence for *LRRK2* common variants associated with PD was not found, so far, in Caucasians [14, 16] but was found in Chinese (this study and [15]). It will be interesting to explore if Gly2385Arg is the biologically relevant variant contained on the PD-associated haplotype reported recently in Chinese patients from Singapore [15].

Allelic association studies are prone to false-positive results, mainly due to small sample sizes, population stratification, and genotyping errors. Our study was based on a large sample; cases and controls were matched for ethnicity; odds ratios were adjusted for age and gender; furthermore, samples from cases and controls were processed using high quality standards and identical genotyping protocols; these considerations argue against the existence of any of the aforementioned causes of false-positive results. However, it will be important to replicate this association in independent, large samples.

In conclusion, we identified novel *LRRK2* variants in the Chinese population, including two novel, potentially pathogenic mutations and the first truncating mutation reported in a PD case. Furthermore, we suggest that the *LRRK2* Gly2385Arg is the first identified, functionally relevant variant, acting as a common risk factor for sporadic PD in the Chinese population. These findings have potential important implications for the dissection of the causes and mechanisms of the common forms of PD.

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We declare that the experiments reported in this paper comply with the current laws of the country in which they were performed.

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Chapter 6 - LRRK2 mutations and Parkinson's disease in Sardinia - A Mediterranean genetic isolate

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LRRK2 mutations and Parkinson's disease in Sardinia—A Mediterranean genetic isolate

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Abstract

The *Leucine-Rich Repeat Kinase 2* (*LRRK2*) Gly2019Ser mutation is frequent among Parkinson's disease (PD) patients from the Arab, Jewish, and Iberian populations, while another mutation, Arg1441Gly, is common in the Basque population. We studied the prevalence of these mutations in Sardinia, a Mediterranean genetic isolate with peculiar structure and similarities with the Basque population. Among 98 Sardinian PD probands we detected one heterozygous Gly2019Ser carrier. This mutation was also found in one of 55 Sardinian controls, an 85-year-old man, later shown to have a positive family history of parkinsonism. No carriers of Arg1441Gly, Arg1441Cys, or Arg1441His mutations were found among cases and controls. Our results suggest that the "Basque" *LRRK2* mutation is absent or very rare in Sardinia. The Gly2019Ser mutation is present but its frequency is lower than that in Iberian, Arab, or Jewish populations. The identification of an 85-year-old, healthy Gly2019Ser carrier supports the concept that this mutation displays incomplete penetrance.
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Keywords: Parkinson's disease; *LRRK2*; Mutation; Sardinia; Genetic isolates

1. Introduction

Mutations in the *Leucine-Rich Repeat Kinase 2* (*LRRK2*) gene are the most frequent known cause of familial and sporadic Parkinson's disease (PD) [1,2], but their prevalence varies markedly across populations [3]. A founder Arg1441Gly mutation is frequent in PD patients from the Basque population and the neighbouring Asturias [2,4], while the Gly2019Ser mutation is present at high frequency in the Iberian populations of Spain and Portugal, (up to 6–18% of familial PD and 3–6% of sporadic cases) [5–7], but it is extremely frequent in North African Arabs and Askenazi Jews (up to 13–41% and 30–37% of sporadic and familial PD cases, respectively) [8,9]. In a large study of 1092 Italian patients with PD, we found the Gly2019Ser mutation in 4.2% of the familial and 1% of the sporadic cases (overall prevalence in Italian PD 1.7%) [10,11], placing the Italian population in an intermediate frequency class between the above-mentioned high-prevalence populations and the low-prevalence ones such as the Northern European [12,13] or the Asian [14,15].

Several forces contributed to shape the complex genetic structure of the modern Mediterranean populations [16]. Different geographical regions, even close islands such as Sicily, Sardinia, Corsica, and the Balearic islands might display different genetic structures, as a result of different founder alleles, prolonged geographical isolation and

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genetic drift [16,17]. The population of Sardinia is one of the most extensively studied genetic isolates, which displays unique features [18–20]. Studies of Sardinian Y chromosome DNA polymorphisms have revealed major contributions from two ancient Paleolithic lineages (termed M173 and M170) of Iberic and central-eastern European origin, respectively, as well as the presence of other lineages, which likely entered Europe as a result of Neolithic migratory waves from the Middle East [16]. There are also intriguing genetic similarities between the Sardinian and Basque populations, suggesting a common ancestry [16,17,21]. For example, the M26 mutation, occurring in the context of the Y-chromosome M170 haplotype, is almost exclusively present in Sardinians and Basques [16]. Due to the central Mediterranean location of Sardinia, the many genetic and historical ties with the Iberic peninsula, North Africa and Middle East, and the genetic similarities with the Basque population, it is of great interest to explore the occurrence of the *LRRK2* Gly2019Ser and Arg1441Gly mutations among Sardinians.

As part of an ongoing genetic study of PD in this isolated population, we report here our findings in an initial, consecutive sample of 153 Sardinian individuals (98 PD cases and 55 controls); we screened for the Gly2019Ser and Arg1441Gly mutations, as well as another two mutations known at *LRRK2* codon 1441: Arg1441Cys, initially found in the so-called family "D" (Western-Nebraska) [1,22], but also present in PD families from the Italian population [10,23], and Arg1441His, which was identified in a few North American and Asian PD cases [24,25].

2. Subjects and methods

Ninety-eight consecutive patients with clinically typical PD and 55 age-matched controls were recruited at the Movement Disorders Unit of the General Hospital "S. Michele" in the town of Cagliari, Southern Sardinia. All patients fulfilled the United Kingdom Brain Bank Criteria for the clinical diagnosis of definite PD [26]. Pathological studies were not available. Cases and controls were only included in the study if they were born and living in Sardinia, and if their parents and four grandparents were also born in Sardinia. A detailed medical history of the nuclear family was taken. Patients were classified as sporadic if they reported no other PD cases among first- and second-degree relatives.

The clinical evaluation of the parkinsonism was performed using the Unified Parkinson's Disease Rating Scale (UPDRS) [27] and the Hoehn–Yahr Scale. The Mini-Mental State Examination (MMSE) was used as a screening test for the cognitive status, and subjects with MMSE score <18 were considered to have dementia. The clinical features of the cases are summarized in Table 1.

The 55 age-matched controls (19 females, 36 males) were mainly spouses of PD cases or subjects examined at the same centre for diseases unrelated to PD. Controls were included only if they reported a negative family history of PD among first- and second-degree relatives. Moreover, at the time of the examination none of the controls showed any signs or symptoms of parkinsonism, dementia, or other neurodegenerative disease. The average age at sampling of the controls was 71.3 ± 10.6 years (range 47–87), slightly older than that of PD cases. The project was approved from the relevant Ethical authorities and written informed consent was obtained from all participating subjects. Genomic DNA was isolated from peripheral blood using standard protocols. Mutations are named according to the *LRRK2* cDNA sequence deposited in Genbank (accession no. AY792511).

Table 1
Clinical features in Sardinian patients with PD

No. of patients	98
Sex	63 males, 35 females
Age at examination (years, mean $\pm$SD)	69.1 ± 9
Age at disease onset (years, mean $\pm$SD)	63.5 ± 10.2
Age at disease onset (years, range)	35–82
Presence of dementia	12/98
Positive family history of PD (among 1st- or 2nd-degree relatives)	13/98
Hoehn & Yahr staging (mean$\pm$SD)	2.1 ± 0.9
UPDRS-III score (mean$\pm$SD)	27.3 ± 13.5

In the whole sample of cases and controls, the *LRRK2* c.G6055A variant in exon 41 (protein effect: Gly2019Ser) was screened by a TaqMan method and Assays-by-Design on an Applied Biosystems 7300 Real Time PCR System. Primers, probes, and experimental conditions are available on request. Positive samples were confirmed by direct sequencing of the *LRRK2* exon 41 in both strands, as described previously [28]. Furthermore, in the whole sample of cases and controls, the three known PD-associated mutations at codon 1441 (exon 31), c.C4321T (Arg1441Cys), c.C4321G (Arg1441Gly), and c.G4322A (Arg1441His) were screened by direct sequencing of this exon, as described previously [23]. Positive and negative controls for the Gly2019Ser and the Arg1441Cys mutations were included in each experiment.

In every subject carrying the Gly2019Ser mutation, we also typed by direct sequencing an intronic single nucleotide polymorphism (SNP), IVS13+104G/A (SNP database accession no. rs28903073), which is in perfect linkage disequilibrium (LD) with the Gly2019Ser mutation [10]. Direct sequencing 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).

3. Results

In this series of 98 consecutive PD patients and 55 controls we found no carriers of the Arg1441Gly, Arg1441Cys, and Arg1441His mutations. One patient was heterozygous carrier of the Gly2019Ser mutation

(representing 1% of our series). This man had a negative family history for PD and clinical features indistinguishable from those of classical PD. The symptoms begun asymmetrically five years ago at the age of 66 years, with prominent rest tremor, rigidity and motor slowness on the right side, and displayed a good response to levodopa. Dementia or atypical neurological signs were absent. The parents of this case died at an age of 78 and 81 years without symptoms or signs of PD.

The Gly2019Ser mutation was also found in heterozygous state in one asymptomatic control aged 85, who initially did not report any family history of PD; however, more careful investigation disclosed that his brother died at the age of 92 years with a diagnosis of parkinsonism.

We found previously that the IVS13 + 104G/A variant (rs28903073) is present in only ~1.5% of the Italian control chromosomes but in 100% of the alleles carrying the Gly2019Ser mutation detected so far [10]. The patient and the control carrying the Gly2019Ser heterozygous mutation in this study were also heterozygous carriers of the IVS13 + 104G/A variant, supporting the notion of the perfect LD between Gly2019Ser and the IVS13 + 104G/A variant, as well as the likely origin of all the Gly2019Ser alleles known so far in Caucasians from a single ancestor.

4. Discussion

This is the first report on the frequency of *LRRK2* mutations in a consecutive series of Sardinian PD patients and controls. The Gly2019Ser is the most frequent PD-causing mutation identified so far [3]. A north–south gradient of distribution is evident: the frequency of this mutation is low in Northern Europe [12,13], high in the Iberian populations [5–7] and huge in North African Arabs and Ashkenazy Jews [8,9]. Interestingly, this mutation is associated with the same haplotype in different populations, indicating the existence of a single, ancient founder [10,29,30]. Here, we show that the Gly2019Ser mutation is present in Sardinia, but we suggest that its frequency is lower than that in Arab, Jewish, or even Iberian populations, and similar to that found in the Italian population (~2% of PD patients overall).

Furthermore, the Arg1441Gly mutation was absent in our series. This mutation was originally identified in 8% of PD cases from the Basque region [2] and later, in 2.7% of patients from the neighbouring area of Asturias [4], as well as in 0.7% of PD from Catalonia [6]. Assuming a common origin of Sardinians and Basque, the absence of Arg1441Gly in our Sardinian cohort suggests that this mutation has likely occurred in the Basque population after the separation of Sardinians and Basque from a common ancestral population.

Together with the observations on the prevalence of the Gly2019Ser mutation, our findings confirm that the Sardinian population is an isolate with peculiar genetic structure [18,19]. However, the Sardinian population is complex and contains many smaller sub-isolates; those located in the internal South Eastern areas seem to be the most ancient [19]. It is possible that the Arg1441Gly or the Gly2019Ser mutations are present or more frequent in some of these sub-isolates, and therefore further screenings seem warranted.

