Autosomal recessive, early-onset Parkinson's disease

Vincenzo Bonifati

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1 Introduction

Chapter 1.1

Genetics of the Mendelian forms, and genome-wide linkage screens in classical forms of Parkinson's disease

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, with a prevalence of 1-2% in the population aged ≥ 65 years.[1] The disease is clinically defined by the presence of parkinsonism (the combination of akinesia, resting tremor, and muscular rigidity), and a good response to dopaminergic therapy. These features are associated at pathological level with neuronal loss and gliosis, mainly in the *substantia nigra pars compacta* but also in other brain areas, and formation of cytoplasmic inclusions called Lewy bodies (LB) and Lewy neurites in the surviving neurons.[2] The role of genetics versus environment in the etiology of PD has been a matter of debate for more than a century, with alternating fortunes.

Genetics versus Environment

Strong support for the "environmental" theories came from the occurrence of post-encephalitic parkinsonism after the pandemia of influenza in the early 1900s, and after the more recent discovery of permanent parkinsonism in humans caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a substance similar to widely used pesticides.[3] However, the disease has survived the era of the post-encephalitic cohort, and despite the epidemiological association of PD with environmental factors such as rural living and occupational exposure to pesticides, a putative MPTP-like toxin causing PD has not been identified yet.

Familial aggregation of PD has been noted for long,[4, 5] re-emerging consistently in the subsequent surveys[6, 7] until the current time,[8, 9] and supporting a role for genetic factors.

Studies in PD twins, however, have produced conflicting results. According to the largest of such studies,[10] the pattern of concordance in monozygotic versus dizygotic twins differs critically on the basis of PD onset age, in keeping with a prominent role for genetic factors in early onset cases (before 50 years of age, according to that study), but a negligible role in late-onset PD, which represent the vast majority of cases. However, the sample size was still relatively small (less than 200 twins with PD), and the study was based on a cross-sectional, clinical survey.[10] The period elapsing before a co-twin develops clinically overt PD, becoming concordant, can be as long as 20 years,[11] indicating that a longer follow-up is important in studying PD twins. Moreover, when positron emission tomography was used to assess the integrity of the nigrostriatal dopaminergic system, the concordance between monozygotic twins appeared significantly higher than that of dizygotic twins, once again supporting the role of genetic factors.[12]

PD appears today as a sporadic disorder in most patients. However, in a significant percentage of cases (10-15% in most studies), the disease runs in families without a clearcut Mendelian pattern of inheritance. Recent segregation analyses found evidence for a major gene with reduced, age-related penetrance.[8, 13] However, these studies might be biased toward segregation patterns consistent with major genes depending on the ascertainment strategy, and the observed scenario also supports the contention of common PD being a complex trait determined by several genetic

as well as non-genetic factors. More rarely, the PD phenotype is transmitted as a Mendelian trait, with either autosomal dominant or recessive inheritance.
In the last few years, family-based linkage analysis and positional cloning has led to the identification of loci and genes for rare monogenic forms (table 1),[14, 15] and more recently, of loci harbouring susceptibility genes for common, non-Mendelian PD forms of late-onset (table 1).[16, 17] Although the monogenic forms so far identified explain a very small fraction of PD cases at the population level, they are proving to be extremely useful in promoting the understanding of the molecular events and the metabolic pathways involved in the common forms of PD as well.
In the light of these findings, the old debate of whether PD is inherited or acquired is gradually loosing ground, and more plausible questions are today:

- is PD a single disease with multiple etiologies, or a group of distinct diseases with similar clinical and/or pathological endpoints?
- how many genes do contribute to determine the susceptibility to common, late-onset PD?
- do different genes play a role in different subgroups of common PD?

Thanks to the knowledge resulting from the Human Genome project[18, 19] and the technological revolution in molecular genetics, it is possible to translate these questions into feasible research programs.
In this chapter, the growing literature about loci and genes for monogenic forms of PD will be reviewed. Moreover, the results of genome-wide screens addressing directly the problem of the genetic bases of the common, non-Mendelian forms of the disease will be discussed.

Table 1.

Current catalogue of genes and loci for PD*

Locus	OMIM	Map position	Gene	Inheritance	Pathology	Ref.
PARK1	#168601	4q21-q23	***α-synuclein***	dominant, high penetrance	LB +	25
PARK2	#600116	6q25-q27	***parkin***	recessive	mostly LB negative	142
PARK3	602404	2p13	unknown	dominant -incomplete penetrance	LB +	61
PARK4	605543	4p15	unknown	dominant, high penetrance	LB +	72
PARK5	191342	4p14	***UCH-L1***	dominant	unknown	79
PARK6	605909	1p36-p35	unknown	recessive	unknown	129
PARK7	#606324	1p36	***DJ-1***	recessive	unknown	130
PARK8	607060	12p11-q13	unknown	dominant - incomplete penetrance	LB negative	94
PARK9	606693	1p36	unknown	recessive	unknown	131
PARK10	606852	1p32	unknown	non-Mendelian	unknown	16
PARK11	607688	2q36-q37	unknown	non-Mendelian	unknown	17
Pending	601828	2q22-q23	***NR4A2 (NURR1)***	dominant	unknown	107

* Named according to HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature)

THE AUTOSOMAL DOMINANT FORMS OF PD

PARK1/*α-synuclein*

A first monogenic PD form was mapped to chromosome 4q21-q23 in 1996, in a large Italian-American family known as the "Contursi kindred" (named after the village of

origin in Southern Italy), with autosomal dominant PD and Lewy body pathology.[20-22] The identification of PARK1 heralded the beginning of the era of genome-wide linkage approaches to the genetic bases of PD. Soon after this first form was localised, the etiological heterogeneity of PD became evident, as linkage to PARK1 was excluded in most autosomal dominant families examined.[23, 24]

One year later, a missense mutation, A53T, was identified in the gene encoding α-synuclein, which co-segregated with PD in the Contursi family and three unrelated, smaller Greek kindreds.[25] The geographical proximity and historical ties between the Southern part of Italy and Greece suggested the possibility of a founder effect for the A53T mutation, which was later supported by haplotype analysis.[26] The A53T mutation has been found in at least fifteen more families, all of Greek ancestry,[27-30] and a second mutation, A30P, was identified in a single German family.[31] However, extensive mutational analysis in large series of sporadic and familial PD cases were negative, in keeping with results from earlier linkage studies, and delineating mutations in *α-synuclein* as a very rare cause of PD.[32-39]

Nevertheless, the discovery of *α-synuclein* mutations in PARK1 was a major breakthrough for the whole PD field. Not only was it the first identified genetic cause of the disease, but especially, it allowed researchers to identify the α-synuclein protein as one of the major components of Lewy bodies in classical PD[40] and Lewy body dementia,[41] and of the glial cytoplasmic inclusions in multiple system atrophy,[42] a group of neurodegenerative disorders also collectively termed "alpha-synucleinopathies".[43]

In recent years, several transgenic models have been generated in rodents, flies, and worms expressing the human wild-type or mutant *α-synuclein* gene carrying the A53T or A30P mutation.[44-50] These models show varying degrees of biochemical, pathological and behavioural abnormalities reminiscent of PD, and they provide further support to the contention that derangements in the α-synuclein pathways are a primary event in the pathogenesis of PD. Although mutations in its gene are very rare, α-synuclein protein plays therefore a central role in PD.

Polymorphisms in the promoter and in a regulatory element 10 kb upstream of the gene influence *α-synuclein* expression in cell culture systems. Alleles conferring increased expression levels could conceivably act as risk factors for PD.[51-53] However, most of the studies published so far found no association between these polymorphisms and PD,[54-57] while the finding of *α-synuclein* haplotypes associated with PD awaits replication in independent datasets.[58]

In comparison with classical PD, the clinical phenotype associated with mutations in *α-synuclein* is characterized by an earlier onset (on average in the mid forties), and reduced prevalence of tremor.[21, 28-30] As tremor is generally less common in early-onset PD, a reduced prevalence of tremor in PARK1 might merely reflect the earlier onset age instead of being a consequence of the specific etiology. The occurrence of cases with rapid progression, dementia, myoclonus, and severe autonomic dysfunctions indicates that PARK1 can present as a more widespread neurodegeneration than classical PD,[21, 28] as confirmed by recent autopsy studies.[59] However, in recent analyses of Greek patients carrying the A53T mutation, clinical features and disease course appear similar to classical PD,[29, 30] and the phenotype is also similar to common PD in the German family with the A30P mutation.[60] The phenotypical spectrum associated with mutations in *α-synuclein* appears therefore broad, and a wide variability of onset ages is observed in the same family.[21, 27, 60] This suggests that other genetic, as well as non-genetic, factors are most likely playing a role as disease modifiers.

PARK3

A second locus for autosomal dominant PD was mapped to chromosome 2p13 in a genome-wide scan using large kindreds of European ancestry.[61] Four of the six families examined yielded support for linkage. The phenotypical spectrum is wide, encompassing typical PD of late onset (average onset age of 59 years) and Lewy body pathology, but also cases with dementia in addition to parkinsonism, and presence of neurofibrillary tangles and senile plaques in addition to Lewy body pathology.[62-64] A 2p13 haplotype is shared by affected individuals in two of the linked families, which originate from Northern Germany and Southern Denmark, suggesting the presence of a founder mutation. Moreover, on the basis of haplotype analysis, a low penetrance (40%) was estimated for the mutation.[61] However, the "founder" haplotype was not carried by other PD cases collected from the regions of origin of the families initially linked to PARK3,[65] and linkage to the 2p13 region has not been replicated in other large kindreds.

Fine mapping studies refined the PARK3 critical region to 2.5 Mb on the physical map, but mutational screening of the genes contained in the region has been negative so far.[66-68] More recently, a genome-wide scan of affected sib-pairs has analysed the onset age of PD (instead of the disease status) as the phenotype of interest.[69] In this study, suggestive evidence for linkage was detected to the PARK3 region, confirming that an important genetic determinant of PD risk and/or modifier of disease onset age might reside in this region.

PARK4

A large kindred of Iowan origin with autosomal dominant parkinsonism-dementia complex and Lewy body pathology could be ascertained by the finding of a common ancestor pair for two independently reported families.[70, 71] A genome-wide search for linkage yielded suggestive evidence for the gene defect being located in a region on chromosome 4p15.[72] The average onset age is 34 years, and average disease duration to death is ~8 years. In addition to parkinsonism, affected individuals also show severe autonomic dysfunctions, body weight loss, and dementia. Pathology shows widespread Lewy bodies, and vacuolisation of brain tissue.[70, 71, 73] The clinical and pathological spectrum in this family is closer to Lewy-body dementia than to classical PD. In addition to patients with the severe clinical form, some carriers of the linked haplotype presented with postural tremor only, suggesting that the genetic defect might be associated with a very wide range of phenotypes including forms similar to essential tremor. This is intriguing in the light of the frequent familial aggregation of PD and essential tremor.[74] However, a cautionary view seems warranted, as the statistical threshold to declare significant linkage at genome-wide level was not reached for PARK4 in the initial report, nor was linkage to the 4p15 region replicated in other studies since then.

PARK5/ubiquitin C-terminal hydrolase-L1 (UCH-L1)

The *UCH-L1 g*ene, located at chromosome 4p14, encodes a 26 kD protein which catalyses the hydrolysis of ubiquityl-peptide conjugates *in vitro*.[75] UCH-L1 was therefore thought to be important for the recycling of free ubiquitin molecules from the ubiquityl-peptides fragments produced by the proteasome. However, recent findings *in vivo* suggest that the function of UCH-L1 is rather to stabilize the neuronal mono-ubiquitin levels by binding mono-ubiquitin and preventing its degradation.[76]

UCH-L1 is abundant in neurons representing 1-2 % of brain proteins, and it has been found in Lewy bodies as well.[77] Moreover, a mouse mutant with a homozygous

intragenic deletion of the *UCH-L1* gene (the gracile axonal dystrophy mouse) develops progressive neuronal degeneration of sensory and motor pathways with accumulation of ubiquitylated inclusions.[78] For all these reasons, *UCH-L1* is considered a candidate gene for PD. In the Iowan family with PD linked to a nearby chromosomal region (4p15, PARK4), the *UCH-L1* gene could be excluded by recombination analysis.[72] Linkage to the region containing the *UCH-L1* gene (4p14) has not emerged in other genome scans for PD.

A single missense mutation (I93M) was found in two German sibs with classical PD and a positive family history suggesting autosomal dominant inheritance and incomplete penetrance.[79] Pathological studies have not been reported in this family. The I93M mutation is associated with a marked decrease of the hydrolase activity of the protein, and could therefore cause disease by haploinsufficiency.[79] However, subsequent screening of this gene for this and other mutations has consistently been negative, suggesting that I93M is either an extremely rare cause of PD or even a neutral polymorphism.[80-83] However, a different polymorphism (S18Y) in this gene is inversely associated with PD in several,[84-87] but not all,[88-90] studies. The Y18 allele could exert a protective role, which seems stronger in early onset cases and for the homozygous carriers.[91, 92]

Last, the report that UCH-L1 also displays a dimerization-dependent ubiquitin ligase activity for α-synuclein *in vitro* lends further support to the contention that UCH-L1 is implicated in the pathogenesis of PD.[93] The Y18 variant exhibits reduced ubiquitin ligase activity for α-synuclein *in vitro*, and it has been proposed that it could protect from PD by altering the balance between the ubiquitin ligase and hydrolase activity of UCH-L1.[93] Another hypothesis, which remains to be tested, is that the Y18 variant influences the recently discovered ubiquitin-stabilising activity of UCH-L1.

PARK8

This novel locus for autosomal dominant PD was identified in a large pedigree, named the "Sagamihara family" from the region of origin in Japan.[94] Clinical features in affected individuals of the kindred are reported to resemble very closely classical PD, with an average disease onset at 51 ± 6 years.[95] Yet, a pattern of "pure nigral degeneration" without Lewy bodies was found at autopsy in four PD patients examined. Moreover, one carrier of the disease-linked haplotype developed pathologically-confirmed striatonigral degeneration, an atypical form of parkinsonism which is known to be associated with α-synuclein-positive brain pathology.[94]

In this family, a genome-wide scan yielded significant evidence for linkage of PD to the centromeric region of chromosome 12. The haplotype analysis suggests an incomplete penetrance of the mutation. This is of interest because an incomplete, age-related penetrance could have masked a Mendelian pattern in other smaller PD families. Interestingly, linkage to the same region was found in a genome scan for Alzheimer's disease (AD), the locus being termed AD5, but this linkage was mostly supported by the subset of AD families containing at least one affected individual with Lewy body pathology.[96] Lastly, within a genome scan in a set of 22 families with *parkin* gene mutations, the centromeric region of chromosome 12 yielded the strongest evidence of linkage.[97] Taken together, these findings suggest that a single locus in this region might control susceptibility to a wide range of clinical and pathological phenotypes (with or without Lewy bodies). Confirmation of linkage to PARK8 in other PD families has not been reported yet, and the defective gene at this locus remains unknown.

The *NR4A2* gene

The *NR4A2* (*NURR1*) gene, located at 2q22-q23, encodes a member of the nuclear receptor superfamily of transcription factors, which is highly expressed in fetal and adult dopaminergic (DA) neurons.[98, 99] Previous studies showed that *NR4A2* is important for the genesis and maintenance of dopaminergic neurons. In *NR4A2* knock-out mice agenesis of DA neurons is observed.[100, 101] The *NR4A2* hemyzygous mice develop a normal set of DA neurons and normal dopamine brain levels, but they show increased susceptibility to the dopaminergic neurotoxin MPTP,[102] which is also a cause of toxic parkinsonism in humans.[3]

NR4A2 has been considered an excellent candidate for PD for long. However, linkage to its chromosomal region has not been found in genome scans, and the *NR4A2* locus was excluded by linkage and haplotype analysis in families with PD compatible with a recessive model of inheritance.[103]

Evidence for association between a polymorphic variant in intron 6 (IVS6+18 insG) and PD has been reported in two studies.[104, 105] However, these were based on small samples, and the association was detected only in homozygous,[104] or only in heterozygous carriers[105] in the different datasets. The interpretation and relevance of these findings remain controversial, also because no data were provided about the functional effects of the IVS6+18 insG polymorphism. A third study did not find association between PD and three polymorphisms in the *NR4A2* gene, including IVS6+18 insG.[106]

More recently, two heterozygous mutations (-291Tdel and –245T→G) in the non-coding region (exon 1) of *NR4A2* were identified in 10 out of 107 patients with familial PD.[107] In most of these ten cases, family history for PD was present in at least two generations, suggesting a dominant pattern of inheritance. Onset age (54 ± 7 years) and other clinical features were those of typical PD in patients carrying the mutation, whereas the associated neuropathology remains unknown. The mutations showed complete co-segregation with the disease in families, and absence from control individuals. Haplotype analysis suggested a founder effect for the more frequent mutation (-291Tdel) in families of German ancestry. The mutations influence the expression of the gene, and are associated with dramatic decreases of the *NR4A2* mRNA levels in both transfected cell cultures and in lymphocytes from patients. Moreover, the mutations are associated with reduced expression of tyrosine hydroxylase, one of the genes targeted by NR4A2.[107] In transfected cell lines and patients lymphocytes, heterozygous mutations are associated with more than 50% reduction in *NR4A2* mRNA levels, suggesting a dominant negative effect for these mutations.[107] While this would fit with the pattern of inheritance observed in these PD families, the mechanism by which the mutation in one allele of *NR4A2* could affect the expression of the other allele remains unknown. One can speculate that a motif in the mRNA might interfere with the other allele, or it might interact with some regulatory DNA- or RNA-binding protein in a dominant negative fashion. The discovery of mutations in *NR4A2* associated with familial PD is an important finding. However, evaluation of this gene in other cohorts of PD families is warranted in order to accurately assess the frequency of these as well as other types of mutations in this gene. The screening of exon 1 of *NR4A2* in independent datasets has been negative so far, suggesting that mutations is this gene, at least those localised in exon 1, are a very rare cause of PD.[108-110]

Synphilin-1 and susceptibility to PD

Synphilin-1 is a 919 amino acid polypeptide with different domains involved in protein-protein interactions, which was first identified in a yeast two-hybrid screen as an interactor of α-synuclein.[111] Like α-synuclein, synphilin-1 is enriched in presynaptic

terminals, and associates with synaptic vesicles, suggesting interaction with α-synuclein.[112] However, the exact function of synphilin-1 remains unknown.
Synphilin-1 is encoded by the *SNCAIP* gene, located on chromosome 5q23, and is expressed in many tissues and brain areas.[113] Immunoreactivity for synphilin-1 is present in Lewy bodies in PD and Lewy body dementia, and in glial cytoplasmic inclusions in MSA, suggesting that deposition of synphilin-1 is a feature of synucleinopathies.[114-116] Overexpression of synphilin-1 alone or in combination with parkin or α-synuclein yields ubiquitin positive, cytoplasmic inclusions suggesting a role for synphilin-1 in promoting protein aggregation.[111, 117, 118]
The observation that parkin and dorfin, two ubiquitin ligases also found in Lewy bodies, interact with, and ubiquitylate synphilin-1, suggests that these ligases and α-synuclein could be involved in a common pathogenic mechanism through the interaction with synphilin-1.[117, 118]
Suggestive evidence for linkage to the chromosomal region containing the *SNCAIP* gene (5q) has been detected in different genome screens of late-onset PD,[16, 97, 119] making the *SNCAIP* locus a candidate gene for susceptibility to classical PD. Earlier mutation screening of the *SNCAIP* gene in PD failed to identify any mutation.[120, 121] However, a heterozygous missense mutation (R621C) has recently been identified in two sporadic German patients with late-onset PD.[122] The mutation was absent in a large number of control chromosomes, and it appeared to be functionally important because in transfected cells it reduced the formation of inclusions in comparison to wild type synphilin-1, and increased the susceptibility to staurosporine-induced cell death. These findings confirm that synphilin-1 promotes the formation of inclusions, and suggest that it protects cells against stress. Haploinsufficiency of synphilin-1 could therefore be a risk factor for neurodegeneration, but further work is needed to clarify whether genetic variation, including alterations in gene copy number, in *SNCAIP* influences susceptibility to PD.

Final remark
It is likely that other autosomal dominant forms of PD will be identified in the future as most of the previous loci have been excluded in additional pedigrees.[123-126] We have recently obtained suggestive evidence for linkage to chromosome 19 in a Cuban family segregating clinically typical PD of late-onset.[127] The pathology of this form remains unexplored.

THE AUTOSOMAL RECESSIVE FORMS OF PD

Autosomal recessive forms of PD are increasingly recognized, especially among cases with early onset (AREP). In Europe, half of the AREP families and almost 20% of isolated cases with onset before age of 45, are explained by mutations in the *parkin* gene (PARK2 locus).[128] The fact that in the remaining AREP families and isolated cases *parkin* mutations are not found, clearly suggests the existence of other genes involved. In fact, three independent studies have detected linkage to close regions on the short arm of chromosome 1 in AREP families, the new loci being termed PARK6, PARK7, and PARK9 (figure 1).[129-131] Yet, linkage exclusion data suggest that additional AREP loci remain to be mapped. The responsible gene at PARK7 locus, *DJ-1*, has been recently identified.[132] The focus on the short arm of chromosome 1 has been further highlighted by the detection of linkage for common PD in the Icelandic population in a fourth,

more centromeric region (1p32, figure 1).[16] This region was also detected in another genome search for determinants of the PD onset age.[133]

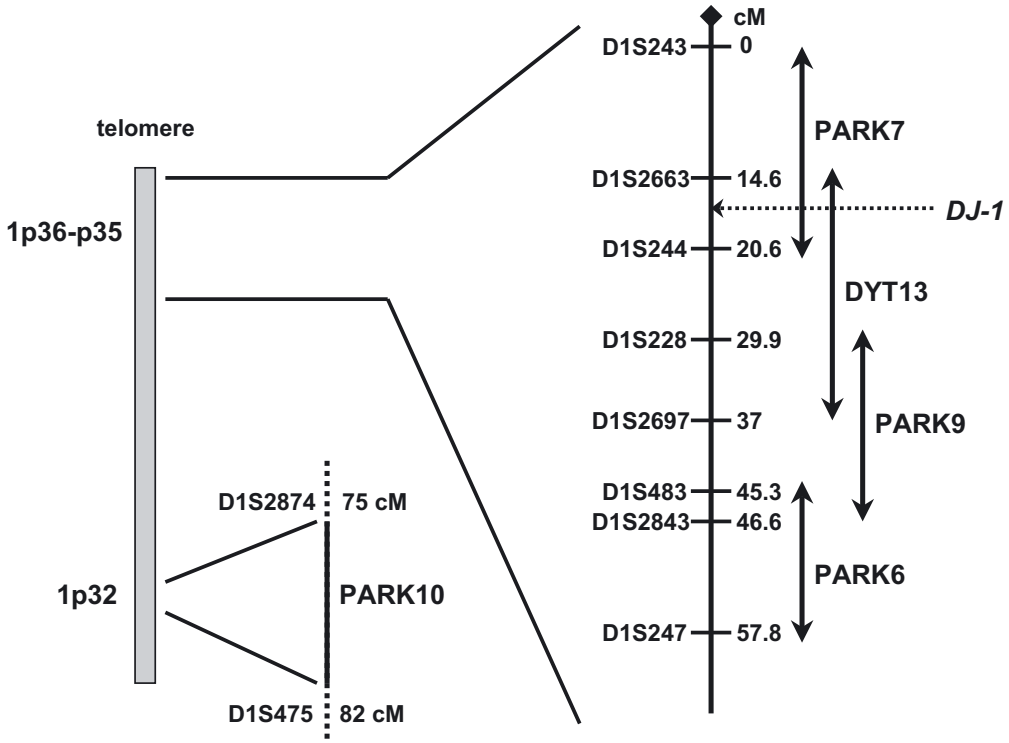

Figure 1. *Chromosome 1p map illustrating the position of the novel PD-related loci.*

PARK2/*parkin*

An autosomal recessive form of juvenile parkinsonism (AR-JP) was first described in Japanese families,[134-136] and a locus for AR-JP was mapped in 1997 to the long arm of chromosome 6.[137] Subsequent studies confirmed the linkage to the same region in families from several ethnic groups, refining the critical interval and delineating PARK2 as an important locus for early-onset parkinsonism worldwide.[138-140]

The defective gene of the PARK2 locus was identified, by positional cloning, within a microdeletion observed in one of the linked families and termed "*parkin*".[141, 142] The gene extends over more than 1 Mb on chromosome 6. It contains 12 exons, and is expressed ubiquitously in the brain, as well as in many extra-cerebral tissues. A survey of human cDNA libraries[142] and RT-PCR amplification of brain mRNA[143] suggest that a splice variant is also expressed that lacks exon 5, and encodes an in-frame protein lacking 28 amino acids. A different variant lacking exons 3-5, found in RT-PCR analysis from human leukocytes, remains unconfirmed.[143] Last, evidence of a splice variant encoding a truncated protein has been found for the mouse *parkin* homolog.[144]

Homozygous exon deletions were initially reported in patients from different Japanese families with AR-JP.[142] A large number of mutations in the *parkin* gene have since been identified in families from different populations.[83, 128, 145-191] In addition to point mutations, large genomic rearrangements (leading to exon deletions and multiplications) are

frequently detected, in homozygous or heterozygous state, indicating the importance of gene dosage techniques for a sensitive screening of *parkin*.[128, 154, 174] Data from large and comprehensive studies indicate that mutations in this gene are responsible for about half of the PD families compatible with recessive inheritance and onset before age of 45 years, as well as about 15-20 % of sporadic cases with onset before 45 (chapter 2.1).[169, 174] Among the sporadic cases, most of the mutations are found in those with a very early-onset (before age of 30) (chapter 2.1)[169], whereas after that age of onset, mutations in this gene are a much rarer finding. In a few families a second mutation has not been found even after gene dosage analysis or sequencing of the promoter region, suggesting that other mutations (likely in intronic or regulatory regions of the gene) still escape detection, or that some of the mutations might be sufficient to cause disease in a heterozygous state.[128, 165]

The *parkin* gene promoter has been characterized,[192, 193] and ten single nucleotide polymorphisms (SNPs) have been identified.[165, 194] One of these variants (-258 T/G) is located in the core promoter region and the "G" allele is associated with a decreased gene expression in cell culture assays.[195] The frequency of the "G" allele was increased among PD cases with borderline significance, suggesting that this variant can increase the risk of late onset PD.

Reports of parkinsonism and heterozygous *parkin* mutation in multiple generations also suggests that parkin-related disease might sometimes be dominantly inherited.[151, 155, 171] PET studies in asymptomatic carriers of heterozygous *parkin* mutations have revealed slight abnormalities of the nigrostriatal pathways, supporting the contention that single heterozygous mutations in parkin might be sufficient to cause subclinical or even overt PD, by haploinsufficiency of dominant negative mechanism.[196, 197] However, pseudo-dominant inheritance could also explain the disease in multigeneration families. This phenomenon has already been documented in different Japanese and Italian families (chapters 2.2, 2.3),[152, 198] once again indicating that *parkin* gene mutations might be frequent in some populations.

Haplotype analyses of the recurrent *parkin* mutations suggest the occurrence of a founder effect for some of the point mutations;[199, 200] on the contrary, the exon rearrangements seem to have arisen from independent mutational events.[199] Several polymorphisms have been identified in the *parkin* gene, including missense changes.[146, 149, 201] The allelic frequencies show wide variations between ethnic groups, with the Ser167Asn and Val380Leu polymorphisms being most frequent among Orientals and Caucasians, respectively.[149, 201] Other variants are virtually population-specific, like the Arg366Trp in Orientals and the Asp394Asn in Caucasians.[149, 201] The results of allelic association studies of these polymorphisms in classical PD have been rather conflicting, but none of the analysed variants seems to have strong effects on the risk of classical PD.[194, 201-209]

Only a few brains from patients with *parkin* mutations have been examined so far.[135, 155, 175, 210-214] The commonly observed pathological features were neuronal loss and gliosis in the *substantia nigra pars compacta* and *locus coeruleus* with deposition of extraneuronal melanin in the *substantia nigra*. In single cases, the neurodegeneration was more widespread, including the *substantia nigra pars reticulata*,[175, 211] and the spinocerebellar system.[213] Lewy bodies were absent in all but one case, who was a compound heterozygous carrier of a deletion of 40 bases within exon 3 and the missense mutation R275W.[155] The last mutation is probably associated with residual protein function.[117] Interestingly, tau-positive inclusions were found in neurons and astrocytes in some of these brains,[210, 211, 213] and recently, clinically and pathologically

confirmed progressive supranuclear palsy, another tauopathy, has been reported in a carrier of a single heterozygous *parkin* mutation.[214] If these represent coincidental findings, or whether tau pathology is in the spectrum of parkin disease remains unclear. It would be interesting to screen the *parkin* gene in tauopathies, and to explore a possible interaction between tau and parkin proteins. Perhaps the first description of tau pathology in the "parkin disease" dates back to 1989, when Rajput and colleagues reported four cases of early-onset levodopa-responsive parkinsonism, with very long disease course, absence of Lewy bodies but presence of neurofibrillary tangles in *substantia nigra* and *locus coeruleus*.[215] The clinical and pathological features of these cases are strikingly similar to those of the patients with *parkin* mutations, but genetic studies on those brains have not been published yet.

Several studies with fluorodopa and positron emission tomography (PET) in parkin disease have been based on small number of cases,[166, 185, 196, 216, 217] and one report on a larger series has been published recently.[218] These studies confirm the presence of presynaptic dopaminergic dysfunction, as in common PD. Moreover, the PET abnormalities in parkin patients display some degrees of left-right asymmetry, although this might be less pronounced than in classical PD, and a clear rostro-caudal gradient (the putamen being more severely affected than caudate), as observed in classical PD.[218] Interestingly, the severity of the PET abnormalities does not correlate with the clinical disability,[218] and the PET abnormalities appear greater than the ones expected on the basis of clinical state. Last, a mild but significant PET abnormality was observed in asymptomatic heterozygous carriers, suggesting the presence of subclinical disease.[196, 197, 219] PET studies also confirmed that the progression in parkin patients was slower than in classical PD.[197] Results using the raclopride tracer suggest that the postsynaptic dopamine receptors might also be abnormal in parkin patients,[196] but this remains to be confirmed in larger studies.

The protein encoded by the *parkin* gene possesses 465 amino acids and it contains an N-terminal domain homologous to ubiquitin (Ub-L), and two RING finger domains separated by an IBR (In-Between-RING) domain in the C-terminal part.[142, 220] The Ub-L domain of parkin adopts the same fold of ubiquitin,[221, 222] and it shares a motif with other Ub-L-containing proteins, which are known to interact with the proteasome.[223] This suggests that parkin might also interact with the proteasome, providing a link between this last structure and the ubiquitylation machinery. The parkin protein has ubiquitin-ligase activity, which is impaired by several mutations found in AREP patients.[224-226]
Different putative substrates for parkin-mediated ubiquitylation have recently been described, but their role *in vivo* remains unclear.[117, 226-230] These substrates are expected to accumulate in neurons of patients with parkin-disease as a result of lack of ubiquitin-ligase activity. This, in turn, might cause neurodegeneration. The discovery of an interaction between parkin and a brain-specific, glycosylated form of α-synuclein, as well as between parkin and synphilin-1 have important implication for Parkinson's disease in general, since these interactions might underlie the deposition of ubiquitylated α-synuclein in Lewy bodies.[117, 228] To the extent that parkin activity is essential for Lewy body formation, its defect can explain the absence of Lewy bodies in the brain of patients with *parkin* mutations.

The clinical phenotype associated with *parkin* mutations is characterized by early onset parkinsonism, good response to levodopa and a benign, slow course. The average onset age was in the early 30s in European patients, but late-onset cases have been

described up to 70 years of age. Motor fluctuations and levodopa-induced dyskinesias are present, whereas marked cognitive or vegetative disturbances seem rare.
The age of disease onset is the most important predictor of *parkin* mutations in that the earlier the onset, the more frequent the mutations (chapters 2.1, 2.4).[169] There are no specific clinical features that identify patients with *parkin* gene mutations from other early-onset forms (chapters 2.1, 2.4).[169] However, symmetrical onset, dystonia at onset and hyperreflexia, slower progression of the disease, and a tendency towards a greater response to levodopa might be more frequent among patients with *parkin* mutations (chapter 2.4). The phenotypical spectrum overlaps with classical PD for late-onset cases (chapter 2.4), and with dopa-responsive dystonia for early-onset ones.[178] Rare atypical presentations have also been described, and a wide variability in onset ages and phenotype is observed even within the same families (chapter 2.4).[171, 184-185, 187, 200, 213] In conclusion, mutations in the *parkin* gene represent a frequent cause of familial early-onset and isolated juvenile parkinsonism. They must therefore be considered in the diagnostic work-up of early onset PD.

PARK7/*DJ-1*
The short arm of chromosome 1 is emerging as a hot spot for PD-related genes (figure 1), as three novel loci for autosomal recessive forms of early onset (AREP) (PARK6, PARK7, and PARK9), and a locus controlling susceptibility to, and/or onset age of late-onset PD (PARK10) have been mapped to chromosome 1p by five different groups.[16, 129-131, 133] Recently, the defective gene at the PARK7 locus, *DJ-1*, has been identified (chapter 2.8).
In the framework of a large research project for gene mapping in a genetically isolated population in the South West of The Netherlands (Genetic Research in Isolated Populations, GRIP), a large consanguineous pedigree was ascertained with four members affected by early-onset parkinsonism (the onset varying from age 27 to 40 years). A genome-wide scan in this family using homozygosity mapping yielded significant evidence for linkage to the chromosome 1p36 region, and this locus was named PARK7 (chapter 2.5). Linkage to the very same region was later confirmed in a consanguineous pedigree from Central Italy with three affected members (chapter 2.6).
Fine mapping studies and a positional cloning strategy led us to the identification of mutations in the *DJ-1* gene in the two definitely linked families (chapter 2.8). All patients of the Dutch kindred carry a homozygous deletion, which removes ~14 kb of genomic sequence, including 4 kb upstream of the *DJ-1* start codon, and a large part of the coding region. In the Italian family, a homozygous missense mutation replaces the Leucine at position 166 with Proline (L166P) in the DJ-1 protein. The homozygous deletion found in the patients of the Dutch family represents a natural knockout of *DJ-1*, clearly indicating that the loss of function of DJ-1 is pathogenic. The L166P mutation is also likely to affect severely the function of DJ-1 because:

(i) it replaces a highly conserved residue;
(ii) it destabilizes a carboxy-terminal α-helix of the DJ-1 protein as predicted by structural models;
(iii) it dramatically changes the subcellular localization of the DJ-1 protein in transfection experiments (chapter 2.8).

Our computer-assisted modelling suggested a globular, α/β-sandwich to be the most likely structure for DJ-1 (chapter 2.8), which has recently been confirmed by four crystallography studies.[231-234]
The human *DJ-1* is organized in eight exons distributed over 24 kb of genomic DNA. The

first two exons are non-coding and alternatively spliced in the two mRNA transcripts.[132, 235] Northern blot analysis showed that *DJ-1* is ubiquitously and highly expressed in the brain areas and extra-cerebral tissues.[132, 235] Interestingly, using Northern analysis the expression seems more abundant in subcortical regions of the brain than in cortical areas. However, further work is needed to explore the regional pattern of DJ-1 expression at the protein level.

The genomic structures of the mouse and human *DJ-1* genes are similar, and human and mouse DJ-1 proteins display 90% amino acid identity.[235] Analysis of promoter activity using deletion constructs and luciferase assays in HeLa cells identified an Sp1 (Specificity Protein1, a transcription factor) dependent site at position –100 from the transcription initiation site contributing most of the promoter activity.[235]

The human DJ-1 protein consists of 189 amino acids and belongs to the ThiJ/PfpI superfamily. Several members of this family of proteins are known in prokaryotes: ThiJ, involved in the thiamine synthetic pathway, PfpI and other proteases, araC and other transcription factors, the glutamine amidotransferase family, which includes some bacterial catalases, and finally a recently recognized family of bacterial chaperones (EcHsp31).

The exact function of the DJ-1 protein is unknown, but the literature suggests a role in the oxidative stress response, or in the response to misfolded proteins.[236-239]. Intriguingly, evidence of oxidative stress and protein misfolding has been documented in the brains of patients with PD,[240, 241] and studies have shown that mutations in *α-synuclein* and *parkin*, two other PD-related genes, might also be linked to oxidative stress.[242, 243]

To date only a few mutational analyses in larger series of families and isolated early onset PD cases have been performed, and a total number of nine different mutations in *DJ-1* have been identified so far, including missense, truncating, splice site mutations, and large deletions (figure 2).[132, 244-246] The identification of novel, compound heterozygous mutations (including one truncating and one splice site mutation) in a further PD patient provides the strongest evidence so far, after our initial report,[132] that *DJ-1* mutations are pathogenic in early onset PD.[244] For other mutations, especially the single heterozygous ones, a pathogenic role is not definitely demonstrated yet. While these findings delineate *DJ-1* mutations as the second most frequent identifiable genetic cause of PD after *parkin*, the frequency of *DJ-1* mutations seems low, being estimated at about 1-2 % in early onset PD. The occurrence of heterozygous genomic rearrangements in *DJ-1* emphasizes the importance of gene dosage assays for a sensitive screening. Gene dosage has been performed in only one study so far,[246] and the other studies might have underestimated the frequencies of mutations. The identification of larger numbers of *DJ-1*-related patients will allow accurate genotype-phenotype correlation studies.

An autosomal dominant form of focal-segmental dystonia maps to 1p36 (the DYT13 locus).[247] The DYT13 critical interval overlaps for 6 cM with the PARK7 interval, and the *DJ-1* gene is located in the region of overlap (figure 1). So far, linkage to this region has not been replicated in other dystonia families, and parkinsonian features have not been detected in the original DYT13-linked family. However, dystonic features such as laterocollis and blepharospasm are present in both the *DJ-1*-related families. This suggests that despite the differences in transmission pattern and clinical phenotypes, PARK7 and DYT13 might be allelic disorders; a mutational screening of the *DJ-1* gene is therefore warranted in DYT13-linked families.

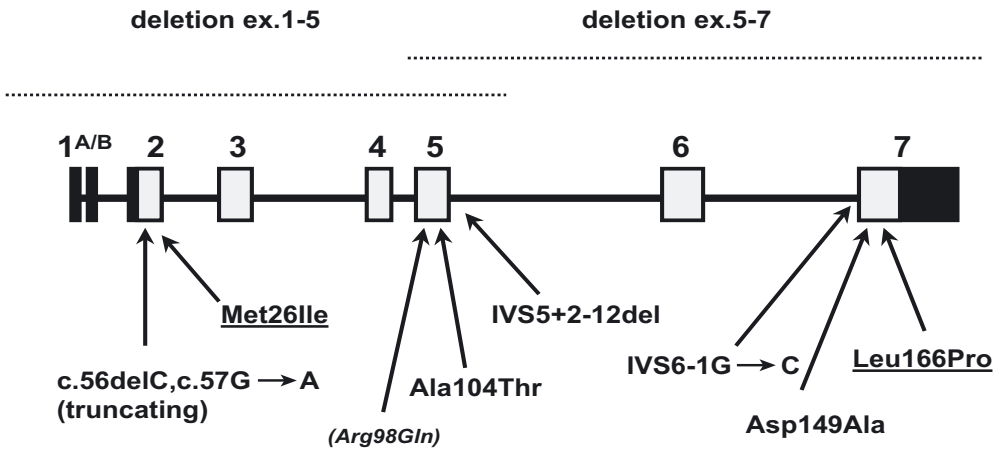

Figure 2. *Genomic structure of the DJ-1 gene and mutations identified in PD. The dark and light boxes indicate non-coding and coding exonic sequence, respectively. Two missense mutations observed in homozygous form are underlined. The Arg98Gln change is a rare, polymorphic variant observed in ~1.5% of control chromosomes in Caucasians.*[246]

Pathological analysis of brains from patients with *DJ-1*-related forms is of great importance, but brain material is not currently available. Our recent finding that DJ-1 immunoreactivity co-localizes with pathological tau inclusions in different neurodegenerative disorders,[248] suggests the involvement of this protein in the pathogenesis of tauopathies, and it raises the question of whether *DJ-1*-related parkinsonism is also associated with tau pathology in the brain. If this would be the case, it would suggest the existence of a common pathological signature in *DJ-1*- and *parkin*-related forms, as tau pathology has been found in brains with parkin disease.[210, 211, 213] It would also be interesting to investigate the expression of the DJ-1 protein in the brain with parkin disease and vice versa.

PARK6

This locus was first identified in a large Italian consanguineous family originating from Sicily.[129] Four family members developed early-onset PD with onset ages ranging from 32 to 48 years. A genome-wide scan provided significant evidence for linkage to the short arm of chromosome 1, in the chromosomal region 1p36-p35. Haplotype analysis delineated a 12.5 cM homozygously inherited region in the affected members, flanked by the markers D1S483 telomerically and D1S247 centromerically. A subsequent study analysed 28 smaller European AREP families, which did not carry mutations in the *parkin* gene. Eight out of 28 families supported linkage to PARK6, suggesting this as a frequent locus.[249] Of particular interest is a second consanguineous family from Central Italy, in which a recombination event would reduce the critical region to 9 cM. However, none of these families independently had enough statistical power to detect significant linkage, and some of them also support linkage to the other recessive loci (PARK2 and PARK7) (chapter 2.6). Considering the genetic heterogeneity of AREP and PD, the assignment of these families to PARK6 should therefore be considered as provisional.

Relationships between PARK2, PARK6 and PARK7

Early onset of parkinsonism, slow disease progression, and good response to levodopa are unifying clinical features in different AREP forms, including *parkin*-related, *DJ-1*-

related, and PARK6-linked families.[128, 130, 250-253] This will make their differentiation difficult on clinical grounds, indicating the importance of genetic testing. In comparison with classical PD, the phenotype in the Dutch and Italian *DJ-1*-related families is characterized by an early onset and a slow progression (chapters 2.6 and 2.7). Interestingly, psychiatric disturbances (including severe anxiety in three cases, and psychotic episodes in one), early behavioural disturbances (one case), and dystonic features (including blepharospasm in two cases) have been reported in both families, and they might represent "red flags" for suspecting *DJ-1* involvement.[130, 251, 253] The presence of severe anxiety and panic attacks has indeed been noted in two further *DJ-1*-related patients identified in a recent screen.[245] However, a note of caution is warranted, as these psychiatric disturbances are non-specific, they are well known to occur in PD in general,[254] and they are also described in patients with mutations in the *parkin* gene.[138, 171] The analysis of larger case series is therefore needed in order to investigate whether psychiatric disturbances are more frequent among *DJ-1*-related form than other PD forms. The average age at onset is in the early thirties for the *parkin*- and *DJ-1*-related families, whereas the onset in PARK6-linked families might be slightly later (late thirties - early forties). The pathology of PARK6-linked and *DJ-1*-related forms is unknown.

Recent positron emission tomography studies in PARK6-linked and *DJ-1*-related patients showed: more uniform patterns of caudate/putamen dopaminergic terminal dysfunction in comparison with the one observed in classical PD; greater dopaminergic dysfunction than the one expected on the basis of clinical severity; a mild dysfunction in asymptomatic heterozygous PARK6 carriers.[250, 253] These features also resemble the pattern observed in *parkin*-related disease, suggesting that these three AREP forms might be more similar to each other than to classical PD on pathophysiological grounds (chapter 2.7).

The similarities between PARK6- and *DJ-1*-associated phenotypes, and the proximity of the two loci on chromosome 1p, raised the question of whether mutations in similar or functionally related genes underlie both forms. An alternative possibility is that, due to a large genomic rearrangement occurring in one of the linked families, linkage is found in seemingly different regions, while there is only one underlying, defective gene. To explore this possibility, a mutational analysis of the *DJ-1* gene should also be performed in the original PARK6-linked family.

PARK9

This form has been initially described in a consanguineous family from a small community in the North of Jordan (Kufor-Rakeb region).[255] In this kindred, five siblings developed a multisystemic, neurodegenerative disease resembling the so-called pallido-pyramidal syndromes, and clinically characterized by onset below age of 20, akinetic-rigid parkinsonism (without tremor), pyramidal tract signs, supranuclear ophtalmoparesis, and cognitive deterioration. The progression is reported to be rapid, but the parkinsonian symptoms responded very well to levodopa therapy. Brain MRI showed progressive atrophy, starting from the pallidum and pyramids but later generalized. No pathology studies have been reported.[255] A genome-wide scan in this family detected significant evidence for linkage to the 1p36 region,[131] and despite the fact that the multisystemic "Kufor-Rakeb syndrome" appears clinically quite different from PD, this locus has been designated as PARK9. Furthermore, the PARK9 critical interval overlaps with the DYT13 and PARK6 critical regions at the telomeric and the centromeric border, respectively (figure 1). PARK9 can therefore be allelic to one of these disorders. Identification of the responsible genes at these loci will provide the answer to this question.

GENOME-WIDE SCREENS IN NON-MENDELIAN PD FORMS

Families segregating PD as a monogenic trait, and large enough to be suitable for classical linkage mapping are very rare. Moreover, for the vast majority of cases, parkinsonism runs in families without a clearcut Mendelian pattern and a highly variable expression, suggesting a complex multifactorial etiology. In this scenario, it has been suggested that it might be more feasible to perform genome-wide scans in series of small PD families containing 2 affected relatives (sibling pairs or other relative pairs).[256] Such families are not rare, and collecting large numbers is not difficult in the framework of multi-centric consortia. In these studies, non-parametric, model-free methods are often used. These methods are very robust because they do not require a genetic model to be specified.[256] The methods are based on the general principle of comparing the observed number of alleles shared identical-by-descent (IBD) between pairs of affected relatives versus the numbers expected under the null hypothesis of no linkage.[256] For example, at a given locus, the distribution of IBD sharing by sibling pairs under the null hypothesis is: 0 alleles (in 25% of the pairs), 1 allele (50% pairs), and 2 alleles (25% pairs). Any deviation from this distribution in the direction of an excess of allele sharing is considered as evidence of linkage, and the results can be expressed as LOD scores as in classical linkage analysis. As for all available methods this approach is not free from limitations and caveats. Since a large number of families are used, genetic heterogeneity influences greatly the outcome of such studies. Depending on the number and magnitude of genetic risk factors involved, and the risk allele frequencies, large sample sets are often needed to obtain meaningful data.[256-258]

The estimates of risk ratios for PD in first-degree relatives of PD patients compared to general population range widely (from 1.3 to 9.7),[259] but in several studies the figures cluster around the value of 3. Specific recurrence risks for siblings (λ_{sibs}) have been estimated only rarely.[9] Assuming the contribution of a single genetic effect and λ_{sibs} values around 3, the number of affected sibling pairs (ASPs) needed to have enough power to detect linkage at 0.05 genome-wide significance level is estimated to be in the order of 200-300. However, the number of ASP needed increases sharply (to the order of thousands) if several genetic effects (each of smaller magnitude) are involved.[256-258]

Modelling the disease definition and phenotype boundaries is another critical issue in PD genetics. Models with narrower or broader disease definitions can be used. The statistical corrections needed to declare significant linkage using multiple comparisons when several clinical models using the same dataset, or when subsets are analysed after stratification of the whole dataset are still debated. Replication of positive findings between different datasets is therefore important in order to identify genuine linkages. Furthermore, regions found using these approaches are usually very large (because ASPs are closely related so the region shared IBD is large) and therefore, follow-up studies to pinpoint the location of the relevant gene are difficult.

The results of four genome-wide scans in families with affected relative pairs (ARP) with classical PD have been published so far (table 2).[16, 97, 119, 260] Moreover, two of the same datasets have been reanalysed in search of modifiers of PD onset age.[69, 133] Comparing the results of these studies is not straightforward, because the studies differ in clinical and genetic designs, sample size, density of markers typed, and especially in statistical analyses. Significant linkage was detected to a new region on chromosome 1p32 (PARK10) in one scan,[16] and to the *parkin* gene locus on 6q (PARK2) in another scan;[119] moreover, the region on 1p32 was significantly linked to PD onset age in another study (figure 3).[133]

A list of additional regions has been generated, with values of LODs ranging from 1 to around 2 (figure 3). These findings represent non-significant, "interesting" or "suggestive" linkage signals that need to be further evaluated in future studies. Several of these signals are non-consistent across the studies, and they might be false positive results. However, the consistency of findings for a few regions suggests that these might represent additional genuine linkages, though the power of the separate studies was insufficient to achieve statistical significance.

Table 2.

The genome-wide screens performed in the common forms of PD

Study	Dataset	Sample size	Marker density	Software packages
OBJECT: determinants of PD risk				
De Stefano et al. ([260])	sib-pairs (*Gene*PD study)	113 ASP (102 families)	11 cM (339 markers)	GENEHUNTER
Scott et al. ([119])	families with ≥ 2 aff. relatives	260 ARP (174 families)	10 cM (344 markers)	VITESSE/HOMOG (par.) GENEHUNTER-PLUS
Pankratz et al. ([97])	sib pairs (*Parkinson Study Group*)	170 ASP (160 families)	8.6 cM (400 markers)	MAPMAKER-SIBS
Hicks et al. ([16])	isolated population *(Iceland)*	117 patients (51 families)	< 5 cM (781 markers)	ALLEGRO
OBJECT: determinants of PD onset age (AAO)				
Li et al. ([133])	families with ≥ 2 aff. relatives	see Scott et al. [119]	see Scott et al. [119]	SOLAR
De Stefano et al. ([69])	sib-pairs (*Gene*PD study)	see De Stefano et al.[260]	see De Stefano et al.[260]	GENEHUNTER

A first study (the *Gene*PD study) performed on 113 ASPs yielded no significant results, probably because of the limited sample size.[260] A second study was performed on a slightly larger sample of 174 families with ≥1 ARPs, including 184 ASPs and 75 ARPs of other type (cousins, parent-child, avuncular).[119] Again, in the overall dataset, no significant linkage was found. However, within a subset of 18 families containing at least 1 PD case with onset ≤ 40 years (early-onset families), significant evidence for linkage (LOD>5) was detected at the PARK2 locus (*parkin)*. While this is not surprising, given the frequency of *parkin* mutations in early onset PD, it is noteworthy that a signal to the PARK2 region was not detected by the analysis on the overall dataset, highlighting the potential pitfalls in linkage analysis of multiple families with a genetically heterogeneous disease. A third study (Parkinson Study Group) analysed 182 families with ASPs.[97] In order to reduce genetic heterogeneity, 22 families with *parkin* gene mutations were excluded from the analysis. Moreover, affection status was modelled according to a narrower (model I) and a broader PD definition (model II). Analyses under model I yielded no significant LOD scores but re-analysis according to the model II (broader PD definition) increased the LODs at regions on 2q and Xq (LODs 2.5 and 2.7 respectively). The finding on chromosome 2q36-q37 was followed up in a larger dataset and now reached significance.[17]

The fourth study was performed in the genetically isolated population of Iceland.[16] Genetically isolated populations are increasingly recognized as a powerful framework for research aimed at the identification of genetic factors underlying complex disorders.[261] Since isolated populations originate from a limited number of founders, they are characterized by a simpler genetic make-up compared to the general

population; this can greatly facilitate the identification of genes for complex diseases, such as PD. The caveat is that genetic determinants identified in genetic isolates might not be relevant in the general population.

A significant familial aggregation of PD was previously shown in Iceland.[9] Using a population-based approach, fifty-one families were ascertained with ≥1 ARPs with PD, yielding a total of 117 patients. Non-parametric, model-free analysis generated significant evidence for linkage to a region on chromosome 1p32 (max LOD=4.9), and this locus was termed PARK10.[16] In this dataset, maximizing the LODs under a variety of parametric models (dominant, additive, multiplicative) did not allow a particular mode of inheritance to be definitely delineated. However, the best fitting model, which incorporated an additive inheritance pattern and given values of penetrances and at-risk allele frequency, allowed to estimate that the PARK10 gene could explain a sibling recurrence risk ratio ranging from 3.2 to 4.6. Given the sibling relative risks for PD of 6.7 observed in Iceland,[9] the authors conclude that PARK10 alone can account for a substantial fraction of the familial aggregation of PD in this population.[16] While this is an important significant linkage finding, its relevance for the other populations remains unknown, and none of the previous three genome-wide scans had detected linkage to 1p32.[97, 119, 260]

Among the "interesting" regions detected across the four genome-wide scans, the most consistent appear those on chromosomes 5q, 9q, 17q, and Xq (figure 3). These regions are very large and contain many genes, including obvious candidates: the gene encoding synphilin-1, an alpha-synuclein interactor (on 5q)[111], the gene encoding dopamine β-hydroxylase, and *DYT1*, the gene causing generalized torsion dystonia (on 9q),[262, 263] and the gene for a monogenic, X-linked dystonia-parkinsonism form (on Xq).[264] The peak detected on 17q is close to the gene for microtubule associated protein tau, an important locus for neurodegeneration and a known risk factor for progressive supranuclear palsy and PD.[265, 266] The *NR4A2* gene maps more than 70 cM centromerically to the linkage peak detected in the study of Pankratz et al.[97]. Despite its intriguing functional links to PD,[102, 107] *NR4A2* is therefore not a likely candidate for the reported 2q36-q37 region.