Another major finding of our study is the identification of the Gly2019Ser mutation in a healthy subject aged 85, with a positive family history of parkinsonism. An age-dependent, likely incomplete penetrance was already suggested in the *PARK8* mapping studies [31,32], and later confirmed after the *LRRK2* gene was identified [1,2,28,29]. The penetrance of the Gly2019Ser mutation was likely overestimated in the earliest studies due to selection bias [23,29], and a lifetime penetrance as low as ~32% was estimated more recently for this mutation among the Ashkenazy Jews [9], also in keeping with the occurrence of this mutation in ~1.5% of controls in that high-prevalence population.

However, to our knowledge, there are only very few previous examples of clear non-penetrance in Caucasians subjects more than 80 years old, who are documented by gene testing to carry the Gly2019Ser mutation [6,33]. These observations confirm that also in Caucasians, being a Gly2019Ser carrier does not imply a 100% lifetime risk of developing clinically overt PD. Although an *LRRK2* mutation represents a major susceptibility factor to develop PD, other factors are probably critical for the expression of a clinically relevant pathology, including perhaps some genetic or environmental risk or protective factors; this represents an important aspect for future research. The accurate estimate of the penetrance of the different *LRRK2* mutations, and of Gly2019Ser in particular, will also be crucial for appropriate genetic counselling. Further research in asymptomatic carriers of *LRRK2* mutations might also provide a unique opportunity to approach the pre-symptomatic phase of the disorder in order to analyse the compensatory mechanisms that retard or prevent symptoms and to evaluate the rate at which pre-synaptic dopaminergic dysfunction progresses [34].

In conclusion, our results suggest that the Gly2019Ser mutation as well as mutations at *LRRK2* codon 1441 do not represent a frequent cause of PD in Sardinian patients. Yet, other *LRRK2* mutations might exist in this population, and further investigations are warranted. A lower prevalence of PD was reported in Sardinia compared to the other European populations [35]. It has been suggested that genetic isolates with low prevalence of a certain disease might be more useful than others, for gene mapping purposes [36,37]. Thus,

Sardinia might be particularly valuable for dissecting the complex genetic aetiology of PD.

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Chapter 7 - High Prevalence of LRRK2 Mutations in Familial and Sporadic Parkinson's Disease in Portugal

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Chapter 7

High Prevalence of *LRRK2* Mutations in Familial and Sporadic Parkinson's Disease in Portugal

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Abstract: **Mutations in the *Leucine-Rich Repeat Kinase 2* (*LRRK2*) gene are the most frequent known cause of Parkinson's disease (PD), but their prevalence varies markedly between populations. Here we studied the frequency and associated phenotype of four recurrent *LRRK2* mutations (R1441C, R1441G, R1441H, and G2019S) in familial and sporadic PD from a single referral center in Lisbon, Portugal. Among 138 unrelated PD probands, we identified 9 heterozygous G2019S carriers (6.52%) and 1 heterozygous R1441H carrier (0.72%). The G2019S mutation was present in 4 of the 107 sporadic (3.74%) and in 5 of the 31 familial probands (16.1%). Mutations were not found among 101 Portuguese controls. The G2019S mutation was present on a single haplotype and displayed reduced penetrance. Heterozygous *parkin* gene mutations were also found in 2 G2019S-positive probands, but their pathogenic role is unclear. The clinical phenotype in patients with *LRRK2* mutations was indistinguishable from that of typical PD, including impaired sense of smell. The G2019S mutation is a very common genetic determinant among the Portuguese patients with PD, and the R1441H mutation is also present in this population. These data have important implications for the diagnostic work-up and genetic counseling of patients with this disease in Portugal.** © 2007 Movement Disorder Society

Key words: Parkinson's disease; Portugal; *LRRK2*; parkin; mutation.

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The relevance of the *Leucine-Rich Repeat Kinase 2* (*LRRK2*) gene for Parkinson's disease (PD) is unprecedented because of the high frequency of mutations in both familial and sporadic patients,[1,2] and because the clinical and pathologic phenotypes are in most cases indistinguishable from those of typical PD.[3-9]

Importantly, the prevalence of *LRRK2* mutations among PD varies markedly between populations. A first founder mutation, p.R1441G, is especially frequent in PD from the Basque population.[1] Moreover, the emerging data delineate the Iberic population among those with the highest prevalence of the p.G2019S mutation. Three studies have been recently performed in Spain, in the regions of Cantabria, Asturias, and Catalonia.[10-12] However, only a single report is available so far from Portugal.[13]

Here we assess the prevalence of *LRRK2* mutations and their phenotype in an independent series of PD cases from a single referral center, the Lisbon University Hospital, Portugal. We screened for the G2019S and R1441G mutations, as well as other mutations occurring at this codon: R1441C and R1441H. Furthermore, in recent studies, *parkin* mutations were identified in a few patients carrying the *LRRK2*-G2019S mutation, suggesting a digenic mechanism of PD inheritance.[6,14,15] Therefore, all patients with *LRRK2* mutations identified in this study were also screened for *parkin* mutations.

SUBJECTS AND METHODS

The appropriate Institutional Review Board approved the study and all participants gave written informed consent. PD patients were consecutively recruited from the movement disorders outpatient clinic of the Lisbon University Hospital. Patients fulfilled the criteria of the UK Brain Bank for idiopathic PD.[16] The patients with signs or symptoms suggesting an atypical parkinsonian syndrome were excluded. Familial PD cases were defined as patients who reported other PD cases among their first- or second-degree relatives. Definition of PD for the patients' relatives obliged to the confirmation of PD by personal examination, or a formal diagnosis of PD made by a medical doctor. Efforts were also made to examine and collect DNA samples from additional affected and at-risk relatives for cosegregation studies. Controls were unrelated subjects from the surroundings of cases (e.g. spouses), free from PD, and matched to the patients by age.

Patients and controls were interviewed and examined by a specialist in movement disorders. A structured interview was used to obtain detailed information on PD history, antiparkinsonian treatments, comorbidity and comedication. The severity of PD was assessed using the Unified Parkinson's Disease Rating Scale (UPDRS) and Hoehn & Yahr scales. Cognitive status was assessed using the Mini Mental State Examination scale. Last, we evaluated the sense of smell using the University of Pennsylvania Smell Identification Test (UPSIT).[17]

Genomic DNA was isolated from peripheral blood using standard protocols. Mutations are named according to the *LRRK2* and *parkin* cDNA sequences deposited in Genbank (accession n. *LRRK2*: AY792511; *parkin*: AB009973), counting from the "A" of the "ATG" translation initiation codon. In the whole sample of cases and controls, the *LRRK2* c.G6055A variant in exon 41 (protein effect: G2019S) was screened by a TaqMan allelic discrimination method, as described.[18] Positive samples were confirmed by direct sequencing of the *LRRK2* exon 41 in both strands, as reported.[3] Furthermore, in the whole sample of cases and controls, the three known mutations at codon 1441 (exon 31), c.C4321T (R1441C), c.C4321G (R1441G), and c.G4322A (R1441H) were screened by direct sequencing.[3] Positive and negative controls for the G2019S and the R1441C mutations were included in each experiment.

In every subject carrying the G2019S mutation, we also typed by direct sequencing an intronic single nucleotide polymorphism (SNP), IVS13 + 104G/A (SNP database accession n. rs28903073), which is in linkage disequilibrium (LD) with the G2019S mutation.[19] In every patient carrying *LRRK2* mutations, the complete coding region of the *parkin* gene was sequenced, as described.[20] Direct sequencing was performed using Big Dye Terminator chemistry. Fragments were loaded on an ABI3100 and analyzed with DNA Sequencing Analysis and SeqScape (Applied Biosystems).

RESULTS

We recruited 138 consecutive, unrelated PD probands, including 107 with sporadic and 31 with familial PD. A second PD case was recruited from 6 of these 31 families, bringing the total to 144 PD patients. Samples from 101 unrelated controls, which were free from PD signs, were also collected. All cases and controls were of Caucasian ethnicity. Demographic characteristics are detailed in Table 1. Among the 31 PD families studied, 12 had at least two consecutive generations affected with PD; 11 were composed of only siblings affected by PD; the remaining 8 families showed only second- and third-degree relatives with PD. A large Portuguese family with PD and the *LRRK2*-G2019S mutation, previously published by us,[21] is not included in this study.

Among the 138 unrelated PD probands, we identified 9 heterozygous carriers of the G2019S (6.52%), 1 heterozygous carrier of the R1441H (0.72%), and no carri-

TABLE 1. *Demographic characteristics of study subjects*

	Patients	Controls
Total number of subjects included	138	101
Average age, years (range)	67.6 (28–86)	69.0 (46–85)
Gender (males/females)	83/55	43/58
Sporadic PD cases	107	
Average age, years (range)	68.0 (28–86)	
Gender (males/females)	66/41	
Familial PD cases	31	
Average age, years (range)	66.4 (49–80)	
Gender (males/females)	17/14	

ers of the R1441C and R1441G mutations. Homozygous mutation carriers were not detected. The G2019S and the three mutations at codon 1441 were not found in any of the controls subjects tested (202 chromosomes).

The G2019S mutation was present in 4 of the 107 sporadic (3.74%) and in 5 of the 31 familial probands (16.1%). The R1441H mutation was found in 1 familial PD proband (3.2%). The frequency of mutations according to the onset age of patients is reported in Table 2, and the simplified pedigrees of probands carrying *LRRK2* mutations are shown in Figure 1.

Among the 6 affected relatives of familial PD cases available for DNA testing, 3 belong to the families of mutation-positive probands (family Lisb-F15 and Lisb-F24 with G2019S; family Lisb-F2 with R1441H). The presence of the above-mentioned mutations was confirmed in the 3 affected relatives (Fig. 1).

We described previously that the IVS13 + 104G/A variant (rs28903073) is present in only ~1.5% of the Italian control chromosomes but in 100% of the alleles carrying the G2019S mutation detected so far in our PD cohorts.[18,19] In this study, all Portuguese patients carrying the G2019S heterozygous mutation were heterozygous carriers of the IVS13 + 104G/A variant. We also tested 25 Portuguese unrelated subjects (50 chromosomes) who did not carry the G2019S heterozygous mutation; none of these carried the IVS13 + 104G/A variant.

Among 13 patients with *LRRK2* mutations identified in this study (10 probands and 3 affected relatives), 2 carried a single heterozygous mutation in the *parkin*

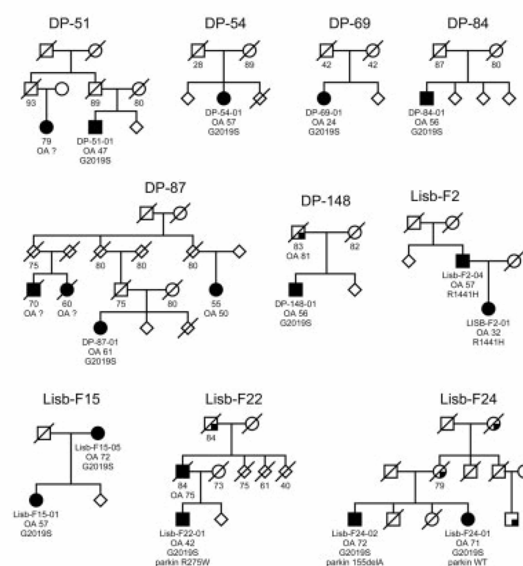

FIG. 1. Simplified pedigrees of 10 Portuguese probands carrying *LRRK2* mutations. To protect confidentiality, the order of individuals in sibships was altered and the gender of some subjects was masked by diamond symbols. No further PD cases are known among the more distant relatives. Full black symbols: individuals affected by PD; symbols with black lower corner: individuals affected only by tremor. Numbers below symbols indicates age at examination or age at death (years). OA – age at symptoms onset (years). Question mark indicates that information is not available.

gene: p.R275W in case Lisb-F22-01, and c.155delA in Lisb-F24-02 (Fig. 1). These are among the *parkin* gene mutations most commonly found in early-onset PD, and they are definitely disease-causing when present in homozygous or compound-heterozygous state.[22,23] DNA samples from affected relatives were not available from family Lisb-F22, whereas in family Lisb-F24 the affected sister (Lisb-F24-01), who carried the *LRRK2*-G2019S, did not carry the *parkin* mutation found in the affected brother (Fig. 1).