All previous studies were designed to identify genetic determinants of PD risk. However, age at disease onset (AAO) might also be genetically controlled, and finding such genetic determinants or modifiers of onset age might have important implications for developing novel therapeutic approaches aiming to delay the onset of disease. In this approach, the available information on AAO of patients and age at examination of unaffected family members can be used to model AAO as a quantitative trait locus (QTL), which is amenable for linkage analysis. Two groups have recently reanalysed their datasets using this approach.[69, 133]

The first study on PD onset age as a QTL[133] reanalysed the dataset published by Scott et al. 2001.[119] According to these results, the estimated heritability of AAO for PD is high (62%), indicating that strong genetic determinants for PD onset age exist. Significant evidence for linkage of AAO was found for markers in the region of chromosome1p32 (LOD=3.41). This region overlaps with the PARK10 region found in the Icelandic study.[16] Additional regions yielding LODs between 1.4 and 1.9 were found on chromosomes 5q, 6p, 10q, 13q, 17p, 20p and 22q (figure 3). Moreover, when the PD dataset was analysed together with a dataset of AD families, evidence was found for the existence of a genetic determinant of onset age for both diseases on chromosome 10q.[133]

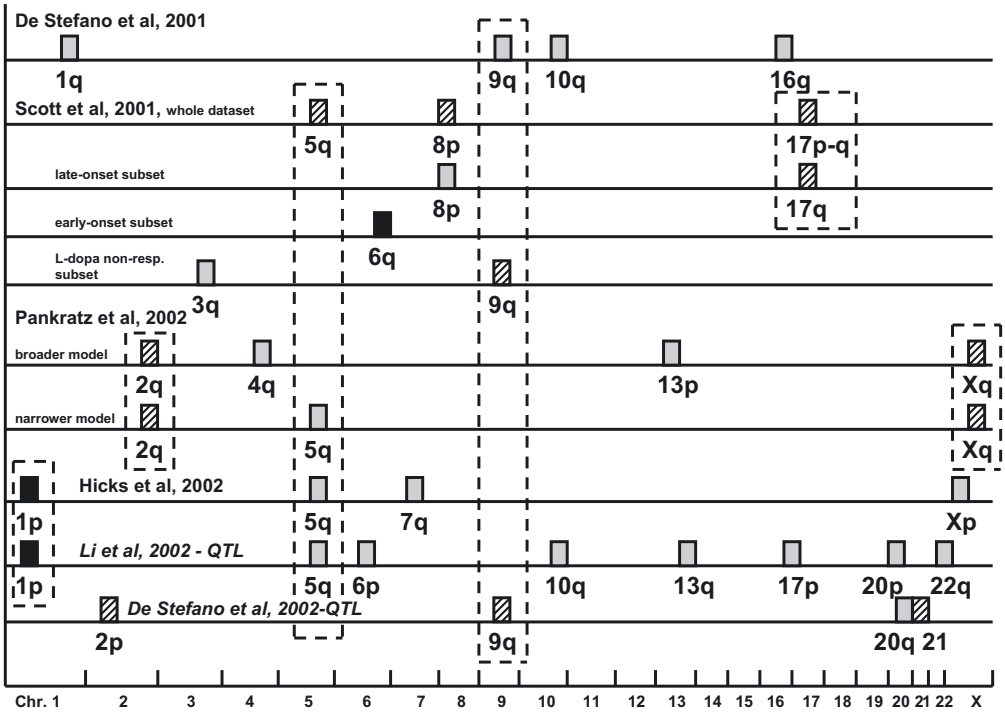

Figure 3. *Overview of results from the genome-wide screens in common forms of PD. The black, shaded, and grey boxes indicate regions with LOD scores values >3, >2, and >1 respectively.*

Although most of the evidence came from the group of AD patients, which was significantly larger then the group of PD patients, this finding is still intriguing since clinical, biochemical and pathological overlaps between PD and AD have been documented, and it is therefore conceivable that common genetic factors might control the pathogenesis and/or the progression in both the diseases. Moreover, together with the results of the Icelandic study, the detection of linkage on 1p32 confirms that an important genetic determinant of PD risk and/or onset age resides in this region.

The other study[69] reanalysed the dataset of the *Gene*PD study using age at onset as the phenotype of interest, and found suggestive evidence (LODs between 1.8 to 2.2) for linkage of AAO to chromosome 2p, 9q, 20q, and 21 (figure 3). The region on 2p coincides with PARK3, a locus for autosomal dominant, late-onset PD,[61] whereas the region on 9q has also been detected in linkage to affection status in the GenePD and in another screen (figure 3). In addition to linkage to PARK3, evidence for association was also observed between a specific allele of D2S1394 (one of the markers within PARK3) and AAO.[69]

In addition to 2p (PARK3) and 1p32 (PARK10), mildly positive LOD scores for other regions like the one on 5q and 9q have been detected by different studies searching for linkage to disease status (genetic risk) and for linkage to AAO (figure 3), illustrating how modelling and statistical analyses can lead to detection of genetic determinants of AAO when studying genetic risk, and vice versa.

With the exception of PARK3, the current results of the genome scans suggest that the genes implicated in the known Mendelian forms are not major loci for common forms of PD. However, the failure to detect significant linkage, and the inconsistency of most peaks across different scans, suggest that most of these studies were underpowered. A still unknown major locus for susceptibility to common forms of PD could have escaped detection just due to power limitations. The results are also compatible with the existence of many weaker genetic risk factors, which might also differ in the different studies, particularly if genetically isolated populations are targeted. In the light of the results of screens published so far for late-onset PD, targeting a genetically isolated population has been more rewarding than scanning series of affected-pairs from the general populations.

CONCLUSIONS

Classical linkage approaches to monogenic forms of PD have been most fruitful in the last few years, but it is anticipated that more and more genome-wide studies will be performed in the coming years in the common form of the disease, using families with affected-pairs, and cohorts of isolated cases from genetically isolated populations. The technology of DNA microarrays (DNA chips) is also anticipated to contribute soon to the identification of PD-related genes. A high level of etiological complexity likely exists for PD; in this scenario, genetic approaches to isolated populations or otherwise-defined more homogeneous groups is convenient in order to try and dissect the complexity of the problem.
This year, at the 50th anniversary of the discovery of the structure of DNA, the finished sequence of the human genome has been released. At the beginning of the post-genomic era, there are motifs to believe that the genome-wide approach will also succeed in deciphering the code of PD.

REFERENCES

1. de Rijk MC, Launer LJ, Berger K, et al. Prevalence of Parkinson's disease in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. Neurology 2000; 54:S21-3.
2. Lang AE, Lozano AM. Parkinson's disease. First of two parts. N Engl J Med 1998; 339:1044-53.
3. Langston JW, Ballard P, Tetrud JW, Irwin I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 1983; 219:979-80.
4. Mjones H. Paralysis agitans: a clinical and genetic study. Acta Psychiatr Neurol 1949; 25 Suppl.54:1-195.
5. Martin WE, Young WI, Anderson VE. Parkinson's disease. A genetic study. Brain 1973; 96: 495-506.
6. Lazzarini AM, Myers RH, Zimmerman TR, Jr., et al. A clinical genetic study of Parkinson's disease: evidence for dominant transmission. Neurology 1994; 44:499-506.
7. Bonifati V, Fabrizio E, Vanacore N, De Mari M, Meco G. Familial Parkinson's disease: a clinical genetic analysis. Can J Neurol Sci 1995; 22:272-9.
8. Maher NE, Currie LJ, Lazzarini AM, et al. Segregation analysis of Parkinson disease revealing evidence for a major causative gene. Am J Med Genet 2002; 109:191-7.
9. Sveinbjornsdottir S, Hicks AA, Jonsson T, et al. Familial aggregation of Parkinson's disease in Iceland. N Engl J Med 2000; 343:1765-70.
10. Tanner CM, Ottman R, Goldman SM, et al. Parkinson disease in twins: an etiologic study. Jama 1999; 281:341-6.

11. Dickson D, Farrer M, Lincoln S, et al. Pathology of PD in monozygotic twins with a 20-year discordance interval. Neurology 2001; 56:981-2.
12. Piccini P, Burn DJ, Ceravolo R, Maraganore D, Brooks DJ. The role of inheritance in sporadic Parkinson's disease: evidence from a longitudinal study of dopaminergic function in twins. Ann Neurol 1999; 45:577-82.
13. Maher NE, Golbe LI, Lazzarini AM, et al. Epidemiologic study of 203 sibling pairs with Parkinson's disease: the GenePD study. Neurology 2002; 58:79-84.
14. Mouradian MM. Recent advances in the genetics and pathogenesis of Parkinson disease. Neurology 2002; 58:179-85.
15. Dawson TM, Dawson VL. Rare genetic mutations shed light on the pathogenesis of Parkinson disease. J Clin Invest 2003; 111:145-51.
16. Hicks AA, Petursson H, Jonsson T, et al. A susceptibility gene for late-onset idiopathic Parkinson's disease. Ann Neurol 2002; 52:549-55.
17. Pankratz N, Nichols WC, Uniacke SK, et al. Significant Linkage of Parkinson Disease to Chromosome 2q36-37. Am J Hum Genet 2003; 72:1053-7.
18. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. Nature 2001; 409:860-921.
19. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. Science 2001; 291:1304-51.
20. Golbe LI, Di Iorio G, Bonavita V, Miller DC, Duvoisin RC. A large kindred with autosomal dominant Parkinson's disease. Ann Neurol 1990; 27:276-82.
21. Golbe LI, Di Iorio G, Sanges G, et al. Clinical genetic analysis of Parkinson's disease in the Contursi kindred. Ann Neurol 1996; 40:767-75.
22. Polymeropoulos MH, Higgins JJ, Golbe LI, et al. Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. Science 1996; 274:1197-9.
23. Scott WK, Staijich JM, Yamaoka LH, et al. Genetic complexity and Parkinson's disease. Deane Laboratory Parkinson Disease Research Group. Science 1997; 277:387-8.
24. Gasser T, Muller-Myhsok B, Wszolek ZK, et al. Genetic complexity and Parkinson's disease. Science 1997; 277:388-9.
25. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 1997; 276:2045-7.
26. Athanassiadou A, Voutsinas G, Psiouri L, et al. Genetic analysis of families with Parkinson disease that carry the Ala53Thr mutation in the gene encoding alpha-synuclein. Am J Hum Genet 1999; 65:555-8.
27. Markopoulou K, Wszolek ZK, Pfeiffer RF, Chase BA. Reduced expression of the G209A alpha-synuclein allele in familial Parkinsonism. Ann Neurol 1999; 46:374-81.
28. Spira PJ, Sharpe DM, Halliday G, Cavanagh J, Nicholson GA. Clinical and pathological features of a Parkinsonian syndrome in a family with an Ala53Thr alpha-synuclein mutation. Ann Neurol 2001; 49:313-9.
29. Papapetropoulos S, Paschalis C, Athanassiadou A, et al. Clinical phenotype in patients with alpha-synuclein Parkinson's disease living in Greece in comparison with patients with sporadic Parkinson's disease. J Neurol Neurosurg Psychiatry 2001; 70:662-5.
30. Bostantjopoulou S, Katsarou Z, Papadimitriou A, Veletza V, Hatzigeorgiou G, Lees A. Clinical features of parkinsonian patients with the alpha-synuclein (G209A) mutation. Mov Disord 2001; 16:1007-13.
31. Kruger R, Kuhn W, Muller T, et al. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet 1998; 18:106-8.
32. Vaughan J, Durr A, Tassin J, et al. The alpha-synuclein Ala53Thr mutation is not a common cause of familial Parkinson's disease: a study of 230 European cases. European Consortium on Genetic Susceptibility in Parkinson's Disease. Ann Neurol 1998; 44:270-3.
33. Vaughan JR, Farrer MJ, Wszolek ZK, et al. Sequencing of the alpha-synuclein gene in a large series of cases of familial Parkinson's disease fails to reveal any further mutations. The European Consortium on Genetic Susceptibility in Parkinson's Disease (GSPD). Hum Mol Genet 1998; 7:751-3.
34. Warner TT, Schapira AH. The role of the alpha-synuclein gene mutation in patients with sporadic Parkinson's disease in the United Kingdom. J Neurol Neurosurg Psychiatry 1998; 65:378-9.

35. Bennett P, Nicholl DJ. Absence of the G209A mutation in the alpha-synuclein gene in British families with Parkinson's disease. Neurology 1998; 50:1183.
36. Wang WW, Khajavi M, Patel BJ, Beach J, Jankovic J, Ashizawa T. The G209A mutation in the alpha-synuclein gene is not detected in familial cases of Parkinson disease in non-Greek and/or Italian populations. Arch Neurol 1998; 55:1521-3.
37. Farrer M, Wavrant-De Vrieze F, Crook R, et al. Low frequency of alpha-synuclein mutations in familial Parkinson's disease. Ann Neurol 1998; 43:394-7.
38. Chan P, Tanner CM, Jiang X, Langston JW. Failure to find the alpha-synuclein gene missense mutation (G209A) in 100 patients with younger onset Parkinson's disease. Neurology 1998; 50:513-4.
39. Chan P, Jiang X, Forno LS, Di Monte DA, Tanner CM, Langston JW. Absence of mutations in the coding region of the alpha-synuclein gene in pathologically proven Parkinson's disease. Neurology 1998; 50:1136-7.
40. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature 1997; 388:839-40.
41. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc Natl Acad Sci U S A 1998; 95:6469-73.
42. Wakabayashi K, Yoshimoto M, Tsuji S, Takahashi H. Alpha-synuclein immunoreactivity in glial cytoplasmic inclusions in multiple system atrophy. Neurosci Lett 1998; 249:180-2.
43. Spillantini MG, Goedert M. The alpha-synucleinopathies: Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy. Ann N Y Acad Sci 2000; 920:16-27.
44. Feany MB, Bender WW. A Drosophila model of Parkinson's disease. Nature 2000; 404:394-8.
45. Masliah E, Rockenstein E, Veinbergs I, et al. Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. Science 2000; 287:1265-9.
46. van der Putten H, Wiederhold KH, Probst A, et al. Neuropathology in mice expressing human alpha-synuclein. J Neurosci 2000; 20:6021-9.
47. Kahle PJ, Neumann M, Ozmen L, et al. Subcellular localization of wild-type and Parkinson's disease-associated mutant alpha-synuclein in human and transgenic mouse brain. J Neurosci 2000; 20:6365-73.
48. Lee MK, Stirling W, Xu Y, et al. Human alpha-synuclein-harboring familial Parkinson's disease-linked Ala-53-Thr mutation causes neurodegenerative disease with alpha-synuclein aggregation in transgenic mice. Proc Natl Acad Sci U S A 2002; 99:8968-73.
49. Giasson BI, Duda JE, Quinn SM, Zhang B, Trojanowski JQ, Lee VM. Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. Neuron 2002; 34:521-33.
50. Lakso M, Vartiainen S, Moilanen AM, et al. Dopaminergic neuronal loss and motor deficits in Caenorhabditis elegans overexpressing human alpha-synuclein. J Neurochem 2003; 86:165-172.
51. Touchman JW, Dehejia A, Chiba-Falek O, et al. Human and mouse alpha-synuclein genes: comparative genomic sequence analysis and identification of a novel gene regulatory element. Genome Res 2001; 11:78-86.
52. Chiba-Falek O, Nussbaum RL. Effect of allelic variation at the NACP-Rep1 repeat upstream of the alpha-synuclein gene (SNCA) on transcription in a cell culture luciferase reporter system. Hum Mol Genet 2001; 10:3101-9.
53. Holzmann C, Kruger R, Saecker AM, et al. Polymorphisms of the alpha-synuclein promoter: expression analyses and association studies in Parkinson's disease. J Neural Transm 2003; 110:67-76.
54. Kruger R, Vieira-Saecker AM, Kuhn W, et al. Increased susceptibility to sporadic Parkinson's disease by a certain combined alpha-synuclein/apolipoprotein E genotype. Ann Neurol 1999; 45:611-7.
55. Khan N, Graham E, Dixon P, et al. Parkinson's disease is not associated with the combined alpha-synuclein/apolipoprotein E susceptibility genotype. Ann Neurol 2001; 49:665-8.
56. Izumi Y, Morino H, Oda M, et al. Genetic studies in Parkinson's disease with an alpha-synuclein/NACP gene polymorphism in Japan. Neurosci Lett 2001; 300:125-7.

57. Pastor P, Munoz E, Ezquerra M, et al. Analysis of the coding and the 5' flanking regions of the alpha-synuclein gene in patients with Parkinson's disease. Mov Disord 2001; 16:1115-9.
58. Farrer M, Maraganore DM, Lockhart P, et al. alpha-Synuclein gene haplotypes are associated with Parkinson's disease. Hum Mol Genet 2001; 10:1847-51.
59. Duda JE, Giasson BI, Mabon ME, et al. Concurrence of alpha-synuclein and tau brain pathology in the Contursi kindred. Acta Neuropathol (Berl) 2002; 104:7-11.
60. Kruger R, Kuhn W, Leenders KL, et al. Familial parkinsonism with synuclein pathology: clinical and PET studies of A30P mutation carriers. Neurology 2001; 56:1355-62.
61. Gasser T, Muller-Myhsok B, Wszolek ZK, et al. A susceptibility locus for Parkinson's disease maps to chromosome 2p13. Nat Genet 1998; 18:262-5.
62. Wszolek ZK, Cordes M, Calne DB, Munter MD, Cordes I, Pfeifer RF. [Hereditary Parkinson disease: report of 3 families with dominant autosomal inheritance]. Nervenarzt 1993; 64: 331-5.
63. Denson MA, Wszolek ZK, Pfeiffer RF, Wszolek EK, Paschall TM, McComb RD. Familial parkinsonism, dementia, and Lewy body disease: study of family G. Ann Neurol 1997; 42: 638-43.
64. Wszolek ZK, Gwinn-Hardy K, Wszolek EK, et al. Neuropathology of two members of a German-American kindred (Family C) with late onset parkinsonism. Acta Neuropathol (Berl) 2002; 103:344-50.
65. Klein C, Vieregge P, Hagenah J, et al. Search for the PARK3 founder haplotype in a large cohort of patients with Parkinson's disease from northern Germany. Ann Hum Genet 1999; 63:285-91.
66. West AB, Zimprich A, Lockhart PJ, et al. Refinement of the PARK3 locus on chromosome 2p13 and the analysis of 14 candidate genes. Eur J Hum Genet 2001; 9:659-66.
67. Zink M, Grim L, Wszolek ZK, Gasser T. Autosomal-dominant Parkinson's disease linked to 2p13 is not caused by mutations in transforming growth factor alpha (TGF alpha). J Neural Transm 2001; 108:1029-34.
68. Lockhart PJ, Holtom B, Lincoln S, et al. The human sideroflexin 5 (SFXN5) gene: sequence, expression analysis and exclusion as a candidate for PARK3. Gene 2002; 285:229-37.
69. DeStefano AL, Lew MF, Golbe LI, et al. PARK3 influences age at onset in Parkinson disease: a genome scan in the GenePD study. Am J Hum Genet 2002; 70:1089-95.
70. Waters CH, Miller CA. Autosomal dominant Lewy body parkinsonism in a four-generation family. Ann Neurol 1994; 35:59-64.
71. Muenter MD, Forno LS, Hornykiewicz O, et al. Hereditary form of parkinsonism--dementia. Ann Neurol 1998; 43:768-81.
72. Farrer M, Gwinn-Hardy K, Muenter M, et al. A chromosome 4p haplotype segregating with Parkinson's disease and postural tremor. Hum Mol Genet 1999; 8:81-5.
73. Gwinn-Hardy K, Mehta ND, Farrer M, et al. Distinctive neuropathology revealed by alpha-synuclein antibodies in hereditary parkinsonism and dementia linked to chromosome 4p. Acta Neuropathol (Berl) 2000; 99:663-72.
74. Pahwa R, Koller WC. Is there a relationship between Parkinson's disease and essential tremor? Clin Neuropharmacol 1993; 16:30-5.
75. Wilkinson KD, Lee KM, Deshpande S, Duerksen-Hughes P, Boss JM, Pohl J. The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. Science 1989; 246: 670-3.
76. Osaka H, Wang YL, Takada K, et al. Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron. Hum Mol Genet 2003; 12:1945-1958.
77. Lowe J, McDermott H, Landon M, Mayer RJ, Wilkinson KD. Ubiquitin carboxyl-terminal hydrolase (PGP 9.5) is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative diseases. J Pathol 1990; 161:153-60.
78. Saigoh K, Wang YL, Suh JG, et al. Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. Nat Genet 1999; 23:47-51.
79. Leroy E, Boyer R, Auburger G, et al. The ubiquitin pathway in Parkinson's disease. Nature 1998; 395:451-2.

80. Lincoln S, Vaughan J, Wood N, et al. Low frequency of pathogenic mutations in the ubiquitin carboxy-terminal hydrolase gene in familial Parkinson's disease. Neuroreport 1999; 10:427-9.
81. Harhangi BS, Farrer MJ, Lincoln S, et al. The Ile93Met mutation in the ubiquitin carboxy-terminal-hydrolase-L1 gene is not observed in European cases with familial Parkinson's disease. Neurosci Lett 1999; 270:1-4.
82. Zhang J, Hattori N, Giladi N, Mizuno Y. Failure to find mutations in ubiquitin carboxy-terminal hydrolase L1 gene in familial Parkinson's disease. 2000; 6:199-200.
83. Hoenicka J, Vidal L, Morales B, et al. Molecular findings in familial Parkinson disease in Spain. Arch Neurol 2002; 59:966-70.
84. Maraganore DM, Farrer MJ, Hardy JA, Lincoln SJ, McDonnell SK, Rocca WA. Case-control study of the ubiquitin carboxy-terminal hydrolase L1 gene in Parkinson's disease. Neurology 1999; 53:1858-60.
85. Wintermeyer P, Kruger R, Kuhn W, et al. Mutation analysis and association studies of the UCHL1 gene in German Parkinson's disease patients. Neuroreport 2000; 11:2079-82.
86. Zhang J, Hattori N, Leroy E, et al. Association between a polymorphism of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) gene and sporadic Parkinson's disease. 2000; 6: 195-197.
87. Momose Y, Murata M, Kobayashi K, et al. Association studies of multiple candidate genes for Parkinson's disease using single nucleotide polymorphisms. Ann Neurol 2002; 51:133-6.
88. Savettieri G, De Marco EV, Civitelli D, et al. Lack of association between ubiquitin carboxy-terminal hydrolase L1 gene polymorphism and PD. Neurology 2001; 57:560-1.
89. Levecque C, Destee A, Mouroux V, et al. No genetic association of the ubiquitin carboxy-terminal hydrolase-L1 gene S18Y polymorphism with familial Parkinson's disease. J Neural Transm 2001; 108:979-84.
90. Mellick GD, Silburn PA. The ubiquitin carboxy-terminal hydrolase-L1 gene S18Y polymorphism does not confer protection against idiopathic Parkinson's disease. Neurosci Lett 2000; 293:127-30.
91. Wang J, Zhao CY, Si YM, Liu ZL, Chen B, Yu L. ACT and UCH-L1 polymorphisms in Parkinson's disease and age of onset. Mov Disord 2002; 17:767-71.
92. Elbaz A, Levecque C, Clavel J, et al. S18Y polymorphism in the UCH-L1 gene and Parkinson's disease: evidence for an age-dependent relationship. Mov Disord 2003; 18: 130-7.
93. Liu Y, Fallon L, Lashuel HA, Liu Z, Lansbury PT, Jr. The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. Cell 2002; 111:209-18.
94. Funayama M, Hasegawa K, Kowa H, Saito M, Tsuji S, Obata F. A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. Ann Neurol 2002; 51:296-301.
95. Hasegawa K, Kowa H. Autosomal dominant familial Parkinson disease: older onset of age, and good response to levodopa therapy. Eur Neurol 1997; 38:39-43.
96. Scott WK, Grubber JM, Conneally PM, et al. Fine mapping of the chromosome 12 late-onset Alzheimer disease locus: potential genetic and phenotypic heterogeneity. Am J Hum Genet 2000; 66:922-32.
97. Pankratz N, Nichols WC, Uniacke SK, et al. Genome screen to identify susceptibility genes for Parkinson disease in a sample without parkin mutations. Am J Hum Genet 2002; 71: 124-35.
98. Saucedo-Cardenas O, Kardon R, Ediger TR, Lydon JP, Conneely OM. Cloning and structural organization of the gene encoding the murine nuclear receptor transcription factor, NURR1. Gene 1997; 187:135-9.
99. Zetterstrom RH, Williams R, Perlmann T, Olson L. Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. Brain Res Mol Brain Res 1996; 41:111-20.
100. Zetterstrom RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T. Dopamine neuron agenesis in Nurr1-deficient mice. Science 1997; 276:248-50.

101. Le W, Conneely OM, Zou L, et al. Selective agenesis of mesencephalic dopaminergic neurons in Nurr1-deficient mice. Exp Neurol 1999; 159:451-8.
102. Le W, Conneely OM, He Y, Jankovic J, Appel SH. Reduced Nurr1 expression increases the vulnerability of mesencephalic dopamine neurons to MPTP-induced injury. J Neurochem 1999; 73:2218-21.
103. Rawal N, Periquet M, Durr A, et al. Exclusion of the Nurr1 gene in autosomal recessive Parkinson's disease. J Neurol 2002; 249:1127-9.
104. Xu PY, Liang R, Jankovic J, et al. Association of homozygous 7048G7049 variant in the intron six of Nurr1 gene with Parkinson's disease. Neurology 2002; 58:881-4.
105. Zheng K, Heydari B, Simon DK. A common NURR1 polymorphism associated with Parkinson disease and diffuse Lewy body disease. Arch Neurol 2003; 60:722-5.
106. Tan EK, Chung H, Zhao Y, et al. Genetic analysis of Nurr1 haplotypes in Parkinson's disease. Neurosci Lett 2003; 347:139-42.
107. Le WD, Xu P, Jankovic J, et al. Mutations in NR4A2 associated with familial Parkinson disease. Nat Genet 2003; 33:85-9.
108. Healy DG, Sleiman P, Sweeney MG, Eunson LH, Wood NW. Exon 1 nurr1 gene mutations are rare in familial Parkinson's disease. Mov Disord 2002; 17(Suppl.5):61.[abstract].
109. Zimprich A, Asmus F, Leitner P, et al. Point mutations in exon 1 of the NR4A2 gene are not a major cause of familial Parkinson's disease. Neurogenetics 2003; 4:219-20.
110. Wellenbrock C, Hedrich K, Schafer N, et al. NR4A2 mutations are rare among European patients with familial Parkinson's disease. Ann Neurol 2003; 54:415.
111. Engelender S, Kaminsky Z, Guo X, et al. Synphilin-1 associates with alpha-synuclein and promotes the formation of cytosolic inclusions. Nat Genet 1999; 22:110-4.
112. Ribeiro CS, Carneiro K, Ross CA, Menezes JR, Engelender S. Synphilin-1 is developmentally localized to synaptic terminals, and its association with synaptic vesicles is modulated by alpha-synuclein. J Biol Chem 2002; 277:23927-33.
113. Engelender S, Wanner T, Kleiderlein JJ, et al. Organization of the human synphilin-1 gene, a candidate for Parkinson's disease. Mamm Genome 2000; 11:763-6.
114. Wakabayashi K, Engelender S, Yoshimoto M, Tsuji S, Ross CA, Takahashi H. Synphilin-1 is present in Lewy bodies in Parkinson's disease. Ann Neurol 2000; 47:521-3.
115. Wakabayashi K, Engelender S, Tanaka Y, et al. Immunocytochemical localization of synphilin-1, an alpha-synuclein-associated protein, in neurodegenerative disorders. Acta Neuropathol (Berl) 2002; 103:209-14.
116. Murray IJ, Medford MA, Guan HP, Rueter SM, Trojanowski JQ, Lee VM. Synphilin in normal human brains and in synucleinopathies: studies with new antibodies. Acta Neuropathol (Berl) 2003; 105:177-84.
117. Chung KK, Zhang Y, Lim KL, et al. Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. Nat Med 2001; 7:1144-50.
118. Ito T, Niwa JI, Hishikawa N, Ishigaki S, Doyu M, Sobue G. Dorfin localizes to Lewy bodies and ubiquitylates synphilin-1. J Biol Chem 2003; 15:15.
119. Scott WK, Nance MA, Watts RL, et al. Complete genomic screen in Parkinson disease: evidence for multiple genes. Jama 2001; 286:2239-44.
120. Farrer M, Destee A, Levecque C, et al. Genetic analysis of synphilin-1 in familial Parkinson's disease. Neurobiol Dis 2001; 8:317-23.
121. Bandopadhyay R, de Silva R, Khan N, et al. No pathogenic mutations in the synphilin-1 gene in Parkinson's disease. Neurosci Lett 2001; 307:125-7.
122. Marx FP, Holzmann C, Strauss KM, et al. Identification and functional characterization of a novel R621C mutation in the synphilin-1 gene in Parkinson's disease. Hum Mol Genet 2003; 12:1223-1231.
123. Gwinn-Hardy KA, Crook R, Lincoln S, et al. A kindred with Parkinson's disease not showing genetic linkage to established loci. Neurology 2000; 54:504-7.
124. Racette BA, Rundle M, Wang JC, et al. A multi-incident, Old-Order Amish family with PD. Neurology 2002; 58:568-74.
125. Nicholl DJ, Vaughan JR, Khan NL, et al. Two large British kindreds with familial Parkinson's disease: a clinico-pathological and genetic study. Brain 2002; 125:44-57.

126. Grimes DA, Grimes JD, Racacho L, et al. Large French-Canadian family with Lewy body parkinsonism: exclusion of known loci. Mov Disord 2002; 17:1205-12.
127. Bertoli Avella AM, Giroud Benitez JL, Bonifati V, et al. Suggestive linkage to chromosome 19 in a large Cuban family with late-onset Parkinson's disease. Mov Disord 2003; in press.
128. Lucking CB, Durr A, Bonifati V, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene. French Parkinson's Disease Genetics Study Group. N Engl J Med 2000; 342:1560-7.
129. Valente EM, Bentivoglio AR, Dixon PH, et al. Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35-p36. Am J Hum Genet 2001; 68:895-900.
130. van Duijn CM, Dekker MC, Bonifati V, et al. Park7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36. Am J Hum Genet 2001; 69:629-34.
131. Hampshire DJ, Roberts E, Crow Y, et al. Kufor-Rakeb syndrome, pallido-pyramidal degeneration with supranuclear upgaze paresis and dementia, maps to 1p36. J Med Genet 2001; 38:680-2.
132. Bonifati V, Rizzu P, van Baren MJ, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 2003; 299:256-9.
133. Li YJ, Scott WK, Hedges DJ, et al. Age at onset in two common neurodegenerative diseases is genetically controlled. Am J Hum Genet 2002; 70:985-93.
134. Yamamura Y, Sobue I, Ando K, Iida M, Yanagi T. Paralysis agitans of early onset with marked diurnal fluctuation of symptoms. Neurology 1973; 23:239-44.
135. Takahashi H, Ohama E, Suzuki S, et al. Familial juvenile parkinsonism: clinical and pathologic study in a family. Neurology 1994; 44:437-41.
136. Ishikawa A, Tsuji S. Clinical analysis of 17 patients in 12 Japanese families with autosomal-recessive type juvenile parkinsonism. Neurology 1996; 47:160-6.
137. Matsumine H, Saito M, Shimoda-Matsubayashi S, et al. Localization of a gene for an autosomal recessive form of juvenile Parkinsonism to chromosome 6q25.2-27. Am J Hum Genet 1997; 60:588-96.
138. Tassin J, Durr A, de Broucker T, et al. Chromosome 6-linked autosomal recessive early-onset Parkinsonism: linkage in European and Algerian families, extension of the clinical spectrum, and evidence of a small homozygous deletion in one family. The French Parkinson's Disease Genetics Study Group, and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Am J Hum Genet 1998; 63:88-94.
139. Jones AC, Yamamura Y, Almasy L, et al. Autosomal recessive juvenile parkinsonism maps to 6q25.2-q27 in four ethnic groups: detailed genetic mapping of the linked region. Am J Hum Genet 1998; 63:80-7.
140. Matsumine H, Yamamura Y, Kobayashi T, Nakamura S, Kuzuhara S, Mizuno Y. Early onset parkinsonism with diurnal fluctuation maps to a locus for juvenile parkinsonism. Neurology 1998; 50:1340-5.
141. Matsumine H, Yamamura Y, Hattori N, et al. A microdeletion of D6S305 in a family of autosomal recessive juvenile parkinsonism (PARK2). Genomics 1998; 49:143-6.
142. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392:605-8.
143. Sunada Y, Saito F, Matsumura K, Shimizu T. Differential expression of the parkin gene in the human brain and peripheral leukocytes. Neurosci Lett 1998; 254:180-2.
144. Kitada T, Asakawa S, Minoshima S, Mizuno Y, Shimizu N. Molecular cloning, gene expression, and identification of a splicing variant of the mouse parkin gene. Mamm Genome 2000; 11:417-21.
145. Lucking CB, Abbas N, Durr A, et al. Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism. The European Consortium on Genetic Susceptibility in Parkinson's Disease and the French Parkinson's Disease Genetics Study Group. Lancet 1998; 352:1355-6.
146. Hattori N, Kitada T, Matsumine H, et al. Molecular genetic analysis of a novel Parkin gene in Japanese families with autosomal recessive juvenile parkinsonism: evidence for variable homozygous deletions in the Parkin gene in affected individuals. Ann Neurol 1998; 44:935-41.

147. Leroy E, Anastasopoulos D, Konitsiotis S, Lavedan C, Polymeropoulos MH. Deletions in the Parkin gene and genetic heterogeneity in a Greek family with early onset Parkinson's disease. Hum Genet 1998; 103:424-7.
148. Hattori N, Matsumine H, Asakawa S, et al. Point mutations (Thr240Arg and Gln311Stop) [correction of Thr240Arg and Ala311Stop] in the Parkin gene. Biochem Biophys Res Commun 1998; 249:754-8.
149. Abbas N, Lucking CB, Ricard S, et al. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Hum Mol Genet 1999; 8:567-74.
150. Nisipeanu P, Inzelberg R, Blumen SC, et al. Autosomal-recessive juvenile parkinsonism in a Jewish Yemenite kindred: mutation of Parkin gene. Neurology 1999; 53:1602-4.
151. Klein C, Pramstaller PP, Kis B, et al. Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: expanding the phenotype. Ann Neurol 2000; 48:65-71.
152. Maruyama M, Ikeuchi T, Saito M, et al. Novel mutations, pseudo-dominant inheritance, and possible familial affects in patients with autosomal recessive juvenile parkinsonism. Ann Neurol 2000; 48:245-50.
153. Munoz E, Pastor P, Marti MJ, Oliva R, Tolosa E. A new mutation in the parkin gene in a patient with atypical autosomal recessive juvenile parkinsonism. Neurosci Lett 2000; 289: 66-8.
154. Hedrich K, Kann M, Lanthaler AJ, et al. The importance of gene dosage studies: mutational analysis of the parkin gene in early-onset parkinsonism. Hum Mol Genet 2001; 10:1649-56.
155. Farrer M, Chan P, Chen R, et al. Lewy bodies and parkinsonism in families with parkin mutations. Ann Neurol 2001; 50:293-300.
156. Lucking CB, Bonifati V, Periquet M, Vanacore N, Brice A, Meco G. Pseudo-dominant inheritance and exon 2 triplication in a family with parkin gene mutations. Neurology 2001; 57:924-7.
157. Bonifati V, Lucking CB, Fabrizio E, Periquet M, Meco G, Brice A. Three parkin gene mutations in a sibship with autosomal recessive early onset parkinsonism. J Neurol Neurosurg Psychiatry 2001; 71:531-4.
158. Pineda-Trujillo N, Carvajal-Carmona LG, Buritica O, et al. A novel Cys212Tyr founder mutation in parkin and allelic heterogeneity of juvenile Parkinsonism in a population from North West Colombia. Neurosci Lett 2001; 298:87-90.
159. Terreni L, Calabrese E, Calella AM, Forloni G, Mariani C. New mutation (R42P) of the parkin gene in the ubiquitinlike domain associated with parkinsonism. Neurology 2001; 56:463-6.
160. Alvarez V, Guisasola LM, Moreira VG, Lahoz CH, Coto E. Early-onset Parkinson's disease associated with a new parkin mutation in a Spanish family. Neurosci Lett 2001; 313:108-10.
161. Nisipeanu P, Inzelberg R, Abo Mouch S, et al. Parkin gene causing benign autosomal recessive juvenile parkinsonism. Neurology 2001; 56:1573-5.
162. Jeon BS, Kim JM, Lee DS, Hattori N, Mizuno Y. An apparently sporadic case with parkin gene mutation in a Korean woman. Arch Neurol 2001; 58:988-9.
163. Nichols WC, Pankratz N, Uniacke SK, et al. Linkage stratification and mutation analysis at the Parkin locus identifies mutation positive Parkinson's disease families. J Med Genet 2002; 39:489-92.
164. Hedrich K, Marder K, Harris J, et al. Evaluation of 50 probands with early-onset Parkinson's disease for Parkin mutations. Neurology 2002; 58:1239-46.
165. West A, Periquet M, Lincoln S, et al. Complex relationship between Parkin mutations and Parkinson disease. Am J Med Genet 2002; 114:584-91.
166. Wu RM, Shan DE, Sun CM, et al. Clinical, 18F-dopa PET, and genetic analysis of an ethnic Chinese kindred with early-onset parkinsonism and parkin gene mutations. Mov Disord 2002; 17:670-5.
167. Kann M, Jacobs H, Mohrmann K, et al. Role of parkin mutations in 111 community-based patients with early-onset parkinsonism. Ann Neurol 2002; 51:621-5.

168. Munoz E, Tolosa E, Pastor P, et al. Relative high frequency of the c.255delA parkin gene mutation in Spanish patients with autosomal recessive parkinsonism. J Neurol Neurosurg Psychiatry 2002; 73:582-4.
169. Periquet M, Latouche M, Lohmann E, et al. Parkin mutations are frequent in patients with isolated early-onset parkinsonism. Brain 2003; 126:1271-1278.
170. Rawal N, Periquet M, Lohmann E, et al. New parkin mutations and atypical phenotypes in families with autosomal recessive parkinsonism. Neurology 2003; 60:1378-81.
171. Khan NL, Graham E, Critchley P, et al. Parkin disease: a phenotypic study of a large case series. Brain 2003; 126:1279-1292.
172. Oliveira SA, Scott WK, Martin ER, et al. Parkin mutations and susceptibility alleles in late-onset Parkinson's disease. Ann Neurol 2003; 53:624-9.
173. Lohmann E, Periquet M, Bonifati V, et al. How much phenotypic variation can be attributed to parkin genotype? Ann Neurol 2003; 54:176-85.
174. Foroud T, Uniacke SK, Liu L, et al. Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. Neurology 2003; 60:796-801.
175. Gouider-Khouja N, Larnaout A, Amouri R, et al. Autosomal recessive parkinsonism linked to parkin gene in a Tunisian family. Clinical, genetic and pathological study. Parkinsonism Relat Disord 2003; 9:247-51.
176. Kim JS, Lee KS, Kim YI, Lee KH, Kim HT. Homozygous exon 4 deletion in parkin gene in a Korean family with autosomal recessive early onset parkinsonism. Yonsei Med J 2003; 44: 336-9.
177. Ujike H, Yamamoto M, Yamaguchi K, Kanzaki A, Takagi M, Kuroda S. [Two cases of sporadic juvenile Parkinson's disease caused by homozygous deletion of Parkin gene]. No To Shinkei 1999; 51:1061-4.
178. Tassin J, Durr A, Bonnet AM, et al. Levodopa-responsive dystonia. GTP cyclohydrolase I or parkin mutations? Brain 2000; 123:1112-21.
179. Kobayashi T, Wang M, Hattori N, Matsumine H, Kondo T, Mizuno Y. Exonic deletion mutations of the Parkin gene among sporadic patients with Parkinson's disease. Parkinsonism Relat Disord 2000; 6:129-131.
180. Bia H, Shao M, Dong X, et al. Preliminary studies on parkin gene deletion at exons 1 to 6 in Chinese patients with praecox Parkinson's disease. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 2000; 17:323-5.
181. Takei K, Yamamoto T, Asano Y, Iwasaki S, Shimazu K. [A case of autosomal recessive juvenile parkinsonism (AR-JP) with onset at the age of 6 years]. Rinsho Shinkeigaku 2000; 40:596-9.
182. Ujike H, Yamamoto M, Kanzaki A, Okumura K, Takaki M, Kuroda S. Prevalence of homozygous deletions of the parkin gene in a cohort of patients with sporadic and familial Parkinson's disease. Mov Disord 2001; 16:111-3.
183. Lu CS, Wu JC, Tsai CH, et al. Clinical and genetic studies on familial parkinsonism: the first report on a parkin gene mutation in a Taiwanese family. Mov Disord 2001; 16:164-6.
184. Kuroda Y, Mitsui T, Akaike M, Azuma H, Matsumoto T. Homozygous deletion mutation of the parkin gene in patients with atypical parkinsonism. J Neurol Neurosurg Psychiatry 2001; 71:231-4.
185. Pramstaller PP, Kunig G, Leenders K, et al. Parkin mutations in a patient with hemiparkinsonism-hemiatrophy: a clinical-genetic and PET study. Neurology 2002; 58: 808-10.
186. Xu Y, Liu Z, Wang Y, Tao E, Chen G, Chen B. [A new point mutation on exon 2 of parkin gene in Parkinson's disease]. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 2002; 19:409-11.
187. Tan LC, Tanner CM, Chen R, et al. Marked variation in clinical presentation and age of onset in a family with a heterozygous parkin mutation. Mov Disord 2003; 18:758-63.
188. Okuma Y, Hattori N, Mizuno Y. Sensory neuropathy in autosomal recessive juvenile parkinsonism (PARK2). Parkinsonism Relat Disord 2003; 9:313-4.
189. Chen R, Gosavi NS, Langston JW, Chan P. Parkin mutations are rare in patients with young-onset parkinsonism in a US population. Parkinsonism Relat Disord 2003; 9:309-12.
190. Inzelberg R, Hattori N, Nisipeanu P, et al. Camptocormia, axial dystonia, and parkinsonism: Phenotypic heterogeneity of a parkin mutation. Neurology 2003; 60:1393-4.

191. Illarioshkin SN, Periquet M, Rawal N, et al. Mutation analysis of the parkin gene in Russian families with autosomal recessive juvenile parkinsonism. Mov Disord 2003; 18:914-9.
192. Asakawa S, Tsunematsu K, Takayanagi A, et al. The genomic structure and promoter region of the human parkin gene. Biochem Biophys Res Commun 2001; 286:863-8.
193. West A, Farrer M, Petrucelli L, Cookson M, Lockhart P, Hardy J. Identification and characterization of the human parkin gene promoter. J Neurochem 2001; 78:1146-52.
194. Mata IF, Alvarez V, Garcia-Moreira V, et al. Single-nucleotide polymorphisms in the promoter region of the PARKIN gene and Parkinson's disease. Neurosci Lett 2002; 329: 149-52.
195. West AB, Maraganore D, Crook J, et al. Functional association of the parkin gene promoter with idiopathic Parkinson's disease. Hum Mol Genet 2002; 11:2787-92.
196. Hilker R, Klein C, Ghaemi M, et al. Positron emission tomographic analysis of the nigrostriatal dopaminergic system in familial parkinsonism associated with mutations in the parkin gene. Ann Neurol 2001; 49:367-76.
197. Khan NL, Brooks DJ, Pavese N, et al. Progression of nigrostriatal dysfunction in a parkin kindred: an [18F]dopa PET and clinical study. Brain 2002; 125:2248-56.
198. Kobayashi T, Matsumine H, Zhang J, Imamichi Y, Mizuno Y, Hattori N. Pseudo-autosomal dominant inheritance of PARK2: two families with parkin gene mutations. J Neurol Sci 2003; 207:11-7.
199. Periquet M, Lucking C, Vaughan J, et al. Origin of the mutations in the parkin gene in Europe: exon rearrangements are independent recurrent events, whereas point mutations may result from Founder effects. Am J Hum Genet 2001; 68:617-26.
200. Lincoln S, Wiley J, Lynch T, et al. Parkin-proven disease: Common founders but divergent phenotypes. Neurology 2003; 60:1605-10.
201. Wang M, Hattori N, Matsumine H, et al. Polymorphism in the parkin gene in sporadic Parkinson's disease. Ann Neurol 1999; 45:655-8.
202. Satoh J, Kuroda Y. Association of codon 167 Ser/Asn heterozygosity in the parkin gene with sporadic Parkinson's disease. Neuroreport 1999; 10:2735-9.
203. Klein C, Schumacher K, Jacobs H, et al. Association studies of Parkinson's disease and parkin polymorphisms. Ann Neurol 2000; 48:126-7.
204. Hu CJ, Sung SM, Liu HC, Lee CC, Tsai CH, Chang JG. Polymorphisms of the parkin gene in sporadic Parkinson's disease among Chinese in Taiwan. Eur Neurol 2000; 44:90-3.
205. Oliveri RL, Zappia M, Annesi G, et al. The parkin gene is not involved in late-onset Parkinson's disease. Neurology 2001; 57:359-62.
206. Mellick GD, Buchanan DD, Hattori N, et al. The parkin gene S/N167 polymorphism in Australian Parkinson's disease patients and controls. 2001; 7:89-91.
207. Eerola J, Launes J, Hellstrom O, Tienari PJ. Apolipoprotein E (APOE), PARKIN and catechol-O-methyltransferase (COMT) genes and susceptibility to sporadic Parkinson's disease in Finland. Neurosci Lett 2002; 330:296-8.
208. Peng R, Gou Y, Yuan Q, et al. Mutation screening and association analysis of the parkin gene in Parkinson's disease patients from South-West China. Eur Neurol 2003; 49:85-9.
209. Oliveira SA, Scott WK, Nance MA, et al. Association study of Parkin gene polymorphisms with idiopathic Parkinson disease. Arch Neurol 2003; 60:975-80.
210. Mori H, Kondo T, Yokochi M, et al. Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology 1998; 51:890-2.
211. Hayashi S, Wakabayashi K, Ishikawa A, et al. An autopsy case of autosomal-recessive juvenile parkinsonism with a homozygous exon 4 deletion in the parkin gene. Mov Disord 2000; 15:884-8.
212. Yamamura Y, Hattori N, Matsumine H, Kuzuhara S, Mizuno Y. Autosomal recessive early-onset parkinsonism with diurnal fluctuation: clinicopathologic characteristics and molecular genetic identification. Brain Dev 2000; 22:S87-91.
213. van de Warrenburg BP, Lammens M, Lucking CB, et al. Clinical and pathologic abnormalities in a family with parkinsonism and parkin gene mutations. Neurology 2001; 56:555-7.
214. Morales B, Martinez A, Gonzalo I, et al. Steele-Richardson-Olszewski syndrome in a patient with a single C212Y mutation in the parkin protein. Mov Disord 2002; 17:1374-80.

215. Rajput AH, Uitti RJ, Sudhakar S, Rozdilsky B. Parkinsonism and neurofibrillary tangle pathology in pigmented nuclei. Ann Neurol 1989; 25:602-6.
216. Broussolle E, Lucking CB, Ginovart N, Pollak P, Remy P, Durr A. [18 F]-dopa PET study in patients with juvenile-onset PD and parkin gene mutations. Neurology 2000; 55:877-9.
217. Portman AT, Giladi N, Leenders KL, et al. The nigrostriatal dopaminergic system in familial early onset parkinsonism with parkin mutations. Neurology 2001; 56:1759-62.
218. Thobois S, Ribeiro MJ, Lohmann E, et al. Young-onset Parkinson disease with and without parkin gene mutations: a fluorodopa F 18 positron emission tomography study. Arch Neurol 2003; 60:713-8.
219. Hilker R, Klein C, Hedrich K, et al. The striatal dopaminergic deficit is dependent on the number of mutant alleles in a family with mutations in the parkin gene: evidence for enzymatic parkin function in humans. Neurosci Lett 2002; 323:50-4.
220. Morett E, Bork P. A novel transactivation domain in parkin. Trends Biochem Sci 1999; 24: 229-31.
221. Tashiro M, Okubo S, Shimotakahara S, et al. NMR structure of ubiquitin-like domain in PARKIN: Gene product of familial Parkinson's disease. J Biomol NMR 2003; 25:153-6.
222. Sakata E, Yamaguchi Y, Kurimoto E, et al. Parkin binds the Rpn10 subunit of 26S proteasomes through its ubiquitin-like domain. EMBO Rep 2003; 4:301-6.
223. Upadhya SC, Hegde AN. A potential proteasome-interacting motif within the ubiquitin-like domain of parkin and other proteins. Trends Biochem Sci 2003; 28:280-3.
224. Shimura H, Hattori N, Kubo S, et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat Genet 2000; 25:302-5.
225. Imai Y, Soda M, Takahashi R. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. J Biol Chem 2000; 275:35661-4.
226. Zhang Y, Gao J, Chung KK, Huang H, Dawson VL, Dawson TM. Parkin functions as an E2-dependent ubiquitin- protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proc Natl Acad Sci U S A 2000; 97:13354-9.
227. Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell 2001; 105:891-902.
228. Shimura H, Schlossmacher MG, Hattori N, et al. Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. Science 2001; 293:263-9.
229. Staropoli JF, McDermott C, Martinat C, Schulman B, Demireva E, Abeliovich A. Parkin is a component of an SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainate excitotoxicity. Neuron 2003; 37:735-49.
230. Corti O, Hampe C, Koutnikova H, et al. The p38 subunit of the aminoacyl-tRNA synthetase complex is a Parkin substrate: linking protein biosynthesis and neurodegeneration. Hum Mol Genet 2003; 12:1427-37.
231. Honbou K, Suzuki NN, Horiuchi M, et al. The crystal structure of DJ-1, a protein related to male fertility and Parkinson's disease. J Biol Chem 2003; 278:31380-4.
232. Tao X, Tong L. Crystal structure of human DJ-1, a protein associated with early onset Parkinson's disease. J Biol Chem 2003; 278:31372-9.
233. Wilson MA, Collins JL, Hod Y, Ringe D, Petsko GA. The 1.1-A resolution crystal structure of DJ-1, the protein mutated in autosomal recessive early onset Parkinson's disease. Proc Natl Acad Sci U S A 2003; 100:9256-61.
234. Huai Q, Sun Y, Wang H, et al. Crystal structure of DJ-1/RS and implication on familial Parkinson's disease. FEBS Lett 2003; 549:171-5.
235. Taira T, Takahashi K, Kitagawa R, Iguchi-Ariga SM, Ariga H. Molecular cloning of human and mouse DJ-1 genes and identification of Sp1-dependent activation of the human DJ-1 promoter. Gene 2001; 263:285-92.
236. Mitsumoto A, Nakagawa Y. DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic Res 2001; 35:885-93.
237. Mitsumoto A, Nakagawa Y, Takeuchi A, Okawa K, Iwamatsu A, Takanezawa Y. Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. Free Radic Res 2001; 35:301-10.

238. de Nobel H, Lawrie L, Brul S, et al. Parallel and comparative analysis of the proteome and transcriptome of sorbic acid-stressed Saccharomyces cerevisiae. Yeast 2001; 18: 1413-28.
239. Trotter EW, Kao CM, Berenfeld L, Botstein D, Petsko GA, Gray JV. Misfolded proteins are competent to mediate a subset of the responses to heat shock in Saccharomyces cerevisiae. J Biol Chem 2002; 277:44817-25.
240. Giasson BI, Ischiropoulos H, Lee VM, Trojanowski JQ. The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. Free Radic Biol Med 2002; 32:1264-75.
241. Zoghbi HY, Botas J. Mouse and fly models of neurodegeneration. Trends Genet 2002; 18: 463-71.
242. Hyun DH, Lee M, Hattori N, et al. Effect of wild-type or mutant Parkin on oxidative damage, nitric oxide, antioxidant defenses, and the proteasome. J Biol Chem 2002; 277: 28572-7.
243. Hashimoto M, Hsu LJ, Rockenstein E, Takenouchi T, Mallory M, Masliah E. alpha-Synuclein protects against oxidative stress via inactivation of the c-Jun N-terminal kinase stress-signaling pathway in neuronal cells. J Biol Chem 2002; 277:11465-72.
244. Hague S, Rogaeva E, Hernandez D, et al. Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation. Ann Neurol 2003; 54:271-4.
245. Abou-Sleiman PM, Healy DG, Quinn N, Lees AJ, Wood NW. The role of pathogenic DJ-1 mutations in Parkinson's disease. Ann Neurol 2003; 54:283-6.
246. Hedrich K, Djarmati A, Schäfer N, et al. DJ-1 mutations are less frequent than parkin mutations in early-onset Parkinson's disease. Submitted to Neurology.
247. Valente EM, Bentivoglio AR, Cassetta E, et al. DYT13, a novel primary torsion dystonia locus, maps to chromosome 1p36.13--36.32 in an Italian family with cranial-cervical or upper limb onset. Ann Neurol 2001; 49:362-6.
248. Rizzu P, Hinkle DA, Zhucareva V, et al. DJ-1 co-localizes with pathological tau inclusions: a possible link between Parkinsonism and dementia. Ann Neurol in press.
249. Valente EM, Brancati F, Ferraris A, et al. PARK6-linked parkinsonism occurs in several European families. Ann Neurol 2002; 51:14-8.
250. Khan NL, Valente EM, Bentivoglio AR, et al. Clinical and subclinical dopaminergic dysfunction in PARK6-linked parkinsonism: an 18F-dopa PET study. Ann Neurol 2002; 52: 849-53.
251. Bonifati V, Breedveld GJ, Squitieri F, et al. Localization of autosomal recessive early-onset parkinsonism to chromosome 1p36 (PARK7) in an independent dataset. Ann Neurol 2002; 51:253-6.
252. Bentivoglio AR, Cortelli P, Valente EM, et al. Phenotypic characterisation of autosomal recessive PARK6-linked parkinsonism in three unrelated Italian families. Mov Disord 2001; 16:999-1006.
253. Dekker M, Bonifati V, Van Swieten J, et al. Clinical features and neuroimaging of PARK7-linked parkinsonism. Mov Disord 2003; 18:751-7.
254. Anderson KE, Weiner WJ. Psychiatric symptoms in Parkinson's disease. Curr Neurol Neurosci Rep 2002; 2:303-9.
255. Najim al-Din AS, Wriekat A, Mubaidin A, Dasouki M, Hiari M. Pallido-pyramidal degeneration, supranuclear upgaze paresis and dementia: Kufor-Rakeb syndrome. Acta Neurol Scand 1994; 89:347-52.
256. Risch N. Evolving methods in genetic epidemiology. II. Genetic linkage from an epidemiologic perspective. Epidemiol Rev 1997; 19:24-32.
257. Risch N, Merikangas K. The future of genetic studies of complex human diseases. Science 1996; 273:1516-7.
258. Risch NJ. Searching for genetic determinants in the new millennium. Nature 2000; 405: 847-56.
259. Foltynie T, Sawcer S, Brayne C, Barker RA. The genetic basis of Parkinson's disease. J Neurol Neurosurg Psychiatry 2002; 73:363-70.
260. DeStefano AL, Golbe LI, Mark MH, et al. Genome-wide scan for Parkinson's disease: the GenePD Study. Neurology 2001; 57:1124-6.

261. Heutink P, Oostra BA. Gene finding in genetically isolated populations. Hum Mol Genet 2002; 11:2507-15.
262. Craig SP, Buckle VJ, Lamouroux A, Mallet J, Craig IW. Localization of the human dopamine beta hydroxylase (DBH) gene to chromosome 9q34. Cytogenet Cell Genet 1988; 48:48-50.
263. Ozelius LJ, Hewett JW, Page CE, et al. The early-onset torsion dystonia gene (DYT1) encodes an ATP-binding protein. Nat Genet 1997; 17:40-8.
264. Wilhelmsen KC, Weeks DE, Nygaard TG, et al. Genetic mapping of Lubag (X-linked dystonia-parkinsonism) in a Filipino kindred to the pericentromeric region of the X chromosome. Ann Neurol 1991; 29:124-31.
265. Heutink P. Untangling tau-related dementia. Hum Mol Genet 2000; 9:979-86.
266. Martin ER, Scott WK, Nance MA, et al. Association of single-nucleotide polymorphisms of the tau gene with late-onset Parkinson disease. Jama 2001; 286:2245-50.

Chapter 1.2

Pathogenesis of Parkinson's disease – the contribution of α-synuclein and parkin

This chapter reviews the available data on the pathogenesis of PD forms caused by mutations in *α-synuclein* and *parkin*, and discusses the implications of this growing body of knowledge for the understanding of the mechanisms underlying the common forms of the disease.

THE CONTRIBUTION OF α-SYNUCLEIN

There is no doubt that the first monogenic form of PD (PARK1) has led to a landmark discovery: α-synuclein is a key player in the pathogenesis of both autosomal dominant PD and idiopathic PD. While missense variants (A53T, A30P) of this protein are a very rare cause of autosomal dominant PD,[1,2] wild type α-synuclein is one of the major components of the Lewy bodies in all forms of PD, and other synucleinopathies.[3,4] In the last three years, several transgenic animal models have been generated that over-express wild type or mutant human α-synuclein. These models display varying degrees of biochemical, pathological and clinical abnormalities reminiscent of PD, and further support the contention that α-synuclein is primarily implicated in the pathogenesis of PD in general.[5-7]
α-Synuclein is a 140 amino acid protein, which has been highly conserved in evolution; it is abundant in neurons and enriched in the presynaptic compartment.[8] Although its exact function remains unknown, an involvement in synaptic plasticity,[8] and in regulation of size[9] and turnover[10] of synaptic vesicles has been suggested. Mice in which the *α-synuclein* gene has been knocked out possess a normal number of dopaminergic neurons and synapses, but show mild reduction in striatal dopamine levels, and abnormalities in the amphetamine-induced responses, suggesting a role for α-synuclein in the regulation of the dopaminergic neurotransmission.[11] *α-Synuclein* KO mice do not develop PD-like pathology, in keeping with the role of this protein in PD being due to a "gain of function" rather than a "loss of function". However, the possibility that loss of the normal function of α-synuclein might contribute to the disease progression cannot be excluded, and the expression of A53T and A30P alleles appears decreased in advanced disease stages.[12,13]
The α-synuclein protein contains a N-terminal amphipathic region, a central region, which includes the amyloidogenic peptide NAC (Non-Aβ Component of Alzheimer's disease amyloid), and a C-terminal acidic region. Six imperfect repeats of 11 amino acids, containing the KTKEGV consensus motif, are present within the first 95 amino acids. These repeats confer a variation in hydrophobicity, which is typical of the amphipathic α-helix of the lipid-binding domain in apolipoproteins.[8,14] And indeed, α-synuclein shows homology with fatty acid binding proteins,[8,15] but also with the 14-3-3 family of molecular chaperones,[16] and it displays inhibitory activity on phospholipases.[17]
α-Synuclein is natively unfolded, and its secondary structure is critically dependent from interaction with lipid membranes, which markedly increase the alpha-helix content.[18]
Biochemical studies have shown that α-synuclein displays a concentration-dependent property to form oligomeric species (also called protofibrils) and amyloid β-sheet fibrils *in vitro*.[19,20] Oligomers are believed to represent the precursors of higher order aggregates (fibrils) *in vivo*, which are therefore assembled in the filamentous structures seen in Lewy bodies and Lewy neurites. In different neurodegenerative diseases, the oligomers, and

not the mature fibrils, are suggested to be the real neurotoxic molecules.[21-25] In support of this view, increased oligomerization, not fibrillization, is the shared property of the two known PD-linked mutations.[26, 27]

It is therefore critical to understand the factors governing the formation, and controlling the steady state levels of oligomers in the common forms of the disease. Several biophysical and biochemical studies investigated α-synuclein aggregation, but only recently, the oligomerization process has been specifically addressed and the factors involved are beginning to be characterized. It is possible that small amounts of α-synuclein oligomers are normally formed in the neuron as well, and evidence for the existence of oligomers in normal conditions has indeed been obtained recently.[28]

Factors enhancing α-synuclein fibrillization *in vitro* include heavy metals and pesticides (implicated in PD on the basis of epidemiological studies),[29-31] oxidative stress,[32] and heparin[33], but how these factors relate to the formation of oligomers is unknown. On the other hand, it is known that molecular crowding,[34] interaction with calcium, possibly via calmodulin[35, 36] interaction with lipid membranes[37] and polyunsaturated fatty acids[28, 38] promote and/or enhance oligomerization, whereas saturated fatty acids decrease the oligomerization.[28]

Furthermore, studies in cell culture and animal models have shown that mitochondrial inhibitors like rotenone and paraquat,[39-41] oxidative[42, 43] and nitrative[44] conditions, and proteasomal inhibition,[45] are all associated with increased α-synuclein fibrillization with formation of intracellular aggregates.

Post-translational modifications of α-synuclein, including transglutaminase mediated cross-linking,[46] phosphorylation,[47], nitration,[48] and mono- and diubiquitylation[49] likely contribute to the formation and/or stabilization of aggregates. On the other hand, magnesium[31], β-synuclein and γ-synuclein[50] appear to inhibit the fibrillization of α-synuclein. β-Synuclein markedly decreased formation of α-synuclein inclusions and prevented neurodegeneration in α-synuclein/β-synuclein double transgenic mouse.[51]

Intrinsic biophysical properties of α-synuclein are probably conferring a high neurotoxic potential to this protein, in analogy to other critical proteins which are pivotal in other neurodegenerative diseases, like tau and β-amyloid for AD, or PrP for prion disease. Recent evidence suggests that interactions between tau and α-synuclein can synergistically promote the polymerization of both proteins,[52] in keeping with reports of co-localization of α-synuclein and tau epitopes in Lewy bodies.[53, 54]

The regulation of α-synuclein neuronal levels is likely subject to tight control, and a major hypothesis is that the primary abnormality in PD is an abnormally increased α-synuclein expression, perhaps giving rise to an excess of oligomers. The factors controlling the expression of *α-synuclein* in human neurons remain largely unexplored and this appears a very promising area for future studies. The expression of *α-synuclein* might be regulated by the transcription factors engrailed-1 and engrailed-2,[55] by nerve growth factor,[56] and by steroid hormones.[57]

Studies of the human *α-synuclein* promoter in cell systems suggest that high- and low-expression alleles exist,[58] but clear evidence of overrepresentation of the high-expression alleles in PD is lacking so far. Intriguingly, increased expression of *α-synuclein* and decreased expression of *β-synuclein* has been reported in brains from patients with diffuse Lewy body disease, suggesting that an imbalance between expression of the different synucleins might be pathogenic,[59] and that smaller peptides mimicking β-synuclein effects might have therapeutic potential in PD.[60] However, another study did not find changes in α-synuclein expression in PD brain.[61]

The other possibility is that in PD there is a primary decrease in the clearance of α-synuclein and/or its oligomers. Direct interaction between α-synuclein and a proteasomal

subunit has been reported.[62, 63] However, whether α-synuclein is degraded by the proteasome or by different systems remains controversial, with evidence in favour,[64, 65] including ubiquitin-independent proteasomal processing,[66] and evidence against it.[65, 67] Other studies suggest that calpain (a cysteine protease),[68] and the lysosomes[44, 65] are also involved in cleavage and degradation of α-synuclein. Intriguingly, reduced activity of the proteasome subunits have been found in PD brains,[69] and two other genes implicated in monogenic PD (*parkin* and *UCH-L1*) suggest an involvement of the ubiquitin-proteasome pathway. Last, proteasomal subunits, ubiquitylated proteins, and molecular chaperones are present in Lewy bodies, further implicating the protein clearance system in the pathogenesis.[7] However, a definite link between α-synuclein and the proteasome remains to be proven, and it is not certain whether α-synuclein (or a modified form) is a parkin substrate *in vivo*. Other pathways might be crucial for α-synuclein degradation and for PD pathogenesis. This is another very important area for future investigation.

How is α-synuclein neurotoxic?

Apart from the questions of how an abnormal oligomerization of α-synuclein is determined in PD, another central problem is how monomeric or oligomeric α-synuclein exerts its toxicity. This remains unclear, but several possibilities have been suggested, including a direct inhibition of the proteasome system,[63, 70, 71] an impairment of mitochondrial function[72] and derangement in cellular trafficking[73]. As a further mechanism, it has been proposed that α-synuclein oligomers can form pore-like structures similar to bacterial toxins, which are able to damage synaptic vesicles.[74, 75] Mutant PD-linked α-synuclein oligomers would have even greater potential to damage vesicles.[76] In the case of the dopaminergic neuron, the release of dopamine in the presynaptic cytosol would lead to oxidative stress.

Any kind of selective interaction between α-synuclein and dopamine has the potential to explain the relative selectivity of the PD neurodegenerative process for the dopaminergic neurons. In this regard, it is interesting that in a neuronal culture system, the toxicity of α-synuclein requires endogenous dopamine production, as it is abolished by tyrosine hydroxylase inhibition, and it seems mediated by reactive oxygen species.[77]

And indeed, α-synuclein and dopamine might be linked at many levels. For example, overexpression of α-synuclein inhibits the activity of tyrosine hydroxylase, the rate-limiting enzyme in the dopamine synthetic pathway,[78] as well as the expression of genes involved in the dopamine biosynthesis, possibly by down-regulating *NR4A2*.[79] This could represent a protective response against the toxicity of excessive α-synuclein levels. Intriguingly, in the last report, both PD-linked *α-synuclein* mutants were unable to down-regulate the expression of dopamine-synthetic enzymes, suggesting that defective down-regulation of the dopamine synthetic pathway is pathogenic.[79] Any links between *α-synuclein* and *NR4A2* pathways is very interesting because *NR4A2* mutations have been described in families with PD. These mutations appear associated with decreased expression of *NR4A2* and its controlled genes,[80] but effects on *α-synuclein* expression are also possible and these remain unexplored.

Other studies suggest that α-synuclein interacts with, and enhances the activity of the dopamine transporter,[81] and it inhibits the monoamine vesicular transporter.[82] Together with a direct damage to vesicles by pore-like oligomers, these effects would converge to the common endpoint of increasing the cytosolic levels of free dopamine, leading to oxidative stress. Moreover, the oxidative metabolite dopamine quinone can form adducts with the same α-synuclein, and these adducts inhibit the conversion of

α-synuclein oligomers to higher aggregates, further reinforcing the oligomer-mediated toxicity.[83] Interestingly, oxidative modifications,[84] including formation of dityrosine cross-links,[27, 85] as well as nitrative modifications,[86] have been independently reported to promote/stabilize α-synuclein oligomers and inhibit the formation of fibrils.
The links between α-synuclein and dopaminergic neurons are also highlighted by evidence that *α-synuclein* overexpression might enhance the toxicity of MPTP,[87] whereas *α-synuclein* KO mice are resistant to the toxicity of MPTP,[88, 89] a selective dopaminergic neurotoxin, which inhibits mitochondrial complex I, but also leads to dopamine redistribution from vesicles to cytosol and dopamine-mediated oxidative stress.[90]

Several studies in cell culture systems have linked the overexpression of wild type and especially mutant *α-synuclein* to mitochondrial dysfunction,[72] oxidative stress,[72, 91-93] susceptibility to dopamine-mediated toxicity,[94, 95] proteasomal inhibition,[71, 93] and apoptosis.[71, 93] It is of interest that in other cell systems, α-synuclein exerted protection against oxidative stress[96] or anti-apoptotic properties,[97] and protected mice from paraquat-induced neurodegeneration.[98] These findings illustrate the complexity of the α-synuclein pathways and its possible functions, as well as the possible confounding effects of the different cell types, levels of expressions, and different experimental paradigms. More importantly, many reciprocal influences are known between protein misfolding, oligomerization, proteasome inhibition, activation of unfolded protein response, oxidative stress, and mitochondria damage, leading to many possible vicious cycles, and ultimately to neuronal cell death. Furthermore, all of these mechanisms have been implicated in the pathogenesis of common PD, but it is difficult to disentangle the primary and secondary events.

The role of Lewy bodies
The role of the highly ordered, filamentous inclusion bodies (Lewy bodies) is controversial, as they might be neurotoxic, inert, or even protective for the neuron. The presence of ubiquitylated proteins, proteasomal subunits, and molecular chaperones in the Lewy bodies, as well as in other aggregates found in neurodegenerative diseases, implicates the protein clearance system in the pathogenesis.
Lewy bodies are observed in the surviving, and otherwise healthy neurons. They can function to eliminate toxic soluble species (oligomers) by sequestering them in insoluble non-toxic form. This could be a compensatory mechanism in situations where the protein quality control system (proteasome, chaperones and other systems) is insufficient or overloaded by misfolded, or toxic proteins. This view is supported by the fact that Lewy bodies, as well as other neurodegenerative disease-associated inclusion bodies, resembles the so-called aggresomes.[99]
Aggresomes are microtubule-dependent inclusion bodies to which aggregated proteins are specifically and actively delivered. They are usually single, perinuclear structures, located close to the centrosome (the microtubule organizing centre). They contain molecular chaperones, proteasomal subunits, and a peripheral cage-shaped ring of intermediate filaments (typically vimentin). Aggresomes are believed to be part of the cell repertoire of responses to protein misfolding and aggregation, as they can sequester oligomers or smaller aggregates of toxic proteins, accumulating them perhaps for more efficient disposal via autophagy.[100] Lewy bodies display many similarities to aggresomes, including the morphology, presence of chaperones, proteasomal subunits, and other aggresomal markers like γ-tubulin and pericentrin.[101, 102] Last, studies in an inducible mice model of Huntington's disease (HD) indicate

that formation of intranuclear inclusions in HD are reversible, suggesting that a continuous influx of misfolded proteins is needed to maintain the inclusions also in other neurodegenerative diseases.[103]

The role of molecular chaperones

Our knowledge about the role of molecular chaperones in neurodegeneration is gaining momentum. These proteins function in assisting the proper folding of nascent polypeptides, and refolding of damaged proteins; they are also involved in targeting and/or delivering of protein to the proteasomal system for degradation.[104] Studies in transgenic animal models and cell systems provided convincing evidence that manipulation of the chaperone and of the ubiquitin systems influences markedly the pathogenesis. In rodent and fly models of different neurodegenerative diseases (including SCA1, SCA3, HD, and α-synuclein), the overexpression of chaperones reduces, whereas interference on the chaperones aggravates neurotoxicity.[105-109] Interestingly, these effects are not accompanied by visible modification of aggregates.[7] On the contrary, interference with the ubiquitylation (namely by inactivating an ubiquitin ligase) enhances pathogenesis but also markedly reduces the formation of aggregates.[110]

Taken as a whole, these studies strongly support the contention that inclusions are not primarily pathogenic, and they might actually be neuroprotective. However, the presence of molecular chaperones, proteasomal subunits, cytoskeletal components, and many other proteins in the Lewy bodies, suggests that the inclusions could also be detrimental for the cell by sequestering these and other important components. Lewy bodies could therefore have a "dual" role, being neuroprotective and neurotoxic, perhaps depending from the disease stage, or the age of the brain. The absence of Lewy bodies in PARK2 is associated with an accelerated loss of nigral neurons and onset of symptoms, but with slower progression of disease, in keeping with such a dual role of the inclusions.

PATHOGENESIS OF PARKIN DISEASE

The discovery of *parkin* mutations in PARK2 has provided a second landmark contribution to the understanding of PD.[111, 112] The results of large and comprehensive studies indicate that mutations in this gene are found in about half of the families with early-onset PD compatible with recessive inheritance, and in about 20% of the sporadic cases with early onset PD (chapters 2.1, 2.4).[113, 114] The fact that *parkin* mutations are a frequent cause of early onset PD supports the contention that the parkin protein is another key player in PD in general.

The function of the parkin protein

The parkin protein is widely expressed in neurons but also in glial cells.[115, 116] In studies using different techniques and different cell types, the parkin protein was localized at the level of the Golgi complex and cytosol,[117] with endoplasmic reticulum and large cytosolic vesicles,[115] in association with actin filaments,[118] synaptic vesicles,[119, 120] postsynaptic densities and lipid drafts,[121] the nuclear matrix,[122] and the outer mitochondrial membrane.[123]

Parkin possesses ubiquitin-ligase activity.[124-126] Covalent attachment of the ubiquitin (Ub) polypeptide (ubiquitylation) tags proteins for proteasomal degradation, and this is a fundamental mechanism for the protein quality control system.[127] The Ub system

involves a series of enzymatic steps: a first ubiquitin-activating enzyme (E1) uses ATP to confer Ub a high-energy state. Then Ub is transferred to an Ub-conjugating enzyme (E2) which in cooperation with an Ub-ligase (E3) transfers the ubiquitin to the final substrate. Only one E1 gene is known in the human genome, whereas there are a few E2 and hundreds of E3 genes. Most of the substrate specificity of the ubiquitylation process appears therefore contributed by the E3s. In some instances, a fourth enzyme (E4) enhances the formation of the poli-ubiquitin chain, which is a preferential signal for proteasomal degradation.[127] Parkin interacts through its RING-IBR-RING domains with different E2 enzymes, including the cytosolic UbcH7 and UbcH8,[124-126] and with the endoplasmic reticulum associated UBC6 and UBC7.[128] Parkin also ubiquitylates itself and promotes its own proteasomal degradation.[126, 129]

Parkin, a protein with many interactors

PARK2 is in most cases a classical recessive disease, suggesting that the loss of parkin function is pathogenic. Moreover, several disease-causing missense mutations in parkin abolish the Ub-ligase activity.[124, 126, 130] To the extent that loss of this E3 function is the culprit in parkin disease, an accumulation of non-ubiquitylated substrates is important in the pathogenesis. The identification of parkin substrates is therefore of great interest.
Yeast two-hybrid screens and other approaches have provided several putative candidates, including: CDCrel-1, a synaptic vesicle associated protein;[126] synphilin-1, an α-synuclein-interacting protein;[130] the putative G protein-coupled transmembrane PAEL-receptor (parkin-associated endothelin receptor-like receptor),[128] αSp22, a new brain-specific glycosylated form of α-synuclein,[131] CASK, a postsynaptic protein,[121] cyclin-E, a protein linked to cell cycle regulation and neuronal apoptosis,[132] p38, a component of the tRNA-aminoacyl synthetase complex,[133] and lastly, α- and β-tubulins.[134]

CDCrel-1 and the septins

The CDCrel-1 protein (sept-5) belongs to a family of proteins (septins) classically involved in cytokinesis, exocytosis and cellular morphogenesis.[135] CDCrel-1 is predominantly expressed in the nervous system where it may regulate synaptic vesicle dynamics. Whether abnormal metabolism of sept-5 might affect the dopaminergic neurotransmission, remains unknown. Moreover, there are other findings linking septins to neurodegeneration: sept-4 is present in Lewy bodies and interacts with α-synuclein,[136] and sept-1, sept-2 and sept-4 are present in neurofibrillary tangles.[137] Last, sept-4 is localized to mitochondria and signals in a pro-apoptotic pathway.[138]

CASK and the postsynaptic compartment

On the basis of the presence of a PDZ (postsynaptic density 95, disc large, zona occludens) binding motif at its C-terminus, parkin was shown to bind the PDZ protein CASK (calcium/calmodulin-dependent serine protein kinase), and to co-localize with CASK at the synapse and post-synaptic densities and lipid drafts in brain.[121] A protein complex formed by CASK, Velis and Mint, is implicated in the transport of the NR2B subunit of the NMDA (*N*-methyl D-aspartate) receptor, which is critical for the postsynaptic plasticity.[139] Indeed, other components involved in the scaffolding or signalling of the NMDA receptor (including PSD-95, the NR2B subunit, the calmodulin-dependent kinase II (CaMKII), and homer 1a) were co-immunoprecipitated with parkin, suggesting that they might associate via CASK.[121] Since parkin does not ubiquitylate CASK, it is possible that CASK serves to scaffold parkin at postsynaptic complexes, in order to target the ubiquitylation of other synaptic proteins.[121] The CDCrel-1 protein

and CASK are once again bringing the attention to the synapse, as α-synuclein did, suggesting synaptic derangement as alternative common pathway in PD. There is increasing evidence that ubiquitylation is important for synaptic function.[140]

PAEL receptor and the endoplasmic reticulum stress

The function of the PAEL receptor is unknown, but when overexpressed, PAEL becomes unfolded and induces endoplasmic reticulum (ER) stress and the unfolded protein response (UPR).[128] The UPR is a complex adaptive cellular pathway, which can be triggered by perturbations in redox state, calcium levels, elevated protein synthesis, altered glycosylation, proteasomal inhibition, and other causes of misfolded protein overload in the ER.[141] The UPR includes the coordinated transcriptional and post-transcriptional regulation of many genes, leading to increased activity of chaperones, increased protein degradation via the ubiquitin-proteasome system, and decreased protein synthesis.[141] In case of failure of these responses to counteract misfolded protein overload, a prolonged activation of UPR leads to cell death by several redundant pathways for caspase activation, including mitochondrial-dependent and direct activation.[141]

Expression profiling in cell systems exposed to toxins which induce dopaminergic neuronal loss and experimental parkinsonism (6-OH-dopamine, MPTP, and rotenone) revealed strong induction of the UPR, lending further support to the contention that UPR is involved in the mechanisms of neuronal cell death in PD.[142, 143] A link between parkin and the UPR was established in a study which showed that parkin expression is induced during the UPR, and that parkin suppresses the UPR–induced cell death through its E3 activity.[125] This suggested that parkin is involved in the UPR response by ubiquitylation and proteasomal degradation of unfolded substrates. A note of caution is warranted here, as parkin expression showed no changes[144] or a mild up-regulation[145] during the UPR in other cell culture studies, and parkin overexpression failed to protect from UPR-induced death in another cell system.[123]

However, subsequent studies showed that parkin overexpression promotes the ubiquitylation and degradation of PAEL and protects from the PAEL-induced toxicity in cell culture as well as in fly models engineered to express human PAEL.[120, 128] Parkin-mediated ubiquitylation of PAEL is promoted by CHIP (carboxyl terminus of the Hsc70-intacting protein), which acts by promoting the dissociation of the chaperone Hsp70 from the parkin-PAEL complex, as well as by directly enhancing the E3 activity of parkin, thereby serving as a E4-like protein.[146]

Finally, there is evidence of PAEL accumulation in the brain of patients with parkin disease,[128] suggesting that PAEL is a substrate for parkin mediated degradation *in vivo*, and that the UPR is implicated in the pathogenesis of parkin disease.[120] The fact that PAEL is highly expressed in nigral neurons provides a first possible explanation for regional selectivity of pathology in parkin disease.[128] However, the panneural expression of PAEL in Drosophila is associated with age-related, selective degeneration of dopaminergic neurons,[120] suggesting that other factors, which are specific of the dopaminergic neurons, determine the selectivity of the pathology.

α-synuclein, synphilin-1 and parkin

Parkin was co-immunoprecipitated with a new O-glycosylated form of α-synuclein (αSp22) from normal brain lysates, but not with the native α-synuclein abundant form. Moreover, wild type parkin (but not disease-linked parkin mutants) ubiquitylate αSp22 *in vitro*, and the levels of αSp22 increase in brain of parkin patients.[131] These findings suggest that αSp22 is a substrate of parkin and identifies a novel pathway, which is

potentially involved in the genesis of Lewy bodies and in the pathogenesis of classical PD. The accumulation of αSp22 in the brain might lead to neurodegeneration in parkin disease.[131] However, the biological relevance of the αSp22 isoform remains unclear. Another study confirmed that parkin does not interact with, nor does it ubiquitylate the native α-synuclein isoform, but the αSp22 species was not detected in neuronal as well as non-neuronal cultures.[130] In this last study, parkin was shown to interact with and ubiquitylate the α-synuclein-interacting protein synphilin-1. Co-expression of parkin, α-synuclein and synphilin-1 in cell cultures results in ubiquitylation of proteins in Lewy body-like cytoplasmic inclusions. Disease-linked mutations in parkin impair the ubiquitylation of synphilin-1 and of the cytoplasmic inclusions.[130]
The reported interaction between parkin and α-synuclein, either direct via αSp22,[131] or indirect via synphilin-1,[130] is intriguing because it would link parkin to α-synuclein, another protein which is central in the pathogenesis of PD. The link between these two proteins has been confirmed in recent studies, which showed that parkin overexpression protects from α-synuclein-induced neurotoxicity in cell culture[147] and in Drosophila.[120] The last report firmly indicates parkin as a potent modifier of the α-synuclein-induced neurodegeneration in Drosophila, once again suggesting that parkin is implicated in the pathogenesis of PD in general.

Parkin and the tubulins
In a recent study, parkin was shown to bind α- and β-tubulins, and microtubules; moreover, parkin was able to ubiquitylate and to promote the degradation of α- and β-tubulins. This suggests that parkin is a microtubule-associated protein, and it plays a protective role in controlling the levels of neurotoxic misfolded tubulins, which originate during the dynamic process of microtubule polymerization and depolymerization.[134]

Cyclin-E, p38 and apoptosis
Two of the latest proposed parkin substrates, cyclin-E and p38 suggest additional and intriguing links between parkin activity and protection of neurons from apoptosis.
According to a recent study, parkin functions in a multiprotein ubiquitin ligase complex, which includes hSel-10 (a F-box/WD repeat protein also termed hCdc4 or Archipelago) and Cullin-1. HSel-10 targets the parkin E3 activity to its interactor cyclin-E.[132]
Cyclin E has been implicated in the regulation of cell cycle entry into S phase, but evidence suggests a role for cyclins including cyclin-E in the regulation of apoptosis in postmitotic neurons, and particularly in the response to excitotoxicity.[148-150] Interestingly, cyclin levels are increased in PD and other neurodegenerative diseases, and cyclin E seems also increased in the brain of patients with parkin disease.[132] Moreover, glutamate excitotoxicity has been implicated in the pathogenesis of neuronal loss in PD.[151] In keeping with this evidence, *parkin* overexpression protected, whereas *parkin* knock down sensitised neuronal cultures to kainate-mediated apoptosis (a model of excitotoxicity), also displaying a relative selectivity for dopaminergic neurons.[132] These results suggest a role for parkin in another pathway of brain neuronal survival, which is potentially implicated in both parkin disease and common PD.
The last putative parkin substrate to be discussed is p38, a key scaffold subunit of the aminoacyl-tRNA synthetase complex.[133] Parkin is able to interact, ubiquitylate and promote the degradation of p38 in cell cultures, and to protect dopaminergic neurons from toxicity associated with p38 overexpression. Moreover, p38 overexpression leads to formation of aggresome-like inclusions also containing parkin, and p38 immunoreactivity was found in Lewy bodies in classical PD.[133] These findings suggest that p38 plays a role in the pathogenesis of parkin disease and of common PD. In

addition to their role in protein translation, several components of the aminoacyl-tRNA synthetase complex might have non canonical functions including cytokine generation and control of apoptosis,[152] suggesting a further putative pathway linking parkin dysfunction to neuronal death.
The links between parkin and apoptosis are supported by findings in the Drosophila *parkin* knock out model.[153] These flies show reduced longevity, male sterility, locomotor defects and mild, age-related structural changes in a particular cluster of dopaminergic brain neurons (the dorsomedial cluster), which is also particularly sensitive to toxicity in the *α-synuclein* transgenic fly.[153] The male sterility is due to mitochondrial defects in spermatogenesis, and the locomotor defects are caused by a mitochondrial myopathy leading to apoptotic cell death.[153] This model failed to reproduce dopaminergic neuronal loss typical of human parkin disease. However, its findings are important in the light of mitochondrial defects observed in classical PD.[154] Reduced male fertility and muscle dysfunctions have not been reported in human parkin disease, but none of these aspects has been specifically addressed in patients, and this would certainly be of interest for future investigations. Intriguingly, the *DJ-1* gene homologue has also been linked to male infertility in rodents,[155, 156] suggesting the possibility that parkin and DJ-1 function is required in a common pathway during spermatogenesis.
Recently, parkin function has been linked to protection from mitochondria-dependent cell death in a cell culture model.[123] Last, oxidative stress and nitric oxide production has been observed as a result of overexpression of disease-linked *parkin* mutations in cell cultures,[157] and the observed accumulation of iron in the *substantia nigra* of parkin patients also supports a role for oxidative stress in the pathogenesis.[158]

Parkin as a multifunctional neuroprotective agent
Whether the accumulation of one or several of the putative parkin substrates is neurotoxic remains unclear. Evidence for accumulation of the proposed parkin substrates in the brain of patients with parkin disease is so far limited to single reports,[128, 131, 132] and they remain to be confirmed in other studies on human brain as well as in animal models of parkin disease. Whatever the initial mechanism is, the final endpoint in parkin disease appears to share evidences of mitochondrial derangement, oxidative stress, and apoptosis,[123, 153, 157] suggesting further links to the pathogenesis of classical PD.
Taken as a whole, the available evidence delineates parkin as a multifunctional neuroprotective protein, which is active against stress induced by several misfolded proteins (α-synuclein, PAEL, α- and β-tubulins, and other proteins, including expanded polyglutamine proteins[159]), as well as against excitotoxicity, and cell death pathways linked to cyclic E or p38. The interaction between parkin and the chaperone Hsp70,[146] now confirmed by an independent group,[159] could mediate the interaction with a variety of misfolded proteins, explaining the apparent broad substrate specificity of parkin.[159] The parkin ubiquitin-ligase activity could protect cells in several ways, including tagging the unfolded substrates for proteasomal degradation, promoting formation of aggresome-like inclusions to sequester excess of substrates in inert forms, and perhaps by signalling in other ubiquitin-mediated pathways related to cell death.

The relation between parkin and Lewy bodies
The presence of α-synuclein,[160] parkin[161, 162] and synphilin-1[163] in Lewy bodies suggests that the Ub-ligase activity of parkin is important for the formation of these inclusions. This could explain the absence of Lewy bodies in the patients with parkin disease. The recent description of a case of heterozygous carrier of *parkin* mutations and Lewy

bodies is in agreement with this view,[164] as one of these mutants possesses residual E3 activity according to another report.[130]
The absence of Lewy bodies in parkin disease (PARK2) is associated with a rather selective, severe loss of dopaminergic neurons, an early onset but a slower progression of symptoms in comparison with common, Lewy body PD. It is tempting to speculate that in common PD the formation of Lewy bodies is initially able to counteract the toxicity of soluble species, slowing down the neurodegenerative process and the loss of dopaminergic neurons, so delaying the onset of symptoms. However, after decades of overload of misfolded or toxic proteins, this system eventually collapses, and the aggregates begin to add to the toxicity of soluble species, so contributing to the more aggressive course, and to the loss of dopaminergic as well as non-dopaminergic neurons, as seen in late-onset PD.

The ubiquitin-proteasome system in parkin disease and PD
While the link between parkin and ubiquitylation is convincingly established, much less so is the implication of the proteasomal degradation. The ubiquitin-like domain (Ub-L) of parkin shares a motif with other Ub-L-containing proteins, which are known to interact with the proteasome.[165] This suggests that parkin might also interact with the proteasome, linking this structure with the ubiquitylation machinery. And indeed, recent evidence suggests that parkin is able to bind proteasomal subunits, such as Rpt6[159] and Rpn10.[166]
However, it is increasingly being recognized that, in addition to the proteasomal degradation, ubiquitin conjugation is a signalling system which regulates a broad range of cellular processes, including gene transcription, endocytosis, and protein sorting.[127, 167] Ubiquitylation is therefore regarded as a form of post-translational protein modification pathway like phosphorylation. Much of the previous functional work has explored the physiological and pathological role of parkin assuming a link to the ubiquitin-proteasome system. However, it is possible that this is not the whole story, and parkin might be linked to neurodegeneration via a different ubiquitin-mediated pathway.

A primary involvement of the ubiquitin system in neurodegeneration is also supported by the association of a single mutation and a polymorphism in *UCH-L1* with PD (chapter 1.1),[168] and a mutation in *mahoganoid,* encoding a ubiquitin ligase, in a spongiform neurodegenerative disease in mice.[169] UCH-L1hydrolyses ubiquityl-peptide conjugates *in vitro*.[170] Although its physiological role remained debated, this protein was deemed important for the recycling of free ubiquitin molecules from the ubiquityl-peptides fragments produced by the proteasome. This contention is not supported by recent evidence, which implicates a different enzyme, POH1, a subunit of the proteasomal regulatory complex, in a rate-limiting de-ubiquitylating reaction taking place before the proteasomal degradation.[171] The UCH-L1 protein displays a second, dimerization dependent, ubiquitin ligase activity for α-synuclein *in vitro*.[172] This ligation uses K63 as opposed to K48 chemistry, and it is therefore unlinked to proteasomal degradation. Whether this reaction takes place *in vivo* and whether it is implicated in PD pathogenesis remains unknown. Last, very recently it has been discovered that UCH-L1 binds mono-ubiquitin in neurons and stabilizes the mono-ubiquitin levels *in vivo* by preventing the degradation of ubiquitin.[173] Interestingly, the PD-associated mutation (I93M) has no effects on this activity, and therefore, if really pathogenic, it acts by another (likely gain-of-function) mechanism.

Mutations in proteasomal subunits have not been linked to human neurodegeneration. Evidence of proteasomal abnormalities has not been reported so far in PD animal models either. It is likely that major defects in this system are not compatible with life. For example, loss of the function of the POH1 homologue Rpn11 is lethal in yeasts. However, mild decreases in proteasomal activity could be compatible with life and could predispose to neurodegeneration.

The available evidence for an involvement of the proteasome system in PD is indirect. Reduced proteasomal activity has been reported in post-mortem studies on PD brains,[69] while another group detected no abnormalities.[174] Overexpression of α-synuclein and other proteins in cell systems leads to proteasomal inhibition with or without aggregate formation.[63, 70, 71, 147] Catecholaminergic neurons seem particularly vulnerable to proteasomal inhibition *in vitro*, suggesting a possible explanation of the selectivity of α-synuclein-associated degeneration.[147] Direct injection of a proteasomal inhibitor in the *substantia nigra* in rats induced neuronal degeneration and formation of inclusions.[175] However, whether the effects observed in post-mortem studies and overexpressing cell models are relevant for *in vivo* conditions remains unclear, and whether a systemic proteasomal inhibition can produce selective dopaminergic neurotoxicity remains to be demonstrated.

The presence of ubiquitylated proteins, molecular chaperones, and proteasomal components in the Lewy bodies is perhaps the strongest evidence implicating the protein clearance system in disease pathogenesis. Moreover, the morphological and biochemical similarities between Lewy bodies and aggresomes[101, 102] once again points to defective or overloaded proteasomal activity, as aggresome formation is considered a cell response to insufficient proteasomal-mediated clearance of misfolded or toxic proteins.[100]

In common PD the proteasome could be primarily defective, for example as a consequence of mutations in its subunits, or it could be inhibited by α-synuclein oligomers or other toxic species, or lastly, it could become insufficient due to chronic overload of misfolded and other damaged proteins. In the case of the proteasomal defect, as for the oxidative stress, the mitochondrial dysfunction, and other abnormalities proposed in the pathogenesis, it is important to distinguish processes that are central and determinant in the cascade leading to neurodegeneration from other collateral effects. Moreover, it is critical to understand why the pathological process in PD affects the dopaminergic neurons with relative selectivity.

TOWARD A UNIFYING THEORY OF PATHOGENESIS

It is possible to reconcile evidences from different research lines assuming that PD (as well as other common neurodegenerative diseases) is a disorder of the protein quality control (PQC) system, which is associated with neuronal accumulation of misfolded proteins and presence of protein aggregates. Major arms in the PQC system are the molecular chaperones,[104] the endoplasmic reticulum associated unfolded protein response pathway,[141] the ubiquitin-proteasome system,[127] and other ubiquitin-like protein conjugation systems, including sumoylation.[176] Studies of the monogenic forms of PD implicate the different components of this system, delineating a coherent pathogenetic framework, which could be at the roots of the common forms of the disease as well.

REFERENCES

1. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 1997; 276:2045-7.
2. Kruger R, Kuhn W, Muller T, et al. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet 1998; 18:106-8.
3. Spillantini MG, Goedert M. The alpha-synucleinopathies: Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy. Ann N Y Acad Sci 2000; 920:16-27.
4. Goedert M. Alpha-synuclein and neurodegenerative diseases. Nat Rev Neurosci 2001; 2: 492-501.
5. Masliah E, Rockenstein E, Veinbergs I, et al. Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. Science 2000; 287:1265-9.
6. Feany MB, Bender WW. A Drosophila model of Parkinson's disease. Nature 2000; 404:394-8.
7. Zoghbi HY, Botas J. Mouse and fly models of neurodegeneration. Trends Genet 2002; 18: 463-71.
8. Clayton DF, George JM. The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. Trends Neurosci 1998; 21:249-54.
9. Murphy DD, Rueter SM, Trojanowski JQ, Lee VM. Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. J Neurosci 2000; 20:3214-20.
10. Cabin DE, Shimazu K, Murphy D, et al. Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. J Neurosci 2002; 22:8797-807.
11. Abeliovich A, Schmitz Y, Farinas I, et al. Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. Neuron 2000; 25:239-52.
12. Markopoulou K, Wszolek ZK, Pfeiffer RF, Chase BA. Reduced expression of the G209A alpha-synuclein allele in familial Parkinsonism. Ann Neurol 1999; 46:374-81.
13. Kobayashi H, Kruger R, Markopoulou K, et al. Haploinsufficiency at the alpha-synuclein gene underlies phenotypic severity in familial Parkinson's disease. Brain 2003; 126:32-42.
14. George JM, Jin H, Woods WS, Clayton DF. Characterization of a novel protein regulated during the critical period for song learning in the zebra finch. Neuron 1995; 15:361-72.
15. Sharon R, Goldberg MS, Bar-Josef I, Betensky RA, Shen J, Selkoe DJ. alpha-Synuclein occurs in lipid-rich high molecular weight complexes, binds fatty acids, and shows homology to the fatty acid-binding proteins. Proc Natl Acad Sci U S A 2001; 98:9110-5.
16. Ostrerova N, Petrucelli L, Farrer M, et al. alpha-Synuclein shares physical and functional homology with 14-3-3 proteins. J Neurosci 1999; 19:5782-91.
17. Jenco JM, Rawlingson A, Daniels B, Morris AJ. Regulation of phospholipase D2: selective inhibition of mammalian phospholipase D isoenzymes by alpha- and beta-synucleins. Biochemistry 1998; 37:4901-9.
18. Davidson WS, Jonas A, Clayton DF, George JM. Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. J Biol Chem 1998; 273:9443-9.
19. Conway KA, Harper JD, Lansbury PT, Jr. Fibrils formed in vitro from alpha-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. Biochemistry 2000; 39:2552-63.
20. Serpell LC, Berriman J, Jakes R, Goedert M, Crowther RA. Fiber diffraction of synthetic alpha-synuclein filaments shows amyloid-like cross-beta conformation. Proc Natl Acad Sci U S A 2000; 97:4897-902.
21. Bucciantini M, Giannoni E, Chiti F, et al. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. Nature 2002; 416:507-11.
22. Lambert MP, Barlow AK, Chromy BA, et al. Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc Natl Acad Sci U S A 1998; 95:6448-53.

23. Sousa MM, Cardoso I, Fernandes R, Guimaraes A, Saraiva MJ. Deposition of transthyretin in early stages of familial amyloidotic polyneuropathy: evidence for toxicity of nonfibrillar aggregates. Am J Pathol 2001; 159:1993-2000.
24. Muchowski PJ, Ning K, D'Souza-Schorey C, Fields S. Requirement of an intact microtubule cytoskeleton for aggregation and inclusion body formation by a mutant huntingtin fragment. Proc Natl Acad Sci U S A 2002; 99:727-32.
25. Walsh DM, Klyubin I, Fadeeva JV, et al. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature 2002; 416: 535-9.
26. Conway KA, Lee SJ, Rochet JC, Ding TT, Williamson RE, Lansbury PT, Jr. Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. Proc Natl Acad Sci U S A 2000; 97:571-6.
27. Krishnan S, Chi EY, Wood SJ, et al. Oxidative dimer formation is the critical rate-limiting step for Parkinson's disease alpha-synuclein fibrillogenesis. Biochemistry 2003; 42:829-37.
28. Sharon R, Bar-Joseph I, Frosch MP, Walsh DM, Hamilton JA, Selkoe DJ. The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson's disease. Neuron 2003; 37:583-95.
29. Uversky VN, Li J, Fink AL. Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular link between Parkinson's disease and heavy metal exposure. J Biol Chem 2001; 276:44284-96.
30. Uversky VN, Li J, Fink AL. Pesticides directly accelerate the rate of alpha-synuclein fibril formation: a possible factor in Parkinson's disease. FEBS Lett 2001; 500:105-8.
31. Golts N, Snyder H, Frasier M, Theisler C, Choi P, Wolozin B. Magnesium inhibits spontaneous and iron-induced aggregation of alpha-synuclein. J Biol Chem 2002; 277:16116-23.
32. Hashimoto M, Hsu LJ, Xia Y, et al. Oxidative stress induces amyloid-like aggregate formation of NACP/alpha-synuclein in vitro. Neuroreport 1999; 10:717-21.
33. Cohlberg JA, Li J, Uversky VN, Fink AL. Heparin and other glycosaminoglycans stimulate the formation of amyloid fibrils from alpha-synuclein in vitro. Biochemistry 2002; 41:1502-11.
34. Shtilerman MD, Ding TT, Lansbury PT, Jr. Molecular crowding accelerates fibrillization of alpha-synuclein: could an increase in the cytoplasmic protein concentration induce Parkinson's disease? Biochemistry 2002; 41:3855-60.
35. Nielsen MS, Vorum H, Lindersson E, Jensen PH. Ca2+ binding to alpha-synuclein regulates ligand binding and oligomerization. J Biol Chem 2001; 276:22680-4.
36. Martinez J, Moeller I, Erdjument-Bromage H, Tempst P, Lauring B. Parkinson's disease-associated alpha -synuclein is a calmodulin substrate. J Biol Chem 2003; 278:17379-87.
37. Cole NB, Murphy DD, Grider T, Rueter S, Brasaemle D, Nussbaum RL. Lipid droplet binding and oligomerization properties of the Parkinson's disease protein alpha-synuclein. J Biol Chem 2002; 277:6344-52.
38. Perrin RJ, Woods WS, Clayton DF, George JM. Exposure to long chain polyunsaturated fatty acids triggers rapid multimerization of synucleins. J Biol Chem 2001; 276:41958-62.
39. Lee HJ, Shin SY, Choi C, Lee YH, Lee SJ. Formation and removal of alpha-synuclein aggregates in cells exposed to mitochondrial inhibitors. J Biol Chem 2002; 277:5411-7.
40. Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA. The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. J Biol Chem 2002; 277:1641-4.
41. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 2000; 3:1301-6.
42. Ostrerova-Golts N, Petrucelli L, Hardy J, Lee JM, Farer M, Wolozin B. The A53T alpha-synuclein mutation increases iron-dependent aggregation and toxicity. J Neurosci 2000; 20:6048-54.
43. Yamin G, Glaser CB, Uversky VN, Fink AL. Certain metals trigger fibrillation of methionine-oxidized alpha-synuclein. J Biol Chem 2003; 16:16.
44. Paxinou E, Chen Q, Weisse M, et al. Induction of alpha-synuclein aggregation by intracellular nitrative insult. J Neurosci 2001; 21:8053-61.

45. Rideout HJ, Larsen KE, Sulzer D, Stefanis L. Proteasomal inhibition leads to formation of ubiquitin/alpha-synuclein-immunoreactive inclusions in PC12 cells. J Neurochem 2001; 78:899-908.
46. Junn E, Ronchetti RD, Quezado MM, Kim SY, Mouradian MM. Tissue transglutaminase-induced aggregation of alpha-synuclein: Implications for Lewy body formation in Parkinson's disease and dementia with Lewy bodies. Proc Natl Acad Sci U S A 2003; 100: 2047-52.
47. Fujiwara H, Hasegawa M, Dohmae N, et al. alpha-Synuclein is phosphorylated in synucleinopathy lesions. Nat Cell Biol 2002; 4:160-4.
48. Giasson BI, Duda JE, Murray IV, et al. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. Science 2000; 290:985-9.
49. Hasegawa M, Fujiwara H, Nonaka T, et al. Phosphorylated alpha-synuclein is ubiquitinated in alpha-synucleinopathy lesions. J Biol Chem 2002; 277:49071-6.
50. Uversky VN, Li J, Souillac P, et al. Biophysical properties of the synucleins and their propensities to fibrillate: inhibition of alpha-synuclein assembly by beta- and gamma-synucleins. J Biol Chem 2002; 277:11970-8.
51. Hashimoto M, Rockenstein E, Mante M, Mallory M, Masliah E. beta-Synuclein inhibits alpha-synuclein aggregation: a possible role as an anti-parkinsonian factor. Neuron 2001; 32:213-23.
52. Giasson BI, Forman MS, Higuchi M, et al. Initiation and synergistic fibrillization of tau and alpha-synuclein. Science 2003; 300:636-40.
53. Arima K, Hirai S, Sunohara N, et al. Cellular co-localization of phosphorylated tau- and NACP/alpha-synuclein-epitopes in lewy bodies in sporadic Parkinson's disease and in dementia with Lewy bodies. Brain Res 1999; 843:53-61.
54. Ishizawa T, Mattila P, Davies P, Wang D, Dickson DW. Colocalization of tau and alpha-synuclein epitopes in Lewy bodies. J Neuropathol Exp Neurol 2003; 62:389-97.
55. Simon HH, Saueressig H, Wurst W, Goulding MD, O'Leary DD. Fate of midbrain dopaminergic neurons controlled by the engrailed genes. J Neurosci 2001; 21:3126-34.
56. Stefanis L, Kholodilov N, Rideout HJ, Burke RE, Greene LA. Synuclein-1 is selectively up-regulated in response to nerve growth factor treatment in PC12 cells. J Neurochem 2001; 76:1165-76.
57. Hartman VN, Miller MA, Clayton DF, Liu WC, Kroodsma DE, Brenowitz EA. Testosterone regulates alpha-synuclein mRNA in the avian song system. Neuroreport 2001; 12:943-6.
58. Chiba-Falek O, Nussbaum RL. Effect of allelic variation at the NACP-Rep1 repeat upstream of the alpha-synuclein gene (SNCA) on transcription in a cell culture luciferase reporter system. Hum Mol Genet 2001; 10:3101-9.
59. Rockenstein E, Hansen LA, Mallory M, Trojanowski JQ, Galasko D, Masliah E. Altered expression of the synuclein family mRNA in Lewy body and Alzheimer's disease. Brain Res 2001; 914:48-56.
60. Windisch M, Hutter-Paier B, Rockenstein E, Hashimoto M, Mallory M, Masliah E. Development of a new treatment for Alzheimer's disease and Parkinson's disease using anti-aggregatory beta-synuclein-derived peptides. J Mol Neurosci 2002; 19:63-9.
61. Wirdefeldt K, Bogdanovic N, Westerberg L, Payami H, Schalling M, Murdoch G. Expression of alpha-synuclein in the human brain: relation to Lewy body disease. Brain Res Mol Brain Res 2001; 92:58-65.
62. Ghee M, Fournier A, Mallet J. Rat alpha-synuclein interacts with Tat binding protein 1, a component of the 26S proteasomal complex. J Neurochem 2000; 75:2221-4.
63. Snyder H, Mensah K, Theisler C, Lee J, Matouschek A, Wolozin B. Aggregated and monomeric alpha-synuclein bind to the S6' proteasomal protein and inhibit proteasomal function. J Biol Chem 2003; 278:11753-9.
64. Bennett MC, Bishop JF, Leng Y, Chock PB, Chase TN, Mouradian MM. Degradation of alpha-synuclein by proteasome. J Biol Chem 1999; 274:33855-8.
65. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. Alpha-synuclein is degraded by both autophagy and the proteasome. J Biol Chem 2003; 28:28.
66. Tofaris GK, Layfield R, Spillantini MG. alpha-synuclein metabolism and aggregation is linked to ubiquitin-independent degradation by the proteasome. FEBS Lett 2001; 509: 22-6.

67. Ancolio K, Alves da Costa C, Ueda K, Checler F. Alpha-synuclein and the Parkinson's disease-related mutant Ala53Thr-alpha-synuclein do not undergo proteasomal degradation in HEK293 and neuronal cells. Neurosci Lett 2000; 285:79-82.
68. Mishizen-Eberz AJ, Guttmann RP, Giasson BI, et al. Distinct cleavage patterns of normal and pathologic forms of alpha-synuclein by calpain I in vitro. J Neurochem 2003; 86:836-847.
69. McNaught KS, Belizaire R, Isacson O, Jenner P, Olanow CW. Altered proteasomal function in sporadic Parkinson's disease. Exp Neurol 2003; 179:38-46.
70. Bence NF, Sampat RM, Kopito RR. Impairment of the ubiquitin-proteasome system by protein aggregation. Science 2001; 292:1552-5.
71. Tanaka Y, Engelender S, Igarashi S, et al. Inducible expression of mutant alpha-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. Hum Mol Genet 2001; 10:919-26.
72. Hsu LJ, Sagara Y, Arroyo A, et al. alpha-synuclein promotes mitochondrial deficit and oxidative stress. Am J Pathol 2000; 157:401-10.
73. Gosavi N, Lee HJ, Lee JS, Patel S, Lee SJ. Golgi fragmentation occurs in the cells with prefibrillar alpha-synuclein aggregates and precedes the formation of fibrillar inclusion. J Biol Chem 2002; 277:48984-92.
74. Volles MJ, Lee SJ, Rochet JC, et al. Vesicle permeabilization by protofibrillar alpha-synuclein: implications for the pathogenesis and treatment of Parkinson's disease. Biochemistry 2001; 40:7812-9.
75. Lashuel HA, Hartley D, Petre BM, Walz T, Lansbury PT, Jr. Neurodegenerative disease: amyloid pores from pathogenic mutations. Nature 2002; 418:291.
76. Volles MJ, Lansbury PT, Jr. Vesicle permeabilization by protofibrillar alpha-synuclein is sensitive to Parkinson's disease-linked mutations and occurs by a pore-like mechanism. Biochemistry 2002; 41:4595-602.
77. Xu J, Kao SY, Lee FJ, Song W, Jin LW, Yankner BA. Dopamine-dependent neurotoxicity of alpha-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. Nat Med 2002; 8:600-6.
78. Perez RG, Waymire JC, Lin E, Liu JJ, Guo F, Zigmond MJ. A role for alpha-synuclein in the regulation of dopamine biosynthesis. J Neurosci 2002; 22:3090-9.
79. Baptista MJ, O'Farrell C, Daya S, et al. Co-ordinate transcriptional regulation of dopamine synthesis genes by alpha-synuclein in human neuroblastoma cell lines. J Neurochem 2003; 85:957-68.
80. Le WD, Xu P, Jankovic J, et al. Mutations in NR4A2 associated with familial Parkinson disease. Nat Genet 2003; 33:85-9.
81. Lee FJ, Liu F, Pristupa ZB, Niznik HB. Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. Faseb J 2001; 15:916-26.
82. Lotharius J, Barg S, Wiekop P, Lundberg C, Raymon HK, Brundin P. Effect of mutant alpha-synuclein on dopamine homeostasis in a new human mesencephalic cell line. J Biol Chem 2002; 277:38884-94.
83. Conway KA, Rochet JC, Bieganski RM, Lansbury PT, Jr. Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. Science 2001; 294:1346-9.
84. Uversky VN, Yamin G, Souillac PO, Goers J, Glaser CB, Fink AL. Methionine oxidation inhibits fibrillation of human alpha-synuclein in vitro. FEBS Lett 2002; 517:239-44.
85. Souza JM, Giasson BI, Chen Q, Lee VM, Ischiropoulos H. Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. J Biol Chem 2000; 275: 18344-9.
86. Yamin G, Uversky VN, Fink AL. Nitration inhibits fibrillation of human alpha-synuclein in vitro by formation of soluble oligomers. FEBS Lett 2003; 542:147-52.
87. Richfield EK, Thiruchelvam MJ, Cory-Slechta DA, et al. Behavioral and neurochemical effects of wild-type and mutated human alpha-synuclein in transgenic mice. Exp Neurol 2002; 175:35-48.
88. Dauer W, Kholodilov N, Vila M, et al. Resistance of alpha-synuclein null mice to the parkinsonian neurotoxin MPTP. Proc Natl Acad Sci U S A 2002; 99:14524-9.