The detailed clinical features of the 13 patients with *LRRK2* mutations are reported in the Table 3. The 11 patients carrying the G2019S mutation showed a male/

TABLE 2. *Frequency of LRRK2 mutations in sporadic and familial PD cases and distribution according to age at onset*

	Total sample n = 138		Patients grouped by age at onset: onset < 50, n= 27		onset ≥ 50, n = 111	
	G2019S	R1441H	G2019S	R1441H	G2019S	R1441H
Total PD sample, n = 138	9 (6.52%)	1 (0.72%)	3/27	1/27	6/111	0/111
Sporadic PD, n = 107	4 (3.7%)	0	1/19	0/19	3/88	0/88
Familial PD, n = 31	5 (16.1%)	1 (3.2%)	2/8	1/8	3/23	0/23

TABLE 3. *Clinical characteristics of patients with LRRK2 mutations*

Patient Identifier	Familial/ Sporadic	Mutation	Age (yrs)	Gender	Age at onset (yrs)	Disease duration (yrs)	Initial symptoms	Last exam UPDRS III ("on")	Last exam HY ("on")	Other concomitant clinical problems	UPSIT	Psych.	Dementia
54	Sporadic	G2019S	69	F	57	12	T	14	2.5	RLS; insomnia; EDS; constipation; orthostatic hypotension; sensory complaints	11	D	No (NE normal)
69	Sporadic	G2019S	48	F	24	24	T	59	3	RLS; insomnia; EDS; constipation; orthostatic hypotension; sensory complaints	20	D	No (MMSE 29/30)
84	Sporadic	G2019S	67	M	56	11	B, R	15	2	RLS; constipation	13	No	No (MMSE 29/30)
148	Sporadic	G2019S	60	M	56	4	T, B	17	2	Insomnia; EDS; constipation; bladder dysfunction; sensory complaints	26	D, A, VD, H	No (MMSE 30/30)
51-01	Familial	G2019S	71	M	47	24	B	42	4	REM sleep behaviour disorder; periodic limb movements during sleep, bladder dysfunction; sensory complaints	9	D	No (MMSE 28/30)
87-01	Familial	G2019S	63	F	61	2	T, B	30	2.5	REM sleep behaviour disorder; insomnia; orthostatic hypotension; bladder dysfunction; sensory complaints	15	No	No (MMSE 22/30) (uninstructed)
15-01	Familial	G2019S	60	F	57	3	T, B	16	1	REM sleep behaviour disorder; insomnia; EDS; constipation; sensory complaints	22	D	No (MMSE 28/30)
15-05	Familial	G2019S	80	F	72	8	B	47	4	Insomnia; constipation; EDS	5	D	No (MMSE 22/30)
22-01	Familial	G2019S	55	M	42	13	T	17	2.5	REM sleep behaviour disorder; EDS; sensory complaints	15	D	No (NE normal)
24-01	Familial	G2019S	80	F	71	9	T	34	3	REM sleep behaviour disorder; insomnia; EDS; constipation	22	D	No (MMSE 30/30)
24-02	Familial	G2019S	74	M	72	2	T	28	2	REM sleep behaviour disorder and RLS; sensory complaints	27	No	No (MMSE 30/30)
2-01	Familial	R1441H	56	F	32	24	B, R	24	2	insomnia; bladder dysfunction; sensory complaints	24	No	No (MMSE 30/30)
2-04	Familial	R1441H	81	M	57	24	B, R	35	3	constipation; sensory complaints	10	H, VD	No (MMSE 27/30)

F, female; M, male; T, tremor; R, rigidity; B, bradykinesia; RLS, restless legs syndrome; EDS, excessive daytime sleepiness; Psych., psychiatric symptoms; D, depression; A, anxiety; VD, vivid dreams; H, hallucinations; NE, Neuropsychological evaluation; MMSE, Mini Mental State Examination.

female ratio of 0.83, a median age for disease onset of 55.9 years (range 24–72), and mean disease duration of 10.2 years.[2-24] Only a minority presented a disease onset before the age of 50 years (Table 3). The predominant phenomenology at onset was tremor and all patients had asymmetric onset of symptoms. The mean UPDRS motor score at last examination in "on" state was 29 (range 14–59), and the Hoehn and Yahr score ranged between 1 and 4 (mean 2.59). No patient had dementia but the majority presented concomitant psychiatric problems, mostly depression. The response to levodopa therapy was good in all cases. Seven of 11 G2019S-positive patients have L-dopa-induced peak-dose dyskinesias, which had started on average 3.7 years (range 0–8) after the beginning of the L-dopa therapy. Six patients presented motor fluctuations that had started on average 5.5 years (range 0–14 years) after the beginning of the L-dopa therapy. One patient noticed motor fluctuations before this therapeutic intervention.

Smell tests using the UPSIT scale showed that 9 of the 11 patients scored below the normal range values for their age. The 2 patients who scored in the normal range belonged to the same PD family. The lowest score belonged to a patient operated for an olfactory sulcus meningioma. Sensory complaints and daytime somnolence was present in 8 and 7 of these 11 G2019S-positive patients, respectively.

The 2 patients (1 man and 1 woman) with the R1441H mutation had symptoms onset at 32- and 57-years, presenting both now 24 years of disease duration. Both predominantly presented rigidity and bradykinesia at disease onset. The UPDRS motor score at the last examination in "on" state was 24 and 35, respectively, and the Hoehn and Yahr score was 2 and 3. None presented relevant cognitive deficits. Both started L-dopa therapy at the time of diagnosis; 1 patient experienced peak-dose dyskinesias and motor fluctuations 8 years after the beginning of L-dopa therapy while in the other patient these complications started 10 and 19 years after the beginning of L-dopa therapy. Both patients performed below the normal range in the UPSIT test.

DISCUSSION

Our findings confirm and expand previous data, delineating a very high prevalence of the *LRRK2*-G2019S mutation, and also identifying a second mutation, R1441H, among Portuguese patients with PD. Furthermore, we provide detailed description of the clinical phenotype, including evaluation of the sense of smell, and we explore the presence of *parkin* mutations in patients with *LRRK2* mutations.

A previous report on the *LRRK2* gene in Portugal was based in Coimbra (~200 km north of Lisbon); it included 124 unrelated PD cases, and estimated a G2019S mutation frequency of 4.9 and 9.1% among 102 sporadic and 22 familial cases, respectively.[13] Our frequency estimate among sporadic cases (3.7%) is very similar, but the frequency among familial cases (16.1%) is higher. We did not include in the calculations the first Portuguese PD family we reported in our initial paper on the G2019S mutation.[21] Including that family, we would obtain an estimate of 18.7% (6 of 32) for the frequency of the G2019S mutation among Portuguese patients with familial PD. A comparison of the frequencies of the G2019S mutation among familial and sporadic PD cases in the Iberic and other populations is presented in Table 4. It must be emphasized that the overall sample size is still relatively small, particularly for the familial PD cases, and studies on larger series are warranted. However, the current scenario indicates the populations of Portugal (this study and[13]) and Spain[10-12] among those with the highest frequency of the G2019S mutation worldwide, after the North African Arabs and Ashkenazi Jews. On the basis of these results, screening of the G2019S mutation is also warranted in other populations of Portuguese and Hispanic ancestry, such as those of Brazil, and of several countries of Central and South America.

There is evidence that the G2019S mutation occurs on at least three different haplotypes. One of them, found in patients of North African, Middle Eastern, and European ancestry, is by far the most common.[19,24,32,33] The other two were found in a minority of patients of European ancestry,[33] and in Japanese patients.[28,34]

In our experience, all cases with the *LRRK2*-G2019S mutation (from Italy, Portugal, Brazil, Greece, and Morocco) are carrying the same, commonest haplotype tagged by the IVS13 + 104G/A variant (rs28903073) we originally identified.[19] All the G2019S cases in this study and none of the subjects who do not carry this mutation, were carriers of the IVS13 + 104G/A variant. These results support the notion of the strong LD between G2019S and the IVS13 + 104G/A variant, as well as the likely origin of most (if not all) the G2019S alleles present in the Mediterranean populations from a single, very old ancestor.

The penetrance of G2019S increases with age but is likely incomplete. Indeed, lifetime penetrance values of 24 to 33% have been recently estimated in Jewish and non-Jewish American, as well as in Italian families.[7,35,36] In this study, samples from at-risk relatives were not available for accurate estimates, but the pedigree structure of G2019S-positive families also suggests a low

TABLE 4. *Frequency of the LRRK2-G2019S mutation in series of PD cases and controls from Portugal and Spain, and, for comparison, in some representative studies from other populations*

	PD-unrelated probands							
	Total PD sample		Familial PD cases		Sporadic PD cases		Controls	
Author, Year (ref.) Region	Sample Size	G2019S carriers No., (%)	Sample Size	G2019S carriers No., (%)	Sample Size	G2019S carriers No., (%)	Sample Size	G2019S carriers No., (%)
Present study Lisbon, Portugal	138	9 (6.52)	31	5 (16.1)	107	4 (3.7)	101	0
Brás et al., 2005 (13) Coimbra, Portugal	124	7 (5.6)	22	2 (9.1)	102	5 (4.9)	126	0
Infante et al., 2006 (11) Cantabria, Spain	105	8 (7.6)	16	3 (18.7)	82	5 (6.1)	310	0
Mata et al., 2006 (12) Asturias, Spain	225	5 (2.2)	50	0	175	5 (2.9)	100	0
Gaig et al., 2006 (10) Catalonia, Spain	302	13 (4.3)	94	6 (6.4)	208	7 (3.4)	not available	
Lesage et al., 2006 (6) North African Arabs	76	30 (39)	27	10 (37)	49	20 (41)	151	2 (1.3)
Ozelius et al., 2006 (7) Ashkenazi Jews	120	22 (18.3)	37	11 (29.7)	83	11 (13.3)	317	4 (1.3)
Goldwurm et al., 2006 (18) Italy	1092	19 (1.74)	236	10 (4.24)	856	9 (1.05)	440[a]	0
Kay et al., 2006 (25) USA	1425	18 (1.3)	329	10 (3)	1096	8 (0.7)	1647	1 (0.06)
Aasly et al., 2005 (26) Norway	433	7 (1.6)	65	6 (9.2)	368	1 (0.27)	519	0
Xiromerisiou et al., 2007 (27) Greece	290	1 (0.34)	55	0	235	1 (0.42)	235	0
Zabetian et al., 2006 (28) Japan	586	2 (0.34)	32	0	554	2 (0.36)	317	0
Punia et al., 2006 (29) India	778	1 (0.13)	60	0	718	1 (0.14)	212	0
Bialecka et al., 2005 (30) Poland	174	0	21	0	153	0	190	0
Tan et al., 2005 (31) Singapore (mostly Chinese)	675	0	58	0	617	0	325	0

[a]Data from Goldwurm et al., 2005 (19).

penetrance. Among the parents of 9 G2019S-positive probands 2 suffered from PD and another 2 had history of tremor only. Of note, most of these parents have lived until advanced age, thus excluding strong censoring bias (Fig. 1). Assuming that 50% of these parents (n = 9) were carriers of the mutation, one estimates a penetrance of ~22% (2/9, including only PD cases) or ~44% (4/9, including PD and tremor cases).