89. Schluter OM, Fornai F, Alessandri MG, et al. Role of alpha-synuclein in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism in mice. Neuroscience 2003; 118:985-1002.
90. Lotharius J, O'Malley KL. The parkinsonism-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation. A novel mechanism of toxicity. J Biol Chem 2000; 275:38581-8.
91. Ko L, Mehta ND, Farrer M, et al. Sensitization of neuronal cells to oxidative stress with mutated human alpha-synuclein. J Neurochem 2000; 75:2546-54.
92. Kanda S, Bishop JF, Eglitis MA, Yang Y, Mouradian MM. Enhanced vulnerability to oxidative stress by alpha-synuclein mutations and C-terminal truncation. Neuroscience 2000; 97:279-84.
93. Lee M, Hyun D, Halliwell B, Jenner P. Effect of the overexpression of wild-type or mutant alpha-synuclein on cell susceptibility to insult. J Neurochem 2001; 76:998-1009.
94. Junn E, Mouradian MM. Human alpha-synuclein over-expression increases intracellular reactive oxygen species levels and susceptibility to dopamine. Neurosci Lett 2002; 320: 146-50.
95. Tabrizi SJ, Orth M, Wilkinson JM, et al. Expression of mutant alpha-synuclein causes increased susceptibility to dopamine toxicity. Hum Mol Genet 2000; 9:2683-9.
96. Hashimoto M, Hsu LJ, Rockenstein E, Takenouchi T, Mallory M, Masliah E. alpha-Synuclein protects against oxidative stress via inactivation of the c-Jun N-terminal kinase stress-signaling pathway in neuronal cells. J Biol Chem 2002; 277:11465-72.
97. Alves Da Costa C, Paitel E, Vincent B, Checler F. Alpha-synuclein lowers p53-dependent apoptotic response of neuronal cells. Abolishment by 6-hydroxydopamine and implication for Parkinson's disease. J Biol Chem 2002; 277:50980-4.
98. Manning-Bog AB, McCormack AL, Purisai MG, Bolin LM, Di Monte DA. Alpha-synuclein overexpression protects against paraquat-induced neurodegeneration. J Neurosci 2003; 23:3095-9.
99. Johnston JA, Ward CL, Kopito RR. Aggresomes: a cellular response to misfolded proteins. J Cell Biol 1998; 143:1883-98.
100. Kopito RR. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol 2000; 10:524-30.
101. Junn E, Lee SS, Suhr UT, Mouradian MM. Parkin accumulation in aggresomes due to proteasome impairment. J Biol Chem 2002; 277:47870-7.
102. McNaught KS, Shashidharan P, Perl DP, Jenner P, Olanow CW. Aggresome-related biogenesis of Lewy bodies. Eur J Neurosci 2002; 16:2136-48.
103. Yamamoto A, Lucas JJ, Hen R. Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. Cell 2000; 101:57-66.
104. Fink AL. Chaperone-mediated protein folding. Physiol Rev 1999; 79:425-49.
105. Cummings CJ, Mancini MA, Antalffy B, DeFranco DB, Orr HT, Zoghbi HY. Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. Nat Genet 1998; 19:148-54.
106. Warrick JM, Chan HY, Gray-Board GL, Chai Y, Paulson HL, Bonini NM. Suppression of polyglutamine-mediated neurodegeneration in Drosophila by the molecular chaperone HSP70. Nat Genet 1999; 23:425-8.
107. Fernandez-Funez P, Nino-Rosales ML, de Gouyon B, et al. Identification of genes that modify ataxin-1-induced neurodegeneration. Nature 2000; 408:101-6.
108. Cummings CJ, Sun Y, Opal P, et al. Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. Hum Mol Genet 2001; 10:1511-8.
109. Auluck PK, Chan HY, Trojanowski JQ, Lee VM, Bonini NM. Chaperone suppression of alpha-synuclein toxicity in a Drosophila model for Parkinson's disease. Science 2002; 295: 865-8.
110. Cummings CJ, Reinstein E, Sun Y, et al. Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. Neuron 1999; 24:879-92.
111. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392:605-8.

112. Lucking CB, Durr A, Bonifati V, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene. French Parkinson's Disease Genetics Study Group. N Engl J Med 2000; 342:1560-7.
113. Periquet M, Latouche M, Lohmann E, et al. Parkin mutations are frequent in patients with isolated early-onset parkinsonism. Brain 2003; 126:1271-8.
114. Foroud T, Uniacke SK, Liu L, et al. Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. Neurology 2003; 60:796-801.
115. Zarate-Lagunes M, Gu WJ, Blanchard V, et al. Parkin immunoreactivity in the brain of human and non-human primates: an immunohistochemical analysis in normal conditions and in Parkinsonian syndromes. J Comp Neurol 2001; 432:184-96.
116. Solano SM, Miller DW, Augood SJ, Young AB, Penney JB, Jr. Expression of alpha-synuclein, parkin, and ubiquitin carboxy-terminal hydrolase L1 mRNA in human brain: genes associated with familial Parkinson's disease. Ann Neurol 2000; 47:201-10.
117. Shimura H, Hattori N, Kubo S, et al. Immunohistochemical and subcellular localization of Parkin protein: absence of protein in autosomal recessive juvenile parkinsonism patients. Ann Neurol 1999; 45:668-72.
118. Huynh DP, Scoles DR, Ho TH, Del Bigio MR, Pulst SM. Parkin is associated with actin filaments in neuronal and nonneural cells. Ann Neurol 2000; 48:737-44.
119. Kubo SI, Kitami T, Noda S, et al. Parkin is associated with cellular vesicles. J Neurochem 2001; 78:42-54.
120. Yang Y, Nishimura I, Imai Y, Takahashi R, Lu B. Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in Drosophila. Neuron 2003; 37:911-24.
121. Fallon L, Moreau F, Croft BG, Labib N, Gu WJ, Fon EA. Parkin and CASK/LIN-2 associate via a PDZ-mediated interaction and are co-localized in lipid rafts and postsynaptic densities in brain. J Biol Chem 2002; 277:486-91.
122. Stichel CC, Augustin M, Kuhn K, et al. Parkin expression in the adult mouse brain. Eur J Neurosci 2000; 12:4181-94.
123. Darios F, Corti O, Lucking CB, et al. Parkin prevents mitochondrial swelling and cytochrome c release in mitochondria-dependent cell death. Hum Mol Genet 2003; 12: 517-26.
124. Shimura H, Hattori N, Kubo S, et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat Genet 2000; 25:302-5.
125. Imai Y, Soda M, Takahashi R. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. J Biol Chem 2000; 275:35661-4.
126. Zhang Y, Gao J, Chung KK, Huang H, Dawson VL, Dawson TM. Parkin functions as an E2-dependent ubiquitin- protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proc Natl Acad Sci U S A 2000; 97:13354-9.
127. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002; 82:373-428.
128. Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell 2001; 105:891-902.
129. Choi P, Ostrerova-Golts N, Sparkman D, Cochran E, Lee JM, Wolozin B. Parkin is metabolized by the ubiquitin/proteosome system. Neuroreport 2000; 11:2635-8.
130. Chung KK, Zhang Y, Lim KL, et al. Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. Nat Med 2001; 7:1144-50.
131. Shimura H, Schlossmacher MG, Hattori N, et al. Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. Science 2001; 293:263-9.
132. Staropoli JF, McDermott C, Martinat C, Schulman B, Demireva E, Abeliovich A. Parkin is a component of an SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainate excitotoxicity. Neuron 2003; 37:735-49.
133. Corti O, Hampe C, Koutnikova H, et al. The p38 subunit of the aminoacyl-tRNA synthetase complex is a Parkin substrate: linking protein biosynthesis and neurodegeneration. Hum Mol Genet 2003; 12:1427-1437.

134. Ren Y, Zhao J, Feng J. Parkin binds to alpha/beta tubulin and increases their ubiquitination and degradation. J Neurosci 2003; 23:3316-24.
135. Field CM, Kellogg D. Septins: cytoskeletal polymers or signalling GTPases? Trends Cell Biol 1999; 9:387-94.
136. Ihara M, Tomimoto H, Kitayama H, et al. Association of the Cytoskeletal GTP-binding Protein Sept4/H5 with Cytoplasmic Inclusions Found in Parkinson's Disease and Other Synucleinopathies. J Biol Chem 2003; 278:24095-102.
137. Kinoshita A, Kinoshita M, Akiyama H, et al. Identification of septins in neurofibrillary tangles in Alzheimer's disease. Am J Pathol 1998; 153:1551-60.
138. Larisch S, Yi Y, Lotan R, et al. A novel mitochondrial septin-like protein, ARTS, mediates apoptosis dependent on its P-loop motif. Nat Cell Biol 2000; 2:915-21.
139. Setou M, Nakagawa T, Seog DH, Hirokawa N. Kinesin superfamily motor protein KIF17 and mLin-10 in NMDA receptor-containing vesicle transport. Science 2000; 288:1796-802.
140. Hegde AN, DiAntonio A. Ubiquitin and the synapse. Nat Rev Neurosci 2002; 3:854-61.
141. Kaufman RJ. Orchestrating the unfolded protein response in health and disease. J Clin Invest 2002; 110:1389-98.
142. Holtz WA, O'Malley KL. Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. J Biol Chem 2003; 278:19367-77.
143. Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D, Greene LA. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. J Neurosci 2002; 22:10690-8.
144. West AB, Gonzalez-de-Chavez F, Wilkes K, O'Farrell C, Farrer MJ. Parkin is not regulated by the unfolded protein response in human neuroblastoma cells. Neurosci Lett 2003; 341: 139-42.
145. Mengesdorf T, Jensen PH, Mies G, Aufenberg C, Paschen W. Down-regulation of parkin protein in transient focal cerebral ischemia: A link between stroke and degenerative disease? Proc Natl Acad Sci U S A 2002; 99:15042-7.
146. Imai Y, Soda M, Hatakeyama S, et al. CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. Mol Cell 2002; 10:55-67.
147. Petrucelli L, O'Farrell C, Lockhart PJ, et al. Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. Neuron 2002; 36:1007-19.
148. Liu DX, Greene LA. Neuronal apoptosis at the G1/S cell cycle checkpoint. Cell Tissue Res 2001; 305:217-28.
149. Padmanabhan J, Park DS, Greene LA, Shelanski ML. Role of cell cycle regulatory proteins in cerebellar granule neuron apoptosis. J Neurosci 1999; 19:8747-56.
150. Verdaguer E, Garcia-Jorda E, Canudas AM, et al. Kainic acid-induced apoptosis in cerebellar granule neurons: an attempt at cell cycle re-entry. Neuroreport 2002; 13:413-6.
151. Lang AE, Lozano AM. Parkinson's disease. First of two parts. N Engl J Med 1998; 339:1044-53.
152. Ivanov KA, Moor NA, Lavrik OI. Non-canonical functions of aminoacyl-tRNA synthetases. Biochemistry (Mosc) 2000; 65:888-97.
153. Greene JC, Whitworth AJ, Kuo I, Andrews LA, Feany MB, Pallanck LJ. Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. Proc Natl Acad Sci U S A 2003; 100:4078-83.
154. Jenner P. Oxidative stress in Parkinson's disease. Ann Neurol 2003; 53:S26-36; discussion S36-8.
155. Wagenfeld A, Gromoll J, Cooper TG. Molecular cloning and expression of rat contraception associated protein 1 (CAP1), a protein putatively involved in fertilization. Biochem Biophys Res Commun 1998; 251:545-9.
156. Welch JE, Barbee RR, Roberts NL, Suarez JD, Klinefelter GR. SP22: a novel fertility protein from a highly conserved gene family. J Androl 1998; 19:385-93.
157. Hyun DH, Lee M, Hattori N, et al. Effect of wild-type or mutant Parkin on oxidative damage, nitric oxide, antioxidant defenses, and the proteasome. J Biol Chem 2002; 277: 28572-7.

158. Takanashi M, Mochizuki H, Yokomizo K, et al. Iron accumulation in the substantia nigra of autosomal recessive juvenile parkinsonism (ARJP). Parkinsonism Relat Disord 2001; 7: 311-314.
159. Tsai YC, Fishman PS, Thakor NV, Oyler GA. Parkin facilitates the elimination of expanded polyglutamine proteins and leads to preservation of proteasome function. J Biol Chem 2003; 278:22044-55.
160. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc Natl Acad Sci U S A 1998; 95:6469-73.
161. Schlossmacher MG, Frosch MP, Gai WP, et al. Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies. Am J Pathol 2002; 160:1655-67.
162. Choi P, Golts N, Snyder H, et al. Co-association of parkin and alpha-synuclein. Neuroreport 2001; 12:2839-43.
163. Wakabayashi K, Engelender S, Tanaka Y, et al. Immunocytochemical localization of synphilin-1, an alpha-synuclein-associated protein, in neurodegenerative disorders. Acta Neuropathol (Berl) 2002; 103:209-14.
164. Farrer M, Chan P, Chen R, et al. Lewy bodies and parkinsonism in families with parkin mutations. Ann Neurol 2001; 50:293-300.
165. Upadhya SC, Hegde AN. A potential proteasome-interacting motif within the ubiquitin-like domain of parkin and other proteins. Trends Biochem Sci 2003; 28:280-3.
166. Sakata E, Yamaguchi Y, Kurimoto E, et al. Parkin binds the Rpn10 subunit of 26S proteasomes through its ubiquitin-like domain. EMBO Rep 2003; 4:301-6.
167. Conaway RC, Brower CS, Conaway JW. Emerging roles of ubiquitin in transcription regulation. Science 2002; 296:1254-8.
168. Leroy E, Boyer R, Auburger G, et al. The ubiquitin pathway in Parkinson's disease. Nature 1998; 395:451-2.
169. He L, Lu XY, Jolly AF, et al. Spongiform degeneration in mahoganoid mutant mice. Science 2003; 299:710-2.
170. Wilkinson KD, Lee KM, Deshpande S, Duerksen-Hughes P, Boss JM, Pohl J. The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. Science 1989; 246: 670-3.
171. Yao T, Cohen RE. A cryptic protease couples deubiquitination and degradation by the proteasome. Nature 2002; 419:403-7.
172. Liu Y, Fallon L, Lashuel HA, Liu Z, Lansbury PT, Jr. The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. Cell 2002; 111:209-18.
173. Osaka H, Wang YL, Takada K, et al. Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron. Hum Mol Genet 2003; 12:1945-1958.
174. Furukawa Y, Vigouroux S, Wong H, et al. Brain proteasomal function in sporadic Parkinson's disease and related disorders. Ann Neurol 2002; 51:779-82.
175. McNaught KS, Bjorklund LM, Belizaire R, Isacson O, Jenner P, Olanow CW. Proteasome inhibition causes nigral degeneration with inclusion bodies in rats. Neuroreport 2002; 13: 1437-41.
176. Kim KI, Baek SH, Chung CH. Versatile protein tag, SUMO: its enzymology and biological function. J Cell Physiol 2002; 191:257-68.

Scope of this thesis

Parkinson's disease (PD) is etiologically heterogeneous, and several monogenic forms have been identified (**chapter 1.1**). Although the monogenic forms so far known explain a very small fraction of PD cases at the population level, they are promoting the understanding of the molecular events and the metabolic pathways involved in the common forms of PD as well (**chapter 1.2**). This thesis aims to study the molecular genetics and the phenotypical aspects of autosomal recessive, early-onset forms of PD.

Mutations in a novel gene termed *parkin* were first identified in families with autosomal recessive early-onset parkinsonism in Japan. In order to accurately assess the frequency of involvement of *parkin* and to characterize the associated mutational spectrum, we analysed this gene in a large number of European families in which early-onset PD was compatible with a recessive model of inheritance, as well as in a large series of isolated cases with early-onset PD (**chapter 2.1**). Mutations were found in almost 50% of families and 20% of isolated cases, delineating *parkin* as a major gene for early onset, recessive PD. To illustrate further the high frequency of the mutations in this gene, we described two families with early-onset PD due to *parkin* gene mutations, and showing pseudo-dominant inheritance or allelic heterogeneity in the same sibship (**chapters 2.2, 2.3**). Last, we collected a large series of patients with PD due to *parkin* gene mutations to perform genotype-phenotype correlation studies. The results are presented in **chapter 2.4**.

In order to identify novel genes causing autosomal recessive, early-onset PD, we undertook a genome-wide linkage mapping study in a large consanguineous family originating from a genetically isolated community of the South West of the Netherlands. This study led us to the identification of PARK7, a novel locus for autosomal recessive, early-onset PD, on the short arm of chromosome 1 (**chapter 2.5**). In order to identify further families linked to PARK7, we performed linkage analysis of candidate regions in an independent dataset of families with early onset PD. This study, presented in **chapter 2.6**, yielded conclusive evidence for linkage to PARK7 in a second family from central Italy. We also undertook detailed clinical and neuroimaging studies in the Dutch family linked to PARK7, to characterise further the phenotypical spectrum associated with this form. The results are shown in **chapter 2.7**.
In order to identify the gene defective at the PARK7 locus, we performed fine mapping and positional cloning studies, which led to the discovery of pathogenic mutations in the *DJ-1* gene in the two families definitely linked to PARK7 (**chapter 2.8**). The function of the protein encoded by *DJ-1* is mostly unknown, and none has previously linked this gene to brain function or disease. We reviewed the literature in order to provide clues to a possible function and formulate hypotheses linking DJ-1 to neurodegeneration (**chapter 2.9**). The discovery of the gene defect in another form of Mendelian PD opens a novel window on the mechanisms underlying human neurodegeneration. More importantly, this discovery might promote our understanding of the pathogenesis of the common forms of PD.

2 Experimental work

Chapter 2.1

Association between early-onset Parkinson's disease and mutations in the *parkin* gene

Lucking CB, Durr A, Bonifati V, Vaughan J, De Michele G, Gasser T, Harhangi BS, Meco G, Denefle P, Wood NW, Agid Y, Brice A, for The European Consortium on Genetic Susceptibility in Parkinson's Disease and The French Parkinson's Disease Genetics Study Group

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ASSOCIATION BETWEEN EARLY-ONSET PARKINSON'S DISEASE AND MUTATIONS IN THE *PARKIN* GENE

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ABSTRACT

Background Mutations in the *parkin* gene have recently been identified in patients with early-onset Parkinson's disease, but the frequency of the mutations and the associated phenotype have not been assessed in a large series of patients.

Methods We studied 73 families in which at least one of the affected family members was affected at or before the age of 45 years and had parents who were not affected, as well as 100 patients with isolated Parkinson's disease that began at or before the age of 45 years. All subjects were screened for mutations in the *parkin* gene with use of a semiquantitative polymerase-chain-reaction assay that simultaneously amplified several exons. We sequenced the coding exons in a subgroup of patients. We also compared the clinical features of patients with *parkin* mutations and those without mutations.

Results Among the families with early-onset Parkinson's disease, 36 (49 percent) had *parkin* mutations. The age at onset ranged from 7 to 58 years. Among the patients with isolated Parkinson's disease, mutations were detected in 10 of 13 patients (77 percent) with an age at onset of 20 years or younger, but in only 2 of 64 patients (3 percent) with an age at onset of more than 30 years. The mean (±SD) age at onset in the patients with *parkin* mutations was younger than that in those without mutations (32±11 vs. 42±11 years, $P<0.001$), and they were more likely to have symmetric involvement and dystonia at onset, to have hyperreflexia at onset or later, to have a good response to levodopa therapy, and to have levodopa-induced dyskinesias during treatment. Nineteen different rearrangements of exons (deletions and multiplications) and 16 different point mutations were detected.

Conclusions Mutations in the *parkin* gene are a major cause of early-onset autosomal recessive familial Parkinson's disease and isolated juvenile-onset Parkinson's disease (at or before the age of 20 years). Accurate diagnosis of these cases cannot be based only on the clinical manifestations of the disease. (N Engl J Med 2000;342:1560-7.)

PARKINSON'S disease is one of the most frequent neurodegenerative disorders, with a prevalence of 1 to 2 percent among persons older than 65 years of age.[1] It is characterized by resting tremor, rigidity, and bradykinesia, all of which respond well to treatment with levodopa. The pathological hallmarks are the presence of Lewy bodies (cytoplasmic eosinophilic hyaline inclusions) and massive loss of dopaminergic neurons in the pars compacta of the substantia nigra.[2] The cause of the disease is still unknown, but the existence of genetic susceptibility factors is strongly suspected.[3,4] Two genes (α-synuclein[5] and ubiquitin carboxy-terminal hydrolase L1 [*UCH-L1*][6]) and two gene loci (on chromosomes 2p13 and 4p14–16.3, respectively[7,8]) have been implicated in the pathogenesis of autosomal dominant Parkinson's disease, but they seem to account for the cases in only a few families. In contrast, mutations in the gene designated *parkin* have recently been identified in several families with autosomal recessive early-onset parkinsonism.[9-14] However, the frequency of mutations in this gene in familial and isolated cases of early-onset parkinsonism has not yet been assessed in large series of patients.

The phenotype associated with mutations in the *parkin* gene has not been clearly established, making the selection of patients for genetic testing difficult. In Japanese patients with *parkin* mutations, the disease is characterized by an early onset, dystonia at

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onset, hyperreflexia, early complications resulting from levodopa treatment, and slow progression.[9-11] In contrast, the clinical characteristics of some European and North African patients with *parkin* mutations were indistinguishable from those of patients with idiopathic Parkinson's disease, with an age at onset of up to 58 years.[13,14] The number of families analyzed so far is small, however, and the correlations between genotype and phenotype are uncertain. Brain tissue from the patients studied did not contain Lewy bodies, suggesting that the pathologic process might differ from that of idiopathic Parkinson's disease.[15,16]

We performed a clinical and molecular study of 73 families with early-onset autosomal recessive Parkinson's disease, including 152 affected family members, and 100 patients with early-onset isolated Parkinson's disease. They were screened for mutations in the *parkin* gene by a semiquantitative polymerase-chain-reaction (PCR) assay designed to detect exon rearrangements (deletions and multiplications) and by genomic sequencing. Correlations between genotype and phenotype were assessed both in patients with *parkin* mutations and in those without such mutations.

METHODS

Patients and Families

We studied 73 families (152 patients with Parkinson's disease and 53 unaffected relatives) that met the following criteria: symptoms of parkinsonism in affected family members that were reduced by at least 30 percent by treatment with levodopa (the response could not be assessed in 3 untreated patients); a mode of inheritance compatible with autosomal recessive transmission (affected siblings without affected parents); an age at onset of 45 years or younger in at least one of the affected siblings; and the absence of extensor plantar reflexes, ophthalmoplegia, early dementia, or early autonomic failure in the family members we examined. Twenty of the families originated from Italy, 14 from France, 12 from Great Britain, 10 from the Netherlands, 9 from Germany, 2 from Portugal, and 1 each from Spain, Algeria, Morocco, Argentina, India, and Vietnam. Eight of the families were consanguineous, and 12 have been described previously.[13,14] In addition, we studied 100 patients with isolated Parkinson's disease with an age at onset of 45 years or younger, most of whom were European, and 8 of whom were from consanguineous marriages. These 100 patients were selected according to the same clinical criteria used for the patients with familial disease but who had no family history of Parkinson's disease. Eight of these 100 patients had never received treatment.

The enrollment of the subjects was random. For each subject, we obtained clinical information from the subject or the subject's records and peripheral blood for DNA analysis. DNA was extracted from peripheral-blood leukocytes according to standard procedures. The study was approved by ethics committees in the countries of all the participating investigators, and written informed consent was obtained from all the study subjects.

Molecular Analysis

Screening for mutations in the *parkin* gene was performed in all index patients (except those known to be homozygous or to have compound heterozygosity for such mutations[13,14]) with the use of a semiquantitative PCR assay for the detection of rearrangements of *parkin* exons. Exons 2 through 12 were amplified simultaneously in three groups by PCR (multiplex PCR): group 1 consisted of exons 4, 7, 8, and 11; group 2 consisted of exons 5, 6, 8, and 10; and group 3 consisted of exons 2, 3, 9, and 12 and an external control, C328, a 328-bp sequence of the transthyretin gene on chromosome 18. The primers used were the same as those described by Kitada et al.[9] except for the primer for exon 3, for which exonic primers were used: 5′AATTGTGACCTGGATCAGC3′ (Ex3iFor) as the forward primer and 5′CTGGACTTCCAGCTGGTGGTGAG3′ (Ex3iRev) as the reverse primer. The C328 forward primer was 5′ACGTTCCTGATAATGGGATC3′ (TTRForHex), and the reverse primer was 5′CCTCTCTCTACCAAGTGAGG3′ (TTR328Rev). All forward primers were fluorescently labeled with HEX-phosphoramitide. The PCR products (2.5 μl) were analyzed by 5 percent denaturing polyacrylamide-gel electrophoresis with an automated sequencer (model ABI 377) and GeneScan version 3.1 and Genotyper version 1.1.1 software (all from Applied Biosystems). All reactions were performed at least twice. The DNA from a patient known to have a heterozygous deletion of exons 8 and 9 was always processed in parallel as an internal control.

We calculated the ratios of all the peak heights in a given reaction and then compared the ratios with the ratios measured in a specimen from a normal subject. This comparison yielded the following rules: values of 0.6 or less were interpreted as indicating a heterozygous deletion of an exon; values of 0.8 to 1.2 were interpreted as normal; values of 1.3 to 1.7 were interpreted as indicating a heterozygous duplication of an exon; values of 1.8 to 2.3 were interpreted as indicating a homozygous duplication or heterozygous triplication of an exon; and values of more than 2.6 were interpreted as indicating a homozygous triplication of an exon. An exon rearrangement was confirmed only if all the ratios concerning this exon were abnormal. The consequence of the rearrangements at the protein level (a frame shift vs. an in-frame rearrangement) was deduced from the exon sequences published by Kitada et al.[9] (DNA Data Bank of Japan accession number AB009973).

Each PCR reaction involved, in a total volume of 25 μl, 40 ng of DNA, with 3 mM magnesium chloride, 0.2 mM of each deoxynucleoside triphosphate, and 1 U of *Taq* polymerase. Denaturation for 5 minutes at 95°C was followed by 23 cycles consisting of 30 seconds of denaturation at 95°C, 45 seconds of annealing at 53°C, and 2.5 minutes of extension at 68°C, with a final period of extension at 68°C for 5 minutes. Primer concentrations were chosen to yield — within the exponential phase of the PCR (data not shown) — similar peak heights in each multiplex reaction and ranged from 0.4 to 1.9 μM. Deletions and insertions of bases were deduced from the size of the PCR products.

In the case of 54 index patients with familial disease and 91 index patients with isolated disease, exon rearrangements or deletions or insertions of bases were not found on both chromosomes and thus did not account for the phenotype. Therefore, the entire coding region of the *parkin* gene (including exon–intron boundaries) was sequenced as described previously[14] in 53 index patients with familial disease and 50 patients with isolated disease.

Whether the *parkin* variants we identified cosegregated with the disease was assessed by genotyping of all available members, affected or not, of the respective families. In addition, 114 chromosomes from mostly European, unrelated controls who had no movement disorders were analyzed for the point mutations and the rearrangements of the exons represented in multiplex group 3. The techniques used were restriction assays, polyacrylamide-gel electrophoresis, and the semiquantitative PCR assay. For two point mutations — the substitution of A for G at nucleotide 939 of complementary DNA (cDNA) and the substitution of T for C at nucleotide 1101 of cDNA — mismatched reverse primers were used in order to create a restriction site that depended on the point mutation being looked for: 5′GGCAGGGAGTAGCCAAGTTGAGGAT3′ for digestion with *Alw*I and 5′AGCCCCGCTCCACAGCCAGCGC3′ for digestion with *Bst*UI (in each primer, the underlined nucleotide differs from the wild-type sequence).

Statistical Analysis

Means (±SD) were compared with the nonparametric Mann–Whitney U test. Frequencies were compared with the chi-square test, with Yates' correction when appropriate.

RESULTS

Frequency of Mutations in the *parkin* Gene

Twenty-five families (56 patients) with autosomal recessive Parkinson's disease had homozygous or compound heterozygous mutations on each allele of the *parkin* gene (Table 1). In addition, 11 families (27 patients) with a mutation in one allele were considered to have *parkin*-related disease, on the basis of the assumption that the second mutation was not detected by the methods used in this study. Thus, mutations in the *parkin* gene were detected in 36 of 73 families (49 percent), including 12 previously described families.[13,14] Among the 100 patients with isolated Parkinson's disease, 18 (18 percent) had *parkin* mutations. The frequency of mutations among consanguineous patients with isolated Parkinson's disease, a pattern that is suggestive of autosomal recessive inheritance, was similar to that among consanguineous patients with familial disease (50 percent vs. 62 percent). The frequency of mutations in the patients with isolated Parkinson's disease decreased significantly with increasing age at onset: mutations were detected in 10 of 13 patients (77 percent) with an age at onset of disease of 20 years or younger, but only in 2 of 64 patients (3 percent) with an age at onset of 31 to 45 years (Table 2). Sequencing of the *parkin* gene in 22 of the 64 patients with isolated Parkinson's disease who were older than 30 years at the onset of symptoms revealed a point mutation in only 1 patient. In 14 families in which the affected family members had *parkin* mutations on both chromosomes, none of 28 unaffected siblings had two *parkin* mutations, indicating the high penetrance of the mutations.

TABLE 1. FREQUENCY OF MUTATIONS IN THE *PARKIN* GENE IN 73 FAMILIES WITH AUTOSOMAL RECESSIVE EARLY-ONSET PARKINSON'S DISEASE AND 100 PATIENTS WITH ISOLATED EARLY-ONSET PARKINSON'S DISEASE.*

No. of Mutations	Families with Autosomal Recessive Disease (N=73)		Patients with Isolated Cases (N=100)	
	No. of Families	No. of Consanguineous Families	No. of Patients	No. of Consanguineous Patients
Two	25	5	14	4
One	11†	0	4	0
None identified	37	3	82‡	4
Total no. with mutations (%)	36 (49)	5 (62)	18 (18)	4 (50)

*Early onset was defined as an onset at or before 45 years of age (in at least one of the affected siblings in affected families).

†The *parkin* gene was not sequenced in one family.

‡The *parkin* gene was not sequenced in 41 patients in whom the onset of disease was after 30 years of age.

TABLE 2. FREQUENCY OF MUTATIONS IN THE *PARKIN* GENE IN 100 PATIENTS WITH ISOLATED EARLY-ONSET PARKINSON'S DISEASE, ACCORDING TO THE AGE AT ONSET.

Age at Onset	Patients with Homozygous or Heterozygous Mutations	Consanguineous Patients with Homozygous or Heterozygous Mutations
	no. of patients/total no. (%)	
≤20 yr	10/13 (77)*	2/3 (67)
21–30 yr	6/23 (26)†	2/2 (100)
31–40 yr	1/49 (2)‡	0/2
41–45 yr	1/15 (7)§	0/1
Total no. of patients (%)	18/100 (18)	4/8 (50)

*P=0.003 for the comparison with patients with an age at onset of 21 to 30 years.

†P=0.005 for the comparison with patients with an age at onset of 31 to 40 years.

‡The *parkin* gene was not sequenced in 35 patients.

§The *parkin* gene was not sequenced in six patients.

Clinical Studies

The 36 families with Parkinson's disease and *parkin* mutations and the 18 patients with isolated Parkinson's disease and *parkin* mutations came from a variety of regions: France (in 15 cases), Italy (in 13), Great Britain (in 7), the Netherlands (in 3), Spain (in 3), Germany (in 3), Portugal (in 2), Algeria (in 2) and Lebanon, India, Pakistan, Vietnam, Japan, and Argentina (in 1 case each).

As a group, the 100 patients with *parkin* mutations had a mean (±SD) age at onset of 32±11 years (range, 7 to 58); the age at onset was not known for 1 patient (Table 3). Among the patients with an age at onset of 45 years or younger, the onset of the disease was earlier in the 18 patients with isolated Parkinson's disease and *parkin* mutations than in the 75 patients with familial Parkinson's disease and mutations (mean age, 21±9 vs. 32±9 years; median, 20 vs. 33 years; $P<0.001$). This difference was not due to selection bias, because the mean ages at onset were similar in the two groups when all initially included patients with an age at onset of 45 years or younger were compared, whether or not they had *parkin* mutations (age at onset in 118 patients with familial disease, 34±9 years; in 100 patients with isolated disease, 32±9 years). The mean age at onset was significantly younger in the patients with *parkin* mutations than in those without mutations, both in the total sample (Table 3) and in the group with familial cases alone (34±10 years for 82 patients with familial disease and mutations and 43±12 years for 65 patients with familial disease but without mutations; $P<0.001$).

The initial manifestations of the disease in most patients with *parkin* mutations were tremor (65 per-

TABLE 3. CHARACTERISTICS OF PATIENTS WITH PARKINSON'S DISEASE ACCORDING TO THE PRESENCE OR ABSENCE OF MUTATIONS IN THE *PARKIN* GENE.*

CHARACTERISTIC	PATIENTS WITH *PARKIN* MUTATIONS (N=101)	PATIENTS WITHOUT *PARKIN* MUTATIONS (N=85)	P VALUE
Sex (F/M)	49/52	31/54	
Age at onset (yr)	32±11	42±11	<0.001
Duration of disease (yr)	17±11	13±11	0.002
Clinical signs at onset (%)			
Micrography	30	47	0.02
Bradykinesia	63	65	
Tremor	65	75	
Dystonia	42	22	0.02
Asymmetric signs	89	98	0.02
Clinical signs at examination			
Bradykinesia (%)	95	98	
Rigidity (%)	92	99	
Resting tremor (%)	74	80	
Postural tremor (%)	54	47	
Urinary urgency (%)	11	25	0.01
Hyperreflexia (%)	44	21	0.04
No progression or slow rate of progression (%)	88	72	
Motor scale of UPDRS score†			
Without treatment	41±22	43±16	
During treatment	23±18	26±15	
Hoen–Yahr assessment without treatment‡			
Mean stage	3.2±1.0	3.1±0.8	
Interval from onset to stage 2 (yr)	11±9	5±3	
Interval from onset to stage 3 (yr)	19±10	17±8	
Interval from onset to stage 4 (yr)	26±8	33±2	
Interval from onset to stage 5 (yr)	40±19	44	
Mini–Mental State Examination score§	29±3	28±2	
Clinical signs during treatment			
Percent improvement with levodopa	72±20	64±17	0.03
Daily dose of levodopa (mg)	500±340	600±400	
Duration of levodopa treatment			
Months	123±102	111±99	
Years	10	9	
Levodopa-induced dyskinesia			
Percentage of patients	77	63	0.04
Months of treatment	64±65	60±55	
Levodopa-induced fluctuations in symptoms			
Percentage of patients	79	65	
Months of treatment	64±61	61±54	
Dystonia			
Percentage of patients	58	45	
Months of treatment	65±72	54±40	

*Plus–minus values are means ±SD. Among the patients with *parkin* mutations, 83 had familial disease and 18 had isolated disease. Among the patients without *parkin* mutations, 57 had familial disease and 28 had isolated disease.

†The motor scale of the Unified Parkinson's Disease Rating Scale (UPDRS)[17] assesses 14 motor functions of patients with Parkinson's disease. Some of the functions were tested separately for each side of the body, the arms and legs, the face, and the trunk, resulting in 27 subtests. Each subtest was scored on a scale from 0 (no impairment) to 4 (severe impairment), resulting in a total score ranging from 0 to 108.

‡The Hoehn and Yahr stages are used to describe the degree of functional disability of patients with Parkinson's disease. Stage 1 indicates mild unilateral symptoms, stage 2 bilateral or axial symptoms, stage 3 impairment of postural reflexes, stage 4 strongly disabling disease (the patient is able to stand and walk unassisted but is markedly incapacitated), and stage 5 severely disabling disease (the patient cannot stand or walk without assistance and is therefore confined to a wheelchair or bed).[18]

§The Mini–Mental State examination assesses orientation; short-term memory; attention span; and naming, copying, reading, writing, spatial, and constructive capacities with respect to 30 tasks, all scored as either 1 (succeeded) or 0 (failed). The maximal score is 30; dementia was considered to be present if the score was below 24.

cent) and bradykinesia (63 percent) (Table 3). The patients with *parkin* mutations had significantly higher frequencies of dystonia and symmetric symptoms at onset and of hyperreflexia at onset or later, as well as a better response to levodopa despite having had the disease for a longer period (Table 3) than those with no *parkin* mutations. Dystonia began in the lower limbs in 28 of 31 patients with mutations, but 2 patients first had torticollis and 1 had right-arm dystonia. Dyskinesia as a result of levodopa treatment was significantly more common in patients with mutations than in those with no mutations, but such dyskinesia occurred in both groups, on average, after nearly 5 years of treatment (range, 1 month to 20 years). There were no significant differences between the 24 patients with at least one missense mutation and the 52 patients with two truncating mutations; the 25 patients with single heterozygous truncating mutations were not assigned to either group, since the nature of the suspected second mutation was unknown.

Nineteen different homozygous and heterozygous exon rearrangements were found in 35 index patients, including 4 from previously described families with homozygous deletions of exons (Table 4 and Fig. 1A).[13,14] In addition to identifying the suspected deletions of an exon, our approach provided evidence of four duplications of an exon and one triplication of an exon. The results were highly reproducible and confirmed by cosegregation analysis. Rearrangements of exons 2, 3, 9, and 12 were not found in the controls.

Sixteen different exonic point mutations were found in 28 index patients, including 8 from previously described families[14] (Table 4 and Fig. 1B). In addition, an intronic deletion of 5 bp (IVS8 −21 to −17del) was detected. All point mutations cosegregated with the disease, and none were found in any control. The amino acids modified by mutations were conserved in the *parkin* orthologues in rats[20] and mice (Gene Bank accession numbers AF210434 and AB019558, respectively). However, in two patients from one family, the homozygous point mutation Arg334Cys was associated with the homozygous intronic 5-bp deletion and the heterozygous Asp280Asn mutation, so that the pathogenicity of the latter two mutations cannot be ascertained.

Many of the exon rearrangements were found repeatedly among the index patients, particularly deletions of exon 3 (in 10 patients), exon 2 (in 4), exon 4 (in 4), and exons 3 and 4 (in 4) (Fig. 1A). Six point mutations were found in more than one index patient: the deletion of A at nucleotide 255 of cDNA (in six index patients), the deletion of A and G at nucleotide 202 to 203 of cDNA (in five), Arg275Trp (in five), the insertion of G and T between nucleotide 321 and nucleotide 322 of cDNA (in two), Lys211Asn (in two), and Gly430Asp (in two) (Fig. 1).

TABLE 4. FREQUENCY OF HOMOZYGOUS, COMPOUND HETEROZYGOUS, AND SINGLE HETEROZYGOUS MUTATIONS AMONG 45 INDEX PATIENTS WITH FAMILIAL OR ISOLATED PARKINSON'S DISEASE, ACCORDING TO THE TYPE OF MUTATION.

TYPE OF MUTATION	EXON REARRANGEMENT	POINT MUTATION	EXON REARRANGEMENT PLUS A POINT MUTATION*	TOTAL NO. (%)
	no. of index patients (no. of consanguineous index patients)			
Homozygous	9 (6)	10 (3)	NA	19 (35)
Compound heterozygous	8	3	9	20 (37)
Single heterozygous	9†	6	NA	15 (28)

*NA denotes not applicable.

†The *parkin* gene was not sequenced in one index patient.

DISCUSSION

We detected mutations in the *parkin* gene in almost half the families with autosomal recessive Parkinson's disease in which at least one affected member was 45 years of age or younger at the onset of symptoms. The frequency of such mutations was lower in a group of patients with isolated early-onset Parkinson's disease.

On average, patients with *parkin* mutations began to have symptoms in their early 30s, but the age at onset ranged widely, from 7 to 58 years. The fact that the onset occurred at an earlier age in patients with isolated Parkinson's disease and *parkin* mutations than in those with familial Parkinson's disease and *parkin* mutations suggests that among patients who are older than 30 years at the onset of isolated Parkinson's disease, the disease is mainly due to causes other than *parkin* mutations.

Can patients with *parkin* mutations be distinguished clinically from patients with early-onset Parkinson's disease from other causes? As a group, those with *parkin* mutations had an earlier onset of disease, were more likely to have dystonia and symmetric signs at onset, as well as hyperreflexia at onset or later, and were more likely to have a better response to levodopa, but were also more likely to have dyskinesia during treatment, than were patients without *parkin* mutations. These signs were less frequent, however, than in previous reports[11,21] and could not be used specifically to identify patients with mutations. Furthermore, the clinical manifestations of the *parkin* mutations were independent of the age at onset.

In addition, patients with late-onset disease who have mutations can be difficult to distinguish from those with idiopathic Parkinson's disease. In general, however, the disease progressed slowly in the patients with mutations. Despite having had symptoms for

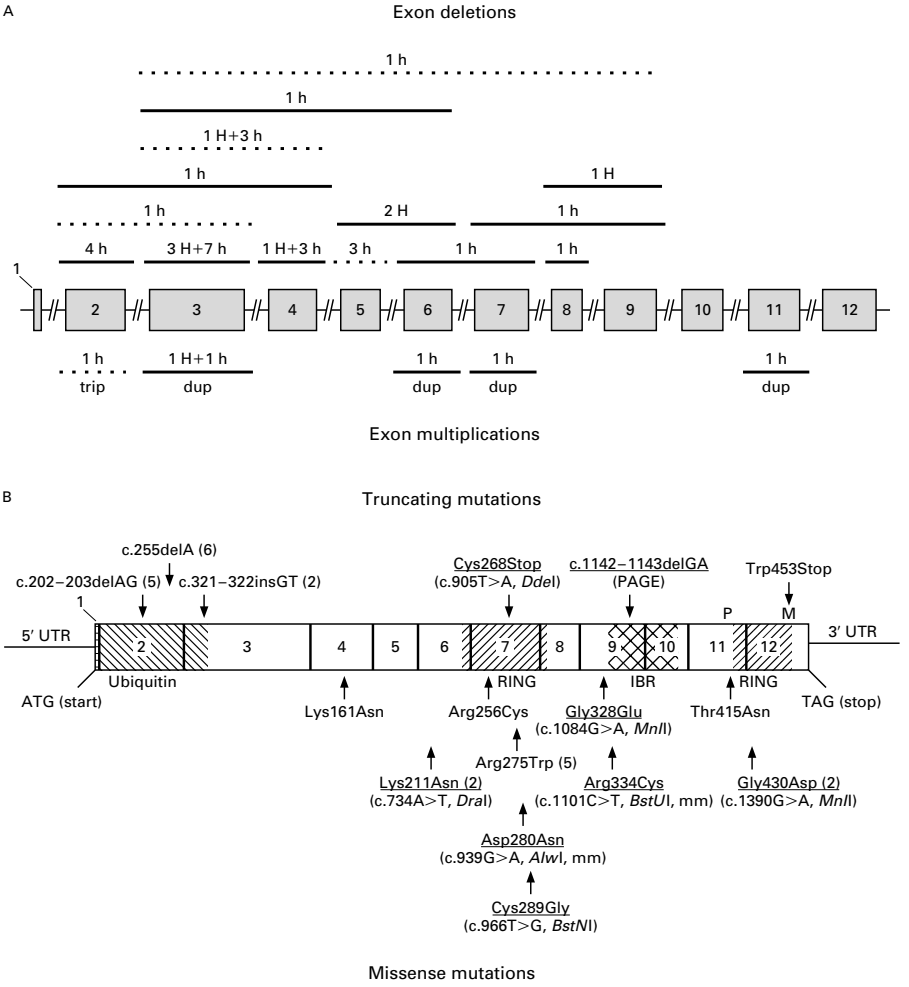

Figure 1. Mutations in the *parkin* Gene.

Panel A shows the exon rearrangements identified. Deletions are indicated above the sequence, and duplications (dup) and triplications (trip) are indicated below the sequence. Their deduced effect on the protein is represented by a dotted line for in-frame rearrangements and by a solid line for frame-shift rearrangements. The number of index patients with the rearrangement and the type of mutation — heterozygous (h) or homozygous (H) — are indicated above each mutation. Panel B shows the point mutations resulting in truncation of the sequence of 12 exons or in a missense mutation. The hatched regions indicate the ubiquitin-like domain and the RING–IBR–RING finger motif.[19] The 6 truncating mutations are indicated above the sequence, and the 10 missense mutations are indicated below the sequence. For mutations identified in more than one index patient, the number of index patients with the mutation is given in parentheses. The nucleotide change and the restriction enzymes used to screen family members and unrelated control subjects without movement disorders are given in parentheses below the mutation (mm denotes the mismatch primer used for the PCR, and PAGE polyacrylamide-gel electrophoresis). Mutations that were not based on published sequences[14] are underlined. Nucleotides prefaced by a small c indicate the numbers in the complementary DNA sequences described by Kitada et al.[9] The ATG of the initiator methionine codon begins at nucleotide 102. The putative site of phosphorylation (P) and an *N*-myristoylation site (M) affected by the Thr415Asn and Trp453Stop mutations, respectively, are indicated. UTR denotes untranslated region.

many years, the majority of patients with *parkin* mutations had good responses to low doses of levodopa. Although levodopa-induced dyskinesia was reported to develop early,[9,11,21] the mean delay in our patients was about 5 years, with a maximum of 20 years. This time frame was similar to that for the patients without *parkin* mutations.

Finally, dementia was rare among the patients with mutations. This might be explained by a less widespread neuronal loss in patients with mutations, in whom the substantia nigra and, to a lesser extent, the locus caeruleus are selectively affected, as compared with patients with idiopathic Parkinson's disease.[15,16] However, the low frequency of dementia in the patients with mutations could also be due to a younger mean age at examination or to the exclusion of patients who had dementia early in the course of the disease.

There were no clinical differences between patients with missense mutations and those with truncating mutations. This finding was surprising, since missense mutations might be expected to interfere less with the function of the *parkin* protein than truncating mutations and therefore to result in a milder phenotype. We therefore assume that the 10 conserved amino acids that were affected by the missense mutations are of crucial importance for the function of the protein or that their modification results in decreased protein synthesis or more rapid degradation.[22] In addition, the wide range of clinical signs, even within single families with mutations (e.g., variation of up to 20 years in the age at onset) suggests that additional factors contribute to the phenotype.

The chief histopathological differences between patients with *parkin* mutations and those with idiopathic Parkinson's disease that have been detected so far are the absence of Lewy bodies and the restriction of neuronal cell loss to the substantia nigra and the locus caeruleus in the patients with *parkin* mutations.[16] Thus, *parkin* gene mutations are responsible for the death of selective cells, the mechanism of which might differ from that in idiopathic Parkinson's disease.

The PCR-based technique that we used revealed numerous rearrangements of exons, including those identified in eight families in which no mutations were found by direct sequencing.[14] In combination with genomic sequencing, this technique greatly improves the sensitivity of the molecular diagnosis in patients with *parkin* gene mutations. The various combinations of exon deletions, the exon multiplications, and the newly identified point mutations increase the already wide variety of disease-related mutations identified in the *parkin* gene. The position of the mutations indicates functionally important protein regions such as the RING–IBR–RING domain, as does conservation of the corresponding amino acids in mice and rats.[20]

The presence of both deletions and multiplications of some exons (e.g., exon 2 and 3) suggests that a mechanism such as unequal recombination might be involved. The observation that 13 of the mutations were found repeatedly in as many as 10 families raises the possibility of a founder effect. However, many of the mutations were found in families from different European countries, suggesting that these alterations are recurrent. The point mutations that accounted for the disease in approximately 40 percent of our patients seem to be less frequent among Japanese patients.[11] Finally, the identification of 15 index patients with single heterozygous mutations indicates that other mutations remain to be discovered, perhaps in noncoding regions of the *parkin* gene.

In conclusion, mutations of the *parkin* gene are frequent among patients with autosomal recessive Parkinson's disease. Although dystonia at the onset of disease, hyperreflexia, and a slow rate of disease progression are characteristic features of patients with *parkin* mutations, there are no specific clinical signs that distinguish these patients from patients with other causes of Parkinson's disease. The wide spectrum of mutations in the *parkin* gene renders molecular diagnosis difficult, but the relatively simple semiquantitative PCR method that we used detected approximately 70 percent of the mutations found in this series of patients.

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APPENDIX

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REFERENCES

1. de Rijk MC, Tzourio C, Breteler MMB, et al. Prevalence of parkinsonism and Parkinson's disease in Europe: the EUROPARKINSON Collaborative Study: European community concerted action on the epidemiology of Parkinson's disease. J Neurol Neurosurg Psychiatry 1997;62:10-5.
2. Fearnley JM, Lees AJ. Ageing and Parkinson's disease: substantia nigra regional selectivity. Brain 1991;114:2283-301.
3. Wood N. Genes and parkinsonism. J Neurol Neurosurg Psychiatry 1997;62:305-9.
4. Piccini P, Burn DJ, Ceravolo R, Maraganore D, Brooks DJ. The role of inheritance in sporadic Parkinson's disease: evidence from a longitudinal study of dopaminergic function in twins. Ann Neurol 1999;45:577-82.

5. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 1997;276:2045-7.
6. Leroy E, Boyer R, Auburger G, et al. The ubiquitin pathway in Parkinson's disease. Nature 1998;395:451-2.
7. Gasser T, Müller-Myhsok B, Wszolek ZK, et al. A susceptibility locus for Parkinson's disease maps to chromosome 2p13. Nat Genet 1998;18: 262-5.
8. Farrer M, Gwinn-Hardy K, Muenter M, et al. A chromosome 4p haplotype segregating with Parkinson's disease and postural tremor. Hum Mol Genet 1999;8:81-5.
9. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998;392:605-8.
10. Hattori N, Matsumine H, Asakawa S, et al. Point mutations (Thr240Arg and Ala311Stop) in the *parkin* gene. Biochem Biophys Res Commun 1998;249:754-8. [Erratum, Biochem Biophys Res Commun 1998;251:666.]
11. Hattori N, Kitada T, Matsumine H, et al. Molecular genetic analysis of a novel parkin gene in Japanese families with autosomal recessive juvenile parkinsonism: evidence for variable homozygous deletions in the parkin gene in affected individuals. Ann Neurol 1998;44:935-41.
12. Leroy E, Anastasopoulos D, Konitsiotis S, Lavedan C, Polymeropoulos MH. Deletions in the parkin gene and genetic heterogeneity in a Greek family with early onset Parkinson's disease. Hum Genet 1998;103: 424-7.
13. Lücking CB, Abbas N, Dürr A, et al. Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism. Lancet 1998;352:1355-6.
14. Abbas N, Lücking CB, Ricard S, et al. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. Hum Mol Genet 1999;8:567-74.
15. Takahashi H, Ohama E, Suzuki S, et al. Familial juvenile parkinsonism: clinical and pathologic study in a family. Neurology 1994;44:437-41.
16. Mori H, Kondo T, Yokochi M, et al. Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology 1998; 51:890-2.
17. Fahn S, Elton RL, UPDRS Development Committee. Unified Parkinson's Disease Rating Scale. In: Fahn S, Marsden CD, Goldstein M, Calne DB, eds. Recent developments in Parkinson's disease. Vol. 2. Florham Park, N.J.: Macmillan Healthcare Information, 1987:153-63.
18. Hoehn MM, Yahr MD. Parkinsonism: onset, progression and mortality. Neurology 1967;17:427-42.
19. Morett E, Bork P. A novel transactivation domain in parkin. Trends Biochem Sci 1999;24:229-31.
20. Gu W-J, Abbas N, Lagune-Zarate M, et al. Cloning of rat parkin cDNA and distribution of parkin in rat brain. J Neurochem 2000;74:1773-6.
21. Ishikawa A, Tsuji S. Clinical analysis of 17 patients in 12 Japanese families with autosomal-recessive type juvenile parkinsonism. Neurology 1996; 47:160-6.
22. Bross P, Corydon TJ, Andresen BS, Jorgensen MM, Bolund L, Gregersen N. Protein misfolding and degradation in genetic diseases. Hum Mutat 1999;14:186-98.