In recent studies, *parkin* mutations were identified in a few patients carrying the G2019S mutation, suggesting a digenic mechanism of PD inheritance.[6,14,15] We identified 2 cases carrying a G2019S and a *parkin* heterozygous point mutation. We did not test for *parkin* genomic rearrangements, and we could have missed other mutations. However, in the family Lisb-F24 (Fig. 1) the *parkin* mutation did not cosegregate with PD, and the PD onset age in the two siblings with and without *parkin* mutation was similar. The presence of *parkin* mutations might be a coincidental finding, as carriers of a single heterozygous *parkin* mutation are frequent, particularly in some populations.[37] A rigorous test of the hypothesis of *LRRK2-parkin* digenic inheritance of PD will require the comprehensive screening of *parkin* in a large series of PD patients with and without *LRRK2* mutations, and large series of controls. However, our findings, together with those of a previous study,[15] exclude the presence of *parkin* point mutations or homozygous exon deletions in the majority of patients with *LRRK2* mutations. Whether patients with *LRRK2* mutations have mutations in *PINK1* or in other PD-causing genes remains unknown.

In agreement with other studies,[6,7,9,18,35] the clinical phenotype among our G2019S carriers appears indistinguishable from typical, idiopathic PD, and a wide range of onset ages is present (from 24- to 72-years-old). Most patients had a typical parkinsonian phenotype with an asymmetric onset, tremor as the most frequent sign at onset, a good response to therapy, slow disease progression, absence of atypical features and no relevant cognitive impairment. They frequently presented depression, sensory complaints, restless legs syndrome and daytime somnolence. Like in typical PD, impaired sense of smell was very common among *LRRK2* mutation carriers (Table 3).

The R1441G is a founder mutation present in ~8% of PD patients from the Basque population,[1] in 2 to 3% of cases from the neighboring Spain region of Asturias,[38] and rarely in patients from Catalonia.[10] On the other hand, this mutation was not observed in Cantabria,[11] nor in Portugal, in this and a previous study.[13] The "Basque" *LRRK2* mutation seems therefore very rare (or absent) in Portugal. On the contrary, our identification of a novel PD family with the R1441H mutation, the first to be reported in Portugal, extends the presence of this mutation to this population. R1441H has been previously identified in one North American family with European ancestry,[39] one Taiwanese family,[8] and one family from Crete.[40]

Taken as a whole, our data add to a growing body of evidence placing the Portuguese population among those with the highest prevalence of *LRRK2* mutations, with relevance for the diagnosis and genetic counseling of PD. Because of the important ethical, psychological, and social implications for the patients and their relatives, genetic testing for diagnostic and especially for predictive purpose, should be always considered in the context of a multidisciplinary team, including neurologists and medical geneticists, and assisted by proper counseling.[41,42] The PD risk assessment for mutation carriers is currently difficult because of the limited knowledge about the penetrance of *LRRK2* mutations, and the large variability of the onset age and other associated clinical features. Clearly, more studies are required.

From the research standpoint, the ascertainment of etiologically homogeneous cohorts of PD cases and at-risk individuals, now feasible in populations with high-prevalence of the G2019S mutation (such as the Portuguese), will promote research into the genetic and nongenetic modifiers of the PD phenotype, and yield cohorts of individuals of crucial importance for testing future neuroprotective interventions.

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Chapter 8 - Neuropathology of Parkinson's Disease Associated With the LRRK2 Ile1371Val Mutation

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Neuropathology of Parkinson's Disease Associated With the *LRRK2* Ile1371Val Mutation

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***Abstract:* *Leucine-Rich Repeat Kinase 2* (*LRRK2*) gene mutations are the most common known cause of Parkinson's disease (PD), but neuropathological studies are available on very few patients with *LRRK2* mutation. The reported findings range from Lewy body–positive pathology to different pathologies, including nigral degeneration without distinctive features, neuronal loss with only ubiquitin-positive inclusions, and tau-positive–only pathology. Here we report the first neuropathological study in an Italian PD case carrying a different *LRRK2* mutation, Ile1371Val, and showing typical ubiquitin- and α-synuclein–positive Lewy body pathology. These findings support the concept that the neurodegeneration associated with *LRRK2* mutations might be clinically and pathologically indistinguishable from typical PD.** © 2006 Movement Disorder Society

Key words: Parkinson's disease; *LRRK2*; Ile1371Val mutation; neuropathology

Mutations in the *Leucine-Rich Repeat Kinase 2* (*LRRK2*, *PARK8*) gene are the most common known cause of familial autosomal dominant Parkinson's disease (PD).[1,2] Moreover, due to incomplete penetrance, *LRRK2* mutations are also found in few patients with sporadic PD.[3–5] Neuropathological studies have been performed so far on very few patients with *LRRK2* mutations, and the results showed a pleomorphic range of pathologies with or without Lewy bodies (LBs).[1,3,6–12]

A new *LRRK2* mutation in exon 29, A4111G (predicted protein effect: Ile1371Val), has been recently detected in two PD families of Indian and Italian origin, respectively.[13,14] We report here the first extensive neuropathological study of one patient bearing the Ile1371Val mutation from the Italian pedigree (individual MI-007-03 in Di Fonzo and colleagues[14]), showing that this mutation is associated with a pathological phenotype indistinguishable from that of typical sporadic PD.

Neuropathological Methods

General autopsy findings were unremarkable. The brain was fixed in formalin; large blocks were subsequently dissected and embedded in paraffin; 10 micron thick sections were stained with hematoxylin and eosin, Luxol Fast Blue, and silver methenamine. For immunohistochemistry, small blocks from medulla oblongata, pons, midbrain, cerebellum, hippocampal formation, amygdala, gyrus cinguli, temporal cortex, insular cortex, caudate, putamen, and globus pallidus were processed. Immunostaining was carried out for glial fibrillary acidic protein (MAb; 1:100; DakoCytomation, Glostrup, Denmark), Tau (AT8; 1:400; Innogenetics, Gent, Belgium), ubiquitin (polyclonal; 1:200; microwave pretreatment; DakoCytomation), and α-synuclein by using three distinct reagents (KM51, 1:40, microwave pretreatment, Novocastra, Newcastle upon Tine, U.K.; LB509, 1:400, Zymed, San Francisco, CA; 42, 1:300, Becton-Dickinson, Biosciences, Pharmingen). Reactions were visualized with the ABC complex (DakoCytomation) and diaminobenzidine (Roche Diagnostics, Basel, Switzerland).

CASE REPORT

This woman died of pulmonary embolism at the age of 71. Neurological symptoms started 10 years earlier (at the age of 61) as unilateral motor impairment and tremor. She was diagnosed with PD and treated with levodopa, with a good response. During the following years, the symptoms became bilateral and motor fluctuations, visual hallucinations, and cognitive disturbances were noted. However, these last were not severe enough to make a formal diagnosis of dementia according to DSM-IV criteria. Detailed clinical and molecular genetic findings in this family (MI-007) have been reported.[14] The family history is peculiar in that the husband of the patient described here also suffered from late-onset PD, while one of their three children developed early-onset PD (onset age 33 years).

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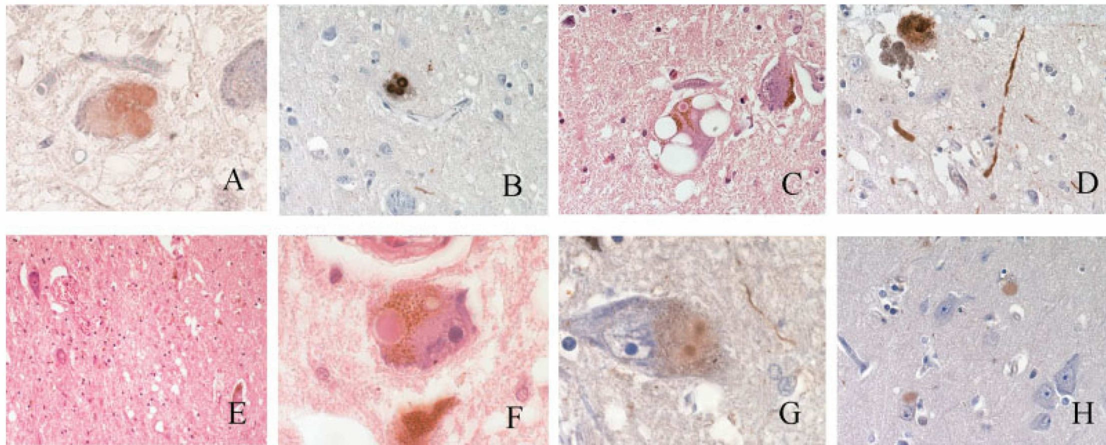

FIG. 1. Pathological features in the *LRRK2*-positive case. Staining methods: α-synuclein immunohistochemistry (**A**, **B**, and **D**); ubiquitin immunohistochemistry (**G** and **H**); and ematoxilin–eosin (**C**, **E**, and **F**). Original magnifications: 1,000× (A, F, and G); 400× (B, C, D, and H); 200× (E). A: α-synuclein–positive neuronal inclusions in dorsal vagus complex, (B) Lewy bodies, (C) neuronal vacuolation, and (D) Lewy neurites in the locus coeruleus. E: Loss of neurons, gliosis, and extracellular pigment in the substantia nigra, ventrolateral part. F: Lewy body and pale body in a neuron of the substantia nigra. G: Multiple ubiquitin-positive Lewy bodies in substantia nigra. H: Cortical Lewy bodies in the anterior cingulated gyrus.

Neuropathological Findings

The external aspect of the brain was unremarkable. There was no brain atrophy, but depigmentation of the substantia nigra was seen on macroscopic examination. In the dorsal glossopharyngeus–vagus complex, a moderate neuronal loss was found. Eosinophilic irregular intraneuronal inclusions and typical LBs, as well as extraneuronal eosinophilic inclusions, were present. Some inclusions were stained by antiubiquitin, and sometimes by anti–α-synuclein antibodies (Fig. 1A), and appeared similar to the "pale bodies," which have been proposed as precursors of LBs.[15] Numerous enlarged neuronal processes (Lewy neurites) were also present in this area. A moderate neuronal loss was found in the locus coeruleus; the residual pigmented neurons displayed single or multiple typical LBs (Fig. 1B), occasionally accompanied by other eosinophilic inclusions. Occasional neurons were affected by prominent vacuolation (Fig. 1C). Dystrophic and enlarged neuronal processes (Lewy neurites; Fig. 1D) and LBs were strongly stained by antiubiquitin and α-synuclein antibodies. The substantia nigra was very severely affected. Neuronal loss, gliosis, and extracellular pigment were prominent in the ventrolateral part (Fig. 1E), while clusters of pigmented neurons were found in the dorsomedial part. LBs, pale bodies, and large vacuoles were variously combined within the cytoplasm of neurons (Fig. 1F). The LBs and Lewy neurites were stained by antiubiquitin and α-synuclein antibodies (Fig. 1G). The striatum and globus pallidus appeared normal. Only rare tau-positive neurofibrillary tangles and rare ubiquitin-positive dystrophic neurites belonging to senile plaques were found in the hippocampus and amygdala. No other tau-positive pathology was observed, and argentophilic amyloid deposition was not found outside the hippocampus. Occasionally, cortical neurons of the anterior cingulated gyrus and insula showed ubiquitin-positive and α-synuclein–positive intracytoplasmic inclusions, similar to cortical LBs (Fig. 1H). Overall, the neuropathology of this case was indistinguishable from that of typical PD. The distribution and frequency of cortical LBs according to McKeith and colleagues[16] were consistent with the transitional category.