Chapter 2.2

Three *parkin* gene mutations in a sibship with autosomal recessive early onset parkinsonism

Bonifati V, Lucking CB, Fabrizio E, Periquet M, Meco G, Brice A.

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SHORT REPORT

Three parkin gene mutations in a sibship with autosomal recessive early onset parkinsonism

V Bonifati, C B Lücking, E Fabrizio, M Periquet, G Meco, A Brice

Abstract
The objective was to describe a family with autosomal recessive, early onset parkinsonism, with affected siblings carrying three different exon rearrangements in the parkin gene.
The living affected siblings were personally examined. Molecular genetic analyses included exon dosage of the parkin gene using a semiquantitative multiplex polymerase chain reaction (PCR) protocol and haplotype analysis.
The index case was a compound heterozygote with a deletion of exon 5 and a duplication of exon 3. His affected sister was a compound heterozygote for a deletion of exon 5 and a deletion of exons 3–9. Haplotype analysis confirmed the presence of three mutant alleles at the parkin locus. The phenotype was early onset parkinsonism with marked response to levodopa, and a very slow, prolonged course.
In conclusion, the frequency of mutations in the parkin gene in certain populations might be high enough to cause allelic heterogeneity in the same sibship.
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Keywords: early onset parkinsonism; parkin gene; exon rearrangements

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Mutations in the parkin gene are a frequent cause of autosomal recessive, early onset parkinsonism. Various mutations, including exon rearrangements (deletions and multiplications) and point mutations (truncating and missense) have been identified.[1–10] The mutational screening is probably still incomplete, as only one mutant allele has so far been detected in 15 out of 54 European families with parkin mutations, even after exon dosage and sequence analysis have been performed.[7] The parkin protein has recently been shown to possess ubiquitin ligase activity, which is lost when mutated.[11]

The finding of a pedigree with parkin mutation in affected persons from two different generations raised the question of a possible dominant negative effect of some parkin mutations.[9] A pseudodominant transmission is an alternative explanation which cannot be ruled out until mutational screenings in parkin become more sensitive. To date, a pseudodominant transmission of parkin related disease has been reported in a single Japanese family segregating three mutant alleles in different sibships (two alleles carrying an exon 4 deletion, and the third allele carrying the Cys431Phe point mutation in exon 12).[10]

We describe a sibship with early onset, slowly progressive parkinsonism whose members are compound heterozygotes for three different exon rearrangements in the parkin gene.

Family report

There is no known consanguinity between the parents of the patients, who originated from different regions in Italy.

The index case (RM611, born 1922) first noticed slowness of movements in the right leg at the age of 35. The clinical course was very slow. He was diagnosed with Parkinson's disease and treated with levodopa from 1970, with marked response. Owing to motor fluctuations and levodopa induced dyskinesias, since 1994 levodopa has been replaced by oral dopamine agonist monotherapy (0.5 mg lisuride four times/day), with a satisfactory response for years.

Our examination in 1998 (at age 76, after 40 years of disease course) showed a flexed posture, axial akinesia and gait difficulties; moderate generalised rigidity, and mild diffuse bradykinesia in all four limbs, although more severe in the upper limbs and on the left side. Tremor was absent and the tendon reflexes were normal. Dysarthria and hypomimia were mild, and a Meyerson's sign was present. Cognition was normal and there was no severe autonomic disturbance. His on state Hoehn and Yahr stage was III. Brain MRI was normal. He was still treated with oral lisuride (0.5 mg four times/day) and deprenyl (5 mg/day).

The sister (RM612, born 1909) first experienced resting tremor in the upper limbs at the age of 31. Tremor was bilateral, although more severe on the right side. When bradykinesia and rigidity developed later, she was diagnosed with Parkinson's disease. The disease was slowly progressive, affecting both sides of the body, although more severely on the right side. In the late 1960s, after more than 25 years, the symptoms had worsened to the extent that she underwent stereotactic left thalamotomy, which satisfactorily controlled the contralateral

tremor. She was treated with levodopa from 1970, with good response. After several years she developed motor fluctuations and levodopa induced dyskinesias. We were able to examine her at the age of 89, after 58 years of disease course, a few months before her death; she had a severe akinetic rigid syndrome, with Hoehn and Yahr stage IV in the on, and V in the off states, respectively. Only mild resting tremor was evident in both upper limbs. Tendon reflexes were brisk. She also had a memory deficit, visual hallucinations, and frank confusional episodes, which were reported to have been present during the past 3 years. At the

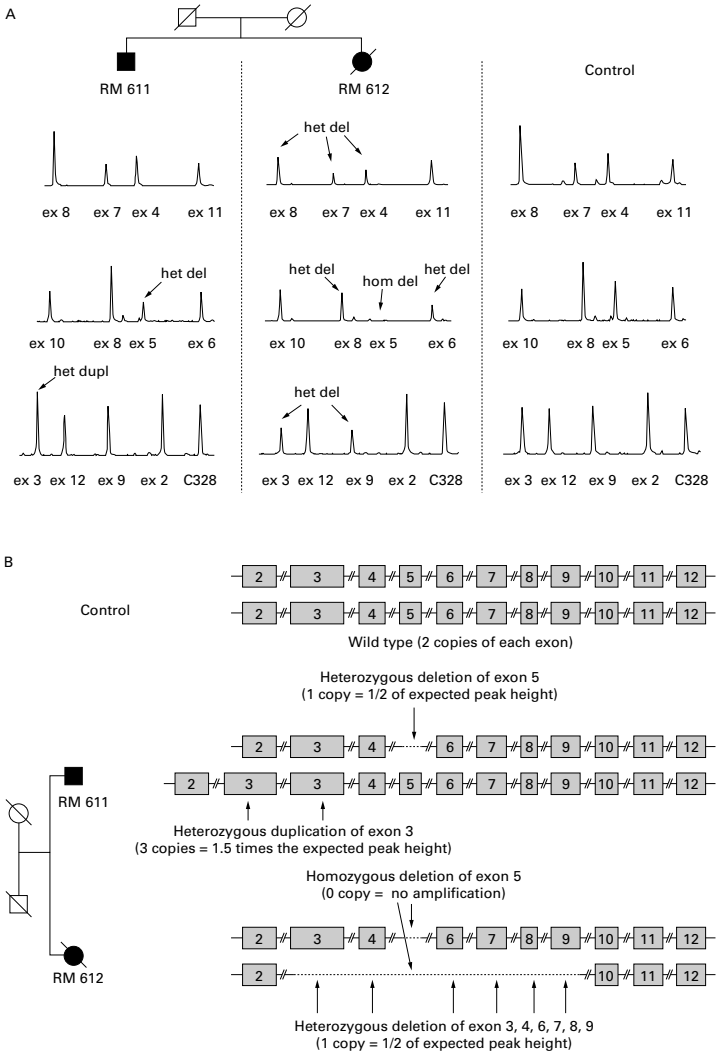

Figure 1 Exon rearrangements detected in the parkin gene. (A) Electropherograms showing fluorescently labelled multiplex PCR products (see methods). The peak height reflects the quantity of template exon DNA. Het del=heterozygous exon deletion, indicated by half of the expected peak height; hom del=homozygous exon deletion, indicated by no amplification product; het dupl=heterozygous duplication, indicated by 1.5 times the expected peak height. (B) Schematic representation of the exon rearrangements at the genomic level. Exons 2–12 within the pair of homologous chromosomes are depicted as boxes, introns as solid lines. As the size of the introns is not known, double slashes separate the exons. Exon deletions are indicated as dotted lines. The in tandem position of the duplicated exon 3 is hypothetical.

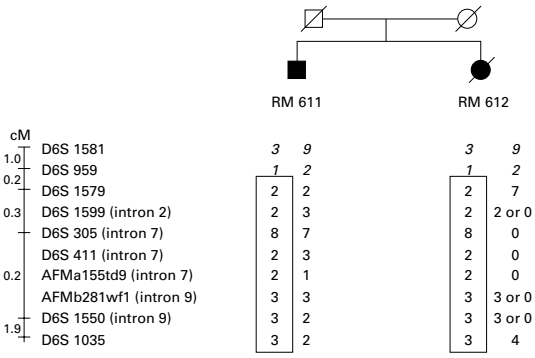

Figure 2 Haplotype analysis. Six intragenic (the intronic position is given in parenthesis) and four flanking markers were analyzed. The haplotype associated with the exon 5 deletion is boxed. The alleles for markers D6S1581 and D6S959 were not attributable to either haplotype and are indicated in italics.

time of our examination she was still receiving levodopa (375 mg/day), amantadine (200 mg/day), and biperiden (5 mg/day). Brain imaging was not available, and pathological studies could not be performed.

Another sister (born 1913) was diagnosed with Parkinson's disease with onset at the age of 31. She died in 1969, after 25 years of disease course. Very limited information is available on the parental and previous generations. The father of the patients died at the age of 54, whereas the mother of the patients died at the age of 67, with no reported evidence of parkinsonism.

Molecular genetic studies

DNA was extracted using standard techniques from peripheral blood leucocytes after informed written consent. Exon rearrangements of the parkin gene were detected using a semi-quantitative multiplex polymerase chain reaction (PCR) protocol described elsewhere.[7] Exons 2–12 were amplified simultaneously in three combinations by multiplex PCR using HEX labelled forward primers. A 328 base pair sequence of the transthyretin gene on chromosome 18 (C328) was also amplified as an internal control.[7] The PCR products were analyzed on an ABI377 automated sequencer with the Genescan 3.1 and Genotyper 1.1.1 software (Applied Biosystems). All possible peak height ratios were calculated between the peaks in given multiplex reactions. The peak height ratios of the family members were compared with a normal control. Differences of a factor of 0.5 were interpreted as indicating a heterozygous deletion, and those of a factor of 1.5 as indicating a heterozygous exon duplication. All reactions were carried out at least in duplicate. This method has been validated by cosegregation studies in several families.[7]

The consequences of exon rearrangements at the level of parkin protein were predicted according to the published exonic sequences (Genome database accession number AB009973).

Haplotypes of the parkin gene region were constructed manually using the following DNA repeat markers: D6S1581, D6S959, D6S1579, D6S1599, D6S305, D6S411, AFMa155td9, AFMb281wf1, D6S1550, D6S1035 (intragenic markers underlined). They were amplified with the primers specified in the Genome database, except for D6S1599 which was amplified using "forward" 5'-GGG TGTGCTTGGATTCCTTCATG-3' and "reverse" 5'-TAGCATGTGGACTGCATAT CAAC-3'. PCR conditions were 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds in 35 cycles. The PCR products were analysed on an ABI377 automated sequencer (Applied Biosystems), and marker alleles were assigned in increasing order.

Molecular findings

The index case is a carrier of heterozygous deletion of exon 5 and heterozygous duplication of exon 3. His sister carries a heterozygous deletion of exon 5, and a heterozygous deletion of exons 3 to 9 (fig 1).

At the level of the parkin protein, the exon 5 and exon 3–9 deletions lead to in-frame rearrangements. By contrast, assuming that the duplicated exons 3 are in tandem (fig 1 B), the mutation introduces a frameshift rearrangement and a complete loss of function of the parkin protein.

Reconstructed haplotypes using intragenic and flanking polymorphic markers are shown in figure 2. As expected, the two sibs only share one haplotype, the one associated with exon 5 deletion. In addition, for the intragenic markers D6S305, D6S411, and AFMa155td9 patient RM612 was an obligate hemizygote, because the deletion of exons 3–9 encompasses intron 7, where these markers are located (fig 1 B). For the remaining intragenic markers D6S1599 (located in intron 2), and AFMb281wf1 and D6S1550 (both located in intron 9), it was not possible to determine whether patient RM612 was homozygote or hemizygote because parental genotypes were not available.

Discussion

The young onset, very slow disease progression and good response to levodopa in this family suggested the involvement of parkin gene mutations.[7] In addition, the prolonged satisfactory response to dopamine agonist monotherapy in case RM611 is of interest. A sustained response to combined therapy with bromocriptine and biperiden has been reported in a patient with parkin mutations.[8] Further studies are warranted to characterise the response to dopaminergic stimulation in patients with parkin mutations.

Dementia is not considered as part of the phenotype in patients with parkin mutations.[7 12] In the largest series so far analyzed, this may have been due to the young age of patients at the examination or exclusion from screening of cases with early dementia.[7] Patient RM612 is the first described with marked cognitive disturbances, which might be ascribed either to the extremely long course of parkin

disease, or to a coincidental Alzheimer-type pathology. However, they more likely represent side effects of the antiparkinsonian drugs in an old subject who also underwent stereotactic thalamotomy.

The presence of three different mutations indicate that one of the parents was also a compound heterozygous carrier of parkin mutations. Neither the father nor mother was known to be affected when they died at the ages of 54 and 67, respectively. This finding may represent a case of as yet undescribed non-penetrance of parkin mutations, or more likely reflect the age dependent penetrance of the disease. Parkin related disease displays wide intrafamilial variability of ages at onset,[5–7] and the latest age at onset reported so far in patients with proved parkin mutations is 64 years.[9] Moreover, owing to the mild severity of symptoms in the first years from onset, the disease of one parent might not have been recognised. Mistaken paternity could be another explanation, but is unlikely because one would have to assume that both biological fathers were heterozygous carriers of different parkin mutations.

Both scenarios therefore suggest that mutations in the parkin gene are frequent enough in some populations to lead to allelic heterogeneity in the same sibship, as described here, or to pseudodominant transmission, as recently reported.[10]

Owing to the presence of three mutant alleles, linkage and haplotype analyses could have led to an erroneous exclusion in this family of the parkin locus. Therefore, parkin gene mutation in patients with levodopa responsive parkinsonism, onset before the age of 40 and slow disease progression should be tested by direct analyses.

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1 Kitada T, Asakawa S, Hattori N, *et al*. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 1998;**392**:605–8.
2 Hattori N, Kitada T, Matsumine H, *et al*. Molecular genetic analysis of a novel parkin gene in Japanese families with autosomal recessive juvenile parkinsonism: evidence for variable homozygous deletions in the parkin gene in affected individuals. *Ann Neurol* 1998;**44**:935–41.
3 Leroy E, Anastasopoulos D, Konitsiotis S, *et al*. Deletions in the parkin gene and genetic heterogeneity in a Greek family with early onset Parkinson's disease. *Hum Genet* 1998;**103**:424–7.
4 Hattori N, Matsumine H, Asakawa S, *et al*. Point mutations (Thr240Arg and Gln311Stop) [correction of Thr240Arg and Ala311Stop] in the parkin gene. *Biochem Biophys Res Commun* 1998;**249**:754–8. [Published erratum appears in *Biochem Biophys Res Commun* 1998;**251**:666]
5 Lücking CB, Abbas N, Dürr A, *et al*. Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism. *Lancet* 1998;**352**:1355–6.
6 Abbas N, Lücking CB, Ricard S, *et al*. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. *Hum Mol Genet* 1999;**8**:567–74.
7 Lücking CB, Dürr A, Bonifati V, *et al*. Association between early-onset Parkinson's disease and mutations in the parkin gene. *N Engl J Med* 2000;**342**:1560–7.
8 Muñoz E, Pastor P, Martí MJ, *et al*. A new mutation in the parkin gene in a patient with atypical autosomal recessive juvenile parkinsonism. *Neurosci Lett* 2000;**289**:66–8.
9 Klein C, Pramstaller PP, Kis B, *et al*. Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: expanding the phenotype. *Ann Neurol* 2000;**48**:65–71.
10 Maruyama M, Ikeuchi T, Saito M, *et al*. Novel mutations, pseudo-dominant inheritance, and possible familial affects in patients with autosomal recessive juvenile parkinsonism. *Ann Neurol* 2000;**48**:245–50.
11 Shimura H, Hattori N, Kubo S, *et al*. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat Genet* 2000;**25**:302–5.
12 Yamamura Y, Hattori N, Matsumine H, *et al*. Autosomal recessive early-onset parkinsonism with diurnal fluctuation: clinicopathologic characteristics and molecular genetic identification. *Brain Dev* 2000;**22**(suppl.1):87–91.

Chapter 2.3

Pseudo-dominant inheritance and exon 2 triplication in a family with *parkin* gene mutations

Lucking CB, Bonifati V, Periquet M, Vanacore N, Brice A, Meco G.

Neurology 2001; 57: 924-927.

Pseudo-dominant inheritance and exon 2 triplication in a family with *parkin* gene mutations

Article abstract—The authors report an Italian family with pseudo-dominant inheritance of parkinsonism attributable to parkin gene mutations. The father (disease onset at age 57 years) was homozygous for a triplication of exon 2 that is so far unique. The unaffected mother was heterozygous for deletions of exons 3 and 4, and the son (onset at age 31 years) was a compound heterozygote carrying both mutations. Thus, pseudo-dominant inheritance of *parkin* gene mutations has to be considered in early-onset parkinsonism, and sensitive screening techniques, such as semiquantitative multiplex PCR, should be applied.

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Parkin gene mutations cause autosomal recessive early-onset parkinsonism. To date, many mutations of different types have been described.[1-7] The associated phenotypes vary widely and include late-onset PD[6,7] as well as phenotypes resembling Dopa-responsive dystonia.[8] Although transmission is reported to be autosomal recessive (implicating loss of function of the parkin protein as pathogenic mechanism), questions have been raised concerning possible dominant negative effects of a subgroup of *parkin* mutations for which patients in two generations were heterozygous.[7] However, in families with apparently dominant inheritance, pseudo-dominant transmission has to be considered. This has been described only once in a family in Japan,[9] a country where the frequency of *parkin* gene mutations might be particularly high.

Here we report the molecular and clinical findings in an Italian family with pseudo-dominant inheritance of parkinsonism due to *parkin* gene mutations.

Patients and methods. The proband (RM 330) of our pedigree (figures 1 through 3) was screened for *parkin* gene mutations[6] because of early onset (at age 31 years) and slow progression of parkinsonism. The affected father (RM 347), whose neurologic signs (onset at age 57 years) were initially thought to result from small vascular lesions detected on brain CT scan, was genotyped subsequently.

DNA was extracted from peripheral blood leukocytes by use of standard techniques after informed written consent. Rearrangements of exons 2 through 12 of the *parkin* gene were detected by semiquantitative multiplex PCR, as described.[6] In brief, we first calculated, for each case and each control, the ratios of the peak heights of each exon to every other exon in a given exon combination (PCR done in duplicate). Then, each ratio of the controls was divided by the respective ratio of the cases, yielding normalized ratios (NR) for the cases. The NR thus could be compared among three independent experiments for exon combination A (figure 1), allowing calculation of mean normalized ratios (MNR). Deviations of the NR/MNR from 1 were interpreted as exon rearrangement. Haplotypes of the *parkin* gene region were constructed manually by using the microsatellite markers D6S1581, D6S959, D6S1579, D6S1599, D6S305, D6S411, AFMa155td9, AFMb281wf1, D6S1550, D6S1035, amplified with the primers specified in the Genome database (GDB), except for D6S1599, which was amplified by using "Forward" 5'-GGGTGTGCTTGGATTCC-TTCATG-3' and "Reverse" 5'-TAGCATGTGGACTGCATAT-CAAC-3'. PCR conditions were 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 45 seconds in 35 cycles. PCR products were analyzed on an ABI377 automated sequencer using GeneScan 3.1 and Genotyper 1.1.1 software (Applied Biosystems, Weiterstadt, Germany), and marker allele numbers were assigned in increasing order from the smallest to the largest PCR product.

Results. *Molecular findings.* In 15 different controls tested for exon combination A, the NR varied between 0.83 and 1.2; mean, 1.0 ± 0.09 (SD of the ratio with the highest SD). On this basis, confirmatory Southern blot was not performed, because this technique—using parkin exons as

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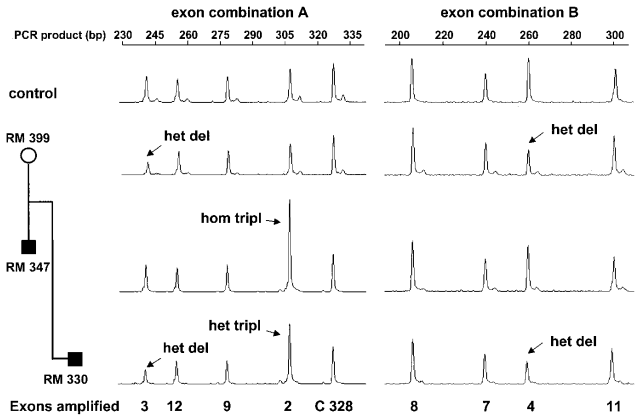

Figure 1. Multiplex PCR detection of exon rearrangements in the parkin *gene. The exons indicated at the bottom were amplified in two multiplex combinations (A and B) with fluorescently labeled forward primers and analyzed on an automated sequencer (ABI 377) with GeneScan 3.1 and Genotyper 1.1.1 software (Applied Biosystems). The size of the PCR products in bp is indicated at the top. The electrophoregrams of the index case (RM 330) and his parents (RM 347, RM 399) are aligned with the control. Peak heights represent the quantity of PCR product that reflects the quantity of template exon DNA. Peak heights of heterozygous deletions (het del) are half the expected height. Peak heights of the heterozygous triplication (het tripl) are 2 times the expected height and of the homozygous triplication (hom tripl) are 3 times the expected height. C328 is a sequence of the transthyretin gene (chromosome 18) serving as reference PCR product.*

probe—was considered less reliable. Figures 1 and 2 show representative PCR profiles and a schematic presentation of the *parkin* gene alleles. In RM 330, the peaks for exons 3 (MNR: 0.52 to 0.55 ± 0.08) and 4 (NR: 0.45 to 0.5) were half the expected size (theoretical value [TV]: 0.5), consistent with heterozygous deletions, whereas the peak for exon 2 was twice the expected size (MNR: 1.9 to 2.0 ± 0.1; TV: 2.0), consistent with four copies of exon 2. RM 330 was heterozygous for all but one intragenic markers (figure 3). RM 347 was homozygous for eight consecutive markers, with a peak for exon 2 that was three times higher than expected (MNR: 2.8 to 3.0 ± 0.3; TV: 3.0). Thus, the PCR profile would be explained by the presence of a homozygous triplication of exon 2 in RM 347 (six copies of the exon), and the combination of a heterozygous triplication of exon 2 and a heterozygous deletion of exons 3 and 4 in RM 330. The deletion was transmitted from RM 399 (NR, exon 3: 0.5 to 0.63; NR, exon 4: 0.51 to 0.57). The deletion included D6S1599, because RM 330 did not receive a maternal allele (figure 3), indicating the large size of the deletion (>72 kb, figure 2).

Clinical findings. The index patient (RM 330) first noticed symmetric resting and postural tremor in the upper limbs and generalized slowness of movement at age 31 years. Two years later, our examination showed mild parkinsonian signs (hypomimia, resting and postural tremors, and bradykinesia) and brisk tendon reflexes. The Hoehn–Yahr stage was II. The cognitive status was normal, but he was very anxious. He remained untreated.

His father (RM 347) noticed resting tremor and motor slowness in his left leg at age 57 years. A year later, brain CT performed in another hospital was reported to be normal. A "pyramidal-extrapyramidal syndrome, and arterial hypertension" were diagnosed, and he was successfully

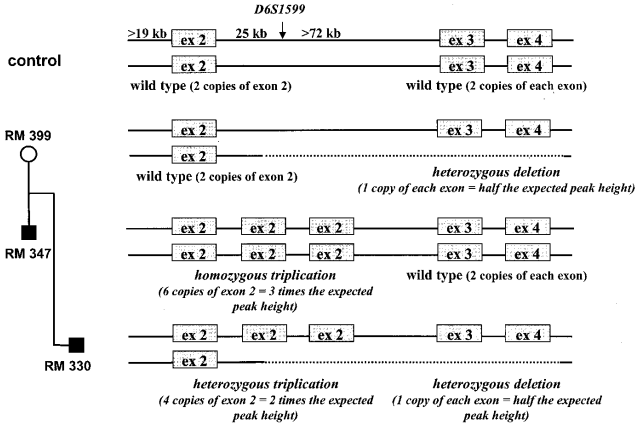

Figure 2. Schematic representation of exons in the family and the control. Exons within the pair of homologous chromosomes are depicted as boxes, introns as solid lines. As the size of the introns is not known, the lines are of arbitrary length. Exon 2 and the marker D6S1599 are positioned according to the PAC clone HS292F10 (116 kb, sequenced by the Sanger Center) with the distances specified in kilobases (kb). Exon deletions are indicated as dotted lines. The sequential localization of the triplicated exon 2 is hypothetical. The type of exon rearrangements and the factors by which the peak heights differ from the control are given below each pair of homologous chromosomes.

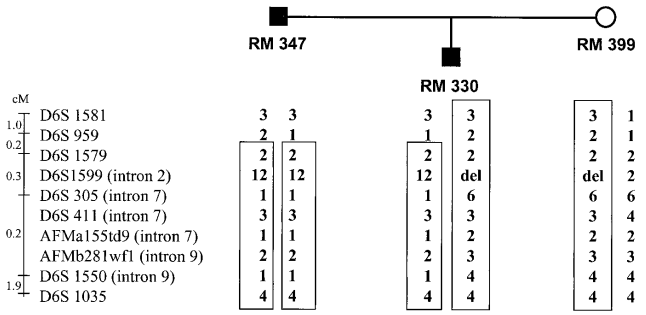

Figure 3. Haplotype analysis of the family. Six intragenic markers (the intronic position is given in parenthesis) and four flanking markers were analyzed. The estimated genetic distance between the markers is indicated in centi-Morgan (cM). Haplotypes linked to exon rearrangements are boxed. Patient RM 347 is homozygous for 8 consecutive markers indicating that his parents are related, and patient RM 330 carries two different haplotypes. Note that marker D6S1599 is deleted (del) on the allele carrying deletions of exons 3 and 4.

treated with the anticholinergic drug trihexyphenidyl (1 mg three times daily). At age 59 years, after transient left hemiparesthesia, a CT scan indicated a small ischemic lesion in the right thalamus. His clinical condition remained stable for 8 years under anticholinergic therapy; then the parkinsonian symptoms worsened. At the time of our first examination (age 69 years, with the same dosage of trihexyphenidyl), he had a flexed posture, generalized rigidity and bradykinesia, more severe on the left side, resting tremor in the left leg, and gait difficulties caused by postural disturbances and motor blocks. The Hoehn–Yahr stage was III. He also had brisk tendon reflexes at all four limbs, and Babinski sign in the left foot. The brain CT showed mild, diffuse cerebral atrophy, and small ischemic lesions in right thalamus, left caudate nucleus, and left pons. Levodopa was then added (100 mg three times daily). It improved bradykinesia, gait, and especially resting tremor. There was no known consanguinity between the parents of the father. However, they came from the same very small Italian village.

The mother (RM 399) was healthy at age 67 years.

Discussion. This is the first European family with established pseudo-dominant transmission of parkinsonism caused by *parkin* gene mutations, and the second family worldwide.[9] Three of the four *parkin* gene alleles in this family harbored mutations. Autosomal dominant transmission in families with *parkin* gene mutations should therefore only be considered after complete exploration of the *parkin* gene. However, even with a combination of exon dosage and genomic sequencing, approximately 15% of mutant alleles remain undetected and might be located in promoter or intronic regions of the gene.[6]

Whereas the proband showed early-onset and slowly progressive parkinsonism, a phenotype usually associated with *parkin* gene mutations, disease onset was later in the father and was complicated by vascular brain lesions. However, later onset of *parkin*-related disease has been described, and the clinical picture can be indistinguishable from idiopathic PD.[5-7] Although ages at onset might vary considerably within the same sibship,[5] they only differed for more than 20 years in less than 10% of our series of 39 sibpairs with *parkin* gene mutations (unpublished observation). Thus, in this family, the difference in age at onset might be attributable to the fact that father and son have only one disease-causing allele in common, and the dissimilar mutations might be more or less deleterious. However, additional genetic or environmental modifying factors are not excluded.

The deletion of exons 3 and 4 is thought to cause a frameshift in the mRNA (as deduced from the published cDNA sequence[1]), resulting in a seriously truncated parkin protein. In contrast, the mechanisms by which the so far unique exon 2 triplication leads to loss of parkin function needs further examination. We were never able to amplify a corresponding reverse transcription (RT)-PCR product (Periquet et al., manuscript in preparation), indicating the resulting mRNA might be unstable. Interestingly, exon 2 has been found to be both triplicated or deleted in different families,[6] suggesting that unequal crossing-over after misalignment of repeated sequences might give rise to both of these mutations.

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References

1. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998;392:605–608.
2. Hattori N, Kitada T, Matsumine H, et al. Molecular genetic analysis of a novel *parkin* gene in Japanese families with autosomal recessive juvenile parkinsonism: evidence for variable homozygous deletions in the *parkin* gene in affected individuals. Ann Neurol 1998;44:935–941.
3. Leroy E, Anastasopoulos D, Konitsiotis S, Lavedan C, Polymeropoulos MH. Deletions in the parkin gene and genetic heterogeneity in a Greek family with early onset Parkinson's disease. Hum Genet 1998;103:424–427.
4. Lücking CB, Abbas N, Dürr A, et al. Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism. Lancet 1998;352:1355–1356.
5. Abbas N, Lücking CB, Ricard S, et al. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. Hum Mol Genet 1999;8:567–574.
6. Lücking CB, Dürr A, Bonifati V, et al. Association between

early-onset Parkinson's Disease and mutations in the *parkin* gene. N Engl J Med 2000;342:1560–1567.
7. Klein C, Pramstaller PP, Kis B, et al. Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: expanding the phenotype. Ann Neurol 2000;48:65–71.
8. Tassin J, Dürr A, Bonnet AM, et al. Levodopa-responsive dystonia: GTP cyclohydrolase I or parkin mutations? Brain 2000; 123:1112–1121.
9. Maruyama M, Ikeuchi T, Saito M, et al. Novel mutations, pseudo-dominant inheritance, and possible familial affects in patients with autosomal recessive juvenile parkinsonism. Ann Neurol 2000;48:245–250.

Chapter 2.4

How much phenotypic variation can be attributed to *parkin* genotype?

Lohmann E, Periquet M, Bonifati V, Wood NW, De Michele G, Bonnet AM, Fraix V, Broussolle E, Horstink MW, Vidailhet M, Verpillat P, Gasser T, Nicholl D, Teive H, Raskin S, Rascol O, Destee A, Ruberg M, Gasparini F, Meco G, Agid Y, Durr A, Brice A, The French Parkinson's Disease Genetics Study Group and The European Consortium on Genetic Susceptibility in Parkinson's Disease

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How Much Phenotypic Variation Can Be Attributed to *parkin* Genotype?

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To establish phenotype–genotype correlations in early-onset parkinsonism, we have compared the phenotype of a large series of 146 patients with and 250 patients without *parkin* mutations. Although no single sign distinguished the groups, patients with mutations had significantly earlier and more symmetrical onset, dystonia more often at onset and hyperreflexia, slower progression of the disease, and a tendency toward a greater response to levodopa despite lower doses. After forward stepwise multiple logistic regression analysis, dystonia at onset and brisk reflexes were not longer significantly different but were correlated with age at onset rather than the presence of the *parkin* mutation. Age at onset in carriers of *parkin* mutations varied as did the rate of progression of the disease: the younger the age at onset the slower the evolution. The genotype influenced the phenotype: carriers of at least one missense mutation had a higher United Parkinson's Disease Rating Scale motor score than those carrying two truncating mutations. The localization of the mutations was also important because missense mutations in functional domains of *parkin* resulted in earlier onset. Patients with a single heterozygous mutation had significantly later and more asymmetrical onset and more frequent levodopa-induced fluctuations and dystonia than patients with two mutations.

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In the last few years, five genes and four loci have been identified in families with Parkinson's disease (PD). Mutations in the *alpha-synuclein* gene on chromosome 4q,[1] the *UCH-L1* gene on chromosome 4p,[2] and the *NR4A2* gene mapped to chromosome 2q22-23[3] are autosomal dominant, whereas mutations in the *parkin* gene on chromosome 6q[4] and the *DJ-1* gene on chromosome 1p[5] are autosomal recessive. Four other loci have been localized on chromosomes 2p13,[6] 4p14-16,[7] 1p35-36,[8] and 12p11-q13.[9] Other susceptibility factors, such as the Icelandic 1p32 locus,[10] the polymorphic variant S18Y of the UCH-L1 gene,[11] or the A0 allele of the tau gene,[12] have been reported. Whereas mutations in *alpha-synuclein* and *UCH-L1* have been found in only a few families,[1,2,13] mutations in the *parkin* gene have been described in many patients with autosomal recessive juvenile parkinsonism (ARJP).[4,14–16] Pathological studies have shown several differences be-

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tween ARJP with *parkin* mutations and idiopathic PD: in four cases there was an absence of Lewy bodies and a severe generalized loss of dopaminergic neurons from the substantia nigra pars compacta.[17–19] One of these four brains showed additional involvement of the substantia nigra pars reticulata,[19] the second showed additional neurofibrillary tangles and argyrophilic astrocytes in cerebral cortex and brainstem nuclei,[18] the third showed neuronal loss in parts of the spinocerebellar system,[17] and the fourth case, with a single heterozygous mutation, showed additional tau aggregates consistent with progressive supranuclear palsy.[20] Only one case, carrying compound heterozygous mutations of the *parkin* gene, has been described as having Lewy bodies.[21] The *parkin* gene, which contains 12 exons, spans more than 1.5Mb and encodes a protein of 465 amino acids with similarity to ubiquitin at the amino terminus (UBL domain) and a RING-in between ring (IBR)–RING motif at the carboxy terminus. Like many other proteins with a RING-finger domain, *parkin* has E3 ubiquitin ligase activity, linking *parkin*-associated PD to a defect in the ubiquitin-proteasome system.[22] Because mutations in *parkin* are associated with recessively inherited parkinsonism, they presumably result in reduction or loss of its E3 ligase activity.[22–25] This might interfere with the ubiquitin-mediated proteolysis of certain substrates, the accumulation of which might lead to neuronal death. Why dopaminergic neurons in the substantia nigra are particularly vulnerable to the loss of *parkin* function remains to be determined.

Many different *parkin* mutations have been found in familial as well as in sporadic cases of different origins. They are reported to be associated with phenotypes including typical early-onset parkinsonism, but also late-onset PD[14,26,27] and phenotypes resembling Dopa-responsive dystonia,[28] hemiparkinsonism-hemiatrophy,[29] forms with cerebellar ataxia,[17,30] pyramidal tract dysfunction,[30] or peripheral neuropathy.[31] However, no clinical correlations have been established so far between a given phenotype and specific mutations in the *parkin* gene. In addition, in some families the disease is associated with heterozygous *parkin* mutations that appear to be transmitted dominantly.[21,26] This suggests that carriers of some single *parkin* mutations might be at risk for developing PD.[32–34] This differs from the pseudodominant inheritance described in rare families in which all patients carry two mutations.[35–37]

The aim of our study was to compare large series of patients with and without *parkin* mutations and to assess the influence of the number and the nature of the mutations on the phenotype of the patients.

Patients and Methods

Patients

Five hundred patients with isolated or familial parkinsonism with ages at onset of up to 55 years (in at least one affected family member for the familial cases) were included as in our previous studies,[14,38,39] even though mutations in the *parkin* gene also have been reported in patients with onset up to 72 years of age.[27] The diagnostic criteria for PD in our study were at least two of the Parkinsonian triad of signs (bradykinesia, rigidity, rest tremor) and at least 30% improvement after L-dopa therapy in familial or isolated cases. Exclusion criteria were the existence of extensor plantar reflexes, ophthalmoplegia, early dementia, or early autonomic failure. A standardized form was used to assess the history of the disease and of the family, the clinical signs and the response to treatment. The response to treatment was calculated only in patients who had United Parkinson's Disease Rating Scale III assessment both "on" and "off" treatment. The percentage of improvement was obtained by comparing the "off" and "on" values. Quality control of clinical information was performed by one clinician.

One hundred and four patients were excluded because clinical information was incomplete. Among the remaining 396 patients, there were 210 cases from 76 families, including 207 compatible with autosomal recessive inheritance and 3 families with pseudodominant inheritance, two of which have been reported already.[36,37] The other 186 had no known family histories of PD in first or second-degree relatives, but in 15 cases there was consanguinity. The families were mostly of European descent (n = 329), including patients from France (n = 165), Italy (n = 73), the Netherlands (n = 26), United Kingdom (n = 26), Germany (n = 20), Portugal (n = 11), Spain (n = 4), and Eastern Europe (n = 4). There were also patients from North Africa (n = 27), South America (n = 15), and North America (n = 2), Asia (n = 9), Russia (n = 6), Middle East (n = 1) and Near East (n = 3), and Turkey (n = 4). Most families were white (91%).

Molecular Analysis

All index cases were screened for exon rearrangements in the *parkin* gene with a semiquantitative multiplex polymerase chain reaction assay, in which several exons of the *parkin* gene were coamplified together in the same reaction. To identify the mutations, we analyzed the polymerase chain reaction products on denaturing polyacrylamide gels on an ABI 377 automated sequencer with the GENESCAN 3.1 and GENOTYPER 1.1.1 softwares. The ratios between the heights of the peaks of each of the exons amplified in a given reaction were calculated and then compared with the ratios for nonrearranged exons in the control sample from a normal subject (for detailed description of this method, see Lücking and Brice[40]). The consequences of the exon rearrangements at the protein level (in-frame or frameshift) were deduced from published exon sequences (DNA Data Bank of Japan, accession no. AB009973). In the patients in whom the assay detected only one or no mutations, the entire coding sequence was analyzed by sequencing as previously described.[41]

To establish phenotype–genotype correlations, we considered all exon rearrangements, nonsense mutations, and small

insertions/deletions resulting in frameshifts to be truncating mutations. Although all of the truncating mutations do not have the same consequences on the protein, they all affect one of the functional domains of the *parkin* protein. All other mutations resulting in single amino acid changes were classified as missense mutations. In addition, patients were considered to carry a single *parkin* mutation only when one unambiguous *parkin* mutation was detected; this excludes all patients with rearrangements of consecutive exons where it cannot be concluded whether there are multiple exon rearrangements on a single allele or two different mutations, one on each allele. Patients with single heterozygous mutations were genotyped for the −258 parkin promoter single nucleotide polymorphism (SNP) according to West and colleagues.[42]

Statistical Analyses

Comparisons were first made between *parkin* carriers and patients without *parkin* mutations. Then, comparisons were made among subgroups of carriers of *parkin* mutations. Because age at onset younger than 40 years usually defines early-onset parkinsonism, we compared patients with age at onset younger than 40 years with those with age at onset 40 years or older. Patients with one or two missense mutations were compared with those carrying two truncating mutations. Patients who carried at least one missense mutation outside of the known functional domains were compared with those carrying two missense mutations within functional domains, such as the UBL and the RING-IBR-RING domains. Finally, patients with only one detected mutation in the *parkin* gene, as defined above, were compared with those with two known mutations. We used the χ^2 test or the Fisher's exact test when appropriate for comparison of proportions and analysis of variance for comparison of means. Forward stepwise multiple logistic regression analysis was performed to sort out the independent variables of items that defined significantly between patients with and without *parkin* mutations (Hoehn and Yahr "off" was not included in this analysis because the available data were unevenly distributed among groups and the sample size was too small in the last step of the analysis). No corrections were applied for multiple testing.

Results

Forty-five of 396 patients are reported for the first time including five isolated cases without *parkin* mutation and 40 cases with family histories including 18 carriers of *parkin* mutations. The remaining patients have been reported previously.[14,34,38,39] The proportion of patients with *parkin* mutations from families with autosomal recessive parkinsonism decreased significantly as a function of age at onset, ranging from 82% before age 20 years to 28% between 46 and 55 years (Fig, $p < 0.01$). This decrease already has been shown in isolated cases in which the difference was even greater.[14,38]

The clinical comparison between *parkin* carriers and noncarriers is shown in Table 1. The groups differed in four respects: (1) mean age at onset in patients with *parkin* mutation (31.4 ± 11.9 years) was earlier (38.1 ± 11.2 years, $p < 0.001$). The range was similar,

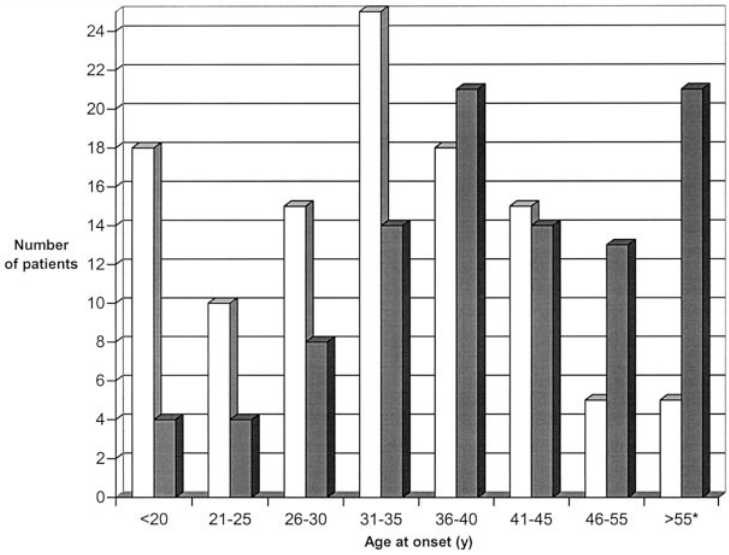

Fig. Frequency of familial parkin cases according to the age at onset. (unfilled bars) *patients with parkin mutations;* (filled bars) *patients without parkin mutations.* (asterisk) *Ages at onset: 56, 58, 68, 68, and 70 years.*

however, in both groups (7–70 vs 12—76 years); (2) dystonia and symmetry at onset and brisk reflexes at examination were more frequent in *parkin* carriers; (3) despite longer mean durations of disease (16.7 ± 10.4 vs 11.8 ± 9.2 years, $p < 0.001$) and L-dopa treatment (11.5 ± 8.3 vs 8.7 ± 7.7 years, $p < 0.005$), the daily dose of L-dopa was lower (497 ± 312 vs 610 ± 405mg, $p < 0.05$), and the percentage of improvement tended to be greater (62 ± 20 vs 55 ± 23%, not significant) in patients with *parkin* mutations; (4) the mean rate of progression, assessed by the Hoehn and Yahr score "off" (without treatment) adjusted for disease duration was lower in the *parkin* group (0.23 ± 0.27, n = 57 vs 0.40 ± 0.40, n = 87, $p = 0.01$) independent of age at onset. The gender of patients had no influence of the phenotype.

The regression analysis showed that age at onset ($p < 0.01$), disease duration ($p < 0.01$), asymmetry at onset ($p < 0.05$), and daily dose of L-dopa ($p < 0.05$) are significant independent variables. Interestingly, dystonia at onset as well as brisk reflexes were no longer independent after forward stepwise multiple logistic regression analysis. However, there was a significant correlation between age at onset and dystonia at onset ($p < 0.01$).

Age at onset was highly variable among *parkin* carriers, ranging from 7 to 70 years. We therefore analyzed the clinical features according to the age at onset (<40 vs ≥40 years). The rate of progression was greater in the group with age at onset after 39 (0.5 ± 0.62, n = 8 vs 019 ± 0.12, n = 49, $p < 0.01$). Presenting signs were also different because dystonia at onset was more frequent in the early-onset than in the late-onset group (30/95 vs 2/22, $p < 0.05$). There were no significant differences according to the age at onset for other clinical features.

To explain the differences in age at onset, we investigated the possible influence of the nature and the localization of the mutation in the *parkin* gene. We first compared patients with at least one missense with those with two truncating mutations (Table 2), reasoning that missense mutations usually have less functional consequences than truncating mutations in autosomal recessive disorders.[43] Patients carrying at least one missense mutation had higher United Parkinson's Disease Rating Scale motor score without treatment than patients carrying two truncating mutations (51.1 ± 20.7 vs 31.3 ± 16.5, $p < 0.01$) after similar disease durations. There were no other differences between the groups. This suggests that missense mutation carriers have a more severe disease than those carrying two truncating mutations.

We then compared phenotypes of patients with missense mutations according to their location with regard to functional domains (Table 3). Patients carrying two missense mutations within the functional domains (RING-IBR-RING and UBL domains) developed symptoms earlier than patients with one mutation outside the functional domains (29.9 ± 13.2 vs 39.5 ± 8.6 years, $p < 0.05$). The latter therefore were significantly older at examination (59.2 ± 9.7 vs 45.5 ± 13.1 years, $p < 0.05$).

Because several observations supported the hypothesis that the presence of a single *parkin* mutation might represent a risk factor for PD,[21,26,32–34] we compared patients with only a single *parkin* mutation to those carrying two mutations (Table 4). Patients with a single mutation showed symptoms later than those with

Table 1. Phenotype of 396 Patients with and without Parkin Mutations

Characteristic	*Parkin* (n = 146)	No. of Patients	Non-*Parkin* (n = 250)	No. of Patients
Gender ratio (M:F)	74:72	146	138:112	250
Age, yr (range)	48.6 ± 12.8 (16–89)	140	49.9 ± 12.6 (17–80)	250
Age at onset, yr[a] (range)	31.4 ± 11.9 (7–70)	146	38.1 ± 11.2 (12–76)	250
Disease duration, yr[a] (range)	16.7 ± 10.4 (1–58)	140	11.8 ± 9.2 (1–54)	250
Signs at onset				
Asymmetry[a]	87%	137	96%	231
Dystonia[a]	27%	117	10%	204
Clinical signs at examination				
Rigidity	92%	143	96%	233
Bradykinesia	94%	144	97%	231
Rest tremor	73%	141	74%	235
Hoehn & Yahr "off"[a] (range)	3.3 ± 1.1 (1.5–5)	57	2.9 ± 1.2 (1–5)	87
Treatment				
Daily dose of L-dopa, mg[a] (range)	497 ± 312 (62–1500)	118	610 ± 405 (50–3,000)	192
Duration of treatment, yr[a] (range)	11.5 ± 8.3 (0.2–40)	115	8.7 ± 7.7 (0.08–55)	182
Other signs				
Brisk reflexes[a]	33%	112	19%	190

[a]Two-tailed $p < 0.05$.

Table 2. Comparison of Parkin Patients with At Least One Missense Mutation and Parkin Patients with Two Truncating Mutations

Characteristic	At Least One Missense Mutation (n = 42)	No. of Patients	Two Truncating Mutations (n = 76)	No. of Patients
Gender ratio (M:F)	23:19	42	38:38	76
Age, yr (range)	49.8 ± 13.6 (16–75)	42	48.6 ± 12.9 (21–89)	70
Age at onset, yr (range)	32.9 ± 12.7 (9–68)	42	30.2 ± 10.0 (7–58)	76
Disease duration, yr (range)	16.9 ± 11.2 (1–42)	42	17.4 ± 10.8 (2–58)	70
Clinical signs at examination				
UPDRS III "off"[a]	51.1 ± 20.7 (25–87)	15	31.3 ± 16.5 (4–62)	23
Treatment				
Daily dose of L-dopa, mg (range)	538 ± 354 (150–1,400)	35	453 ± 286 (62–1,500)	60
Dyskinesia	76%	37	75%	65

[a]Two-tailed $p < 0.01$.

UPDRS = United Parkinson's Disease Rating Scale.

two mutations (37.3 ± 11.3 vs 30.5 ± 10.7 years, $p < 0.05$). L-Dopa–induced fluctuations and dystonia were more frequent in the latter (94% vs 69%, $p < 0.05$, and 70% vs 40%, $p < 0.05$, respectively) and clinical signs were more frequently asymmetrical at onset (100 vs 82%, $p < 0.05$). One of 34 relatives of patients with known *parkin* mutations carrying heterozygous R275W mutations had PD with onset at age 68 years. Among these relatives, there were 16 siblings with a mean age at examination of 41 ± 7.9 years (ranging from 28 to 50 years) and 18 parents with a mean age at examination of 71.1 ± 6.9 years (ranging from 63 to 84 years). None of these individuals were examined at an age younger than the age at onset of their affected relatives. Recently, a genetic association was described between idiopathic PD and the −258 T/G promoter SNP of the parkin gene.[34] The −258G allele, associated with reduced *parkin* expression in in vitro experiments, is also suspected to contribute to early-onset parkinsonism.[34] To determine whether the status of heterozygous *parkin* mutation carrier could be responsible for parkinsonism in conjunction with a *parkin* polymorphism, we genotyped the −258 T/G *parkin* promoter SNP by polymerase chain reaction restriction in the 23 patients with a single *parkin* mutation. The −258 G allele was observed in 45.6% of alleles inherited by heterozygous *parkin* carriers compared with 14% of Northern European controls ($p < 0.001$).[34]

Because some mutations were recurrent, we compared seven groups of patients with the same genotype. No specific clinical pattern could be associated with any of the genotypes. Mean disease durations were significantly different, but not ages at onset or at examination, which were highly variable (Table 5). Patients homozygous for the exon 3 deletion had ages at onset that differed by as much as 34 years. Patients carrying the R275W missense mutation tended to have an earlier age at onset than those from all other groups with two truncating mutations ($p = 0.09$). This suggests that the R275W mutation, located within the RING-IBR-RING domain, could have a more deleterious effect than truncating mutations. Unfortunately, the disease durations in patients with this mutation were very short, precluding the assessment of the severity of disease progression.

Atypical manifestations in *parkin* patients are re-

Table 3. Comparison of Parkin Patients with and without Mutations in Functional Domains (UBL and RING-IBR-RING)

Characteristic	Mutation in Functional Domains (n = 29)	No. of Patients	Mutation outside the Functional Domains (n = 13)	No. of Patients
Gender ratio (M:F)	17:12	29	6:7	13
Age, yr[a] (range)	45.5 ± 13.1 (16–70)	29	59.2 ± 9.7 (44–75)	13
Age at onset, yr[a] (range)	29.9 ± 13.2 (9–68)	29	39.5 ± 8.6 (18–55)	13
Disease duration, yr (range)	15.7 ± 10.5 (2–40)	29	19.7 ± 12.5 (1–42)	13
Clinical signs at examination				
Hoehn and Yahr "on" (range)	2.3 ± 0.7 (1–4)	24	2.6 ± 0.9 (1–4)	12
Treatment				
Daily dose of L-dopa, mg (range)	523 ± 309 (150–1,400)	25	577 ± 466 (150–1,400)	10

[a]Two-tailed $p < 0.05$.

UBL = ubiquitin at the amino terminus; IBR = in between ring.

Table 4. Comparison of Parkin Patients with a Single Mutation and Parkin Patients with Two Mutations

Characteristic	Single (n = 23)	No. of Patients	Two (n = 109)	No. of Patients
Gender ratio (M:F)	10:13	23	55:54	109
Age, yr	51.5 ± 9.2 (31–70)	23	48.3 ± 13.3 (16–89)	103
Age at onset, (yr)[a] (range)	37.3 ± 11.3 (18–68)	23	30.5 ± 10.3 (7–58)	109
Disease duration, yr (range)	14.2 ± 8.2 (2–37)	23	17.2 ± 10.9 (1–58)	103
Signs at onset				
Asymmetry[a]	100%	23	82%	100
Treatment				
Daily dose of L-dopa, mg (range)	461 ± 283 (125–1400)	22	476 ± 310 (62–1500)	84
Dyskinesia	82%	22	74%	92
Fluctuation[a]	94%	17	69%	83
Dystonia[a]	70%	17	40%	70

[a]Two-tailed $p < 0.05$.

ported in Table 6. In nine patients from different families, psychiatric disorders were evident and included psychosis, panic attacks, depression, disturbed sexual behavioral and obsessive-compulsive behaviors. Four patients had abnormal magnetic resonance imaging findings, but no evident pattern could be seen. A single patient had axonal polyneuropathy.

Discussion

We report the first evidence to our knowledge of phenotype–genotype correlations in patients with *parkin* mutations in a large cohort of 396 patients. Our previous studies had shown that *parkin* mutations accounted for 49% (36/73 families) of ARJP and 14% (20/146 patients) of isolated cases with an age at onset younger than 45 years.[14,38] The relative frequencies of *parkin* mutations in ARJP or isolated cases with age at onset at 55 years or younger found in this study are similar to those in our previous report: 61% (54/89 families) and 19% (35/186 patients), respectively. However, in familial cases with ages at onset 20 years or younger, greater than 80% of the patients have *parkin* mutations, whereas only 28% of those between the age of 46 and 55 are *parkin* carriers. This confirms that *parkin* mutations are much more frequent in early-onset patients.