DISCUSSION

In spite of the high prevalence of *LRRK2* mutations in familial and sporadic PD, the neuropathology of *LRRK2*-associated neurodegeneration has been investigated so far in a very limited number of cases, and the results have shown a pleomorphic picture. In four PD patients from the original PARK8-linked Japanese family (Sagamihara), the autopsy revealed nigral degeneration without LBs.[6,7] However, in the same family, one carrier of the disease haplotype received a pathological diagnosis of striatonigral degeneration, a form of multiple-system atrophy known to be associated with α-synuclein–positive glial cytoplasmic inclusions.[7]

Detailed pathological studied are available in two large families included in one of the original *LRRK2* cloning papers: Family "A" with the Tyr1699Cys muta-

TABLE 1. *Pathological findings reported in patients with LRRK2 mutations*

Mutation	Protein domain	Number of brains	Brain pathology*	Reference
Ile1371Val	ROC	1	LBs	This study
Arg1441Cys	ROC	4	LBs (2), neurofibrillary tangles (1), Ub-only inclusions (1)	Wszolek and colleagues[9]; Zimprich and colleagues[1]
Tyr1699Cys	COR	2	Ub-only inclusions	Wszolek and colleagues[8]; Zimprich and colleagues[1]
		1	LBs	Khan and colleagues[10]
Gly2019Ser	Kinase	3	LBs	Gilks and colleagues[3]
		8	LBs	Ross and colleagues[12]
		3	LBs (2), LRRK2-positive neurites (1)	Giasson and colleagues[11]
Ile2020Thr	Kinase	4	No inclusions	Funayama and colleagues[7]

*In addition to neuronal loss and gliosis in the *substantia nigra*
Ub-only inclusions, immunoreactive only for ubiquitin; ROC, Ras of complex proteins (GTPase domain); COR, C-terminal of ROC domain.

tion, and Family "D" with the Arg1441Cys mutation.[1,8,9] The common feature in all these cases was the nigral cell loss with gliosis, delineating this feature as the main pathological correlate of the parkinsonism.[1] However, a very heterogeneous scenario emerged when the pattern of neuronal inclusions was characterized.

Both cases analyzed in Family A and one from Family D did not show any α-synuclein– or tau-positive pathology, but only ubiquitin-positive cytoplasmic and nuclear inclusions. Another two cases in Family D had LB-positive pathology, while the fourth case showed a pattern of tau-positive pathology similar to that of progressive supranuclear palsy. Alzheimer-type pathology was also present in several patients, and in Family A neuronal loss in the anterior horn of the spinal cord was also noted, correlating with the presence of amyotrophy in those patients.[1,8,9] It could be argued that in Family A the atypical pathology correlates with the presence of a particular mutation (Tyr1699Cys). However, one autopsy performed more recently in a British family with PD and the same mutation, the Lincolnshire kindred, revealed LB-positive pathology in the brain and olfactory bulb, as observed in classical PD.[10]

The most common *LRRK2* mutation known so far is Gly2019Ser, present in up to 6% to 18% of familial PD and in 3% to 6% of sporadic cases in some European populations[4,17–20] and in many more cases among Arab and Ashkenazy Jewish populations.[21,22] Presence of LB-positive pathology was briefly mentioned in three cases included in one of the earliest descriptions of this mutation.[3]

More recently, in a large screen of 405 LB-positive brains, 8 turned out to be carriers of the Gly2019Ser mutation (~2%), including 4 with brainstem-type, three with transitional-type, and 1 with diffuse LB pathology.[12] Alzheimer-type pathology was also present in some, but in only two Gly2019Ser-positive brains, it was of enough severity to make a concomitant pathological diagnosis of Alzheimer's disease. On the other hand, only two carriers of Gly2019Ser were identified among 1,179 LB-negative brains (including 326 progressive supranuclear palsy, 43 multiple-system atrophy, 654 Alzheimer's disease, and 156 healthy control brains), supporting a causal relationship between this mutation and the LB-positive pathology.

In the last available study, 80 brains with PD or LB dementia were screened for the Gly2019Ser mutation, and 3 were found to be carriers.[11] Typical brainstem-type LB-positive pathology was found in one, while the Lewy body variant of Alzheimer's disease was diagnosed in the second. The third brain showed only cell loss in the substantia nigra and locus coeruleus, but no α-synuclein inclusions were detected. There were only rare tau-positive tangles and occasional plaques. No other ubiquitin-positive inclusions were present either. Interestingly, only in this case, LRRK2-positive neurites were identified in the substantia nigra using an LRRK2 antibody.[11]

From this literature synopsis (Table 1), it can be summarized that LB-positive pathology is the most common pattern associated to pathogenic *LRRK2* mutations, particularly the common Gly2019Ser mutation. However, other pathologies might be seen, including nigral cell loss without distinctive inclusions, or with inclusions, which are stained only with ubiquitin or only with tau antibodies.

Here we report the first neuropathological study in one PD case carrying a different *LRRK2* mutation, Ile1371Val, showing a pattern consistent with classical LB-positive pathology. Two groups reported the identification of the Ile1371Val mutation in two unrelated PD families, the first from eastern India[13] and the second from Italy.[14] The mutation is located in a Rab family motif within one of the most conserved functional domains of LRRK2 (the ROC domain). Isoleucine and Valine are both aliphatic amino acids, but Isoleu-

cine1371 is highly conserved among the LRRK2 protein homologues.[14] While these considerations suggest that the Ile1371Val mutation is pathogenic, we acknowledge that the genetic support for this view remains limited. Due to the small family size, the evidence for cosegregation of the mutation with PD was present but limited in the Italian family, while cosegregation could not be examined in the Indian family. Furthermore, ethnically matched controls from the Indian population were not assessed.[13] We found this mutation in only 1 of 208 Italian controls (416 chromosomes).[14] Future work, including case–control studies and functional analyses, might help to better understand the pathogenic relevance of the Ile1371Val mutation. However, the observations reported here represent a unique opportunity to explore the pathological correlate of the Ile1371Val mutation in the human brain, adding to the growing knowledge on the *LRRK2*-associated neurodegeneration.

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Chapter 9 - The LRRK2 Arg1628Pro variant is a risk factor for Parkinson's disease in the Chinese population

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ORIGINAL ARTICLE

The LRRK2 Arg1628Pro variant is a risk factor for Parkinson's disease in the Chinese population

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Abstract The c.G4883C variant in the *leucine-rich repeat kinase 2* (*LRRK2*) gene (protein effect: Arg1628Pro) has been recently proposed as a second risk factor for sporadic Parkinson's disease in the Han Chinese population (after the Gly2385Arg variant). In this paper, we analyze the Arg1628Pro variant and the associated haplotype in a large sample of 1,337 Han subjects (834 patients and 543 controls) ascertained from a single referral center in Taiwan. In our sample, the Arg1628Pro allele was more frequent among patients (3.8%) than among controls (1.8%; p = 0.004, OR 2.13, 95% CI 1.29–3.52). Sixty heterozygous and two homozygous carriers of the Arg1628Pro variant were identified among the patients, of which only one was also a carrier of the *LRRK2* Gly2385Arg variant. We also show that carriers of the Arg1628Pro variant share a common, extended haplotype, suggesting a founder effect. Parkinson's disease onset age was similar in patients who carried the Arg1628Pro variant and in those who did not carry it. Our data support the contention that the Arg1628Pro variant is a second risk factor for Parkinson's disease in the Han Chinese population. Adding the estimated effects of Arg1628Pro (population attributable risk [PAR] ~4%) and Gly2385Arg variants (PAR ~6%) yields a total PAR of ~10%.

Keywords Parkinson's disease · Genetics · LRRK2 · Association · Risk allele

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Introduction

The identification of mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) gene as a cause of autosomal dominant Parkinson's disease (PD) opened a novel era for the understanding of the causes and mechanisms of this disorder [1, 2]. *LRRK2* (particularly, the Gly2019Ser mutation) gave proof of principle of a common genetic cause of typical late-onset PD, but it also yielded the first variant, Gly2385Arg, a frequent polymorphism in different Asian populations, which acts as a risk factor for the common, sporadic form of PD in those populations [3].

In search for population-specific biologically relevant variants, we sequenced the complete *LRRK2* coding region in a number of PD patients from Taiwan [4]. This work led to our initial identification of the association of Gly2385Arg with PD, a finding that has been rapidly and

consistently replicated in Chinese populations from Singapore [5], Taiwan [6, 7], mainland China [8, 9], and in the Japanese population [10].

Very recently, a different *LRRK2* missense variant, Arg1628Pro, was proposed as a second risk factor for sporadic PD in the Han Chinese population (OR 1.84, 95% CI 1.20–2.83, p = 0.006) [11]. In this study, we report the results of the analysis of the Arg1628Pro variant in a large, independent sample of PD cases and controls of Han Chinese ethnicity.

Materials and methods

The study sample consisted of 1,377 subjects of Han Chinese ethnicity, ascertained at a single referral center in Taiwan, and included 834 patients with a clinical diagnosis of idiopathic PD and sporadic pattern of presentation (343 women, 491 men) and 543 control subjects (319 women, 224 men). For the patients, the average ages at last examination and at disease onset were 65.7 ± 11.8 years (range 24–97) and 55.6 ± 12.1 years (range 12–94), respectively. The average disease duration was 10.1 ± 6.24 years (range 1–30). The clinical diagnosis of PD was established according to published criteria [12]. A first subgroup of 608 PD cases was previously screened by us for the *LRRK2* Ile2012Thr, Gly2019Ser, and Ile2020Thr mutation and proved to be negative [13]. In cases with disease onset before the age of 40 years, the *parkin* and *PINK1* genes were screened by single-strand conformation polymorphism, and no mutations were found. The control subjects were free from PD, matched to the patients by ethnicity, collected at the same center, and originating from the same geographical areas. They were ascertained among the spouses of PD patients, spouses of patients with unrelated diseases, and healthy volunteers. The mean age at sampling for controls was 51.9 ± 18.4 years, range 11–87, which is slightly, but significantly younger than the average age at onset in the cases. The project was approved from the local ethical authorities, and written informed consent was obtained from all subjects.

Genomic DNA was isolated from peripheral blood using standard protocols. In the whole sample of cases and controls, the *LRRK2* c.G4883C variant in exon 34 (single nucleotide polymorphism [SNP] accession no. rs33949390, predicted protein effect: Arg1628Pro) was screened by a TaqMan allelic discrimination method and Assays-by-Design on an Applied Biosystems 7300 Real Time PCR System. Primers, probes, and experimental conditions are available on request.

For genotyping quality control, multiple positive and negative controls for the c.G4883C variant were included in each polymerase chain reaction plate. Furthermore, 176 cases and 181 controls were also genotyped for Arg1628-Pro and other variants located in exon 34 by direct sequencing in both strands, as described previously [14], yielding 100% concordance with the results of the TaqMan assay for the Arg1628Pro variant.