There is no individual sign or symptom that distinguished between *parkin* carriers and noncarriers with early-onset parkinsonism. However, patients with *parkin* mutations tended to have earlier and more symmetrical onset, slower progression of the disease, and greater response to L-dopa despite lower doses. Nevertheless, our *parkin* carriers did not present all of the signs associated with ARJP previously reported: coarse tremor, foot dystonia, and diurnal fluctuations were absent.[44] They have, however, consistently slower disease progression and a marked response to L-dopa, but they do not have more complications from treatment, such as dyskinesias and fluctuations, than patients without *parkin* mutations, as reported in several previous studies.[44–46] The younger the age at onset, the more frequently dystonia is observed at onset. Thus, dystonia does not represent a specific sign of carriers of *parkin* but rather is associated with young onset parkinsonism regardless of the genetic origin. Age at onset among patients carrying *parkin* mutations varies (range, 7–70 years), as does the rate of progression of the disease: the younger the age at onset the slower the disease. In addition, atypical signs also were observed, such as psychiatric manifestations, cerebellar signs, neuropathy, and abnormal findings on brain magnetic resonance imaging, enlarging the spectrum of the disease (see Table 6). Behavioral disorders in *parkin* disease have been mentioned previously, but only in a few reports.[31,47,48] We did not find a homogenous psychiatric pattern in our *parkin* patients, and, because psychiatric manifestations are frequent in the general population and delirium and hypersexuality can be triggered by antiparkinsonism drugs, it cannot yet be concluded whether they are part of the phenotype or if they occurred by chance in several patients. Systematic psychiatric evaluation of *parkin* patients and their relatives will be required to resolve this question. Additional neurological manifestations, such as axonal neuropathy or cerebellar ataxia, were observed in several of our cases. Cerebellar signs,[17,30] pyramidal tract dysfunction[30] and peripheral neuropathy[31] have been reported previously, but not the presence of abnormalities on brain magnetic resonance imaging.

Mutations in the *parkin* gene are polymorphous and often private. To date, 79 different *parkin* mutations have been described: 45 point mutations including 20 truncating, 22 missense, and 3 splice mutations, as well as 34 exon rearrangements including 24 deletions and 10 multiplications.[4,14,15,21,25–27,32–35,37,41,47–58] The large spectrum of *parkin* gene defects, which differ in their predicted consequences on the function of the protein, raises the question of their role in the variabil-

ity of the phenotype. We indeed have found some differences. Despite the fact that the age at onset is similar in patients with at least one missense mutation and those with two truncating mutations, the phenotype is significantly more severe in the former. Although missense mutations usually have less functional consequences than truncating mutations in autosomal recessive disorders,[43] the consequences of several types of mutations is very difficult to predict (eg, missense mutations might have effects on splicing and therefore they can have more drastic consequences at the protein level).[59] Furthermore, the location of the mutation within the gene plays a role in the phenotype because mutations in functional domains resulted in onset of the disease approximately 9 years earlier than mutations in domains not known to be essential for *parkin* function. However, the influence of the nature and position of the mutation on the phenotype appears to be of limited importance, because no evident pattern emerged from comparison of patients with the same genotype. Furthermore, for most genotypes, there was great variability (up to 34 years) in age at onset despite the presence of the same mutations. The number of cases in each of the seven genotype groups was too low for a specific clinical pattern to emerge, but it appears that the mean age at onset of the patients who carry both the R275W mutation and a truncating mutation tended to be earlier than in those with two truncating mutations. This result suggests that the R275W mutation does not result in a simple loss of function as postulated for missense mutations. The R275W mutation might have dominant negative effect, which would account for the greater severity of the phenotype. However, this hypothesis is not supported by the fact that four heterozygous relatives (aged 28–70 years) are not affected by PD. Another explanation is that that R275W might confer a partial loss of function which, in conjunction with an additional, still unknown, predisposing factor is more deleterious than complete ablation of the gene. However, this discussion remains speculative, and the results must be confirmed by analyzing more patients.

Single mutations were detected in several patients[36,37] despite thorough molecular analyses of the gene including the promoter region.[34] Furthermore, families with apparently true autosomal dominant inheritance[21,26] or decreased striatal F-DOPA uptake in asymptomatic carriers of heterozygous *parkin* mutations[60] have been described. This prompted us to compare the phenotype in *parkin* carriers as a function of the number of mutations detected. Patients with a single *parkin* mutation were characterized by later and more asymmetrical onset and more frequent fluctuations and dystonias than patients with two characterized mutations. Their age at onset extends up to 68 years, 10 years later than in our series of patients with two *parkin* mutations. This suggests that the presence of a single *parkin* mutation might represent a risk factor for later onset PD. This result should be interpreted with caution, however, because the size and the complexity of the *parkin* gene may cause some mutations to escape detecting by our current screening techniques.[34]

However, note that among the 34 heterozygous carriers who were relatives of patients with two identified mutations only one was affected with onset at age 68 years. This may be coincidental or may suggest reduced penetrance or a slightly higher risk of developing PD in heterozygous carriers, even though the age at examination was earlier in siblings than parents, and they may develop symptoms in the future. In contrast, all their relatives with two mutations were affected. This observation indicates that being carrier of a single *parkin* mutation is not sufficient to produce the phenotype in all individuals and that other genetic or environmental factors are probably necessary in combination with the mutation. In this regard, note that the frequency of the −258 G allele of the *parkin* promoter SNP was significantly higher in our patients who carry a single heterozygous mutation than in European controls. These findings are consistent with a previous study[34] and suggest that the −258 G allele also may contribute to early-onset parkinsonism, particularly in individuals who also carry a heterozygous *parkin* mutation.

Table 5. Comparison of Parkin Patients with the Same Genotype

Characteristic	ex3del/ex3del (n = 8)	ex3-4del/ex3-4del (n = 4)	ex2del/ex3del (n = 6)
Gender ratio (M:F)	3:5	3:1	3:3
Age, yr (range)	46.9 ± 9.8 (35–59)	52.5 ± 6 (44–58)	49 ± 6.8 (42–59)
Age at onset, yr (range)	35.4 ± 10.6 (21–55)	37 ± 10.6 (23–47)	33.2 ± 10.4 (14–42)
Duration of disease, yr (range)[a]	11.5 ± 5.3 (4–17)	15.5 ± 6.2 (7–21)	15.8 ± 10.6 (3–33)

[a]Significant differences among the groups ($p < 0.05$).

ex = exon, del = deletion.

Table 6. Parkin Patients with Atypical or Additional Features

Patient	First Mutation	Second Mutation	Age at Onset (yr)	Age at Examination (yr)	Atypical Features
FPD-171-3	Exon 6 duplication	c255delA	26	29	Obsessive-compulsive behavior and depression
FPD-192-6	Exon 3 deletion	Exon 7 duplication	27	53	Psychosis
SPD-70	c255delA	c255delA	19	30	Delirium
SPD-92	Exon 3 to 6 deletion	R275W	16	46	Panic attacks
SPD-169	Exon 2 deletion	Exon 3 deletion	29	42	Verbal aggressiveness
BHAM-3	Exon 5 to 6 deletion	Exon 5 to 6 deletion	29	59	Hypersexuality, behavioral changes
BHAM-18	Exon 3, 4, 5 deletion[a]	?	18	53	Hypersexuality and depression
UK-4720	c202-203delAG	c202-203delAG	29	53	Psychosis
SAL-711-4	Exon 3 deletion	Exon 3 deletion	33	50	Depression, anxiety, hysterical episodes, and conversional symptoms. Cerebral MRI: T2 periventricular white matter hyperintensities
SAL-711-5	Exon 3 deletion	Exon 3 deletion	40	55	Cerebral MRI: important dilatation of the lateral ventricles and T2 hyperintensities in the frontal white matter
SAL-711-6	Exon 3 deletion	Exon 3 deletion	28	41	Axonal polyneuropathy
FPD 235-7	Exon 3 to 6 deletion	c255delA	27	55	Presenting sign: resting tremor in both legs that increased markedly when standing and was absent when walking
IT-48-97	Exon 3 to 4 deletion	Exon 3 to 4 deletion	43	58	Orthostatic tremor diagnosed before PD. Cerebral cortical atrophy at MRI, T2 hyperintensities in the upper pons and midbrain.
JMP-28	Exon 3 deletion	Exon 3 deletion	28	34	Cerebellar signs (nystagmus, slight left limb dysmetria). Cerebral MRI: bilateral sickle-shaped abnormal signals (decreased in T_1-, increased in T_2-weighted images) in the cerebellum.

[a]The phase of transmission was not known for this patient, and we could not determine if the deletion was present on one or both alleles.
MRI = magnetic resonance imaging; PD = Parkinson's disease.

Conclusion

There were significant clinical differences between patients with and without *parkin* mutations: *parkin* carriers tended to have earlier onset, more dystonia at onset, and brisk reflexes. They also took lower doses of L-dopa despite longer durations but did not have more

Table 5. (Continued)

c255delA/c255delA (n = 4)	c202-203delA/c202-203delA (n = 2)	ex3-4del/c255delA (n = 2)	ex3del/R275W (n = 3)
3:1	1:1	0:2	1:2
48.8 ± 16 (30–62)	48 ± 7.1 (43–53)	33 ± 17 (21–45)	25.3 ± 8.3 (16–32)
28.5 ± 13.1 (17–45)	26 ± 4.2 (23–29)	26.5 ± 17.7 (14–39)	21.3 ± 8.1 (12–27)
20.3 ± 7.9 (11–29)	22 ± 2.8 (20–24)	6.5 ± 0.7 (6–7)	4 ± 1 (3–5)

complications from treatment than noncarriers. There were no clinical signs that permit identification of a patient with *parkin* mutations, but *parkin* carriers represented a specific subgroup of early-onset parkinsonism. The phenotype differs with the genotype: patients with missense mutations had a more severe disease than those with truncating mutations, suggesting that missense mutations result in more than a loss of function. A dominant negative effect, as suggested for the R275W mutation, would produce a more severe clinical course. Not unexpectedly, missense mutations in functional domains resulted in an earlier onset than mutations in other regions of the protein. Finally, patients with two detected mutations had earlier onset than those with only one detected mutation, although the interpretation of this result is subject to caution. It is not yet clear whether the second mutation was not detected by the techniques used or whether the presence of a single mutation constitutes a real risk factor for PD.

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Appendix

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References

1. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 1997;276:2045–2047.
2. Leroy E, Boyer R, Polymeropoulos MH. Intron-exon structure of ubiquitin c-terminal hydrolase-L1. Nature 1998;5:397–400.
3. Le W, Xu PY, Jankovic J, et al. Mutations in NR4A2 associated with familial Parkinson disease Nat Genet 2003;33:85–89.
4. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998;392:605–608.
5. Bonifati V, Rizzu P, van Baren MJ, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 2003;10:256–259.
6. Gasser T, Muller-Myhsok B, Wszolek ZK, et al. A susceptibility locus for Parkinson's disease maps to chromosome 2p13. Nat Genet 1998;18:262–265.
7. Farrer M, Gwinn-Hardy K, Muenter M, et al. A chromosome 4p haplotype segregating with Parkinson's disease and postural tremor. Hum Mol Genet 1999;8:81–85.
8. Valente EM, Bentivoglio AR, Dixon PH, et al. Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35–p36. Am J Hum Genet 2001;68:895–900.
9. Funayama M, Hasegawa K, Kowa H, et al. A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. Ann Neurol 2002;51:296–301.
10. Hicks AA, Petursson H, Jonsson T, et al. A susceptibility gene for late-onset idiopathic Parkinson's disease. Ann. Neurol 2002; 52:549–555.
11. Maraganore DM, Farrer MJ, Hardy JA, et al. Case-control study of the ubiquitin carboxy-terminal hydrolase L1 gene in Parkinson's disease. Neurology 1999;53:1858.
12. Golbe LI, Lazzarini AM, Spychala JR, et al. The Tau A0 allele in Parkinson's disease. Mov Disord 2001;16:442–447.
13. Kruger R, Kuhn W, Muller T, et al. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet 1998;18:106–108.
14. Lücking CB, Durr A, Bonifati V, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene. French Parkinson's Disease Genetics Study Group. N Engl J Med 2000;342:1560–1567.
15. Hedrich K, Kann M, Lanthaler AJ, et al. The importance of gene dosage studies: mutational analysis of the parkin gene in early-onset parkinsonism. Hum Mol Genet 2001;10: 1649–1656.
16. Hattori N, Kitada T, Matsumine H, et al. Molecular genetic analysis of a novel Parkin gene in Japanese families with autosomal recessive juvenile parkinsonism: evidence for variable homozygous deletions in the Parkin gene in affected individuals. Ann Neurol 1998;44:935–941.
17. van de Warrenburg BP, Lammens M, Lucking CB, et al. Clinical and pathologic abnormalities in a family with parkinsonism and parkin gene mutations. Neurology 2001;56:555–557.
18. Mori H, Kondo T, Yokochi M, et al. Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology 1998;51:890–892.
19. Hayashi S, Wakabayashi K, Ishikawa A, et al. An autopsy case of autosomal-recessive juvenile Parkinsonism with a homozygous exon 4 deletion in the Parkin gene. Mov Disord 2000;15: 884–888.
20. Morales B, Martinez A, Gonzalo I, et al. Steele-Richardson-Olszewski syndrome in a patient with single C212Y mutation in the Parkin protein. Mov Disord 2002;17:1374–1380.
21. Farrer M, Chan P, Chen R, et al. Lewy bodies and parkinsonism in families with parkin mutations. Ann Neurol 2001;50: 293–300.
22. Shimura H, Hattori N, Kubo S, et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat Genet 2000;25:302–305.
23. Imai Y, Soda M, Takahashi R. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. J Biol Chem 2000;275:35661–35664.
24. Zhang Y, Gao J, Chung KK, et al. Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proc Natl Acad Sci USA 2000;97:13354–13359.

25. Terreni L, Calabrese E, Calella AM, et al. New mutation (R42P) of the parkin gene in the ubiquitinlike domain associated with parkinsonism. Neurology 2001;56:463–466.
26. Klein C, Pramstaller PP, Kis B, et al. Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: expanding the phenotype. Ann Neurol 2000;48:65–71.
27. Nichols WC, Pankratz N, Uniacke SK, et al. Linkage stratification and mutation analysis at the parkin locus identifies mutation positive Parkinson's disease families. J Med Genet 2002; 39:489–492.
28. Tassin J, Durr A, Bonnet AM, et al. Levodopa-responsive dystonia. GTP cyclohydrolase I or parkin mutations? Brain 2000; 123:1112–1121.
29. Pramstaller PP, Kunig G, Leenders K, et al. Parkin mutations in a patient with hemiparkinsonism-hemiatrophy: a clinical-genetic and PET study. Neurology 2002;58:808–810.
30. Kuroda Y, Mitsui T, Akaike M, et al. Homozygous deletion mutation of the parkin gene in patients with atypical parkinsonism. J Neurol Neurosurg Psychiatry 2001;71:231–234.
31. Tassin J, Durr A, de Broucker T, et al. Chromosome 6-linked autosomal recessive early-onset Parkinsonism: linkage in European and Algerian families, extension of the clinical spectrum, and evidence of a small homozygous deletion in one family. The French Parkinson's Disease Genetics Study Group, and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Am J Hum Genet 1998;63:88–94.
32. Hedrich K, Marder K, Harris J, et al. Evaluation of 50 probands with early-onset Parkinson's disease for Parkin mutations. Neurology 2002;58:1239–1246.
33. Hoenicka J, Vidal L, Morales B, et al. Molecular findings in familial Parkinson disease in Spain. Arch Neurol 2002;59: 966–970.
34. West A, Periquet M, Lincoln S, et al. Complex relationship between Parkin mutations and Parkinson disease. Am J Med Genet 2002;114:584–591.
35. Maruyama M, Ikeuchi T, Saito M, et al. Novel mutations, pseudo-dominant inheritance, and possible familial affects in patients with autosomal recessive juvenile parkinsonism. Ann Neurol 2000;48:245–250.
36. Bonifati V, Lücking CB, Fabrizio E, et al. Three parkin gene mutations in a sibship with autosomal recessive early onset parkinsonism. J Neurol Neurosurg Psychiatry 2001;71:531–534.
37. Lücking CB, Bonifati V, Periquet M, et al. Pseudo-dominant inheritance and exon 2 triplication in a family with parkin gene mutations. Neurology 2001;57:924–927.
38. Periquet M, Latouche M, Lohmann E, et al. Parkin mutations are frequent in patients with isolated early-onset parkinsonism. Brain (in press)..
39. Rawal N, Periquet M, Lohmann E, et al. New Parkin mutations and atypical phenotypes in families with autosomal recessive parkinsonism. Neurology 2003;60(8):1378–1381.
40. Lücking CB, Brice A. Semiquantitative PCR for the detection of exon rearrangements in the parkin gene. In: Methods in molecular medicine–neurogenetics: methods and protocols. Potter N, ed. Totowa, NJ: Humana Press, 2003:13–26.
41. Abbas N, Lucking CB, Ricard S, et al. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Hum Mol Genet 1999;8: 567–574.
42. West AB, Maraganore D, Crook J, et al. Functional association of the parkin gene promoter with idiopathic Parkinson's disease. Hum Mol Gen 2002;11:2787–2792.
43. Strachan T, Read AP. Human molecular genetics. 1st ed. Oxford, UK: Bios Scientific Publishers, 1996.
44. Ishikawa A, Tsuji S. Clinical analysis of 17 patients in 12 Japanese families with autosomal-recessive type juvenile parkinsonism. Neurology 1996;47:160–166.
45. Yamamura Y, Sobue I, Ando K, et al. Paralysis agitans of early onset with marked diurnal fluctuation of symptoms. Neurology 1973;23:239–244.
46. Matsumine H, Yamamura Y, Kobayashi T, et al. Early onset parkinsonism with diurnal fluctuation maps to a locus for juvenile parkinsonism. Neurology 1998;50:1340–1345.
47. Yamamura Y, Hattori N, Matsumine H, et al. Autosomal recessive early-onset parkinsonism with diurnal fluctuation: clinicopathologic characteristics and molecular genetic identification. Brain Dev 2000;22(suppl 1):S87–S91.
48. Wu RM, Shan DE, Sun CM, et al. Clinical, 18F-dopa PET, and genetic analysis of an ethnic Chinese kindred with early-onset parkinsonism and parkin gene mutations. Mov Disord 2002;17:670–675.
49. Matsumine H, Saito M, Shimoda-Matsubayashi S, et al. Localization of a gene for an autosomal recessive form of juvenile Parkinsonism to chromosome 6q25.2-27. Am J Hum Genet 1997;60:588–596.
50. Hattori N, Matsumine H, Asakawa S, et al. Point mutations (Thr240Arg and Gln311Stop) [correction of Thr240Arg and Ala311Stop] in the Parkin gene. Biochem Biophys Res Commun 1998;249:754–758.
51. Leroy E, Anastasopoulos D, Konitsiotis S, et al. Deletions in the Parkin gene and genetic heterogeneity in a Greek family with early onset Parkinson's disease. Hum Genet 1998;103:424–427.
52. Lücking CB, Abbas N, Durr A, et al. Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism. The European Consortium on Genetic Susceptibility in Parkinson's Disease and the French Parkinson's Disease Genetics Study Group. Lancet 1998;352:1355–1356.
53. Nisipeanu P, Inzelberg R, Blumen SC, et al. Autosomal-recessive juvenile parkinsonism in a Jewish Yemenite kindred: mutation of Parkin gene. Neurology 1999;53:1602–1604.
54. Munoz E, Pastor P, Marti MJ, et al. A new mutation in the parkin gene in a patient with atypical autosomal recessive juvenile parkinsonism. Neurosci Lett 2000;289:66–68.
55. Pineda-Trujillo N, Carvajal-Carmona LG, Buritica O, et al. A novel Cys212Tyr founder mutation in parkin and allelic heterogeneity of juvenile Parkinsonism in a population from North West Colombia. Neurosci Lett 2001;298:87–90.
56. Periquet M, Lücking C, Vaughan J, et al. Origin of the mutations in the parkin gene in Europe: exon rearrangements are independent recurrent events, whereas point mutations may result from Founder effects. Am J Hum Genet 2001;68:617–626.
57. Alvarez V, Guisasola LM, Moreira VG, et al. Early-onset Parkinson's disease associated with a new parkin mutation in a Spanish family. Neurosci Lett 2001;313:108–110.
58. Nisipeanu P, Inzelberg R, Abo MS, et al. Parkin gene causing benign autosomal recessive juvenile parkinsonism. Neurology 2001;56:1573–1575.
59. Hutton M, Lendon C, Rizzu, et al. Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17. Nature 1998;393:702–705.
60. Hilker R, Klein C, Hedrich K, et al. The striatal dopaminergic deficit is dependent on the number of mutant alleles in a family with mutations in the parkin gene: evidence for enzymatic parkin function in humans. Neurosci Lett 2002;19:50–54.

Chapter 2.5

PARK7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36

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Report

PARK7, a Novel Locus for Autosomal Recessive Early-Onset Parkinsonism, on Chromosome 1p36

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Although the role of genetic factors in the origin of Parkinson disease has long been disputed, several genes involved in autosomal dominant and recessive forms of the disease have been localized. Mutations associated with early-onset autosomal recessive parkinsonism have been identified in the *Parkin* gene, and recently a second gene, *PARK6,* involved in early-onset recessive parkinsonism was localized on chromosome 1p35-36. We identified a family segregating early-onset parkinsonism with multiple consanguinity loops in a genetically isolated population. Homozygosity mapping resulted in significant evidence for linkage on chromosome 1p36. Multipoint linkage analysis using MAPMAKER-HOMOZ generated a maximum LOD-score of 4.3, with nine markers spanning a disease haplotype of 16 cM. On the basis of several recombination events, the region defining the disease haplotype can be clearly separated, by ⩾25 cM, from the more centromeric *PARK6* locus on chromosome 1p35-36. Therefore, we conclude that we have identified on chromosome 1 a second locus, *PARK7,* involved in autosomal recessive, early-onset parkinsonism.

Parkinson disease (PD [MIM 168600]) is a neurodegenerative disorder characterized by bradykinesia, resting tremor, muscular rigidity, and postural instability. The cerebral pathology includes loss of dopaminergic neurons, in particular at the substantia nigra and cytoplasmatic eosinophilic inclusions (i.e., Lewy bodies). The role of genetic factors in the origin of PD has long been disputed. However, several families segregating the disease as a dominant or recessive trait have been identified. At present, four genes implicated in autosomal dominant forms of parkinsonism have been identified or localized, including the *α-synuclein* gene (Polymeropoulos et al. 1997), the *ubiquitin carboxy-terminal hydrolase-L1* gene (Leroy et al. 1998), and two yet-unidentified genes—*PARK3* on chromosome 2p13 (Gasser et al. 1998) and *PARK4* on chromosome 4p14-16.3 (Farrer et al. 1999). Until now, most mutations have been found in the *Parkin* gene (Kitada et al. 1998) and have been found to lead to a recessive form of juvenile parkinsonism (ARJP [MIM 600116]). The onset age may vary from 20 to 65 years, but in most patients the onset is at age <40 years (Lücking et al. 2000). *Parkin* mutations are estimated to explain up to 50% of familial patients with ARJP (Lücking et al. 2000). Recently, a new locus, *PARK6* (MIM 605909), involved in autosomal recessive early-onset parkinsonism was localized on chromosome 1p35-36 in a single Italian family (Valente et al. 2001). In the present report, we describe a family segregating early-onset parkinsonism with multiple consanguinity loops that we identified in a genetically isolated population. We report the findings of a genome search using homozygosity mapping in an effort to localize the gene involved in the disease.

Patients were from a genetically isolated community in the southwestern region of The Netherlands. The study is part of a larger research program named "Genetic Research In Isolated Populations" (GRIP). The isolated population was founded ~1750, by ~150 individ-

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uals. The scientific protocol of GRIP has been approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam. From the population, initially three patients (VII-2, VII-3 and VII-7) diagnosed with early-onset parkinsonism were ascertained, including two affected siblings and one apparently sporadic patient (fig. 1). The patients and their first-degree relatives were personally examined by the research physician, a neurologist, and a clinical geneticist. Clinical diagnosis of parkinsonism required the presence of at least two of the three cardinal signs (i.e., resting tremor, bradykinesia, and muscular rigidity) and absence of atypical features and of signs of involvement of other neurological systems (pyramidal, cerebellar, or autonomic). The extrapyramidal signs were evaluated by Hoehn-Yahr staging (HY) (Hoehn and Yahr 1967) and the Unified Parkinson's Disease Rating Scale (UPDRS; maximum of motor subscale = 108) (Fahn et al. 1987). Furthermore, structural and functional brain-imaging studies were performed. The clinical examinations of available relatives revealed a fourth affected individual (VII-6), who was not known to have the diagnosis of parkinsonism.

The clinical details of the four affected subjects are reported in table 1. Onset of symptoms was at age ⩽40 years in three subjects (VII-2, VII-3, and VII-7). The fourth affected individual (VII-6), who was age 40 years at the time of the clinical examination, was newly diagnosed with parkinsonism and could not indicate the

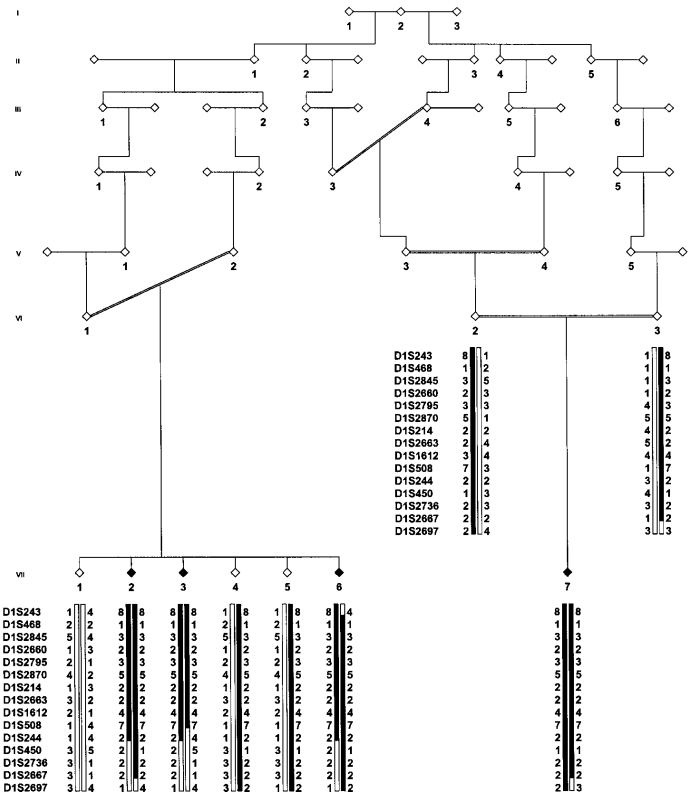

Figure 1 Pedigree of family with autosomal recessive early-onset parkinsonism, and haplotypes of marker loci spanning the linked region on chromosome 1p36. To protect patient confidentiality, a diamond symbol has been used to mask the sex of all family members. Individuals VI-2, VI-3, and VII-1–VII-7 were examined clinically. Black symbols indicate definitely affected individuals; black bars denote the disease-associated haplotype.

Table 1

Clinical Phenotype of Four Patients with Parkinsonism

Patient	Age at (years)		Symptom[a]						Therapy	HY	UPDRS	Other Characteristics
	Time of Study	Onset[b]	RT	PT	B	R	P	AS				
VII-2	50	40			X	X	X	X	Untreated	II	29	Psychotic episodes
VII-3	48	31	X	X	X	X	X	X	Pergolide	III	57	Neurotic signs, blepharospasm
VII-6	40	NA		X	X	X	X		Untreated	III	22	Neurotic signs
VII-7	38	27		X	X	X	X	X	L-dopa/IDD, entacapone	III	42	Wearing-off, dyskinesias, off dystonia

[a] RT = resting tremor; PT = postural tremor; B = bradykinesia; R = muscular rigidity; P = loss of postural reflexes; AS = asymmetric onset of symptoms.
[b] NA = not available.

age at onset of the disease. None of the patients exhibited atypical features or signs of involvement of additional neurological systems. The parkinsonian signs responded well to therapy with L-dopa or dopamine agonists in two patients (VII-3 and VII-7); at the time of clinical examination, the two other patients still had not been treated with antiparkinsonian drugs. One patient (VII-7) showed motor fluctuations of "wearing-off" type, L-dopa–induced dyskinesias in the "on" phase, and "off"-phase dystonia (i.e., laterocollis). Mild blepharospasm was present in patient VII-3, who receives dopamine-agonist therapy. Tendon reflexes in lower limbs were brisk in three of the patients (VII-3, VII-6, and VII-7). Babinski sign was absent. Two of the patients also showed neurotic signs, and one had suffered from psychotic episodes. In all patients, the progression of disease was slow. Structural brain images were unremarkable, in agreement with the diagnosis of idiopathic parkinsonism. Brain single-positron–emission computed tomography with dopamine-transporter tracer, performed in patients VII-2 and VII-3, showed severe abnormalities consistent with presynaptic dysfunction of nigrostriatal dopaminergic systems. Autopsy data are not available. At the time of clinical examination, the ages of unaffected individuals VII-1, VII-4 and VII-5 were 54, 50, and 46 years, respectively.

The genealogical history of the isolate has been computerized as far back as 1750. Although the patients were not aware of consanguinity in their family, the pedigree of each of the two sibships showed multiple consanguinity loops (fig. 1). The pedigree is drawn on the basis of the two shortest consanguinity loops for both parent pairs (VI-1/V-2 and VI-2/VI-3), which links the two sibships to a common ancestor six generations ago. However, other connections between the sibships exist, including a second loop linking VI-1 and V-2 to a common ancestor, who is also related to VI-2.

The pedigree structure is consistent with an autosomal recessive mode of inheritance, and the kindred therefore was considered to be suitable for homozygosity mapping (Lander and Botstein 1987). For all patients and available first-degree relatives, genomic DNA was isolated from peripheral blood, according to the method described by Miller et al. (1988). For the systematic genome scan, short tandem-repeat polymorphisms (STRPs) from the ABI PRISM Linkage Mapping Set MD-10 (Applied Biosystems) were used. Additional markers for fine-mapping and the *PARK6* region were obtained from the Généthon and Marshfield (Center for Medical Genetics, Marshfield Medical Research Foundation) genetic-marker sets. Marker order and distances were obtained from the Marshfield (Center for Medical Genetics, Marshfield Medical Research Foundation) integrated linkage map. Markers were amplified from genomic DNA, according to methods specified by the manufacturers. PCR products were pooled and loaded onto an ABI377 automated sequencer (filter set D; 5% denaturing FMC LongRanger acrylamide gel), and data were analyzed by ABI GENESCAN3.1 and ABI GENOTYPER2.1 software.

In the initial screens, only three definitely affected individuals (VII-2, VII-3, and VII-7) were genotyped. We first tested for linkage of our family to known PD loci (Polymeropoulos et al. 1997; Gasser et al. 1998; Kitada et al. 1998; Leroy et al. 1998; Farrer et al. 1999), using STRPs from the ABI PRISM Linkage Mapping Set MD-10. At chromosome 4q22, the markers D4S414 and D4S1572 were assessed; at chromosome 4p14, markers D4S419 and D4S405; at chromosome 4p14-16.3, markers D4S419 and D4S1592; at chromosome 6q25.2-q27, markers D6S1581 and D6S264; and, at chromosome 2p13, markers D2S337 and D2S2368. The analysis of the markers flanking the known PD genes did not show evidence for homozygosity, indicating that the disease in the family cannot be explained by one of these known genes (data not shown). We then performed a systematic genome screen. LOD scores were generated by the program MAPMAKER-HOMOZ (Kruglyak et al. 1995), under the assumptions of equal recombination for males and females, autosomal recessive inheritance, and gene

frequency .004. In the initial genome screen, the family structure used in the analysis is depicted in figure 1, and allele frequencies were estimated on the basis of the DNA of 51 spouses of patients participating in the various studies in GRIP. The three individuals included in the initial screen (VII-2, VII-3, and VII-7) were homozygous for six markers, located on chromosomes 1, 5, 11, 17, and 21. Only for the chromosome 1 region, two adjacent markers (D1S468 and D1S214) were found to be homozygous in all patients, leading to a maximum LOD score of 2.8.

The newly diagnosed patient (VII-6) was identified after this initial screen and was typed, together with unaffected first-degree relatives (VI-2, VI-3, VII-1, VII-4, and VII-5), only for the five regions at which the first three patients were homozygous.

Homozygosity remained only for markers on chromosomes 1, 5, and 17. On chromosomes 5 and 17, homozygosity was found with very-frequent (34% and 42%, respectively) alleles of the markers D5S1981 and D17S944. For closely linked (i.e., <10 cM distant) markers, all four patients were heterozygous, indicating that the observed alleles for these markers could be identical by state (IBS). In contrast, the population frequency of the homozygous allele at D1S468 was 9%. The chances that the homozygous allele in our distantly related patients is IBS are very small. In addition, all four patients were homozygous for the flanking marker D1S214, indicating that the alleles were most likely identical by descent. We therefore saturated the region surrounding D1S468 and D1S214 with 13 additional markers and found that all patients were homozygous for 9 of the markers studied, whereas the unaffected parents and two unaffected siblings were all either heterozygous or noncarriers of the disease haplotype (fig. 1). When LOD scores for the disease haplotype were calculated, equal allele frequencies for each marker were assumed, because population allele frequencies were not available. Penetrance was assumed to be 100% by age 40 years. The most conservative analysis—that is, under the assumption that there is a second-degree relationship between the parents of the sibships—resulted in a maximum multipoint LOD score of 4.3. Subsequent haplotype analysis showed homozygosity of all four patients, for a region of ~16 cM on the sex-averaged linkage map. The first recombination events were observed for marker D1S243 on the telomeric side in individual VII-6 and for marker D1S244 on the centromeric side in individual VII-3.

Because of Valente et al.'s (2001) recent report of linkage of an early-onset autosomal recessive form of parkinsonism to chromosome 1p35-36, we investigated whether we could exclude linkage to the *PARK6* region in the family that we studied; we tested nine markers from the *PARK6* region (table 2). The patients in this family showed no homozygosity at any of these markers.

Table 2

Genotypes of Affected Individuals, for Markers of the *PARK6* Region

	Genotype of Affected Individual			
Marker[a]	VII-2	VII-3	VII-6	VII-7
D1S199	2 5	3 5	2 5	3 4
D1S483	1 1	1 2	1 1	2 2
D1S478	3 5	1 3	3 5	2 3
D1S2828	4 1	4 5	4 1	4 3
D1S2732	2 4	2 3	2 4	1 4
D1S2702	2 4	2 3	2 4	4 1
D1S2734	5 4	5 5	5 4	4 1
D1S2885	4 1	4 2	4 1	1 3
D1S247	4 2	1 6	4 2	3 5

Source.—Valente et al. (2001).

[a] Order is according to the Marshfield (Center for Medical Genetics, Marshfield Medical Research Foundation) integrated linkage map.

The *PARK6* critical region, between markers D1S483 and D1S247, is localized ⩾25 cM centromeric of our critical region. Considering the evidence that the critical region in the family that we studied does not overlap with the region encompassing the *PARK6* gene identified by Valente et al. (2001) and taking into account the recommendations that Lander and Kruglyak (1995) have made with regard to the reporting of linkage findings, we report a significant linkage finding for the presence, on chromosome 1p, of a second locus for autosomal recessive early-onset parkinsonism; and we propose to name it "*PARK7*."

Both *PARK6* (Valente et al. 2001) and *PARK7* were identified by homozygosity mapping. Although this is a very powerful approach in consanguineous pedigrees (Lander and Botstein 1987), some possible methodological problems recently have been raised in articles published in the *Journal* (Miano et al. 2000). Miano et al. (2000) have shown that patients may be homozygous for large regions by chance—in particular if there are multiple loops in the pedigree, such as occur in the pedigree that we studied. However, our most conservative analysis, based on an analysis assuming a second-degree relationship, still yielded a LOD score >4. In addition, the fact that the affected individuals from the two sibships share an identical homozygous haplotype, whereas none of the unaffected first-degree relatives was homozygous for this haplotype, makes a false-positive finding less likely.

Using homozygosity mapping, we have identified a third locus for autosomal recessive early-onset parkinsonism: *PARK7*. Similar to what has been reported in other studies of early-onset parkinsonism and *Parkin* mutations (Lücking et al. 2000), the disease in the patients of the family that we studied is characterized by

a slow progression. Dystonic features, including blepharospasm and laterocollis, and brisk tendon reflexes have also been found in patients with *Parkin* mutations (Abbas et al. 1999; Lücking et al. 2000; Tassin et al. 2000). *PARK7* is the second locus, in the chromosome 1p36 region, involved in autosomal recessive, early-onset parkinsonism. The region defining the disease haplotype can be clearly separated from that of the more centromeric *PARK6* locus. Although the genomic sequence of the critical region is far from complete and many gaps remain, a first analysis of the Project Ensembl and Human Genome Project Working Draft at UCSC databases showed that inside the critical region ⩾25 genes are located, including 7 genes with an unknown function. Possible candidate genes are the gene encoding the vesicle-associated membrane protein 3 (*VAMP3*) and the gene encoding protein kinase C, zeta (*PRKCZ*). Telomeric of D1S243, a ubiquitin-conjugating enzyme has been localized. Although this would be an obvious candidate, its location is outside the candidate region. The observation, on chromosome 1p, of two closely linked loci for early-onset parkinsonism raises the question whether there might have been a duplication of this chromosomal region during evolution; however, comparison of the gene contents from the *PARK6* and *PARK7* critical regions and the syntenic region in the mouse does not support this idea.

Recessive mutations in the *Parkin* gene have been shown to be a common cause of early-onset parkinsonism (Lücking et al. 2000). Our findings and those of Valente et al. (2001) strongly suggest there may be other recessive mutations, in at least two unidentified genes involved in the disease. Analysis of additional families with early-onset parkinsonism lacking *Parkin* mutations will reveal the importance of these two new loci for early-onset parkinsonism.

The frequency of recessive mutations in early-onset parkinsonism may even have masked the genetic origin of PD for a long time, since familial aggregation and the identification of consanguinity require extensive genealogical research. Therefore, it remains to be determined whether these common recessive mutations are involved in late-onset forms of the disease as well. Studies of inbred and/or isolated populations such as GRIP may therefore help to further dissect the pathogenesis of PD.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Center for Medical Genetics, Marshfield Medical Research Foundation, http://research.marshfieldclinic.org/genetics/ (for information on polymorphic markers and localization of polymorphic markers)
Généthon, http://www.genethon.fr/ (for information on polymorphic markers and localization of polymorphic markers)
Human Genome Project Working Draft at UCSC, http://genome.cse.ucsc.edu/ (for identification of candidate genes *VAMP3* and *PRKCZ*)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for PD [MIM 168600], ARJP [MIM 600116], and *PARK6* [MIM 605909])
Project Ensembl, http://www.ensembl.org/ (for identification of candidate genes *VAMP3* and *PRKCZ*)

References

Abbas N, Lücking CB, Ricard S, Durr A, Bonifati V, De Michele G, Bouley S, Vaughan JR, Gasser T, Marconi R, Broussolle E, Brefel-Courbon C, Harhangi BS, Oostra BA, Fabrizio E, Bohme GA, Pradier L, Wood NW, Filla A, Meco G, Denefle P, Agid Y, Brice A (1999) A wide variety of mutations in the *Parkin* gene are responsible for autosomal recessive parkinsonism in Europe: French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Hum Mol Genet 8:567–574

Fahn S, Elton RL, UPDRS Development Committee (1987) Unified Parkinson's disease rating scale. In: Fahn S, Marsden CD, Calne DB (eds) Recent developments in Parkinson's disease. Vol 2. MacMillan Healthcare Information, Florham Park, NJ, pp 153–163

Farrer MJ, Gwinn-Hardy K, Muenter M, DeVrieze FW, Crook R, Perez-Tur J, Lincoln S, Maraganore D, Adler C, Newman S, MacElwee K, McCarthy P, Miller C, Waters C, Hardy J (1999) A chromosome 4p haplotype segregating with Parkinson's disease and postural tremor. Hum Mol Genet 8: 81–85

Gasser T, Müller-Myhsok B, Wszolek ZK, Oehlmann R, Calne DB, Bonifati V, Bereznai B, Fabrizio E, Vieregge P, Horstmann RD (1998) A susceptibility locus for Parkinson's disease maps to chromosome 2p13. Nat Genet 18:262–265

Hoehn MM, Yahr MD (1967) Parkinsonism: onset, progression and mortality. Neurology 17:427–442

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N (1998) Mutations in the *Parkin* gene cause autosomal recessive juvenile parkinsonism. Nature 392:605-608

Kruglyak L, Daly MJ, Lander ES (1995) Rapid multipoint linkage analysis of recessive traits in nuclear families, including homozygosity mapping. Am J Hum Genet 56: 519–527

Lander ES, Botstein D (1987) Homozygosity mapping: a way to map human recessive traits with the DNA of inbred children. Science 236:1567–1570

Lander ES, Kruglyak L (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 11:241–247

Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, Harta G, Brownstein MJ, Jonnalagada S, Chernova T, Dehejia A, Lavedan C, Gasser T, Steinbach PJ, Wilkinson KD, Polymeropoulos MH (1998) The ubiquitin pathway in Parkinson's disease. Nature 395:451–452

Lücking CB, Durr A, Bonifati V, Vaughan J, De Michele G, Gasser T, Harhangi BS, Meco G, Denefle P, Wood NW, Agid Y, Brice A (2000) Association between early-onset Parkinson's disease and mutations in the *Parkin* gene: French Parkinson's Disease Genetics Study Group. N Engl J Med 342: 1560–1567

Miano MG, Jacobson SG, Carothers A, Hanson I, Teague P, Lovell J, Cideciyan AV, Haider N, Stone EM, Sheffield VC, Wright AF (2000) Pitfalls in homozygosity mapping. Am J Hum Genet 67:1348–1351

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the α-synuclein gene identified in families with Parkinson's disease. Science 276:2045–2047

Tassin J, Durr A, Bonnet AM, Gil R, Vidailhet M, Lücking CB, Goas JY, Durif F, Abada M, Echenne B, Motte J, Lagueny A, Lacomblez L, Jedynak P, Bartholome B, Agid Y, Brice A (2000) Levodopa-responsive dystonia. GTP cyclohydrolase I or *Parkin* mutations? Brain 123:1112–1121

Valente EM, Bentivoglio AR, Dixon PH, Ferraris A, Ialongo T, Frontali M, Albanese A, Wood NW (2001) Localization of a novel locus for autosomal recessive early-onset parkinsonism, *PARK6*, on human chromosome 1p35-p36. Am J Hum Genet 68:895–900

Chapter 2.6

Localization of autosomal recessive early-onset parkinsonism to chromosome 1p36 (*PARK7*) in an independent dataset

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Localization of Autosomal Recessive Early-Onset Parkinsonism to Chromosome 1p36 (*PARK7*) in an Independent Dataset

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Two new loci, *PARK6* and *PARK7*, for autosomal recessive early-onset parkinsonism have recently been identified on chromosome 1p, in single large pedigrees. Among 4 autosomal recessive early-onset families analyzed here, 2 supported linkage to *PARK7*, 1 with conclusive evidence. These data confirm localization of autosomal recessive early-onset parkinsonism to *PARK7*, suggesting it to be a frequent locus. Assignment of families to either *PARK6* or *PARK7* might be difficult because of the proximity of the two loci on chromosome 1p.

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The etiology of Parkinson's disease (PD) remains unknown in most cases. However, rare mendelian forms have been identified, and genetics appears to play a prominent role in cases with early onset.[1,2]

Autosomal recessive forms of early onset parkinsonism (AR-EP) are caused by mutations in the *parkin* gene (*PARK2* locus) on chromosome 6q.[3] In Europe, mutations in the *parkin* gene explain for almost half of the families with recessive parkinsonism and onset before age 45.[4] These cases show good response to levodopa, slow progression, frequent levodopa-induced motor fluctuations and dyskinesias, and no severe cognitive disturbances.[4] Late-onset forms can also result from *parkin* mutations, being clinically indistinguishable from classical PD.[4–5] However, Lewy bodies, the PD pathological hallmarks, have not been found in the few brains with parkin mutations analysed, suggesting a different pathological process.[6,7]

Two new loci for AR-EP have recently been identified on the short arm of chromosome 1,[8,9] located close to each other but clearly separated by more than 25cM. Both new loci (*PARK6* and *PARK7*) were found in single large consanguineous families, using homozygosity mapping. The frequency of involvement of both loci remains to be evaluated in larger series of families.

We analyze here 4 families with AR-EP, confirming linkage to *PARK7* locus in an independent dataset. In 1 family we found evidence supporting linkage to all three AR-EP loci (*PARK2*, *PARK6*, and *PARK7),* illustrating the complexity and limitations of linkage analysis in heterogeneous disorders like AR-EP.

Patients and Methods

We studied 4 families with early-onset parkinsonism compatible with autosomal recessive inheritance (Fig 1, Table), of which 3 are consanguineous (Families 1–3). The families originate from Italy (Families 1 and 2), Uruguay (Family 3), and the Netherlands (Family 4). Living members were personally examined and blood samples obtained after written informed consent. The clinical diagnosis of parkinsonism required the presence of two of the three cardinal signs (resting tremor, bradykinesia, muscular rigidity), absence of atypical features (dementia early in disease course, ophtalmoplegia, pyramidal or cerebellar system involvement), and no identifiable causes. Brain imaging (computed tomography and/or magnetic resonance imaging) were unremarkable.

Genomic DNA was isolated from peripheral blood using standard protocols. For the linkage studies we typed polymorphic short tandem repeat markers linked to the three known AR-EP loci: *PARK2* (6q25.2–q27, D6S441, D6S1581, D6S305, D6S1277, D6S264, D6S446); *PARK6* (1p35–p36, D1S483, D1S199, D1S2732, D1S478, D1S2828, D1S2702, D1S2734, D1S2885, D1S234, D1S247); *PARK7* (1p36, D1S243, D1S468, D1S2845, D1S2660, D1S2795, D1S2870, D1S214, D1S2663, D1S2666, D1S508, D1S1612, D1S503, D1S244, D1S450, D1S2736, D1S2667).

Markers were obtained from the Généthon and Marshfield Center sets, and they were ordered according to the Marshfield integrated linkage map and the April 2001 physical assembly of the Human Genome (http://genome.ucsc.edu).

Markers were typed by radioactive polymerase chain reaction, essentially according to Weber and May.[10] Fluorescently labeled markers were used as specified by the manufacturers and analyzed using an ABI377 automated sequencer with Genescan 3.1 and Genotyper 2.1 software (Applied Biosystems, Foster City, CA).

The entire coding region of the *parkin* gene,[3] and exons

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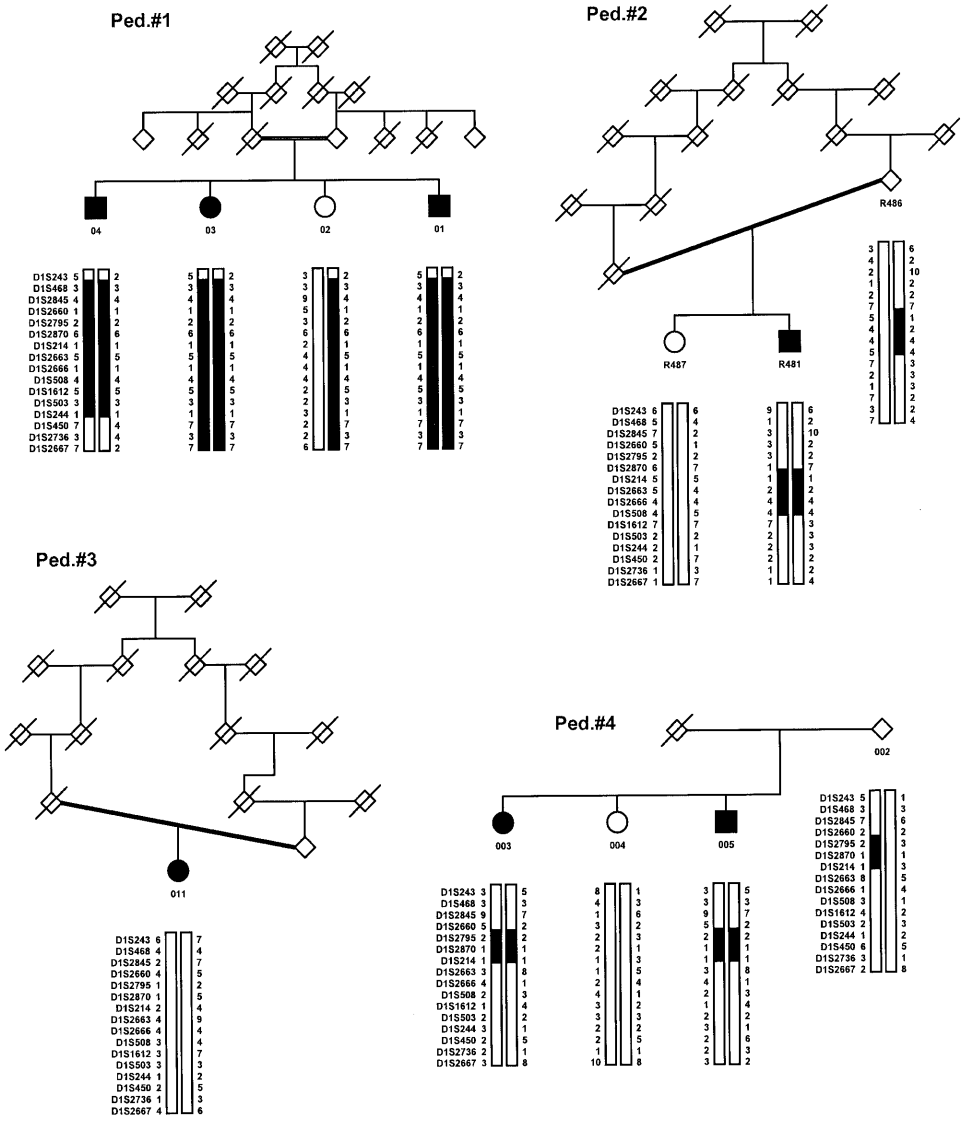


Fig 1. Simplified pedigrees of the 4 families with haplotypes for the PARK7 *region. Homozygous regions are indicated by* black bars. *Markers are ordered according to the Marshfield integrated linkage map, and the April 2001 draft assembly of human genome (http://genome.ucsc.edu). The gender of relatives has been masked to protect family confidentiality.*

3–4 of α-*synuclein* gene (where mutations have been reported in familial PD)[11,12] were sequenced in probands from pedigrees 1 and 2, using Big Dye Terminator chemistry (Applied Biosystems).

Linkage analyses were performed using MAPMAKER-HOMOZ, a program that allows rapid generation of multipoint log of the odds (LOD) scores in both consanguineous and nonconsanguineous pedigrees.[13] In the calculations we

Table. Clinical Features

Family	Patient	Onset Age	Disease Duration (yr)	T	B	R	L-DOPA	H-Y	Dyt	Other Remarks
1	01	28	18	+	+	+	+	III	+	Blepharospasm
1	03	35	21	+	+	+	+	III	+	Behavioral disturbances at onset
1	04	27	32	+	+	+	+	IV	+	Visual hallucinations (after L-DOPA)
2	R481	37	18	+	+	+	+	IV	+	Severe LID, DBS
3	011	35	9	+	+	−	+	II	+	LID
4	003	53	12	+	+	+	+	III	−	LID
4	005	37	17	−	+	+	+	III	+	LID, DBS

Phenotype at examination: T = resting tremor; B = bradykinesia; R = rigidity; L-DOPA = response to levodopa; H-Y = Hoehn-Yahr staging; Dyt = presence of dystonia at onset; LID = levodopa-induced dyskinesias; DBS = patient treated with deep brain stimulation.

assumed autosomal recessive inheritance, gene frequency of 0.006, complete penetrance by age 55, and a phenocopy rate of 0.00004. Equal recombination rates for men and women and equal marker allele frequencies were used. Haplotypes were constructed manually based on a minimal number of recombination events.

Results

The main clinical features of the affected individuals in the 4 families are summarized in the Table.

Two consanguineous families (1 and 2) showed evidence for linkage to *PARK7*. Family 1 generated a multipoint LOD score >2 across the whole *PARK7* region (maximum 2.50), whereas Family 2 supported linkage for a small part of the *PARK7* region (maximum 0.76; Fig 2). Haplotype analysis (see Fig 1) showed an extended homozygous interval in all affected individuals from Family 1, flanked by heterozygous markers D1S243 on the telomeric side and D1S450 on the centromeric side, which overlaps completely with the region found in the original *PARK7*-linked family.[9] The patient from Family 2, where consanguinity is more distant, showed a smaller homozygous interval flanked by markers D1S2870 (telomeric) and D1S1612 (centromeric).

In these kindreds (1 and 2), the multipoint LOD scores were negative and all affected individuals were heterozygous across the *PARK6* and *PARK2* regions (see Fig 2, and data not shown). Sequencing the coding region of *parkin* and exons 3–4 of α-*synuclein* gene in the probands did not reveal any mutations.

Family 3 did not support linkage to any of the tested regions (maximum LOD −0.16 in *PARK7* region) (see Fig 2, and data not shown).

Family 4 showed positive LOD scores, and the 2 patients share haplotypes for all three AR-EP loci (maximum 0.73 in *PARK7* region) (see Figs 1 and 2, and data not shown).

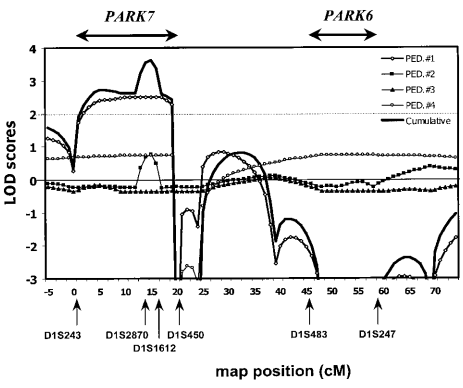
A maximum cumulative multipoint LOD score of 3.63 was obtained with markers of the *PARK7* region, including all the 4 typed families (see Fig 2).