For haplotype analysis, we first examined the complete coding region of *LRRK2* and exon–intron boundaries, sequenced previously by us in two heterozygous carriers of the c.G4883C (Arg1628Pro) variant [4]. All the variants detected were tested in one additional heterozygous and two homozygous Arg1628Pro carriers detected in this study.

Furthermore, five coding SNPs located in exon 5 (Leu153Leu, rs10878245), exon 34 (Gly1624Gly, rs1427263; Lys1637Lys, rs11176013; Ser1647Thr, rs11564148), and exon 49 (Met2397Thr, rs3761863) were typed by direct sequencing in 46 PD patients who were carriers of the Arg1628Pro variant, as well as in 87 Taiwanese subjects (44 PD patients and 43 controls) who were not carrying Arg1628Pro. Haplotype analysis was performed using Haploview ver. 1.4 [15]. Lastly, the c. G4883C (Arg1628Pro) variant was typed by direct sequencing in 202 Caucasian subjects (106 PD patients and 96 controls, total 404 chromosomes). Sequencing was performed using Big Dye Terminator chemistry ver.3.1 (Applied Biosystems). Fragments were loaded on an ABI3100 and analyzed with DNA Sequencing Analysis (ver.3.7) and SeqScape (ver.2.1) software (Applied Biosystems). All variants are named according to the *LRRK2* complementary DNA sequence deposited in GenBank (accession no. NM_198578).

For predicting the secondary structure of the region of the LRRK2 protein containing the Arg1628 residue, we used the PSIPRED ver.2.6 [16] and NPS [17] programs. Statistical analyses included contingency tables and Student's test as appropriate. For the calculation of the population attributable risk (PAR), the following formula was used: $P(\mathrm{OR} - 1)/1 + P(\mathrm{OR} - 1)$, where P is the frequency of the risk genotype (heterozygous Arg1628Pro) in the controls, considered to approximate the frequency of the risk genotype in the general population and OR is the odds ratio obtained in the case control comparison, used to estimate the relative risk of PD for the carriers of the risk genotype.

Results

The results of the association analysis are reported in the table. Controls and cases were both in Hardy–Weinberg equilibrium. The Arg1628Pro variant was detected in 62 cases (7.4%, two homozygous and 60 heterozygous) and in 20 controls (3.7%, all heterozygous). The Arg1628Pro allele was significantly more frequent among patients (p =

Table 1 Distribution of G4883C (Arg1628Pro) allelic and genotypic frequencies

	Cases (n=834)					Controls (n=543)				
	Genotypes			Alleles		Genotypes			Alleles	
	G/G	G/C	C/C	G	C	G/G	G/C	C/C	G	C
Number	772	60	2	1,604	64	523	20	0	1,066	20
Percentage	0.926	0.072	0.002	0.962	0.038	0.963	0.037	0.0	0.982	0.018

p value=0.004—comparison of allelic frequency in PD cases versus controls
p value=0.006—comparison of genotype frequency in PD cases versus controls (heterozygous and homozygous Arg1628Pro carriers combined)
p value=0.009—comparison of genotype frequency in PD cases versus controls (only heterozygous Arg1628Pro carriers)
Chi-square with Yates correction

0.004, OR 2.13, 95% CI 1.29–3.52; Table 1). Using this observed value of OR to estimate the relative risk and the frequency of the risk genotype (heterozygous Arg1628Pro) among controls as an estimate of its frequency in the general population yields a PAR of ~4%.

The sequencing of *LRRK2* exons and exon–intron boundaries in two homozygous and three heterozygous carriers of the Arg1628Pro variant revealed several, previously reported intronic and exonic SNP variants (Fig. 1a). Haplotypes in the two homozygous patients are identical (Fig. 1a), and those in the three heterozygous cases are also compatible with the presence of the same shared haplotype across the whole *LRRK2* gene. Furthermore, in 46 Taiwanese patients, who carried the Arg1628-Pro variant, the analysis by Haploview detected association of this variant always with the same haplotype (formed by the C-A-G-A-C alleles of the Leu153Leu, Gly1624Gly, Lys1637Lys, Ser1647Thr, and Met2397Thr variants). On the other hand, this specific C-A-G-A-C haplotype was estimated by Haploview at a frequency of 0.283 and 0.291 among Taiwanese PD patients and controls, who did not carry the Arg1628Pro variant.

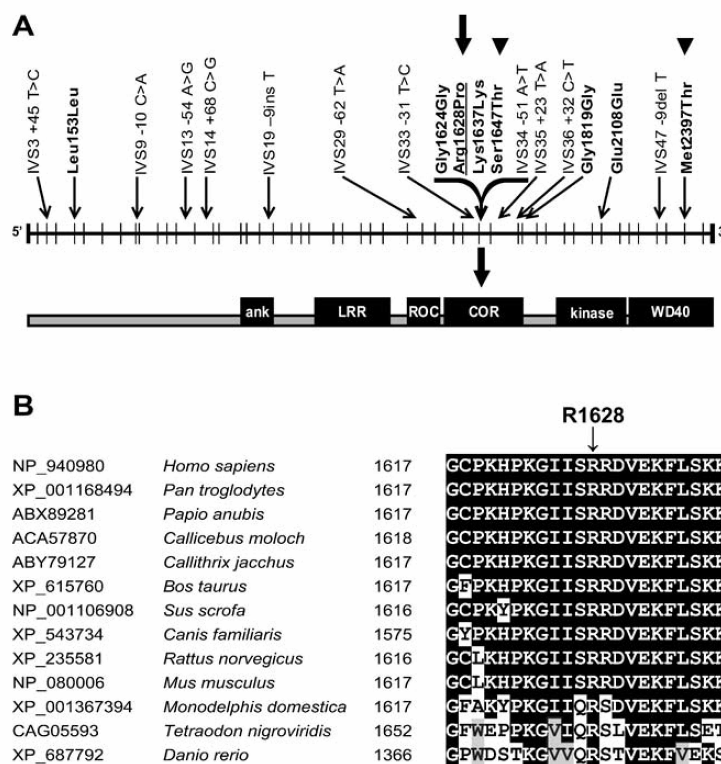

Fig. 1 **a** Haplotype containing the Arg1628Pro variant. Exonic variants are *bolded* and two non-synonymous variants are indicated by *arrowheads*. The position of the Arg1628Pro variant is indicated by an *arrow* in the haplotype and in the LRRK2 domain structure shown *below*. *ank* ankyrin-like repeat region, *LRR* leucine-rich repeat region, *ROC* Ras in complex proteins, *COR* C-terminal of Roc, *WD40* WD40 repeat-like domain. **b** Alignment of LRRK2 protein homologues in the region corresponding to the human Arg1628 residue

All the patients who were carriers of the Arg1628Pro variant were tested for the presence of the Gly2385Arg variant, by direct sequencing of *LRRK2* exon 49. Only one of the carriers of the Arg1628Pro variant (heterozygous) was also a carrier of the heterozygous Gly2385Arg variant. We did not detect the c.G4883C (Arg1628Pro) variant in any of 404 Caucasian chromosomes (212 from PD patients and 192 from controls).

PD average onset age in the 60 heterozygous carriers of the Arg1628Pro variant was 55.4±10.3 years (range 35–80), while in the 772 non-carriers, the onset age was 55.6±12.2 (12–94; p=NS). Disease duration was 10.9±7.1 years (1–30) and 10.1±6.2 (1–30) in carriers and non-carriers, respectively (p=NS). The two patients homozygous carriers of Arg1628Pro had PD onset at ages 38 and 55 and disease duration of 15 and 18 years.

The mean age at examination in the 20 controls, who were carriers of the Arg1628Pro heterozygous variant, was 49.2 years±18.3 (22–72), which is younger but not significantly different than the mean onset age of PD in the heterozygous carriers (p=0.064, Student's t-test); however, half of the control carriers were younger than the mean onset age of PD in heterozygous carriers.

Discussion

Our results support the contention that the *LRRK2* Arg1628Pro variant is a second risk factor for the common forms of sporadic PD in the Chinese population (after the Gly2385Arg variant). We neither detected the Arg1628Pro variant in any of the 404 Caucasian chromosomes, nor was the variant reported in the other *LRRK2* studies in Caucasians. Similar to Gly2385Arg [3], Arg1628Pro might be therefore considered as a PD-associated population-specific allele in the Han Chinese population. Interestingly, in the previous report [11], the Arg1628Pro variant was not detected among Japanese subjects, but the sample size was limited (N=246), and further studies are warranted.

The results of our haplotype analyses, in keeping with the data from the previous study [11], strongly suggest that most carriers of the Arg1628Pro variant share an extended haplotype, indicative of a founder effect.

Any given disease-associated variant might merely represent a marker of another, biologically relevant variant, which lies in linkage disequilibrium on the risk allele. While Arg1628Pro is a non-conservative change in a highly conserved residue (Fig. 1b), the associated haplotype includes 11 intronic, five exonic silent, and two conservative variants (Ser1647Thr and Met2397Thr; Fig. 1a). However, all these variants display much higher allele frequencies than Arg1628Pro (our data not shown), and, more importantly, these variants are also present in Caucasian populations, where association with PD was not detected in previous studies examining common *LRRK2* variants using haplotype-tagging SNPs [18, 19]. We also explored the frequency of three variants located in exon 34: Gly1624Gly (rs1427263), Lys1637Lys (rs11176013), and Ser1647Thr (rs11564148) in 176 cases and 181 controls from this study (more than 700 chromosomes), but we detected no association with PD (Gly1624Gly minor allele frequency [MAF] 0.45 in cases and 0.53 in controls; Lys1637Lys MAF 0.44 in cases and 0.49 in controls; Ser1647Thr MAF 0.33 in cases and 0.32 in controls). From all these arguments, we conclude that the Arg1628Pro variant is most likely the biologically relevant variant, responsible for the association with PD detected here and in the previous, independent study [11].

Results of allelic association studies might be biased, mainly by the use of small sample sizes, population stratification, and genotyping errors [20], and replication in independent, large samples is important. In order to minimize these sources of biases, we tested a large sample of ethnically matched cases and controls, recruited at only one referral center; we adopted strict PD diagnostic criteria, and we excluded familial PD cases; genotyping was performed in only one laboratory using standard and uniform genotyping platforms and rigorous systematic quality controls. These considerations argue against the existence of the above-mentioned biases. Furthermore, as some of the control carriers of the variant might still develop PD at later ages, the actual effect size (OR) of the Arg1628Pro variant might be higher.

Contrary to the Arg2385Gly, the Arg1628Pro variant replaces a highly conserved residue in the COR domain of the LRRK2 protein (Fig. 1a,b). The COR domain (C-terminal of Roc) is unique for the LRRK2 protein family [21], where it is thought to mediate intramolecular signaling between the N-terminal Roc-GTPase and the C-terminal kinase domain of LRRK2 (Fig. 1a). Only one definitely PD-causing mutation is known so far in the COR domain (Tyr1699Cys). The Arg1628Pro variant replaces the positively charged arginine with a nonpolar Proline. Moreover, Proline might change the local folding, as it is typically acting by breaking α-helical conformation in proteins. Analyses using the PSIPRED and NPS programs indicate that the Arg1628 and the following 7–10 amino acids are likely to adopt an α-helical conformation. Loss of the net positive charge, change in α-helical content, or both might affect the structure and function of the COR domain, with the final effect of altering the regulation of the downstream kinase activity of LRRK2. Functional studies are warranted to understand the mechanisms of action of the Arg1628Pro variant. As it is the case for the other PD-associated variant in *LRRK2* (Arg2385Gly), onset age of PD in cases who are carriers and non-carriers of the Arg1628Pro variant does

not differ, but further study might identify differences in other clinical or patho-physiological features.

With only one exception, in our experience, the cases that carry Arg1628Pro do not carry Arg2385Gly and vice versa. These two variants are therefore located on different alleles, and their impact at the population level will add to each other. The patient who carries both *LRRK2* risk variants has a 16-year history of tremor-predominant PD, which started at the age of 65 and responded well to L-dopa, but with L-dopa-induced psychosis developing 7 years after PD onset. DAT scan (^{99m}Tc-TRODAT SPECT) showed decreased dopamine transporter activity. Before the onset of motor symptoms, the patient developed anosmia but no rapid eye movement (REM) sleep behavior disorder. We also detected two cases who are homozygous carriers of Arg1628Pro. These cases had PD onset at ages 38 and 55 and disease duration of 15 and 18 years. The first has tremor-predominant, while the other has akinetic-rigid-predominant PD type. Both responded well to L-dopa, but L-dopa-induced psychosis developed 13 years after onset of PD in the second. DAT scan (^{99m}Tc-TRODAT SPECT) showed decreased dopamine transporter activity in both. None of these developed hyposmia or REM sleep behavior disorder. Further homozygous cases need to be identified in order to answer the question whether carrying two copies of the Arg1628Pro variant yields a younger or more aggressive phenotype.

The PAR is the percentage of cases in a given population, which are attributable to the effect of a given etiologic factor (in this case, the risk allele), and which could be prevented if the risk factor could be ideally removed from the population. Using the observed frequency of the risk genotype (heterozygous Arg1628Pro) among controls (0.037) and the observed value of OR (2.1) to estimate the risk genotype frequency in the general population and the relative risk, one can estimate a PAR of ~4%. In the most populous nations of the world, the number of PD cases among individuals over age 50 is projected to more than double from the current approximately four to nine million by the year 2030, with the Chinese population contributing more that 50% of these patients [22]. The Gly2385Arg variant is present in ~5% of controls from Taiwan, and its size effect (OR) as a risk factor for PD is around 2.2 in our Taiwanese study [4] and in replication studies [3], allowing an estimate of PAR of ~6% for the Gly2385Arg variant. Therefore, considering the Arg1628Pro and Gly2385Arg variants together, the coding variability in *LRRK2* might account for ~10% of PAR for this disease in China. The identification of Arg1628Pro as a second risk factor for PD constitutes therefore a further important step forward in the dissection of the causes and mechanisms of PD. Additional risk variants in *LRRK2* or different genes might exist in other populations, and further, extensive analyses are warranted.

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Chapter 10 - General Discussion

In the past decade significant progress has been made in the dissection of the molecular biology of Parkinson's disease. An important contribution to this understanding was made by the discovery of genes implicated in familial forms of the disease.

Among these genes, the role of *LRRK2* is remarkable since its mutations have been reported, for the first time and with an unprecedented high frequency, not only in familial, but also in sporadic cases.

The G2019S mutation alone accounts for up to 6% to 16% of familial PD and in 1% to 4% of sporadic cases in some European populations and in many more cases among Arab and Ashkenazy Jewish populations, representing the most frequent genetic cause of PD known so far[1]. The prevalence of this mutation is strongly population specific, since it is particularly frequent in North Africa, where it probably originated, and in the surrounding countries wherein it was spread during the centuries.

Further studies on the whole coding region of this large gene are warranted in order to identify *LRRK2* mutations that might be the counterpart of the G2019S in other populations. This is the case for the R1441G mutation, for example, which is specific of the Basque and Northern Spain regions[2-4].

To make this picture even more complex, the G2019S itself and other *LRRK2* mutations as the R1441C and R1441H have been found in the context of different haplotypes, suggesting independent mutational events[5, 6].

The presence of different mutational hot-spots and a high polymorphic content suggest that the *LRRK2* gene is particularly unstable and prone to mutations. Many intronic and exonic nucleotide changes have been reported so far in PD patients and controls. Some of them

represent non-pathogenic polymorphisms. However, for a consistent group of variants the pathogenic role has still to be elucidated.

Another specific characteristic of *LRRK2* mutations is the reduced penetrance, especially of the common G2019S, which has been estimated to be ~30% in some populations[7-9]. Such a low penetrance is in line with the high prevalence of this mutation among sporadic patients and the rare occurrence in controls (~1%), particularly among the populations with the highest mutation frequencies such as Arabs and Ashkenazi Jews.

An important role is also played by the G2385R variant, which in Chinese increases the risk to develop PD of 2.5 fold[10]. This variant represents the first known genetic risk factor for the common PD form, at least in the Asian population, and so far the most frequent genetic determinant of PD worldwide, especially considering the large and expanding size of the Chinese population.

The discovery of the frequent low-penetrance G2019S mutation and the frequent G2385R risk allele provide for the first time etiological links between the familial and the sporadic forms of PD, and has revolutionized the previous beliefs on the contribution of genetics in the aetiology of this frequent and complex disorder.

However, how *LRRK2* mutations result in the death of dopaminergic neurons selectively in the substantia nigra has yet to be elucidated. Although exciting functional results on the physiologic function and consequence of the pathogenic mutations have been provided so far, the results are still preliminary and must be still considered with caution. The major limitation of the current studies is the specificity of most antibodies in detecting the endogenous protein, especially in its physiologic context. LRRK2 is a large peptide and

likely exists as dimer or even larger complexes. Due to its multiple domain structure, LRRK2 is predicted to have multiple functions. However, biological substrates/targets of LRRK2 are still undiscovered; their identification will certainly help to better understand its normal role, the effect of the mutations and the mechanisms that lead to cell death.

To date, most of the functional studies proposed a gain of function effect of the pathogenic mutations identified in PD cases, by either increasing the kinase activity or reducing the intra-molecular signalling action of the GTPase domain[11-14]. Interestingly, the mechanism whereby mutations in different LRRK2 domains cause clinical PD may be different. Further studies on mammalian models will facilitate our understanding on the pleomorphic consequences of the *LRRK2* mutation in humans.

The most important question remains whether the *LRRK2*-associated PD represents the same entity of the so-called “idiopathic” PD. The evidences provided by the studies conducted so far are in favour of this hypothesis. The clinical phenotype of *LRRK2* mutation carriers is identical to classical PD, including a broad age of disease onset. Neuropathological studies also revealed that LB-positive pathology is the most common pattern associated to pathogenic LRRK2 mutations, particularly the common G2019S mutation. Rarely other clinical findings, as dementia and amyotrophy, and different pathologies, including nigral cell loss without distinctive inclusions, or with inclusions, which are stained only with ubiquitin or only with tau antibodies, might be seen. However, those exceptions, which do not fulfil the current clinical and neuropathological diagnostic criteria for PD, probably recapitulate the heterogeneity of PD itself, which can be a more complex disease that what we thought. From the etiopathological point of view, we might

hypothesize that this variability is due to a cumulative effect of different gene-environment and/or gene–gene interactions. In this scenario, the way to classify PD as clinical and neuropathological entity should be reconsidered. The *LRRK2* associated PD represents an exceptional opportunity to retrace the definition of this disorder, since it give us the opportunity to study patients who share the same genetic etiology.

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SUMMARY

This thesis focuses on the role of the leucine rich repeat kinase 2 (LRRK2) gene in Parkinson's disease (PD). PD is the second most frequent human neurodegenerative disorder after Alzheimer's disease. The etiology of PD remains unknown in most cases, but several genetically-determined forms have been recently identified, which are greatly promoting our understanding of the disease mechanisms. Mutations in the LRRK2 gene were initially identified through positional cloning within the PARK8 locus in families with autosomal dominant inheritance of PD. Subsequent studies, included some of those described in this thesis, have delineated LRRK2 mutations as the most frequent, known cause of familial and sporadic PD.

In *Chapter 1*, the studies dealing with genetic and functional aspects of LRRK2 are reviewed. A detailed overview of the frequency of LRRK2 mutations in familial and sporadic PD cases from different countries is reported. In particular, the prevalence of the G2019S mutation, the most common genetic determinant of PD identified so far, appears to be strongly population specific, since it is particularly frequent in North Africa (where it probably originated) and in the surrounding countries, where it has spread during the centuries. Of note, this mutation has a low penetrance, explaining its considerable prevalence also among sporadic PD cases.

Furthermore, the data concerning the LRRK2 variants reported until now are discussed in order to classify them as definitely pathogenic, possibly pathogenic, or as neutral variants (polymorphisms). An important role is played by two LRRK2 polymorphisms, G2385R and R1628P, since both have been consistently associated with an increased risk to develop PD in the Asian population.

In most patients with LRRK2 mutations, the phenotype is very similar to, if not indistinguishable from the so-called "idiopathic PD". In some cases the associated pathology has been shown to be pleomorphic, raising the question whether a broader pathological spectrum might also be associated to the classic form of the disease. The functional aspects of the human LRRK2 protein are also dealt with. The results of the

overexpression studies of the wild type and mutant protein are in line with a gain of function effect of the mutations identified in PD patients. However, several caveats regarding the experimental procedures are highlighted, suggesting that more physiological models to study the effects of the mutations on the function of the LRRK2 protein are required.

In *Chapter 2 and 4,* extensive analyses of the LRRK2 gene in PD families are described. The sequence of the entire LRRK2 coding region led us to identify the heterozygous G2019S mutation in four of 61 (6·6%) unrelated families with autosomal dominant Parkinson's disease. The families originated from Italy, Portugal, and Brazil, indicating the presence of the mutation in different populations. The heterozygous R1441C mutation was also identified in two Italian families. The associated phenotype was broad, including early and late disease onset. The mutations co-segregated with the disease status within the families and were absent in population-specific control subjects, strongly supporting their pathogenic role.

In *Chapter 3* we report a genetic screening of the recurrent LRRK2 mutations in a large sample of PD patients recruited from a single Italian clinical Center. Among 629 probands, 13 (2.1%) were heterozygous carriers of the G2019S mutation. The mutation frequency was higher among familial (5.1%) than among sporadic probands (0.9%) (p=0.002). There was no difference in the frequency of the G2019S mutation in probands with early vs late disease onset. Among 600 probands, one heterozygous R1441C was also detected. Haplotype analysis in families from five countries suggested that the G2019S mutation originated from a single ancient founder. The G2019S mutation was associated with the classical Parkinson's disease phenotype and a broad range of onset ages (34 to 73 years).

In *Chapter 5 and 9* we report the analyses of two LRRK2 variants for association with disease in a large sample of PD cases and ethnically matched controls of Chinese Han ancestry. We showed for the first time that heterozygosity for the G2385R variant was significantly more frequent among PD patients than controls (nominal p value=0.004, corrected for multiple comparisons=0.012, gender- and age-adjusted odds ratio=2.24, 95% C.I.: 1.29–3.88). The R1628P allele was also more frequent among patients (3.8%) than among controls (1.8%; p = 0.004, OR 2.13, 95% CI 1.29–3.52). Our findings suggest that the LRRK2 G2385R and R1628P are the first identified, functionally relevant variants,

which act as common risk factors for sporadic PD in the large, expanding Chinese population.