Discussion

Our current findings confirm the existence of the *PARK7* locus in an independent dataset of AR-EP families. Two out of 3 *parkin*-negative consanguineous families show evidence for linkage to *PARK7*, and one (Family 1) exceeds the accepted level of significance required for conclusive confirmation of linkage according to the guidelines of Lander and Kruglyak.[14] Because only a small number of markers is analyzed whithin one interval, the corrections needed for multiple testing are minimal. As a consequence, a LOD score of +2.1 (nominal p value = 0.001) is sufficient for linkage confirmation at interval-wide significance level.[14] Family 1 is therefore the second pedigree with significant evidence for linkage to *PARK7* after our original report.[9]

Family 2 has limited statistical power because of its small size, and the maximum observed LOD (0.76) did not achieve interval-wide significance ($p > 0.05$). However, this family is of interest because of the small homozygous region present in the patient, which

Fig 2. Multipoint log of the odds (LOD) score plots for the PARK6 *and* PARK7 *regions. The* PARK6 *and* PARK7 *critical intervals are indicated according to references 8 and 9.*



would narrow down the critical region to less than 3cM, flanked by the heterozygous markers D1S2870 and D1S1612.

In Family 4, the 2 affected individuals share haplotypes at all three regions implicated in AR-EP (*PARK2*, *PARK6*, and *PARK7)*. This family has previously been described to provide evidence for linkage to *PARK6* by some of us (unpublished research), but the positive linkage findings to the other loci should also be considered before definitely assigning the family to a single locus. Although *parkin* gene mutations were not detected in this family by genomic sequencing and exon dosage analysis,[4] other types of mutations cannot be excluded. The presence of homozygosity for a small part of the *PARK7* region is also of interest (see Fig 1), but because there is no known consanguinity in Family 4, this might also be a chance finding. Family 4 therefore illustrates some of the potential pitfalls and limitations in linkage analysis of diseases such as AR-EP in which small, nonconsanguineous sibships are often studied, and there is genetic heterogeneity. In addition, because of the proximity of the *PARK7* and *PARK6* loci, some families might support linkage to both because of presence of extended regions of homozygosity or haplotype sharing.

In a conservative estimate (excluding Family 2 and 4), the *PARK7* critical interval spans about 20cM between marker D1S243 and D1S244.[9] According to the April 2001 draft assembly of human genome, about 60 genes are present in this interval, including several with unknown function.

The phenotype of *PARK7*-linked families appears similar to the *parkin*-related and *PARK6*-linked forms. The average age at onset was 32.1 years (range 27–40) for *PARK7* (7 patients from Families 1 and 2 in this study, and data from van Duijn and colleagues[9]) versus 32 years (range 7–58) in *parkin* disease (100 patients).[4] The onset appears slightly later (average 40.7 years, range 32–48, 4 patients) in the *PARK6*-linked family.[8] Other clinical features, including a good, prolonged response to levodopa and slow progression are shared by patients from all three AR-EP loci. This will make their differentiation difficult on clinical grounds, indicating the importance of genetic testing.

Interestingly, behavioral and psychic disturbances at onset or early in the disease course, and focal dystonias including blepharospasm, were noticed in the original *PARK7* kindred[9] and in Family 1 described here. Though occasionally described in families with *parkin* mutations, these features could represent clinical "red flags" for suspecting *PARK7*. However, larger case-series are needed for accurate genotype-phenotype correlations. An autosomal dominant form of focal-segmental dystonia (*DYT13*) maps to chromosome 1p36,[15] and its critical interval (telomeric border D1S2663) overlaps for 6cM with the centromeric part of *PARK7*. Despite differences in phenotype and transmission pattern, *DYT13* and *PARK7* might be allelic disorders.

No pathology data are so far available in *PARK7* families. Presynaptic dopaminergic abnormalities were detected by functional imaging in the original *PARK7* kindred.[9]

In conclusion we confirm the localization of AR-EP to the *PARK7* locus in an independent dataset, suggesting *PARK7* as an important locus for early onset parkinsonism.

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References

1. Zhang Y, Dawson VL, Dawson TM. Oxidative stress and genetics in the pathogenesis of Parkinson's disease. Neurobiol Dis 2000;7:240–250.
2. Tanner CM, Ottman R, Goldman SM, et al. Parkinson disease in twins: an etiologic study. JAMA 1999;281:341–346.
3. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998;392:605–608.
4. Lücking CB, Dürr A, Bonifati V, et al. Association between early-onset Parkinson's Disease and mutations in the parkin gene. N Engl J Med 2000;342:1560–1567.
5. Klein C, Pramstaller PP, Kis B, et al. Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: expanding the phenotype. Ann Neurol 2000;48:65–71.
6. Mori H, Kondo T, Yokochi M, et al. Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology 1998;51:890–892.
7. van de Warrenburg BP, Lammens M, Lucking CB, et al. Clinical and pathologic abnormalities in a family with parkinsonism and parkin gene mutations. Neurology 2001;56:555–557.
8. Valente EM, Bentivoglio AR, Dixon PH, et al. Localization of a novel locus for autosomal recessive early-onset parkinsonism, *PARK6*, on human chromosome 1p35–p36. Am J Hum Genet 2001;68:895–900.
9. van Duijn CM, Dekker MC, Bonifati V, et al. *PARK7*, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36. Am J Hum Genet 2001;69:629–634.
10. Weber JL, May PE. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 1989;44:388–396.
11. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 1997;276:2045–2047.
12. Krüger R, Kuhn W, Müller T, et al. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet 1998;18:106–108.
13. Kruglyak L, Daly MJ, Lander ES. Rapid multipoint linkage analysis of recessive traits in nuclear families, including homozygosity mapping. Am J Hum Genet 1995;56:519–527.
14. Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 1995;11:241–247.
15. Valente EM, Bentivoglio AR, Cassetta E, et al. DYT13, a novel primary torsion dystonia locus, maps to chromosome 1p36.13–36.32 in an Italian family with cranial-cervical or upper limb onset. Ann Neurol 2001;49:362–366.

Chapter 2.7

Clinical features and neuroimaging of *PARK7*-linked parkinsonism

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Clinical Features and Neuroimaging of *PARK7*-Linked Parkinsonism

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Abstract: We recently reported linkage to chromosome 1p36 (the *PARK7*-locus) in a family with early-onset parkinsonism. Linkage to this locus has since been confirmed in an independent data set. We describe clinical and neuroimaging features of the 4 patients in the original *PARK7*-linked kindred. Age at onset of parkinsonism varied from 27 to 40 years. Clinical progression was slow, and response to dopaminergic therapy good. The clinical spectrum ranged from mild hypokinesia and rigidity, to severe parkinsonism with levodopa-induced dyskinesias and motor fluctuation. Three of four patients with *PARK7*-linked parkinsonism exhibited psychiatric disturbances. Structural neuroimaging was unremarkable, but functional imaging of the brain, carried out in 3 patients, showed significant evidence for a presynaptic dopamine deficit, and assessment of cerebral glucose metabolism, as carried out in 1 patient, showed possible cerebellar involvement. © 2003 Movement Disorder Society

Key words: autosomal-recessive parkinsonism; *PARK7*; genetics; phenotype; neuroimaging

The cause of Parkinson's disease (PD), one of the most common neurodegenerative diseases in the elderly, is largely unknown. In recent years, attempts to dissect the genetic background of familial, mostly early-onset, PD have been successful.[1] In PD with an autosomal dominant pattern of inheritance, five genes or loci have been identified.[2–6] For autosomal recessive early-onset PD, one gene and two loci have been reported so far. The *parkin* gene (chromosome 6q25.2–27)[7] encodes a ubiquitin-protein ligase and accounts for a large part of early-onset PD.[8] The range of ages at onset in patients with mutations in *parkin* is very wide, and the clinical picture varies from parkinsonism to levodopa (L-dopa)–responsive dystonia, with varying severity. Response to L-dopa therapy is good and progression of disease is slow. After administration of L-dopa, motor fluctuations and dyskinesias occur frequently.[8] *PARK6* (chromosome 1p35–p36) is a second locus for autosomal-recessive parkinsonism, and is associated with sustained response to L-dopa and slow progression of symptoms.[9] In *PARK6*-linked parkinsonism, symptoms often reported in patients with early-onset autosomal-recessive parkinsonism, such as early dystonia and sleep benefit, were not observed.[10]

We recently mapped a third locus (*PARK7*) in a consanguineous pedigree with early-onset autosomal recessive parkinsonism.[11] The *PARK7* critical region lies on chromosome 1p36, and is clearly separated from *PARK6* by 25 cM. Since then, linkage to *PARK7* has been

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confirmed in an independent set of families.[12] In this study, the clinical and neuroimaging features of the original *PARK7*-linked family are described and compared to other forms of autosomal recessive early-onset parkinsonism.

PATIENTS AND METHODS

Patients

This study was carried out within the framework of a larger research program named "Genetic Research in Isolated Populations," or GRIP. The scientific protocol of GRIP has been approved by the Medical Ethics Committee of the Erasmus Medical Centre. Genealogical information of the *PARK7*-linked family was obtained and extended with local municipal and church records, as well as computerized registers. Patients were ascertained through their general practitioners, who informed them about the study and requested their cooperation.

Neurological Examination

A neurological examination in each patient and available relative was carried out by 2 neurologists, blinded to the genotype status. The clinical diagnosis of parkinsonism was established by the presence of at least two of the three cardinal signs (resting tremor, muscular rigidity, and bradykinesia) without atypical features or signs of other neurological systems (autonomic, pyramidal and cerebellar) being involved. The extent of motor dysfunction was evaluated by Unified Parkinson's Disease Rating Scale (UPDRS)[13] and Hoehn and Yahr scores.[14] One patient had previously undergone an extensive work-up for mitochondrial encephalomyopathy, dopamine-responsive dystonia, and other metabolic disease (screen for mitochondrial-DNA point mutations, biopterin-excretion assessment, bone-marrow and muscle biopsies), which did not yield evidence for those disorders.

Neuroimaging

During the assessment of neuroimaging results, the researchers were unaware of the genetic status of the patients. Structural brain-imaging data were obtained and reviewed. Functional brain-imaging was carried out in 3 of 4 patients. In all 3 patients, the presynaptic nigrostriatal system was assessed. Furthermore was assessed the postsynaptic nigrostriatal system in 2 patients, and brain glucose-metabolism in 1 patient. The scans were made in three medical centres in The Netherlands over a 6-year period because patients were treated by different neurologists. For this reason, the methods of neuroimaging could vary from one patient to the next. One patient (Patient VII-2) had a ^{123}I-*N*-omega-fluoropropyl-2β-carbomethoxy-3β-(4-iodophenyl) nortropane single-photon emission CT (FP-CIT SPECT) scan made. In another patient (Patient VII-3), ^{123}I-iodobenzamide (IBZM) and FP-CIT SPECT scans were made. In the third patient (Patient VII-7), IBZM, 2-β-carboxymethoxy-3-β-(4-iodophenyltropane (β-CIT) SPECT, ^{18}F-deoxyglucose (FDG) and ^{18}F-dopa (F-DOPA) positron emission tomography (PET) scans were carried out.[15–17]

RESULTS

Patients

The *PARK7* kindred consisted of two nuclear families, indicated as Family 1 and Family 2.[11] A genealogical study linked the patients to one common ancestor six generations ago in a pedigree that contained several consanguineous loops (Fig. 1). All 4 patients were homozygous, and 2 of 3 unaffected siblings as well as the parents of Patient VII-7 were heterozygous for the disease haplotype. The third unaffected sibling (VII-1) did not carry the disease haplotype.

Patient VII-3 from Family 1 and Patient VII-7 from Family 2 were receiving neurological treatment at the time of the study (pergolide in Patient VII-3 and L-dopa with entacapone in Patient VII-7). Neurological examination of the rest of the sibship of Patient VII-3 (consisting of 5 other presumedly unaffected siblings) showed 2 more affected individuals not previously known to have parkinsonism (Patients VII-2 and VII-6). The patient in the second sibship (Patient VII-7, Family 2) had no other affected close relatives.

Neurological Examination

Neurological examination in the parents and 2 siblings heterozygous for the disease haplotype, and in the sibling not carrying the haplotype yielded no evidence of neurological abnormalities. Clinical features of the 4 patients are listed in Table 1. Ages at onset of disease varied from 27 to 40 years. Patient VII-6 was 40 years old at first neurological examination (which established the diagnosis of parkinsonism). All 4 patients showed the cardinal signs of parkinsonism without atypical (pyramidal, cerebellar, autonomic) features. Two patients had signs of focal dystonia: blepharospasm occurred in Patient VII-3, and laterocollis and foot dystonia in Patient VII-7, the latter worsening in the off-phase of medication. Patient VII-3 could not indicate whether the blepharospasm preceded therapy for parkinsonism, or only arose afterward. In Patient VII-7 the laterocollis occurred within a month of initiation of treatment. Patients VII-3 and VII-7 were

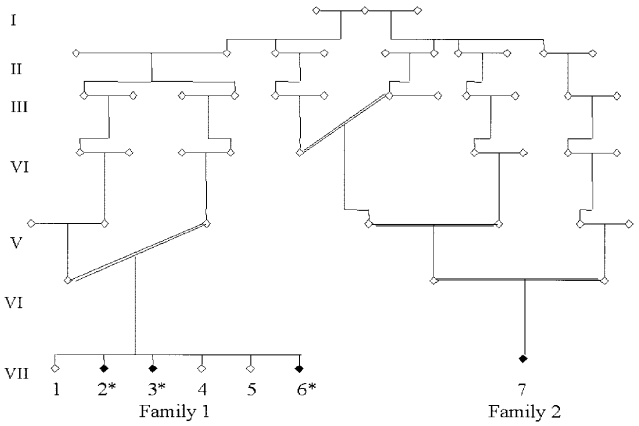

FIG. 1. Pedigree of the PARK7-kindred. The symbol on top represents the common ancestor, and the symbols on the bottom row the examined individuals. Solid symbols indicate affected individuals. *Additional psychiatric features.

treated with pergolide and L-dopa with entacapone, respectively, and newly diagnosed Patients VII-2 and VII-6 did not receive any dopaminergic medication. Patient VII-7 developed dyskinesias and dystonias within 1 month from L-dopa treatment. In addition to parkinsonism, all 3 patients VII-2, VII-3, and VII-6 in Family 1 exhibited varying degrees of anxiety, which was reported to have existed for years before onset of parkinsonism. Patient VII-2 was also known to have suffered from recurrent psychotic episodes with paranoid delusions, for which treatment was often declined. In Patient VII-7 of Family 2, no such psychiatric or behavioral symptoms were noted.

Neuroimaging

Structural brain-imaging was available for Patients VII-3 (computed tomography images) and VII-7 (magnetic resonance images), and was unremarkable. Functional brain-imaging was carried out in 3 of 4 patients (VII-2, VII-3, and VII-7). Results are listed in Table 2.

TABLE 1. *Clinical features of PARK7-linked parkinsonism*

Patient	VII-2	VII-3	VII-6	VII-7
Age at examination (yr)	50	48	40	38
Age at onset (yr)	40	31	Not known	27
Resting tremor	−	+	−	−
Rigidity	+	+	+	+
Bradykinesia	+	+	+	+
Postural instability	+	+	+	+
Asymmetry at onset	+	+	−	+
Dystonia at examination	−	Blepharospasm	−	Laterocollis, foot dystonia
Hyperreflexia	−	+	−	+
Dopaminergic drugs, daily dosage (mg)	−	Pergolide (1.5)	−	L-dopa (+IDD)/entacapone (187.5/600)
Side-effects of levodopa	n.a.	n.a.	n.a.	Dyskinesias, dystonias, motor fluctuations
UPDRS score (*on/off* phase)	29 (n.a.)	57 (*on*)	22 (n.a.)	25 (*on*)
Hoehn and Yahr stage (*on/off* phase)	III (n.a.)	III (*on*)	III (n.a.)	III (*on*)
Psychiatric symptoms	Anxiety, psychotic episodes, paranoid delusions	Anxiety	Anxiety	−
Psychotropic drugs, daily dosage (mg)	Diazepam (5), haloperidol,[a] olanzapine[a]	Temazepam (10)	Diazepam (5)	−

[a]Intermittent usage, last time of administration of at least six months before neurological examination.
UPDRS, Unified Parkinson's Disease Rating Scale; IDD, dopa-decarboxylase inhibitor.

TABLE 2. *Functional neuroimaging in PARK7-linked parkinsonism*

	Patient	Scan performed	Results
Family 1	VII-2	FP-CIT SPECT	Uptake putamen and caudate bilaterally and symmetrically decreased
	VII-3	FP-CIT SPECT	Uptake putamen and caudate bilaterally and symmetrically decreased
	VII-3	IBZM SPECT	Normal dopamine-D2 receptor binding
	VII-6	—	—
Family 2	VII-7	β-CIT SPECT	Uptake putamen bilaterally decreased; uptake caudate unilaterally decreased
	VII-7	IBZM SPECT	Normal dopamine-D2 binding
	VII-7	^{18}F-DOPA PET	Uptake putamen and caudate bilaterally decreased, more pronounced contralateral to most severely affected body side
	VII-7	FDG PET	Diffuse cerebellar hypometabolism

FP-CIT SPECT, ^{123}-I-N-Ω-fluoropropyl-2β-carbomethoxy-3β-(4-iodophenyl) nortropane single photon emission CT; IBZM SPECT, ^{123}I-iodobenzamide SPECT; β-CIT SPECT, 2-β-carboxymethoxy-3-β-(4-iodophenyltropane) SPECT; FDG PET, ^{18}F-deoxyglucose PET.

The fourth patient (VII-6) has not undergone neuroimaging. On SPECT imaging, 2 patients (VII-2 and VII-3) had a significant, symmetrical presynaptic dopaminergic deficit that was present bilaterally in putamen and caudate nucleus. The putamen was more severely affected than the caudate nucleus. Striatal dopamine-D_2 receptor binding (assessed in Patients VII-3 and VII-7) was normal on IBZM neuroimaging. Patient VII-7 showed a strongly reduced presynaptic dopaminergic neurotransmitter function on both sides in putamen and caudate nucleus on PET and SPECT imaging. Uptake was more reduced in the hemisphere contralateral to the clinically more affected body side, and the putamen was more severely affected than the caudate nucleus. Furthermore, the FDG PET scan carried out in Patient VII-7 showed decreased glucose metabolism in the cerebellum. ^{18}F-DOPA PET images of Patient VII-7 and of an age-matched healthy control subject are shown in Figure 2.

DISCUSSION

Based upon observations in this original kindred, *PARK7*-linked parkinsonism is of variable severity. Some of the patients had overt parkinsonism, receiving L-dopa or dopaminergic therapy to which they responded well. Others had mild parkinsonism for which medical attention was never sought. Patients VII-3 and VII-7 had most pronounced motor dysfunction, and were on antiparkinson treatment. The latter 2 patients also had focal dystonia. Patient VII-7, who received L-dopa, developed this within a month of starting dopamine therapy, as well as motor fluctuations, on-phase dyskinesias and off-phase dystonia. In all 4 patients, progression as observed over the 3-year period of our study was very slow. The features in early-onset parkinsonism mentioned above, such as the variable severity, the positive response to, with motor complications of, L-dopa and slow progression of disease, are comparable to the phenotype of *parkin*-related[8] and *PARK6*-linked parkinsonism.[9] Similar observations were made in other *PARK7*-linked kindreds.[12] In families with *PARK6*-linked parkinsonism, however, focal dystonia was not observed and resting tremor prevailed.[10] Although the number of patients studied is too small to draw firm conclusions, *PARK6*- and *PARK7*-linked parkinsonism appear to be entities that are genetically as well as clinically distinct.

The siblings and parents who were heterozygous for the *PARK7*-linked haplotype showed no symptoms of parkinsonism at neurological examination. As for *parkin* and *PARK6*, being a heterozygous carrier of the *PARK7* disease-haplotype is probably not sufficient to confer clinical parkinsonism.

All patients in Family 1 had symptoms of anxiety, for which they had been receiving benzodiazepine treatment for years (Patient VII-3 had been using benzodiazepines even before parkinsonism was diagnosed and treated). Because the 3 siblings were treated by different general practitioners, this common medication cannot have been due to the propensity of 1 physician to prescribe benzodiazepines. In this family, complaints of anxiety may have been superimposed on, or confused with, initial symptoms of parkinsonism. In Family 1, the 3 siblings who were unaffected with parkinsonism (VII-1, VII-4, VII-5) had no psychiatric symptoms either. These psychiatric symptoms may thus constitute part of the *PARK7* phenotype. In 1 patient of the independent family with significant linkage to *PARK7*,[12] psychiatric and behav-

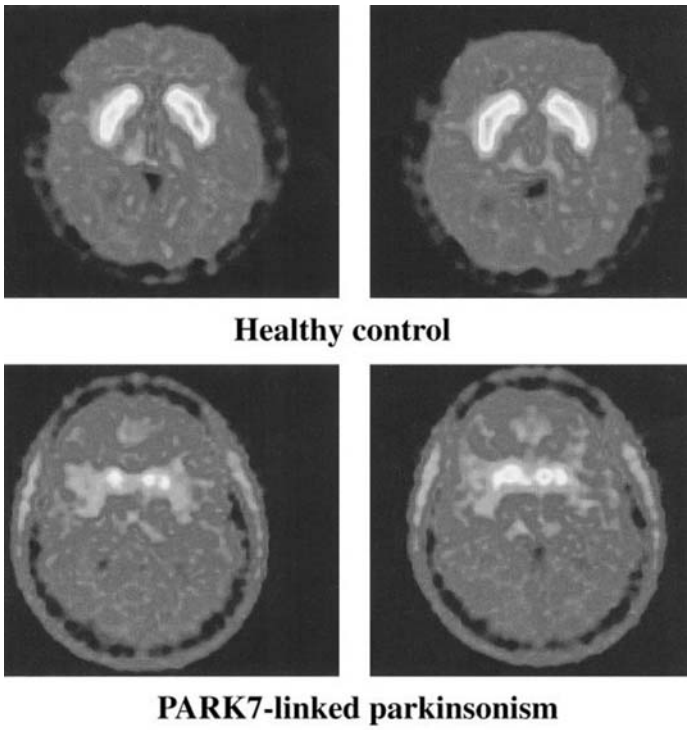

FIG. 2. ^{18}F-DOPA PET images of an age-matched healthy control subject and of Patient VII-7. Two transaxial planes through the brain of an age-matched healthy control subject (**top**) and Patient VII-7 (**bottom**). The subjects are viewed from the feet. The linear color scale is scaled for activity between the subjects. The red color indicates maximum uptake, and purple very low uptake. Compared to the control subject, uptake into putamen and caudate nucleus in Patient VII-7 is reduced markedly on both sides.

ioral problems at onset of disease were also present: aggression, irritability, and obsessive-compulsive disorder (V. Bonifati, personal communication). In *parkin*-linked disease, psychiatric features, such as pronounced anxiety requiring treatment, have been reported occasionally,[18–20] and in classical, idiopathic PD, anxiety is also observed commonly.[21] Due to the consanguineous structure of our pedigree, however, psychiatric symptoms may form a 'private' trait in Family 1 only, and be unrelated to the *PARK7* phenotype. Additional families are therefore required to study psychopathological aspects of *PARK7*-linked parkinsonism.

On FP CIT- and β-CIT imaging in Patients VII-2 and VII-3, there was significant evidence for a symmetrical presynaptic dopaminergic deficit. In Patient VII-7, who underwent PET and SPECT neuroimaging, dopa uptake was greatly reduced bilaterally in both putamen and caudate nucleus. This was more pronounced in the cerebral hemisphere contralateral to the more severely affected body side. ^{18}F-DOPA PET-scan series carried out in *parkin* patients and carriers[22–24] showed a bilaterally and symmetrically decreased dopa uptake, in which putamen as well as caudate nucleus were affected. Furthermore, asymptomatic *parkin*-heterozygotes showed a mild but significant decrease in dopa uptake, suggesting a sub-clinical disease process in heterozygotes.[23] In idiopathic, non-familial PD, dopa uptake is primarily asymmetrically decreased, affecting the putamen much more severely than the caudate nucleus.[25]

The dopa-uptake reduction is symmetrical in 2 patients in our study, but asymmetrical in a third patient, which does not permit straightforward comparison to dopa-uptake patterns in *parkin*-linked parkinsonism or to those in idiopathic PD. The presence of impaired uptake in putamen as well as in the caudate nucleus in our patients resembles *parkin*-linked parkinsonism more

than idiopathic PD.[22–24] More data on *PARK7*-linked parkinsonism are therefore required to further delineate its functional-neuroimaging pattern.

The FDG PET image of Patient VII-7 showed cerebellar hypometabolism of glucose. This may either be a primary phenomenon in the disease process, or occur secondary to striatal deafferentation. A decreased glucose metabolism in the cerebellum was also observed on FDG PET imaging in patients with a deletion in the *parkin* gene,[22] suggesting another feature that *PARK7*- and *parkin*-linked parkinsonism have in common. Furthermore, clinical cerebellar dysfunction and neuropathology have been described in Japanese and Dutch families with *parkin*-related parkinsonism.[26,27] In addition to the observations in *parkin*-related parkinsonism, a SPECT study reported symmetrical cerebellar hypoperfusion in an early-onset patient without *parkin* mutations.[28] Cerebellar involvement in autosomal recessive parkinsonism remains unclear and therefore requires further research.

In PET-neuroimaging studies on *parkin*-linked parkinsonism, ^{11}C-raclopride binding, assessing status of postsynaptic dopamine-D_2 receptors, was shown to be reduced significantly.[23] In Patient VII-7, a C-raclopride PET scan has unfortunately not been carried out, but IBZM imaging in this patient and in Patient VII-3 showed normal dopamine-D_2 receptor binding. Finally, functional-neuroimaging studies of the *PARK7*-positive confirmation families and the *PARK6* families are not yet available, nor are neuropathological data.

The number of patients of this newly described form of parkinsonism is small, but nevertheless demands comparison to other forms of recessive parkinsonism and idiopathic PD. Our first delineation of the *PARK7* phenotype can be refined as more PARK7-linked families become available.

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REFERENCES

1. Gasser T. Genetics of Parkinson's disease. J Neurol 2001;248:833–840.
2. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the α-synuclein gene identified in families with Parkinson's disease. Science 1997;276:2045–2047.
3. Leroy E, Boyer R, Auburger G, et al. The ubiquitin pathway in Parkinson's disease. Nature 1998;395:451–452.
4. Gasser T, Müller-Myhsok B, Wsolek ZK, et al. A susceptibility locus for Parkinson's disease maps to chromosome 2p13. Nat Genet 1998;18:262–265.
5. Farrer MJ, Gwinn-Hardy K, Muenter M, et al. A chromosome 4p haplotype segregating with Parkinson's disease and postural tremor. Hum Mol Genet 1999;8:81–85.
6. Funayama M, Hasegawa K, Kowa H, Saito M, Tsuji S, Obata F. A new locus for Parkinson's disease (*PARK8*) maps to chromosome 12p11.2–q13.1. Ann Neurol 2002;51:296–301.
7. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392:605–608.
8. Lücking CB, Durr A, Bonifati V, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene. French Parkinson's Disease Genetics Study Group. N Engl J Med 2000;342:1560–1567.
9. Valente EM, Bentivoglio AR, Dixon P, et al. Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35–p36. Am J Hum Genet 2001;68:895–900.
10. Valente EM, Brancati F, Ferraris A, et al. Park6-linked parkinsonism occurs in several European families. Ann Neurol. 2002;51: 14–18.
11. van Duijn CM, Dekker MCJ, Bonifati V, et al. PARK7, a novel locus for autosomal-recessive early-onset parkinsonism on chromosome 1p36. Am J Hum Genet 2001;69:629–634.
12. Bonifati V, Breedveld GJ, Squitieri F, et al. Localization of autosomal recessive early-onset parkinsonism to chromosome 1p36 (PARK7) in an independent data set. Ann Neurol 2002;51:253–256.
13. Fahn S, Elton RL, members of the UPDRS Development Committee. Unified Parkinson's Disease Rating Scale. In: Fahn S, Marsden CD, Calne DB, editors. Recent developments in Parkinson's disease, vol 2. Florham Park, NJ: MacMillan Healthcare Information; 1987. pp 153–163.
14. Hoehn MM, Yahr MD. Parkinsonism: onset, progression and mortality. Neurology 1967;17:427–442.
15. Kruger R, Kuhn W, Leenders KL, Sprengelmeyer R, Muller T, Woitalla D, Portman AT, Maguire RP, Veenma L, Schroder U, Schols L, Epplen JT, Riess O, Przuntek H. Familial parkinsonism with synuclein pathology: clinical and PET studies of A30P mutation carriers. Neurology 2001;56:1355–1362.
16. Booij J, Speelman JD, Horstink MW, Wolters EC. The clinical benefit of imaging striatal dopamine transporters with [^{123}I]FP-CIT SPET in differentiating patients with presynaptic parkinsonism from those with other forms of parkinsonism. Eur J Nucl Med. 2001;28:266–272.
17. Marek K, Innis R, van Dyck C, Fussell B, Early M, Eberly S, Oakes D, Seibyl J. [^{123}I]β-CIT SPECT imaging assessment of the rate of Parkinson's disease progression. Neurology 2001;57:2089–2094.
18. Tassin J, Dürr A, de Broucker T, et al. Chromosome 6-linked autosomal recessive early-onset parkinsonism: linkage in European and Algerian families, extension of the clinical spectrum, and evidence of a small homozygous deletion in one family. Am J Hum Genet 1998;63:88–94.
19. Lücking CB, Bonifati V, Periquet M, Vanacore N, Brice A, Meco G. Pseudo-dominant inheritance and exon 2 triplication in a family with parkin gene mutations. Neurology 2001;57:924–927.
20. Yamamura Y, Hattori N, Matsumine H, Kuzuhara S, Mizuno Y. Autosomal recessive early-onset parkinsonism with diurnal fluc-

tuation: clinicopathologic characteristics and molecular genetic identification. Brain Dev 2000;22:S87–S91.
21. Walsh K, Bennett G. Parkinson's disease and anxiety. Postgrad Med J 2001;77:89–93.
22. Portman AT, Giladi N, Leenders KL, et al. The nigrostriatal dopaminergic system in familial early onset parkinsonism with parkin mutations. Neurology 2001;56:1759–1762.
23. Hilker R, Klein C, Ghaemi M, et al. Positron emission tomographic analysis of the nigrostriatal dopaminergic system in familial parkinsonism associated with mutations in the Parkin gene. Ann Neurol 2001;49:367–376.
24. Khan RL, Pavese N, Wood N, Brooks DJ, Piccini P. An ^{18}F-dopa PET study of disease progression and subclinical nigrostriatal dysfunction in a Parkin kindred. Neurology 2001;56:A249.
25. Leenders KL, Salmon EP, Tyrell P, et al. The nigrostriatal dopaminergic system assessed in vivo by positron emission tomography in healthy volunteer subjects and patients with Parkinson's disease. Arch Neurol 1990;47:1290–1298.
26. Kuroda Y, Mitsui T, Akaike M, Azuma H, Matsumoto T. Homozygous deletion mutation of the parkin gene in patients with atypical parkinsonism. J Neurol Neurosurg Psychiatry 2001;71: 231–234.
27. Horstink MW, van de Warrenburg BP, Lammens M, Brice A. Parkin gene related multisystem disorder. J Neurol Neurosurg Psychiatry 2002;72:419–420.
28. Yoshioka M, Shigeto H, Oya Y, Ogawa M, Kawai M. Juvenile parkinsonism with symmetrical hypoperfusion in the cerebellum—a case report. [Japanese] Rinsho Shinkeigaku 2000;40:1008–1011.

Chapter 2.8

Mutations in the *DJ-1* gene associated with autosomal recessive early-onset parkinsonism

Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P.

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Mutations in the *DJ-1* Gene Associated with Autosomal Recessive Early-Onset Parkinsonism

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The *DJ-1* gene encodes a ubiquitous, highly conserved protein. Here, we show that *DJ-1* mutations are associated with PARK7, a monogenic form of human parkinsonism. The function of the DJ-1 protein remains unknown, but evidence suggests its involvement in the oxidative stress response. Our findings indicate that loss of DJ-1 function leads to neurodegeneration. Elucidating the physiological role of DJ-1 protein may promote understanding of the mechanisms of brain neuronal maintenance and pathogenesis of Parkinson's disease.

The causes of Parkinson's disease (PD), a common neurodegenerative disorder, remain largely unknown but genes identified for rare Mendelian forms have increased our understanding of disease pathogenesis (*1*, *2*). By homozygosity mapping in two consanguineous families from genetically isolated communities in the Netherlands and Italy, we localized a gene for autosomal recessive early-onset parkinsonism, PARK7, to a 20-cM interval on chromosome 1p36 (supporting online text) (*3*, *4*).

Fine mapping by typing available and newly developed short tandem repeat (STR) or single nucleotide polymorphism (SNP) markers, reduced the critical region in the Dutch family to a 5.6-Mb region of homozygous sequence, spanning six contigs and five gaps in NCBI build 29 of the human genome (*5*) and containing as many as 90 genes (Fig. 1) (*6*). We sequenced obvious candidate genes from genomic DNA, but no mutations were detected in the two families (supporting online text).

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We then performed a systematic screening of the transcripts in the region, using reverse-transcriptase–polymerase chain reaction (RT-PCR) material obtained from lymphoblastoid cell lines of one patient in each family and a normal control. One of the cDNAs analyzed, corresponding to the whole *DJ-1* gene open reading frame (ORF), could not be amplified in the Dutch patient (fig. S1). Amplification of *DJ-1* exons $1^{A/B}$ to 5 from genomic DNA yielded no products in the same patient, whereas the two more centromeric exons 6 and 7 were normally amplified. This confirmed the presence of a homozygous genomic deletion and placed the centromeric border of the deletion in intron 5 of the *DJ-1* gene. From the telomeric side, we could amplify exons of the neighboring gene *TNFRSF9*, as well as sequences from the intergenic region, delineating *DJ-1* as the only gene involved in the deletion.

Using a PCR-based strategy, we cloned the deletion breakpoint (supporting online text). A novel fragment of about 2000 base pairs (bp) was amplified only from the individuals carrying the disease allele. Sequencing this fragment confirmed the joining of the telomeric and centromeric borders, delineating a deletion of 14.082 bp, which includes 4 kb of sequence upstream of the *DJ-1* gene ORF start. Interestingly, ALU elements flank the deleted sequence on both sides, and the breakpoint occurs within 16 bp of sequence identical in the two ALUs, suggesting that unequal crossing-over was likely at the origin of this genomic rearrangement (fig. S1).

The deletion showed complete cosegregation with the disease allele in the Dutch family (fig. S1), whereas it was absent in 380 chromosomes from the general Dutch population, which supports the position that it is a pathogenic mutation. We also investigated the frequency of the *DJ-1* deletion within the genetically isolated population where the Dutch PARK7 family was identified (*3*). Among 440 independent chromosomes from the genetic isolate we found four heterozygous and no homozygous carriers of the deletion. Moreover, the deletion was absent in 400 chromosomes from the regions closely surrounding the genetic isolate. Together with our results from the general Dutch population, these findings indicate that, although the mutant allele is present at 0.9% within the isolated population, it is likely confined to this population. None of 46 late-onset PD patients from the isolated community carried the deletion, indicating that they have a different etiology.

We then sequenced the *DJ-1* cDNA from the Italian patient and identified a homozygous point mutation (T→C transition at position 497 from the ORF start in cDNA), resulting in the substitution of a highly conserved leucine at position 166 (Leu^{166}) of the DJ-1 protein by a proline (Fig. 2A). This change showed complete cosegregation with the disease allele in the Italian family (fig. S1) and was absent from 320 chromosomes from the general Italian population. No further changes segregating with the disease in the Italian family and absent in the general population were detected.

Although PARK6 and PARK7 seem to be separate, close loci (supporting online text), it will be interesting to search the published PARK6 family for mutations in *DJ-1* (*7*). We have tested a limited number of early-onset familial and sporadic PD patients that did not have mutations in the *parkin* gene; we did not find a mutation in *DJ-1* (supporting online text).

The *DJ-1* gene contains 8 exons distributed over 24 kb (Fig. 1A). The first two exons (1^A and 1^B) are noncoding and alternatively spliced in the *DJ-1* mRNA (*5*). One major transcript of about 1 kb contains

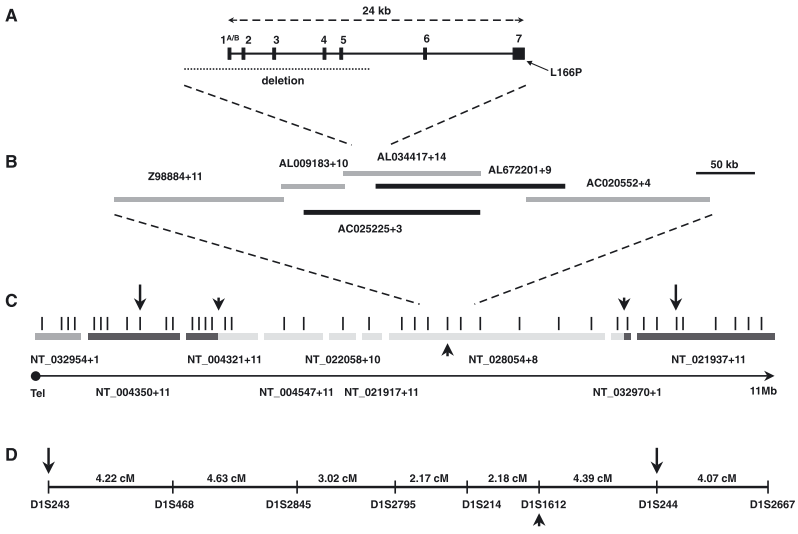

Fig. 1. Genetic and physical maps of the PARK7 region. (**A**) Genomic organization of the *DJ-1* gene. The location of the eight exons and the mutations found in the Dutch and Italian families are shown. (**B**) BAC/PAC clone coverage of a 500-kb region spanning the *DJ-1* gene. Blue and black bars indicate finished and unfinished clones, respectively. (**C**) Physical map of the PARK7 region. Vertical bars above contigs indicate the location of the polymorphic markers typed in this study. Contigs depicted in yellow contain fully informative, homozygous markers in the Dutch family patients. Regions depicted in brown contain heterozygous markers in at least one patient. The most telomeric contig depicted in gray contains homozygous, noninformative markers. The contig NT 022058 contains informative homozygous markers and is therefore placed within the PARK7 critical region. Its position in the NBCI assembly (build 29) is more telomeric. The remaining contigs are ordered as in the NCBI assembly. Heterozygous markers placed in contig NT 004321 telomerically and NT 032970 centromerically, flank 5.6 Mb of homozygous sequence and define the new PARK7 critical interval (black arrowheads). (**D**) Genetic map; the intermarker genetic distance is given in centimorgans, according to the Marshfield integrated sex average linkage map. Markers flanking the PARK7 locus in previous mapping studies are indicated with black arrows. The marker D1S1612, located 80 kb centromeric to the *DJ-1* gene, is indicated by a red arrowhead (C and D).

a 570-bp ORF, encoding a protein of 189 amino acids, that is ubiquitously expressed in body tissues and brain areas, including those more affected in PD (fig. S2). DJ-1 belongs to the ThiJ/PfpI family (pfam01965), which includes ThiJ, a protein involved in thiamine biosynthesis in prokaryotes; PfpI and other bacterial proteases; araC and other bacterial transcription factors; and the glutamine amidotransferases family (including bacterial catalases) (Fig. 2B).

To investigate the structural consequences of the Leu^{166}Pro mutation, a molecular model of DJ-1 was built using the programs WHAT IF (*8*) and YASARA (*9*). With 23% sequence identity to the known structure of protease PH1704 from *Pyrococcus horikoshii* (*10*) and an almost gapless alignment spanning 170 residues, DJ-1 can safely be assumed (*11*) to adopt the same α/β sandwich structure as PH1704 (Fig. 2C). According to the model, Leu^{166} is placed right in the middle of the carboxyl-terminal helix. Proline is a strong helix breaker (*12*) and its presence in the DJ-1 mutant is therefore likely to destabilize the terminal helix. The PH1704 protease was found to form a hexameric ring structure made of a trimer of dimers (*10*). The general pattern of salt bridges and hydrophobic packing in the trimerization region of PH1704 is conserved in the DJ-1 model, which makes it likely that DJ-1 also forms higher aggregates.

To explore the functional consequences of the *DJ-1* mutation, we transfected wild-type and mutant *DJ-1* in cell cultures (Fig. 2, D to I). In COS and PC12 cells transfected with wild-type *DJ-1* we observed diffuse cytoplasmic and nuclear DJ-1 immunoreactivity similar to findings from previous studies (*13*, *14*). Transfection of the *DJ-1* carrying the Leu^{166}Pro mutation showed a similar uniform nuclear staining, whereas the cytoplasmic staining appeared mostly colocalized with mitochondria.

Taken as a whole, our findings indicate that the DJ-1 protein is lacking in the Dutch family and is functionally inactive in the Italian family because of the Leu^{166}Pro change. The mutant DJ-1 (Leu^{166}Pro) is still present in the nucleus, which suggests that the loss of cytoplasmic activities is pathogenic, or that the nuclear activity is affected by the mutation even if the protein retains its translocation capability.

The function of DJ-1 is unknown. Human *DJ-1* was first identified as an oncogene (*13*) and later as a regulatory subunit of an RNA-binding protein (RBP) (*14*). Moreover, DJ-1 binds to PIAS proteins (*15*), a family of SUMO-1 ligases that modulate the activity of various transcription factors (*16*). DJ-1 itself is sumoylated at lysine 130 (*15*). Interestingly, in human and murine cell lines, DJ-1 has been identified as a hydroperoxide-responsive protein that is converted into a variant with a more acidic pI in response to oxidative stimuli like H_2O_2 or the herbicide Paraquat, which suggests a function as an antioxidant protein (*17*, *18*). The transcription of *YDR533C*, a yeast *DJ-1* homolog, is induced together with genes involved in the oxidative stress response (*19*).

Oxidative damage has been implicated in the mechanisms of neuronal death and the pathogenesis of PD (*20*). Normal dopamine metabolism produces reactive oxygen species, making nigral neurons particularly sensitive to oxidative stress, and signs of oxidative stress are found in postmortem studies of PD brains (*2*, *20*). Emerging evidence also links oxidative stress to mutations in α-*synuclein* and *parkin*, two PD-related genes (*21*, *22*).

It is possible that DJ-1 participates in the oxidative stress response by directly buffering cytosolic redox changes, and/or by modulating gene expression at transcriptional and post-transcriptional levels (fig. S3), interacting with RBP complexes and

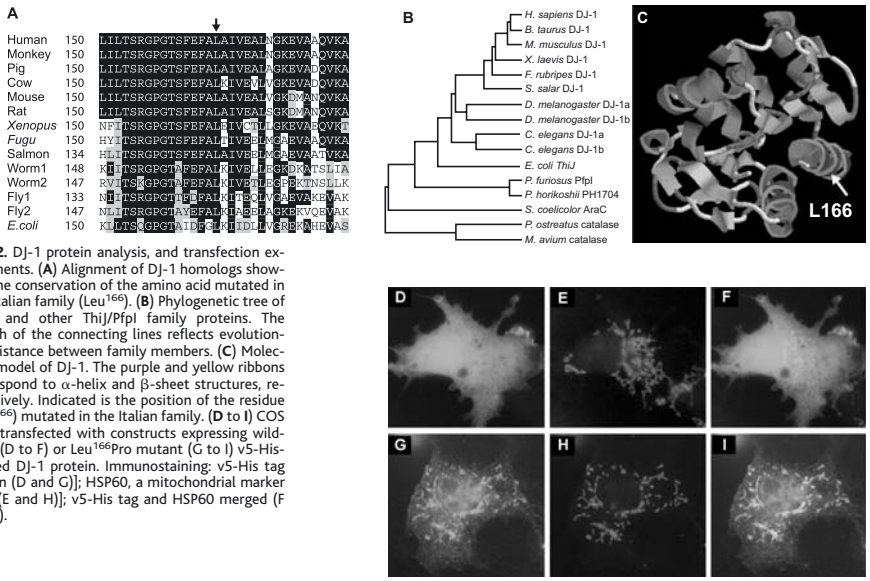

Fig. 2. DJ-1 protein analysis, and transfection experiments. (**A**) Alignment of DJ-1 homologs showing the conservation of the amino acid mutated in the Italian family (Leu^{166}). (**B**) Phylogenetic tree of DJ-1 and other ThiJ/PfpI family proteins. The length of the connecting lines reflects evolutionary distance between family members. (**C**) Molecular model of DJ-1. The purple and yellow ribbons correspond to α-helix and β-sheet structures, respectively. Indicated is the position of the residue (Leu^{166}) mutated in the Italian family. (**D** to **I**) COS cells transfected with constructs expressing wild-type (D to F) or Leu^{166}Pro mutant (G to I) v5-His-tagged DJ-1 protein. Immunostaining: v5-His tag [green (D and G)]; HSP60, a mitochondrial marker [red (E and H)]; v5-His tag and HSP60 merged (F and I).

transcriptional cofactors such as PIAS or other, unknown factors.

Our discovery of *DJ-1* mutations in PARK7 opens new avenues for understanding the neuronal function of DJ-1, that, when lost, causes neurodegeneration. Furthermore, the observation that DJ-1 may be involved in the oxidative stress response links a genetic defect in this pathway to the development of parkinsonism, with possible implications for understanding the pathogenesis of the common forms of PD.

References and Notes

1. A. E. Lang, A. M. Lozano, *N. Engl. J. Med.* **339**, 1044 (1998).
2. M. M. Mouradian, *Neurology* **58**, 179 (2002).
3. C. M. van Duijn *et al.*, *Am. J. Hum. Genet.* **69**, 629 (2001).
4. V. Bonifati *et al.*, *Ann. Neurol.* **51**, 253 (2002).
5. www.ncbi.nlm.nih.gov
6. Materials and methods are available as supporting material on *Science* Online.
7. E. M. Valente et al., *Am. J. Hum. Genet.* **68**, 895 (2001).
8. G. Vriend, *J. Mol. Graph* **8**, 52 (1990).
9. E. Krieger, G. Koraimann, G. Vriend, *Proteins* **47**, 393 (2002).
10. X. Du *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 14079 (2000).
11. B. Rost, *Protein Eng.* **12**, 85 (1999).
12. M. K. Kim, Y. K. Kang, *Protein Sci.* **8**, 1492 (1999).
13. D. Nagakubo *et al.*, *Biochem. Biophys. Res. Commun.* **231**, 509 (1997).
14. Y. Hod, S. N. Pentyala, T. C. Whyard, M. R. El-Maghrabi, *J. Cell Biochem.* **72**, 435 (1999).
15. K. Takahashi *et al.*, *J. Biol. Chem.* **276**, 37556 (2001).
16. N. Kotaja, U. Karvonen, O. A. Janne, J. J. Palvimo, *Mol. Cell. Biol.* **22**, 5222 (2002).
17. A. Mitsumoto *et al.*, *Free Radic. Res.* **35**, 301 (2001).
18. A. Mitsumoto, Y. Nakagawa, *Free Radic. Res.* **35**, 885 (2001).
19. H. de Nobel *et al.*, *Yeast* **18**, 1413 (2001).
20. B. I. Giasson, H. Ischiropoulos, V. M.-Y. Lee, J. Q. Trojanowski, *Free Radic. Biol. Med.* **32**, 1264 (2002).
21. M. Hashimoto *et al.*, *J. Biol. Chem.* **277**, 11465 (2002).
22. D.-H. Hyun *et al.*, *J. Biol. Chem.* **277**, 28572 (2002).

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Supporting Online Material

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Materials and Methods
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Supporting Online Material

Mutations in the *DJ-1* gene associated with autosomal recessive early onset parkinsonism, by V. Bonifati et al.

Materials and methods, Supporting text, Figs. S1, S2, S3, References and notes

Materials and methods

Marker development and genotyping

Sequence data from selected BAC or PAC clones were downloaded from GenBank (*1*). Short Tandem Repeat (STR) sequences were selected by repeat length and type using the program FileProcessor (*2*). Single Nucleotide Polymorphisms (SNPs) were downloaded from NCBI (*3*) or they were generated through use of the Celera Discovery System and Celera's associated databases. STR markers were typed by radioactive polymerase chain reaction, essentially according to Weber and May (*4*). SNP markers were typed using a touch-down PCR protocol and digestion with restriction endonucleases. Heterozygosity of markers was determined using a panel of independent chromosomes from the general population. The complete lists of typed STRs and SNPs is available on request.

Genetic analyses

Genomic DNA was isolated from peripheral blood using standard protocols.
For mutation analysis of the *DJ-1* gene, PCR reactions were performed in 25 or 50 µl containing 1x GibcoBRL PCR buffer, 1.5mM $MgCl_2$, 0.01% W-1, 250µM of each dNTP, 0.4µM forward primer, 0.4µM reverse primer, 2.5 units of Taq DNA polymerase (GibcoBRL) and 50 ng genomic DNA/25 µl of PCR volume. For exon 2, the concentration of each primer was 2µM. For exon 1, DMSO 7% was used.
Cycle conditions were: 5 min at 94°C; 35 cycles of 30 s denaturation at 94°C, 30 s annealing at temperatures reported below, and 90 s extension at 72°C; final extension 5 min at 72°C. Primers, annealing temperatures, and size of amplified fragments were as follows:

Exon	Forward Primer 5'→3'	Reverse Primer 5'→3'	annealing T	size (bp)
$1^{A/B}$	GAGGTAGACTCGGCCGGAC	TTCTGGACGCTTCAGCGTTG	70°→60° (-1°/cycle), then 60°	405
2	TAGGAAGTACTTACTCTGCTTG	TATTTATTCTTATGTCATCTCTG	66°→58° (-1°/cycle), then 58°	359
3	CAGCTGTGTAAACGTTACTC	ATTCTGTATCAAGCAATTGCC	65°→56° (-1°/cycle), then 56°	546
4	CTATCTCCTGTACTTCCCAC	ACAGAACATAAGCAGATGCTG	66°→58° (-1°/cycle), then 58°	254
5	TGAGAAATGCCTTGCTTGGG	GCTATTTGGAATCAAACCATCG	70°→60° (-1°/cycle), then 60°	354
6	TTTGCCAGATGTGCTCAGCAAATCG	ACTGCACTCCAGCCTGGGCGATGG	68°, total:35 cycles	495
7	CACATAGCCCATTAGGATGTC	AGCTGCAAATGAAGGTGATAC	66°→58° (-1°/cycle), then 58°	504

Direct sequencing of both strands was performed using Big Dye Terminator ver. 3.0 chemistry (Applied Biosystems). Fragments were loaded on an ABI3100 automated sequencer and analysed

with DNA Sequencing Analysis (ver 3.7) and SeqScape (ver 1.1) packages (Applied Biosystems).

Analysis of the genomic deletion found in the Dutch family.
For sequencing purposes, a 1761 bp DNA fragment containing the deletion breakpoint was amplified using 2µM forward 5'-GCAAATAGCCAAAAGTGAAGTC-3' and 2µM reverse 5'-CGTACAAGACCTGCCAGGG-3' primers. PCR conditions were as above, with annealing temperatures of 70°→60° (-1°/cycle), then 60°, total 35 cycles.
For testing the cosegregation in the Dutch family and the occurrence in population controls, a 2011 bp fragment containing the deletion breakpoint was amplified using 1µM forward 5'-GCAAATAGCCAAAAGTGAAGTC-3' and 1µM reverse 5'-TACTTTCAGCAGAATCATAAGG-3' primers; a 354 bp fragment containing exon 5 of *DJ-1* gene was also amplified in the same PCR reaction using 0.2µM forward 5'-TGAGAAATGCCTTGCTTGGG-3' and 0.2µM reverse 5'-GCTATTTGGAATCAAACCATCG-3' primers. PCR conditions were as above, with annealing temperatures of 70°→60° (-1°/cycle), then 60°, total 35 cycles. Fragments were resolved using agarose 1.5% gel electrophoresis.

For RT-PCR analyses, lymphoblastoid cell lines were established from one patient of each PARK7-linked family and one unrelated normal control. Total mRNA was isolated, and cDNA was prepared according to standard protocols. The following primers (0.4µM each) were used to amplify the *DJ-1* cDNA: forward-I 5'-GGGTGCAGGCTTGTAAACAT-3', reverse-I 5'-TGACTTCCATACTTCCGCAAA-3', forward-II 5'-CGCGTCCGCAGGAAGAGG-3', reverse-II 5'-TAGACACAATTTGTAGGCTGAG-3', forward-III 5'-GCGTGCTGGCGTGCGTTC-3'. The primers were used in the following combinations: forward-I/reverse-I (product size 741 bp); forward-II/reverse-I (763 bp); forward-III/reverse-I (770 bp); forward-I/reverse-II (780 bp); forward-II/reverse-II (802 bp); forward-III/reverse-II (809 bp). In some experiments, a 285 bp fragment of the *KIAA833* cDNA was amplified as internal PCR control, using 0.4µM forward 5'-AGCTGCCGTCGTGCTGTAC-3' and 0.4µM reverse 5'-GGGTTGGTGCTTGCAATAAC-3' primers. PCR conditions were as above, with annealing temperatures of 66°→58° (-1°/cycle), then 58°, total 35 cycles.