In *Chapter 6 and 7* we report the analyses of the LRRK2 recurrent mutations in sporadic and familial PD patients from Sardinia and Portugal, respectively. Among 98 Sardinian PD probands we detected one heterozygous G2019S carrier, indicating the presence of the mutation in this genetically isolated population, although with a lower frequency than in the Iberian, Arab, or Jewish populations. The same mutation was also found in one of 55 Sardinian healthy controls, an 85-year-old man, later shown to have a positive family history of parkinsonism, supporting the concept that this mutation displays incomplete penetrance. Among 138 unrelated PD probands from Portugal, we identified 9 heterozygous G2019S carriers (6.52%) and 1 heterozygous R1441H carrier (0.72%). The G2019S mutation was present in 4 of the 107 sporadic (3.74%) and in 5 of the 31 familial probands (16.1%). Mutations were not found among 101 Portuguese controls. The G2019S mutation was present on a single haplotype and displayed reduced penetrance.

In *Chapter 8*, we report the first neuropathologic study in an Italian PD case carrying a different LRRK2 mutation, I1371V, and showing typical ubiquitin- and α-synuclein–positive brain Lewy body pathology, supporting the concept that the neurodegeneration associated with LRRK2 mutations might be clinically and pathologically indistinguishable from typical PD.

Finally, a general discussion on the importance of the discovery of LRRK2 mutations in PD, especially of the low-penetrance G2019S and the risk allele G2385R, is reported in *Chapter 10*.

SAMENVATTING

Dit proefschrift beschrijft de rol van het gen leucine rich repeat kinase 2 (LRRK2) in de ziekte van Parkinson. De ziekte van Parkinson is, op de ziekte van Alzheimer na, de meest vaak voorkomende neurodegeneratieve aandoening. De etiologie van de ziekte van Parkinson in veel gevallen onbekend, echter onlangs geïdentificeerde genetische varianten hebben het inzicht in het ontstaan van deze ziekte sterk bevorderd. In families waarin de ziekte van Parkinson autosomaal dominant over erft zijn, m.b.v. positioneel kloneren, mutaties gevonden in het LRRK2 gen. Aanvullende studies, waarvan enkele beschreven in dit proefschrift, hebben aangetoond dat mutaties in het LRRK2 gen de meest frequente bekende oorzaak zijn van familiaire en sporadische vormen van de ziekte van Parkinson.

In *Hoofdstuk 1* wordt een overzicht gegeven van genetische en functionele studies betreffende het LRRK2 gen. In detail worden de frequenties van LRRK2 mutaties beschreven in familiaire en sporadische vormen van de ziekte van Parkinson in verschillende landen, in het bijzonder het voorkomen van de G2019S mutatie, tot op heden, de meest voorkomende genetische determinant van de ziekte van Parkinson. Deze mutatie blijkt zeer populatie specifiek en komt frequent voor in Noord Afrika, waar deze mutatie is ontstaan en van waaruit deze zich heeft verspreidt door de eeuwen heen. Hierbij dient vermeldt te worden dat deze mutatie laag penetrant is, hetgeen een verklaring is voor de grote prevalentie bij sporadische gevallen van de ziekte van Parkinson. Aanvullend wordt op basis van tot op heden bekende data beargumenteerd om de verschillende LRRK2 varianten te classificeren of als definitief pathogeen, of als mogelijk pathogeen of als neutrale varianten (polymorfismen). Een bijzonder rol is weggelegd voor de twee LRRK2 varianten G2385R and R1628P, daar beide sterk geassocieerd zijn met een verhoogd risico voor het verkrijgen van de ziekte van Parkinson in de Aziatische populatie.

In de meeste patiënten met LRRK2 mutaties lijkt het ziektebeeld erg op de “idiopatische ziekte van Parkinson”, of is er zelfs niet van te onderscheiden. In enkele gevallen is de geassocieerde pathologie pleomorf, de vraag oproepend of een breder pathologisch spectrum ook geassocieerd kan zijn met klassieke vormen van de ziekte. De functionele

aspecten van het humane LRRK2 eiwit worden ook behandeld. De resultaten van overexpressie studies van normaal en mutant eiwit liggen in lijn met het veranderende functie effect door de mutaties, geïdentificeerd in patiënten met de ziekte van Parkinson. Echter, enkele hiaten in de experimentele procedures worden besproken die aangeven, dat mogelijk meer fysiologische modellen nodig zijn om het effect van mutaties op de functie van het LRRK2 eiwit te bestuderen.

In *Hoofdstuk 2 en 4* worden uitgebreide analyses van het LRRK2 gen in Parkinson families beschreven. Het bepalen in patiënten van de complete volgorde van het LRRK2 coderende gebied heeft geleid tot de identificatie van de heterozygote G2019S mutatie in 4 van de 61 (6.6%) ongerelateerde families met een autosomaal dominante vorm van de ziekte van Parkinson. Deze families zijn afkomstig uit Italië, Portugal en Brazilië, een duidelijke aanwijzing van de aanwezigheid van dezelfde mutatie in verschillende populaties. De R1441C mutatie werd in twee Italiaanse families gevonden. Het geassocieerde fenotype was breed, waarbij de ziekte op zowel vroege als late leeftijd is begonnen. De pathogene rol van deze mutaties wordt ondersteund doordat de mutaties overerven met het ziektebeeld in de families en niet aanwezig zijn in populatie specifieke controle individuen.

In *Hoofdstuk 3* rapporteren wij een genetische screening van voorkomende LRRK2 mutaties in een groot aantal patiënten afkomstig van een klinisch centrum in Italie. Van de 629 probanden bleken 13 (2.1%) heterozygoot drager van de G2019S mutatie te zijn. De mutatie frequentie was hoger onder probanden in PD families (5.1%) dan onder sporadische probanden (0.9%) (p=0.002). Er werd geen verschil gevonden in de frequentie van het voorkomen van de G2019S mutatie in probanden met een vroeg ontstaan van de ziekte vergeleken met een laat ontstaan van de ziekte. Onder 600 probanden werd ook 1 heterozygote drager van de R1441C mutatie gedetecteerd. Haplotype analyse in families met de G2019S mutatie afkomstig uit vijf verschillende landen ondersteunt de suggestie dat de G2019S mutatie afkomstig is van een enkele voorouder. De G2019S mutatie is geassocieerd met het klassieke Parkinson fenotype met een breed scala van leeftijden waarop de ziekte ontstond (34 tot 73 jaar).

In *Hoofdstuk 5* en *9* rapporteren wij de analyse van twee LRRK2 varianten in een groot aantal Parkinson patiënten met etnisch gelijke gezonde controles van Han Chinese afkomst. Wij toonden voor het eerst aan dat heterozygositeit voor de G2385R variant significant

vaker voor kwam onder Parkinson patiënten dan onder gezonde controles. (nominale p waarde=0.004, gecorrigeerd voor veelvoudige vergelijkingen= 0.012, herkomst en leeftijd aangepaste odds ratio=2.24, 95% C.I.: 1.29–3.88). Het R1628P allel werd ook frequenter gevonden in patiënten (3.8%) dan in controles (1.8%; p = 0.004, OR 2.13, 95% CI 1.29–3.52). Onze bevindingen suggereren dat de G2385R en R1628P varianten in LRRK2 de eerste geïdentificeerde, functioneel relevante varianten zijn die als risico factoren kunnen worden aangewezen in de grote, steeds verder uitbreidende Chinese populatie.

In *Hoofdstuk 6* en *7* beschrijven wij de analyse van voorkomende LRRK2 mutaties in sporadische en familiaire Parkinson patiënten uit respectievelijk Sardinië en Portugal. Onder de 98 Sardinische probanden detecteerden wij een enkele heterozygote G2019S drager, hetgeen aantoont dat deze mutatie ook aanwezig is in deze genetisch geïsoleerde populatie, echter met een lagere frequentie dan in de Iberische, Arabische of joodse populatie. Dezelfde mutatie werd ook aangetroffen in een 1 van de 55 Sardinische gezonde controles, een 85 jarige gezonde man, met wat later bleek een positieve familie geschiedenis met de ziekte van Parkinson. Deze bevinding ondersteunt het concept dat deze mutatie onderhevig is aan incomplete penetrantie. Onder 138 ongerelateerde probanden met de ziekte van Parkinson uit Portugal hebben wij 9 heterozygote G2019S dragers geïdentificeerd (6.52%) and 1 heterozygote R1441H drager (0.72%). De G2019S mutatie was aanwezig in 4 van de 107 sporadische (3.74%) en in 5 van de 31 familiaire probanden (16.1%). Mutaties werden niet aangetroffen onder 101 Portugese controles. De G2019S mutatie was ook hier aanwezig op 1 enkel haplotype en vertoonde ook gereduceerde penetrantie.

In *Hoofdstuk 8* rapporteren wij de neuropathologische studie in een Italiaanse Parkinson patiënt, drager van een andere LRRK2 mutatie, I1371V. Deze patiënt laat een typische ubiquitine en α-synucleine–positieve hersen Lewy body pathologie zien. Dit ondersteunt het concept dat de neurodegeneratie geassocieerd met LRRK2 mutaties klinisch en pathologisch niet is te onderscheiden van wat wordt waargenomen bij typische Parkinson patiënten.

Ten slotte wordt in *Hoofdstuk 10* een algemene discussie beschreven over het belang van de ontdekking van LRRK2 mutaties bij de ziekte van Parkinson, in het bijzonder de laag penetrante G2019S mutatie en het risico allel G2385R.

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PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Alessio Di Fonzo Erasmus MC Department: Clinical Genetics	PhD period: 2004-2009 Promotor(s): Ben Oostra Supervisor: Vincenzo Bonifati	
1. PhD training		
	Year	**Workload (Hours/ECTS)**
Professional academic skills		
- Residency and intern training in clinical neurology, Department of Neurology, University of Milan, Italy	2003-2007	
- Board certified registration as Clinical Neurologist	2008	
Research skills		
- Working in the laboratory of the Department of Clinical Genetics, Erasmus MC Rotterdam	2005-2009	
In-depth courses (e.g. Research school, Medical Training)		
- Genetic linkage Analysis Course at the Erasmus MC Rotterdam	2005`	32
Presentations		
- WPC Junior Scholar Award	2006	128
- Genetics of Parkinson's disease "Il Galliera e le sfide della medicina: la Genetica" , Genova, Italy.	2006	24
International conferences		
- 16th International Congress on Parkinson's Disease and Related Disorders, Berlin, Germany (poster contribution)	2005	32
- 1st World Parkinson Congress WPC 2006**.** Washington D.C., USA (poster contribution)	2006	32
- 10th International Congress of Parkinson's Disease and Movement Disorders, Kyoto**,** Japan (poster contribution)	2006	32
- European Human Genetics Conference 2006 Amsterdam, The Netherlands (poster contribution)	2006	32
- 11th International Congress of Parkinson's Disease and Movement Disorders, Istanbul, Turkey (poster contribution)	2007	32
- 12th International Congress of Parkinson's Disease and Related Disorders, Amsterdam, The Netherlands.	2008	32
Seminars and workshops		
- Update in genetics of Parkinson's disease, Paris, France	2005	16
2. Teaching activities		
	Year	**Workload (Hours/ECTS)**
Supervising practicals and excursions		
- Supervising practical "Virtual gene Cloning" at Erasmus University Rotterdam	2006-2009	16

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1. Del Bo R, Bordoni A, Sciacco M, **Di Fonzo** A, Galbiati S, Crimi M, Bresolin N, Comi GP. Remarkable infidelity of polymerase gammaA associated with mutations in POLG1 exonuclease domain.
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