For allele specific oligo (ASO) hybridization, PCR products containing *DJ-1* exon 7 were blotted into Hybond-N+ membranes (Amersham Biosciences). The blots were hybridized for 1 hour at 37°C in 5X SSPE, 1% SDS and 0.05 mg/ml single strand salmon sperm DNA with either the normal or mutated sequence oligo. Filters were washed until a final stringency of 0.3 x SSC/0.1% SDS at 37°C. The following oligos were used for hybridization: wild-type allele TTTGCGCTTGCAATT, mutated allele (L166P) TTTGCGCCTGCAATT.

Northern blot analysis was performed according to manufacturers protocols (Clontech).

GenBank accession numbers: *DJ-1* genomic sequence: AL034417. *DJ-1* transcripts: AB073864, AF021819, BC008188, D61380. DJ-1 protein: CAB52550, BAB71782, AAC12806, AAH08188, BAA09603.

Bioinformatics of DJ-1 protein
Multiple sequence alignments were produced with ClustalW (*5*) at the European Bioinformatics Institute (http://www2.ebi.ac.uk/clustalw). The phylogenetic tree of DJ-1 protein family was generated with TreeTop at GeneBee (*6*).

Molecular modeling of DJ-1 protein
Initial alignments for DJ-1 were obtained from the 3D-PSSM fold recognition server (*7*) which identified PDB entry 1G2I as the closest homolog of known structure. The alignment and also the model does not cover the N-terminal three and C-terminal 16 residues of DJ-1 protein. Side chains were built using WHAT IF (*8*). The model was energy minimized with the YASARA NOVA force field which was shown to improve models (*9*). Validation of the model with WHAT_CHECK (*10*) showed a Ramachandran Z-score of -2.46 and all other quality Z-scores about one standard deviation below the average high resolution X-ray structure, which is good for a model based on only 23% sequence identity.

***DJ-1* expression vectors**
Wild-type and mutant (L166P) *DJ-1* cDNAs were cloned in pcDNA3.1/V5-His TOPO TA vector (Invitrogen) and checked by sequencing using the Big Dye Terminator ver. 3.0 chemistry (Applied Biosystems).

Cell culture and transfection
COS cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100µg/ml streptomycin and were kept at 37°C in 5% CO_2 (all from GIBCO BRL). PC12 cells were grown in DMEM supplemented with 10% horse serum (Sigma) and 5% fetal calf serum, 100 units/ml penicillin and 100µg/ml streptomycin and kept at 37°C in 10% CO_2. COS and PC12 cells were transiently transfected with the constructs mentioned above using the Lipofectamine + Reagent (Gibco) according to the manufacturer's recommendations. Transfected cells were fixed for immunofluorescence staining 24 and 48 h after transfection.

Immunofluorescence staining
Cells were rinsed with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde (10 min), permeabilized with methanol (15 min), blocked with PBS/5% bovine serum albumin /0.15% glycine (15 min) and incubated for 1h with monoclonal mouse anti-V5 antibody (Invitrogen), dilution 1:500. FITC-conjugated goat anti-mouse immunoglobulin G (1:500 1h) was used as secondary antibody (Sigma). Cells were mounted in Vectashield media (Vector) containing DAPI and examined with a Zeiss Axioplan 2 microscope equipped with digital camera. Mitochondrial staining was performed by using rhodamine-conjugated MitoTrack (1:2000) or by double-labeling immunofluorescence with rabbit anti-HSP60 antibody (1:200, 1h Stressgen) and TRITC-conjugated goat anti-rabbit immunoglobulin (1:500, 1h).

Supporting text

Clinical studies

The Dutch and Italian PARK7-linked families have been described previously (*11,12*). The diagnosis of parkinsonism required the presence of two of three cardinal signs (resting tremor, bradykinesia, muscular rigidity), absence of atypical features (dementia early in disease course, ophtalmoplegia, pyramidal or cerebellar involvement), and no other identifiable causes. Brain imaging with computed tomography and/or magnetic resonance were unremarkable. Response to levodopa or dopamine agonist therapy was present in all patients except two who were untreated at the time of study. Autopsy data are not available. Functional brain imaging studies performed in patients of the Dutch family using dopamine-transporter tracers and SPECT or PET showed severe abnormalities, indicating presynaptic dysfunction of the nigrostriatal dopamine system, the pattern typically observed in Parkinson's disease (PD) (*13*).

In comparison with classical, late-onset PD, the clinical phenotype in the Dutch and Italian PARK7-linked families is characterized by an early onset (in the thirties) and a slow disease progression. Furthermore, behavioral and psychic disturbances, and dystonic features (including blepharospasm) have been reported in both families (*11,12*). However, more detailed genotype-phenotype correlation studies are needed to accurately define the clinical spectrum associated to mutations in *DJ-1* gene.

Fine mapping studies

The PARK7 locus as delineated by initial linkage studies (*11,12*) spans more than 8 Mb in the current physical map of the Human Genome (NCBI, build 29), including 9 contigs separated by 8 gaps, containing a large number of genes.

In the last four NCBI builds of the human genome, released during the course of this study, the order of several contigs inside and in the proximity of the PARK7 region has fluctuated considerably, and for some contigs the internal assembly remains provisional.

In order to overcome these difficulties, we have used extensive homozygosity mapping by typing available STR or SNP markers in the two definitely linked families to characterize each of the contigs located inside and flanking the PARK7 genetic borders. For contigs where STRs or SNPs were not available or not informative we developed new polymorphic STR markers by scanning clones of interest for repetitive sequences.

Analysis of candidate genes

The refinement of PARK7 critical interval allowed us to exclude many genes located in contigs showing heterozygous markers, including obvious candidates: *UBE4B* and *Ubc6p*, encoding enzymes involved in ubiquitination; *ISG15*, encoding an ubiquitin-like protein; *FLJ10782 (PANK4)*, encoding a homolog of the gene mutated in pantothenate kinase-associated neurodegeneration, an autosomal recessive neurodegenerative disease, and *FRAP1*, encoding an immunophilin ligand.

Obvious candidate genes located in contigs showing homozygous markers were analysed by direct sequencing from genomic DNA (primers and PCR conditions are available on request). We detected no mutations in the coding region of the following genes: *WDR8*, encoding a protein containing WD40 repeats, a domain also found in ubiquitin ligase protein complexes; *VAMP3*, encoding a vesicle-associated protein, possibly involved in docking or fusion of synaptic vesicles; *FLJ10737*, encoding a protein with a DnaJ domain, also present in molecular chaperones;

KCNAB2, encoding a subunit of the potassium voltage-gated channel, shaker-related subfamily.

Strategy for cloning the deletion breakpoint

In order to identify the deletion breakpoint, we designed primer sets to amplify fragments of DNA evenly distributed between the minimal deleted area and the two flanking areas of non-deleted sequence. Iteration of this technique reduced the interval between the deleted region and the flanking, non-deleted borders to less than 3 kb. We then used primers placed on the non-deleted borders to amplify a fragment of about 2000 bp across the deletion breakpoint. In the normal chromosomes, these two primers are separated by about 16 kb.

Replacing the reverse primer with another primer located 250 bases upstream yielded a novel fragment of about 1750 bp in the same individuals, confirming the specificity of the reaction and the presence of the deletion (data not shown).

Cloning of the deletion breakpoint allowed us to test for the presence of the mutation in both heterozygous or homozygous state using PCR.

Relationships between PARK6 and PARK7

A different locus for autosomal recessive early onset parkinsonism (PARK6) has been localized 25 cM centromeric of PARK7 on chromosome 1p35-p36, in a single large pedigree (*14*). Given the similarities between PARK6- and PARK7-associated phenotypes, and the proximity of the two loci, it is possible that mutations in similar or functionally-related genes underlie both forms. As an alternative possibility, a large genomic rearrangement could account for both linkage reports.

Linkage analysis of autosomal recessive parkinsonism is complicated by the small size of most recessive pedigrees, presence of further locus heterogeneity, proximity of the two loci on chr.1p. As a result, the assigment of additional smaller families to either PARK6 or PARK7 must be considered as provisional (*12,15*).

Mutation analyses of *DJ-1* gene in smaller families and isolated early onset cases

Nine additional families with a diagnosis of clinically definite idiopathic parkinsonism in at least two siblings, possible autosomal recessive inheritance (affected sibs, unaffected parents), disease onset before age 50 in at least one sibling, exclusion of linkage to PARK2 locus, or *parkin* gene mutations excluded by sequencing and exon dosage, were available for analysis.

By haplotype analysis, four of these families were compatible with linkage to PARK7; three families to both PARK7 and PARK6; and two families to PARK6 only (*16*). None of the above-mentioned families could be definitely linked to either of the two loci.

Furthermore, twenty-two patients with idiopathic parkinsonism, negative family history and onset below age 40 were also studied. In nine cases mutations in *parkin* gene had been excluded by genomic sequencing and exon dosage.

Sequencing the exons and exon-intron boundaries of the *DJ-1* gene in the patients and families described above revealed no pathogenic mutations. Among the same patients and families, there were no carriers of the *DJ-1* deletion identified in the Dutch family. However, detection of compound heterozygosity for different genomic rearrangements requires gene dosage analysis which is currently being developed and therefore will be the subject of future studies.

Fig.S1 Mutation analyses

A. Cosegregation of the genomic deletion in the Dutch family. Duplex PCR from genomic DNA using primers designed to amplify the deletion breakpoint (2011 bp band) and one of the deleted *DJ-1* exons (exon 5 - 354 bp band). Exon 5 is absent from all patients (hom deletion, lanes 2,3,6,8); the deletion breakpoint band is present in homozygous (lanes 2,3,6,8) and heterozygous (lanes 4,5,7,9) carriers of the mutant allele (note the dosage effect). Individual 1 is not carrying the disease haplotype, in agreement with data in ref.*11*.
B. Cosegregation of the L166P mutation in the Italian family. Allele specific oligo hybridization: mut - mutant; wt - wild-type; C - unrelated normal control.
C. Sequence of the deletion breakpoint in the Dutch family showing the joining of the telomeric and centromeric borders. The breakpoint lies within a 16 bp of sequence identical in the Alus and is depicted in a frame. The specific nucleotides in telomeric and centromeric sequences are indicated by upper lines.
D. Electropherograms showing the L166P mutation in the Italian family. Sequencing from genomic DNA and cDNA.
E. Scheme of the genomic rearrangement in the Dutch family.
F. RT-PCR amplification of a 741 bp fragment corresponding to the *DJ-1* cDNA indicates absence of expression in the Dutch patient, and presence of expression in the Italian patient. To exclude the possibility of a polymorphism in one of the primer binding sites this was replicated using six different primer combinations (data not shown). A 285 bp fragment of the *KIAA0833* cDNA is amplified as internal PCR control in the left lanes.

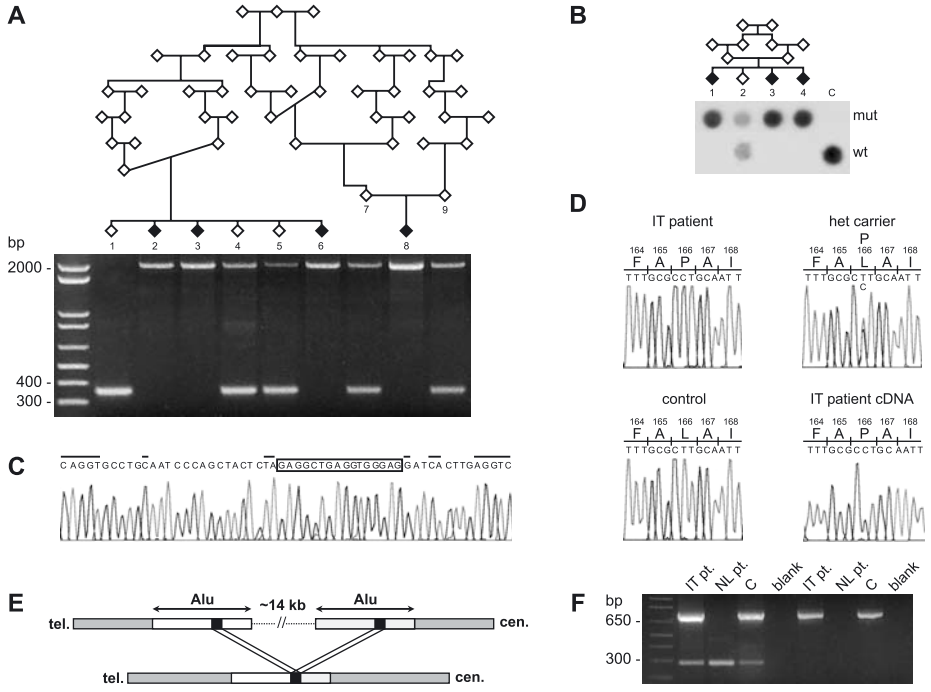


Fig.S2 Northern blot – different human tissues and brain regions
Upper panels: *DJ-1* probe. Lower panels: control probe (*β-actin*). *DJ-1* expression is higher in subcortical regions (caudate nucleus, thalamus, s.nigra) than in cortical regions.

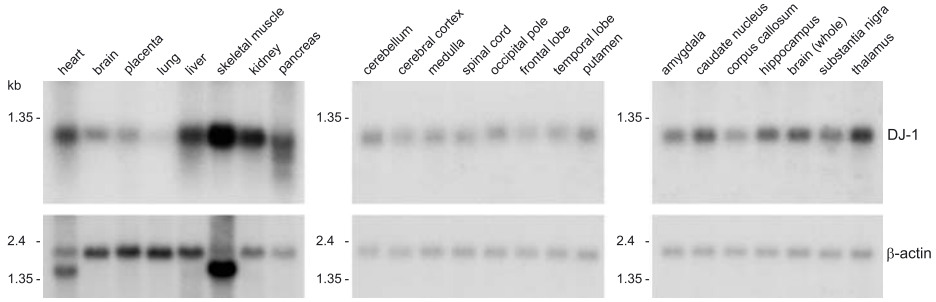
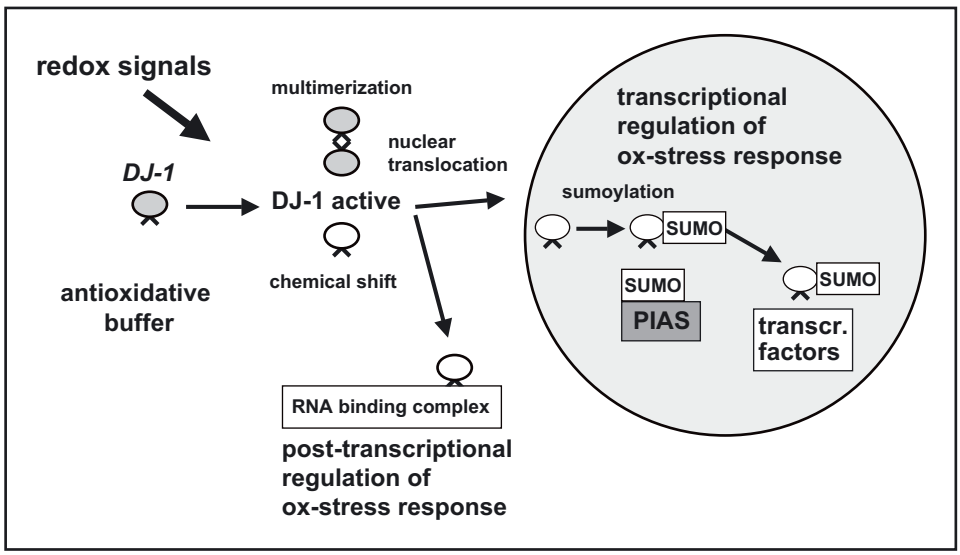


Fig.S3 Model of putative DJ-1 function
Modification of the DJ-1 protein (by chemical or multimerization changes) may directly buffer cytosolic redox fluctuations; activated DJ-1 may influence expression of genes for oxidative stress response post-transcriptionally (binding to RBP complexes); DJ-1 may also translocate to the nucleus, be sumoylated, and influence gene transcription.



8

References and notes

1. http://www.ncbi.nih.gov/Genbank.

2. J.W. van Dongen, 2001. The File-Processor program is available at http://www.eur.nl/fgg/kgen; choose web link: "Databases and Sequence Analysis".

3. http://www.ncbi.nlm.nih.gov/SNP.

4. J. L. Weber, P. E. May, *Am J Hum Genet* **44**, 388 (1989).

5. J. D. Thompson, D. G. Higgins, T. J. Gibson, *Nucleic Acids Res* **22**, 4673 (1994).

6. L. I. Brodskii *et al.*, *Biokhimiia* **60**, 1221 (1995).

7. L. A. Kelley, R. M. MacCallum, M. J. Sternberg, *J Mol Biol* **299**, 499 (2000).

8. G. Vriend, *J Mol Graph* **8**, 52 (1990).

9. E. Krieger, G. Koraimann, G. Vriend, *Proteins* **47**, 393 (2002).

10. R. W. Hooft, G. Vriend, C. Sander, E. E. Abola, *Nature* **381**, 272 (1996).

11. C. M. van Duijn *et al.*, *Am J Hum Genet* **69**, 629 (2001).

12. V. Bonifati *et al.*, *Ann Neurol* **51**, 253 (2002).

13. M. Dekker et al., unpublished data.

14. E. M. Valente et al., *Am J Hum Genet* **68**, 895 (2001).

15. E. M. Valente et al., *Ann Neurol* **51**, 14 (2002).

16. V. Bonifati et al., unpublished data.

Chapter 2.9

Linking DJ-1 to neurodegeneration

The discovery of *DJ-1* mutations in autosomal recessive, early-onset PD establishes that the loss of the DJ-1 function leads to neurodegeneration (chapter 2.8). The exact role of this protein remains unknown but several pieces of information are available in the literature which provide clues to a possible function. Here, the current knowledge about DJ-1 is reviewed, and hypotheses linking DJ-1 to neurodegeneration are formulated. The current evidence suggests an involvement of DJ-1 in processes as different as cell cycle regulation and oncogenesis, sperm maturation and fertilization, control of gene transcription, regulation of mRNA stability, and response to cell stress.

A human cDNA termed *DJ-1* was first cloned in 1997, as a result of a yeast two-hybrid screen for human proteins interacting with C-MYC, the protein encoded by the oncogene *c-myc*. The DJ-1 protein was reported to have oncogenic properties in cooperation with the oncogene ras.[1] The biological relevance of this finding is questioned by the fact that the DJ-1 protein did not bind C-MYC, and the interaction with C-MYC was actually due to translation of a sequence upstream of the *DJ-1* open reading frame in the construct used in the two-hybrid system.[1] Nevertheless, recent proteomic-based studies found increased expression of the DJ-1 protein in various human tumours, once again suggesting an involvement of DJ-1 in oncogenesis.[2-4]

In 1999 a human protein named RS that was identical to DJ-1 was identified as the regulatory (inhibitory) subunit of a 400 kD RNA-binding protein (RBP) complex.[5] DJ-1 immunoreactivity was shown in the cytoplasm, in association with microtubules, and the nucleus, depending on the cell-cycle phase, or the cell-types studied. The authors suggested that DJ-1 might be a multifunctional protein with important role(s), including cytoskeleton-coupled RNA sorting, RNA degradation, and functions in the nucleus as well. Interestingly, in the last study[5] DJ-1 was co-purified with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Whether GAPDH is a genuine DJ-1 interactor remains to be explored. However, this is a first, potentially important link between DJ-1 and neurodegeneration. GAPDH is increasingly recognized as a multifunctional protein, which is not only involved in glycolysis, but also in other processes.[6] Notably, by translocating from the cytoplasm to the nucleus, GAPDH mediates the induction of a neuronal apoptotic pathway.[7, 8] Evidence also links GAPDH to the pathogenesis of classical PD, as nuclear translocation of GAPDH was detected in nigral neurons in post-mortem PD brains, and GAPDH co-localised with α-synuclein in Lewy bodies.[9] In the light of these evidences, the possible interaction between DJ-1 and GAPDH warrants further investigation. Interestingly, the yeast genes encoding DJ-1 and GAPDH homologues are induced during cell stress, together with chaperones, antioxidants and other stress-response genes.[10]

Furthermore, two independent groups identified a rat protein called CAP1 (Contraception Associated Protein 1) or SP22 (Sperm Protein 22), putatively involved in spermatogenesis and fertilization, as the rat DJ-1 homolog.[11, 12] Whether DJ-1 is also involved in these processes in humans remains unclear. However, DJ-1 is present in human sperm, mainly as a flagellum protein, whereas in rats the homologue CAP1 or SP22 is also abundant in sperm heads.[13] In the tail DJ-1 co-localises with β-tubulin, a major axoneme component. An infertile patient with morphological and functional abnormalities, and undetectable levels of DJ-1 in sperm has been reported.[13] It would

be interesting to search whether this individual carries *DJ-1* mutations and will develop a neurological phenotype.
DJ-1 and *parkin* are highly expressed in the testis, in addition to the brain, and loss of function of both DJ-1 and parkin proteins are linked to male infertility in animal species.[11, 12, 14] This could be coincidental, but it could also reflect the involvement in common pathways that could be important in the brain as well. The issue of fertility has not been studied in male patients with parkinsonism due to mutations in these genes, and this should also be actively explored in the future.

In a yeast two-hybrid system, PIASxα/ARIP3 was detected as a DJ-1 binding protein.[15] PIASxα/ARIP3 is expressed predominantly in the testis, and is a modulator of the androgen receptor (AR) transcriptional activity.[16] In cell cultures, the effects of PIASxα/ARIP3 on the AR activity depend from the cell lines and the reporter genes used.[17] However, in most cell lines, including Sertoli cells, PIASxα/ARIP3 is a negative modulator of the AR transcriptional activity.[15] DJ-1 might therefore positively regulate the AR-mediated transcriptional activity by recruiting PIASxα and thereby removing its inhibitory activity.[15]
PIASxα/ARIP3 belongs to a family of proteins (PIAS, Protein Inhibitor of Activated STAT) that modulate the activity of transcription factors by functioning as SUMO-1 (Small Ubiquitin-like MOdifier) ligases.[18] Interestingly, a DJ-1 mutant at Lys130 (K130R) was unable to regulate the AR activity.[15] As the Lysine 130 of DJ-1 seems to be sumoylated,[15] it is possible that this modification is necessary for the full activity of DJ-1.
More recently another DJ-1-binding protein, DJBP, was identified in a yeast two hybrid screen.[19] DJBP is also an AR-binding protein, which is specifically expressed in the testis, and appears to inhibit AR activity by recruiting a histone deacetylase (HDAC) complex.[19] The binding of DJ-1 to the DJBP-AR complex abrogates the HDAC-DJBP interaction, resulting in the enhancement of the AR activity. DJ-1 appears therefore to positively regulate AR by antagonizing the inhibitory effects of PIASxα and DJBP.[15, 19]
DJBP is expressed exclusively,[19] and PIASxα/ARIP3 predominantly in the testis,[16] and therefore, these proteins appear not relevant for the neuronal function of DJ-1. However, other members of the PIAS family, PIAS3 and PIASy, were also shown to bind DJ-1.[15] These interactions remain to be characterized and could be more relevant for the effects of DJ-1 on gene expression in neurons.
Recent observations suggest that sumoylation plays an important role in brain function and neurodegeneration.[20, 21] Most of the known substrates for sumoylation are nuclear proteins, and sumoylation might influence protein function by changing the substrate localization, by competing with ubiquitylation, thereby inhibiting substrate degradation, and lastly, by directly modulating the functional properties of the substrate.[22]
Sumoylation regulates the activity not only of the steroid receptor superfamily, but also of transcription factors mediating the heat-shock response, like HSF1 and HSF2.[22] Another member of the steroid receptor super-family of transcription factors, NR4A2,[23, 24] is mutated in familial forms of PD.[25] It will be interesting to see if these transcription factors are also targeted by DJ-1 either directly or through interaction with PIAS. Future studies will reveal the real targets of DJ-1 activity in the nucleus, but perhaps an important contribution of the discovery of *DJ-1* mutations in PARK7 is the focusing to the nuclear mechanisms and the control of gene expression in PD pathogenesis.

Sumoylation can also influence synaptic function via regulation of calcium/calmodulin-dependent kinase II (CaMKII),[20] suggesting yet another possible link between DJ-1 and neuronal function. There is evidence that sumoylated proteins are increased in the

brain of patients with polyglutamine diseases,[26] and altered sumoylation increases neurodegeneration in Drosophila models of polyglutamine disease.[21] Whether these findings are relevant for other neurodegenerative diseases is unknown. However, by linking sumoylation to the pathogenesis of PD, DJ-1 might extend the involvement of this ubiquitin-like protein-conjugation system in the neurodegenerative diseases characterized by protein aggregation in the cytoplasm.

Recent studies showed that human DJ-1 is converted into a variant having a more acidic pI in response to exogenous oxidative stress (including the herbicide paraquat or hydrogen peroxide) or endogenous reactive oxygen species, suggesting a role for DJ-1 as an antioxidant, or a sensor of oxidative stress.[27, 28] The oxidative conversion of a sulphydryl group at a cysteine residue to a cysteine sulfinic acid is proposed as the most plausible explanation of the observed variation of DJ-1 pI in response to oxidative stress.
Furthermore, the transcription of the yeast *DJ-1* homologue *YDR533C* is up-regulated together with many chaperones and antioxidants during cell stress paradigms, notably the stress induced by weak acids,[10] external pH changes,[29] or by protein misfolding,[30] which in turn are associated with oxidative stress, raising the question of whether DJ-1 also plays a role as a molecular chaperone. Another, recently identified member of the DJ-1-ThiJ-PfpI superfamily, the *E.coli* EcHsp31, is a stress-inducible chaperone.[31]

On the basis of the available evidence, we have proposed that DJ-1 might be involved in the cellular response to stress at multiple levels (chapter 2.8):

- First, it might directly react to stress signals (e.g. redox changes, misfolded proteins) by a chemical shift and/or change in multimerization state.
- Second, DJ-1 might modulate the gene-expression of the stress-response at post-transcriptional level, by the known interaction with RNA-binding protein complexes. Interestingly, the post-transcriptional regulation of gene expression is important for both neuronal function and spermatogenesis.
- Third, DJ-1 might translocate to the nucleus in response to stress signals. In the nucleus, it might interact with PIAS or other co-factors, and modulate the gene expression at the transcriptional level.

Although an involvement of human DJ-1 in the oxidative stress response, or in the response to misfolded protein stress remains to be proven and the exact function of DJ-1 remains unknown, the proposed model is intriguing in the light of the evidence of oxidative stress and protein misfolding documented in the brains of patients with PD.[32, 33]
Recent studies have shown that mutations in α-*synuclein* and *parkin*, two other PD-related genes, might also be linked to oxidative stress.[34, 35] A redox regulation of sperm maturation and motility has been proposed,[36, 37] and such a mechanism of "physiologically controlled oxidative stress" could explain why DJ-1 is involved in the spermatogenesis and fertilization.

One can speculate that DJ-1 signals in a specific pathway regulated by redox state. Reactive oxygen species can also be viewed as regulators of cellular functions by the effect that redox status has on key proteins like JNK and MAPK kinases, and transcription factors, which are activated in response to redox changes. For instance, the c-Jun NH_2-terminal kinases (JNKs) are activated in response to redox and other stressors, and they phosphorylate the transcription factor c-Jun, increasing its activity.[38] The JNK signalling pathway plays an important role in the neuronal apoptotic response, dopamine- and

excitotoxicity-induced apoptosis, and MPTP-model of PD.[39, 40] α-Synuclein might confer neuroprotection from oxidative stress by inactivating JNK.[35] Being targeted by PIAS,[18] c-Jun is also possibly regulated by DJ-1.

It is therefore clear that much work is still ahead to clarify the biology of DJ-1 and the mechanisms of DJ-1-related neurodegeneration. Clarifying these mechanisms might potentially shed light on novel pathways of neuronal survival and promote the understanding of pathogenesis of common forms of PD. In the current scenario, the possible involvement of DJ-1 as an antioxidant and/or a molecular chaperone appears particularly intriguing.

REFERENCES

1. Nagakubo D, Taira T, Kitaura H, et al. DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. Biochem Biophys Res Commun 1997; 231:509-13.
2. Le Naour F, Misek DE, Krause MC, et al. Proteomics-based identification of RS/DJ-1 as a novel circulating tumor antigen in breast cancer. Clin Cancer Res 2001; 7:3328-35.
3. Bergman AC, Benjamin T, Alaiya A, et al. Identification of gel-separated tumor marker proteins by mass spectrometry. Electrophoresis 2000; 21:679-86.
4. Srisomsap C, Subhasitanont P, Otto A, et al. Detection of cathepsin B up-regulation in neoplastic thyroid tissues by proteomic analysis. Proteomics 2002; 2:706-12.
5. Hod Y, Pentyala SN, Whyard TC, El-Maghrabi MR. Identification and characterization of a novel protein that regulates RNA-protein interaction. J Cell Biochem 1999; 72:435-44.
6. Mazzola JL, Sirover MA. Alteration of intracellular structure and function of glyceraldehyde-3-phosphate dehydrogenase: a common phenotype of neurodegenerative disorders? Neurotoxicology 2002; 23:603-9.
7. Ishitani R, Tanaka M, Sunaga K, Katsube N, Chuang DM. Nuclear localization of overexpressed glyceraldehyde-3-phosphate dehydrogenase in cultured cerebellar neurons undergoing apoptosis. Mol Pharmacol 1998; 53:701-7.
8. Fukuhara Y, Takeshima T, Kashiwaya Y, Shimoda K, Ishitani R, Nakashima K. GAPDH knockdown rescues mesencephalic dopaminergic neurons from MPP+ -induced apoptosis. Neuroreport 2001; 12:2049-52.
9. Tatton NA. Increased caspase 3 and Bax immunoreactivity accompany nuclear GAPDH translocation and neuronal apoptosis in Parkinson's disease. Exp Neurol 2000; 166:29-43.
10. de Nobel H, Lawrie L, Brul S, et al. Parallel and comparative analysis of the proteome and transcriptome of sorbic acid-stressed Saccharomyces cerevisiae. Yeast 2001; 18: 1413-28.
11. Welch JE, Barbee RR, Roberts NL, Suarez JD, Klinefelter GR. SP22: a novel fertility protein from a highly conserved gene family. J Androl 1998; 19:385-93.
12. Wagenfeld A, Gromoll J, Cooper TG. Molecular cloning and expression of rat contraception associated protein 1 (CAP1), a protein putatively involved in fertilization. Biochem Biophys Res Commun 1998; 251:545-9.
13. Whyard TC, Cheung W, Sheynkin Y, Waltzer WC, Hod Y. Identification of RS as a flagellar and head sperm protein. Mol Reprod Dev 2000; 55:189-96.
14. Greene JC, Whitworth AJ, Kuo I, Andrews LA, Feany MB, Pallanck LJ. Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. Proc Natl Acad Sci U S A 2003; 100:4078-83.
15. Takahashi K, Taira T, Niki T, Seino C, Iguchi-Ariga SM, Ariga H. DJ-1 positively regulates the androgen receptor by impairing the binding of PIASx alpha to the receptor. J Biol Chem 2001; 276:37556-63.
16. Moilanen AM, Karvonen U, Poukka H, et al. A testis-specific androgen receptor coregulator that belongs to a novel family of nuclear proteins. J Biol Chem 1999; 274: 3700-4.

17. Kotaja N, Aittomaki S, Silvennoinen O, Palvimo JJ, Janne OA. ARIP3 (androgen receptor-interacting protein 3) and other PIAS (protein inhibitor of activated STAT) proteins differ in their ability to modulate steroid receptor-dependent transcriptional activation. Mol Endocrinol 2000; 14:1986-2000.
18. Kotaja N, Karvonen U, Janne OA, Palvimo JJ. PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. Mol Cell Biol 2002; 22:5222-34.
19. Niki T, Takahashi-Niki K, Taira T, Iguchi-Ariga SM, Ariga H. DJBP: A Novel DJ-1-Binding Protein, Negatively Regulates the Androgen Receptor by Recruiting Histone Deacetylase Complex, and DJ-1 Antagonizes This Inhibition by Abrogation of This Complex. Mol Cancer Res 2003; 1:247-61.
20. Long X, Griffith LC. Identification and characterization of a SUMO-1 conjugation system that modifies neuronal calcium/calmodulin-dependent protein kinase II in Drosophila melanogaster. J Biol Chem 2000; 275:40765-76.
21. Chan HY, Warrick JM, Andriola I, Merry D, Bonini NM. Genetic modulation of polyglutamine toxicity by protein conjugation pathways in Drosophila. Hum Mol Genet 2002; 11:2895-904.
22. Kim KI, Baek SH, Chung CH. Versatile protein tag, SUMO: its enzymology and biological function. J Cell Physiol 2002; 191:257-68.
23. Zetterstrom RH, Williams R, Perlmann T, Olson L. Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. Brain Res Mol Brain Res 1996; 41:111-20.
24. Saucedo-Cardenas O, Kardon R, Ediger TR, Lydon JP, Conneely OM. Cloning and structural organization of the gene encoding the murine nuclear receptor transcription factor, NURR1. Gene 1997; 187:135-9.
25. Le WD, Xu P, Jankovic J, et al. Mutations in NR4A2 associated with familial Parkinson disease. Nat Genet 2003; 33:85-9.
26. Ueda H, Goto J, Hashida H, et al. Enhanced SUMOylation in polyglutamine diseases. Biochem Biophys Res Commun 2002; 293:307-13.
27. Mitsumoto A, Nakagawa Y. DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic Res 2001; 35:885-93.
28. Mitsumoto A, Nakagawa Y, Takeuchi A, Okawa K, Iwamatsu A, Takanezawa Y. Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. Free Radic Res 2001; 35:301-10.
29. Kapteyn JC, ter Riet B, Vink E, et al. Low external pH induces HOG1-dependent changes in the organization of the Saccharomyces cerevisiae cell wall. Mol Microbiol 2001; 39: 469-79.
30. Trotter EW, Kao CM, Berenfeld L, Botstein D, Petsko GA, Gray JV. Misfolded proteins are competent to mediate a subset of the responses to heat shock in Saccharomyces cerevisiae. J Biol Chem 2002; 277:44817-25.
31. Quigley PM, Korotkov K, Baneyx F, Hol WG. The 1.6-A crystal structure of the class of chaperones represented by Escherichia coli Hsp31 reveals a putative catalytic triad. Proc Natl Acad Sci U S A 2003; 100:3137-42.
32. Giasson BI, Ischiropoulos H, Lee VM, Trojanowski JQ. The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. Free Radic Biol Med 2002; 32:1264-75.
33. Zoghbi HY, Botas J. Mouse and fly models of neurodegeneration. Trends Genet 2002; 18: 463-71.
34. Hyun DH, Lee M, Hattori N, et al. Effect of wild-type or mutant Parkin on oxidative damage, nitric oxide, antioxidant defenses, and the proteasome. J Biol Chem 2002; 277: 28572-7.
35. Hashimoto M, Hsu LJ, Rockenstein E, Takenouchi T, Mallory M, Masliah E. alpha-Synuclein protects against oxidative stress via inactivation of the c-Jun N-terminal kinase stress-signaling pathway in neuronal cells. J Biol Chem 2002; 277:11465-72.
36. Maiorino M, Ursini F. Oxidative stress, spermatogenesis and fertility. Biol Chem 2002; 383: 591-7.
37. Aitken RJ. Possible redox regulation of sperm motility activation. J Androl 2000; 21:491-6.

38. Weston CR, Davis RJ. The JNK signal transduction pathway. Curr Opin Genet Dev 2002; 12:14-21.
39. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000; 103:239-52.
40. Xia XG, Harding T, Weller M, Bieneman A, Uney JB, Schulz JB. Gene transfer of the JNK interacting protein-1 protects dopaminergic neurons in the MPTP model of Parkinson's disease. Proc Natl Acad Sci U S A 2001; 98:10433-8.

3 General discussion

General discussion

The recent discovery of genes causing rare inherited forms of PD has provided tremendous help for the understanding of the molecular mechanisms of this devastating disease. Furthermore, the genetic dissection of monogenic forms has important implications for the nosology of PD, and for the diagnostic work-up of early-onset patients.

Is Parkinson's disease a single entity?

The classical concept of Parkinson's disease (PD) is based on a clinical-pathological triad, namely:

- presence of parkinsonism (a combination of akinesia, resting tremor, and muscular rigidity),
- good response to dopaminergic therapy,
- presence of neuronal loss and gliosis, mainly in the *substantia nigra pars compacta* but also in other brain areas, with formation of cytoplasmic inclusions called Lewy bodies (LB) and Lewy neurites in the surviving neurons.[1-4]

This definition reflects the common clinical and pathological findings, but it also highlights the current lack of biological markers, and none of the triad components is specific for PD.[1, 2] Parkinsonism can be the clinical correlate of lesions or dysfunctions at various levels in the basal ganglia, including the *substantia nigra* and striatum, which can be caused by neurodegeneration, inflammation, drugs, toxins, tumors, and vascular lesions. Response to l-dopa or synthetic dopamine agonists is characteristic, but again, non-specific of PD, resulting from the loss of nigral dopaminergic neurons in presence of spared post-synaptic striatal dopamine receptors. Last, Lewy bodies are observed in conditions as different as Alzheimer's disease (AD),[5] Lewy body dementia,[6] progressive supranuclear palsy,[7] neurodegeneration with iron accumulation-type 1 (formerly Hallervorden-Spatz disease),[8] and importantly, in a minority of brains from elderly individuals died without neurological disease ("incidental" Lewy bodies).[9]

According to these criteria, PD corresponds to the overlap of two spectra: that of l-dopa-responsive parkinsonian syndromes, and that of Lewy-body-associated conditions. This concept of PD has survived for long, not only because of the lack of a better definition, but also for its practical implications for the clinical work-up and treatment of patients.

However, the lack of a definition in terms of molecular mechanisms, and the lack of biomarkers have been major factors hampering the research for cause(s) and mechanism(s) of PD, and other common neurodegenerative diseases. The problematic disease definition also translates in problematic disease boundaries and debated relationships between PD and other entities like AD. Clinical and pathological overlaps are documented between PD and AD,[10] and common genetic susceptibility factors might also exist,[11] supporting the contention that these seemingly different diseases are closer at the molecular level.

The discovery of mutations in different genes causing rare Mendelian forms of PD has further highlighted the need of a new nosology of the disease. Some of these forms (like PARK1 and PARK3) are associated with Lewy body pathology but the resulting phenotype includes typical PD but also additional motor signs, autonomic disturbances, and dementia in few patients.[12, 13] On the contrary, different monogenic forms (like PARK2 and PARK8) are not associated with Lewy body pathology in most cases,[14, 15] yet they can produce clinically typical PD phenotypes.

What is clear today is that PD, as defined by the classical clinical-pathological criteria,

is etiologically heterogeneous. This leads to two possibilities. A first, "splitting" view is that PD includes many distinct diseases with distinct molecular mechanisms, but similar clinical and/or pathological endpoints. A second, "lumping"view, which in my opinion is more convincing, is that there are few common pathways underlying PD, which can be initiated by several etiologies including Mendelian mutations in rare cases, or combinations of small genetic as well as non-genetic effects in the common forms.

The place of parkin in the context of Parkinson's disease

The discovery of *parkin* mutations in PARK2 provided a second landmark contribution to the understanding of PD, after *α-synuclein*. Soon after the cloning of the *parkin* gene in 1998,[16] other groups confirmed the presence of mutations in PD families.[17-19] However, the frequency of involvement of *parkin* in early onset PD and the mutational spectrum were not fully appreciated until the study described in chapter 2.1, which explored for the first time this gene in a large dataset of families and isolated early-onset cases, including genomic sequencing and gene copy dosage. The results revealed mutations in this gene in about half of the families compatible with recessive inheritance and PD onset before age of 45, and in about 20% of the isolated cases with early onset. Another important finding of the study is that only half of the mutations detected in *parkin* are point mutations and small deletions or insertions, whereas the remaining half consists of a variety of large genomic rearrangements, such as deletions and, less often, multiplications of whole exons. These mutations are present in the patients in any possible combination, and therefore, for a sensitive screening gene copy analysis is needed in addition to gene sequencing. Subsequent studies of large datasets confirmed the frequent involvement of *parkin* in early-onset PD, and the importance of gene dosage assays.[20-23] A caveat is that all of these studies were hospital-based, and the frequency of the *parkin* mutations might be lower in population-based series of PD cases.[24] Nevertheless, a *parkin* mutation is a major cause of early-onset PD, which in turn represents 5-10% of all PD cases.[25]

The involvement of the *parkin* gene in late-onset PD has been explored to a lesser extent, as patients with early onset and/or recessive inheritance were pre-selected in most of the mutational screens performed so far (chapter 2.1).[20, 21] Recently, a large study went beyond because it included PD sib pairs supporting linkage to the PARK2 locus, irrespective of the onset age, and used gene dosage in addition to sequencing. *Parkin* mutations were found in 62% of the selected patients with onset before age 50, but also in 11% of those with onset after that age.[22] In our experience, mutations are found in 28% of recessive families with onset between 46 and 55 years (chapter 2.4). These data clearly implicate the *parkin* gene in a substantial fraction of late-onset families, underlining the importance of gene testing in late-onset PD with family history compatible with recessive inheritance.

A few studies provide evidence that parkinsonism due to a *parkin* mutation might be dominantly inherited in a minority of cases.[26-28] However, the possibility of pseudo-dominant inheritance must be considered as an alternative explanation of the disease in multigeneration families. If the frequency of recessive mutations in a given gene is high in the population, the mating of a homozygous (or a compound heterozygous) and a heterozygous carrier might lead to appearance of the recessive disease in subsequent generations, mimicking a dominant pattern of transmission. This was demonstrated in at least four different studies in Japanese and Italian families (chapters 2.2, 2.3).[29, 30] The occurrence of pseudo-dominant inheritance suggests that *parkin* gene mutations might be frequent in some populations.

However, apart from these pseudo-dominant families, the issue of whether single heterozygous *parkin* mutations can lead to disease, and particularly can increase the risk to develop late–onset forms of PD remains unclear. PET studies showing that heterozygous carriers have mild nigrostriatal dysfunctions, suggest that *parkin* mutations might be co-dominant at least at a subclinical level.[31, 32] In comprehensive mutation screenings, several cases (up to ~50%) remain where only a single mutation has been found, and the onset age appears later in these cases.[20-22, 24, 28, 33] In a recent series, most cases (42/50) with onset after 50 years had only one *parkin* mutation.[22]

Taken as a whole, these studies suggest that a single heterozygous mutation in *parkin* might be sometimes sufficient to cause PD, perhaps in combination with other genetic and non-genetic factors. Mechanisms to explain the disease in these cases might involve haploinsufficiency of the ubiquitin-ligase activity, or dominant negative effects.

However, other evidence militates against this view: first, some studies (even very recent) did not include gene dosage analysis,[23] and therefore might have missed many heterozygous exon rearrangements. Secondly, some of the variants identified in heterozygous form might be rare, neutral polymorphisms. Even the well-known Ser167Asn polymorphism (1% frequent in Caucasians)[19] has been considered a pathogenic mutation in a recent study.[22] Thirdly, in our experience only one of 34 heterozygous relatives of patients with two mutations developed PD (notably of late-onset)(chapter 2.4). Although these 34 heterozygous carriers were examined at an age older than the age of disease onset in their affected relatives, some of them might develop the disease later in life. However, these results suggest that the mutations concerned were strictly recessive. This does not exclude the possibility that other mutations are dominant. Fourth, in another study, among nine early-onset cases remaining with one heterozygous mutation found after sequencing of coding region and of the promoter, and after testing gene copy dosage,[34] all carried mutations which were observed in other families in homozygous form, or in compound heterozygosity with a second mutation. This is somehow confirmed by other studies, which included testing gene copy dosage and sequencing, and found that missense and truncating mutations were similarly represented among carriers of one or two mutations.[21, 22]

All these evidences suggest that most *parkin* mutations are recessive, but in some cases the second mutation still escapes detection by current methods. In this regard, it is interesting that the –258 T/G polymorphism in the *parkin* promoter, associated with reduced expression in *in vitro* assays,[35] is much overrepresented in cases with single mutations, and might also be pathogenic (chapter 2.4).[35] Mutations in the large introns, large inversions, or large deletions in the promoter can also be envisaged, and the possibility that a second heterozygous mutation is present in a different gene encoding a protein involved in the same pathway, cannot be excluded. In this regard, it will be interesting to screen the *DJ-1* gene (as well as other genes to be identified) in cases where only one *parkin* mutation has been found. Perhaps, the definitive answer to the question of whether single *parkin* mutations cause PD will come when (and if) linkage to the *parkin* locus will be found in families with dominantly inherited PD, assuming a dominant model of inheritance in the linkage analysis. This has not happened so far, but preliminary results suggest that it might indeed be the case.[22]

Last, whether the –258 T/G polymorphism in the *parkin* promoter, or heterozygous missense mutations in exon 7, encoding the first RING domain, are risk factors for late onset PD, as it has been proposed,[23, 35] remains to be seen. A recent study performed in a large dataset found no evidence of association between known *parkin* polymorphisms (including –258 T/G) and susceptibility to classical, late-onset PD.[36] However, in order to

fully explore the role of *parkin* in late-onset PD we probably need to understand better how this gene is regulated.

Implications of *parkin* for the clinical work-up of early-onset PD

In addition to the importance for researchers, the mutations in the *parkin* gene have diagnostic implications because of their frequency in early-onset cases. Testing the *parkin* gene is today recommended in the clinical work-up of PD cases, whenever the family history is compatible with a recessive mechanism of inheritance, and especially if the disease onset is before age of 50 (even when only in one of the affected siblings). If the family history is negative, testing *parkin* is still important, especially in the cases with a very early onset. In fact, among the sporadic patients, most of the mutations are found in those with a very early-onset (before age of 30), whereas after that age of onset mutations in this gene are a much rarer finding (chapter 2.1).[21]

An early onset and the presence of a family history compatible with recessive inheritance are therefore critical to prioritise cases for *parkin* testing. The importance of collecting an accurate family history in every PD patient should always be highlighted, because this is still a frequently neglected item by the clinicians who follow PD patients. The presence of consanguinity between parents of the patient is another critical item, for which clinical records are often non-informative, and which can suggest the presence of a recessive mechanism of inheritance in cases presenting without any family history of PD.

Due to the large size of the gene and the wide spectrum of associated abnormalities, the mutation screening of *parkin* is difficult, and it should always include gene copy analysis in addition to genomic sequencing. Clinicians should be aware that limiting the screening to genomic sequencing only, leads to the erroneous exclusion of the parkin gene in many cases carrying heterozygous genomic rearrangements. It is possible that rapid, microarray-based methods will facilitate the diagnostic screening of genomic rearrangements in *parkin* in the near future. Apart from the complexity in diagnostic screening, the genetic counselling of patients with *parkin* mutations and of their relatives is difficult because of the current uncertainties about the role of single heterozygous alleles, and the broad phenotypical spectrum associated, including a very large intra-familial variance of onset age and phenotypes (chapter 2.4).

***Parkin* and genotype phenotype correlations**

Genotype phenotype correlations for parkin disease (as for many disorders) are poorly understood, and there are several potential explanations for this. The spectrum of mutations is very wide and probably incomplete. Due to this, large series of patients are needed for accurate studies. More importantly, we do not understand much of the regulation of the gene itself and of the encoded protein. Due to this, it is difficult to definitely establish if and how a given mutation has a biological effect. However, the analysis of a large number of cases is providing indications that a specific genotype might influence the phenotype (chapter 2.4), suggesting for instance, that missense mutations in functional domains result in earlier onset than mutations in other regions of the protein. Surprisingly, patients with at least one missense mutation did not differ in age of onset but they had a more severe disease than the patients with two truncating mutations, as if some missense mutations result in more than a loss of parkin function (chapter 2.4).

We are starting to disentangle the phenotypical effects of specific genetic etiology from the effects of other variables like aging. For example, dystonia and brisk reflexes are more frequent in patients with *parkin* mutations than in cases without, but both

these clinical features correlate better with young onset age than genetic etiology (chapter 2.4), suggesting that they are not specific of parkin disease, but are rather the phenotypical correlate of the brain dopaminergic deficiency at a younger age.

The wide intrafamilial variability of the phenotype strongly suggests that other genetic, as well as non-genetic modifiers exist for this disease, and support the notion that Mendelian diseases are not always simple traits. For instance, the role of smoking or caffeine intake in parkin disease has not been explored, and it is interesting to see whether a protective effect is also present in this monogenic form like it is in idiopathic PD.[37] Genome scans in families with parkin disease could reveal modifier loci, and the homogeneity of the dataset will likely confer high power to detect additional genetic risk factors. One of such studies performed in a small dataset suggested already candidate regions including the PARK8 locus.[38] The fact that *parkin* mutations are a frequent cause of early onset PD supports the contention that the parkin protein is another key player in PD in general.

The contribution of DJ-1

The *DJ-1* gene has been highly conserved in evolution and is abundantly and ubiquitously expressed in the brain and other body tissues (chapter 2.8). Yet, its function has remained elusive, and nobody had previously linked the DJ-1 protein to brain function or brain disorders. The discovery that mutations in *DJ-1* cause autosomal recessive early-onset forms of PD is therefore relevant for two reasons. First, it establishes that the loss of the DJ-1 function leads to human neurodegeneration. Second, clarifying of mechanisms of *DJ-1*-related disease might potentially shed light on novel mechanisms of brain neuronal maintenance and promote the understanding of pathogenesis of common forms of PD (chapter 2.8).

A total number of nine different mutations in *DJ-1* have been identified so far, including missense, truncating, splice site mutations, and large deletions (table) (chapter 2.8).[39-41] In particular, a PD patient carrying compound heterozygous *DJ-1* mutations (including one truncating and one splice site mutation) (case #8 in the table) provides strong support to the contention that *DJ-1* mutations are pathogenic in early onset PD. For other mutations, especially the single heterozygous ones, a pathogenic role is not definitely demonstrated yet.

On the basis of the first screening in large series of patients, *DJ-1* mutations are the second most frequent identifiable genetic cause of PD after *parkin*, but the frequency of *DJ-1* mutations seems rather low, being estimated at about 1-2 % in early onset PD.[39-41] Testing the *DJ-1* gene should therefore be considered in the clinical work-up of patients with early-onset PD. However, due to the low frequency of involvement, testing the *DJ-1* gene should be recommended only after exclusion of *parkin* gene mutations.

Clinical and genetic features in patients with *DJ-1* mutations

Code	Presentation	Origin	Gender	Onset	Duration	1st mutation	2nd mutation	Reference
#1	Familial	Dutch	F	**31**	17	Δ exon 1-5	Δ exon 1-5	46
#2	Familial	Dutch	M	**40**	10	Δ exon 1-5	Δ exon 1-5	46
#3	Familial	Dutch	M	**<40**	na	Δ exon 1-5	Δ exon 1-5	46
#4	Familial	Dutch	M	**27**	11	Δ exon 1-5	Δ exon 1-5	46
#5	Familial	Italian	M	**28**	18	L166P	L166P	46
#6	Familial	Italian	F	**35**	21	L166P	L166P	46
#7	Familial	Italian	M	**27**	32	L166P	L166P	46
#8	Sporadic	Hispanic	F	**24**	5	IVS6-1G→C	c.56delC, c.57G→A	39
#9	Sporadic	Hispanic	M	**35**	9	A104T	--- *	39
#10	Sporadic	Jewish	na	**39**	na	M26I	M26I	40
#11	Sporadic	Afro-Caribbean	na	**36**	na	D149A	--- *	40
#12	Sporadic	South Tyrolean	M	**42**	17	Δ exon 5-7	---	41
#13	Sporadic	Russian	F	**17**	na	IVS5+2-12del	---	41

*Gene copy dosage not performed.
The c.56delC, c.57G→A mutation leads to frameshift and truncation of DJ-1 protein after the first 18 amino acids.

The occurrence of heterozygous genomic rearrangements in *DJ-1* emphasizes the importance of gene dosage assays for a sensitive screening. This has been performed in only one study so far.[41] Further studies, including gene dosage assays, and promoter analysis, are therefore needed in order to accurately estimate the frequency of this form among early-onset PD, and to characterize the associated phenotype. That the DJ-1 protein exists as a dimer also suggests the possibility of dominant-negative mutations, and therefore a dominant pattern of inheritance should not preclude testing the *DJ-1* gene. The identification of additional disease-linked missense mutations will help pinpointing functionally important domains in the DJ-1 protein, and will complement the biochemical and structural biology studies in the common effort to elucidate the role of DJ-1 and the mechanisms of DJ-1-related disease.
The genomic regions containing *parkin* and *DJ-1* are rich in low-copy-repeat elements (chapter 2.8),[42] which might explain why these regions are prone to rearrangements. Genomic instability might be important in other forms of PD as well, and future studies should target this kind of genetic mechanism more specifically.
Evidence for linkage to the PARK7 region was not found in genome scans for late onset familial PD, suggesting that *DJ-1*, like the other known genes for Mendelian PD, is not a major locus in common familial forms. However, whether genetic variation in *DJ-1* modifies the susceptibility to, or modulates the expression of sporadic, late-onset PD or different neurodegenerative diseases remains an important question, which will require further genetic work.
Investigating the presence of the DJ-1 protein (and its acidic variant) in brain from patients with Lewy body disease, as well as other neurodegenerative diseases is urgently warranted. These studies might provide important clues on the involvement of *DJ-1* in common forms of neurodegeneration. We have recently explored the

presence of the DJ-1 protein in brain from patients with common neurodegenerative diseases. While we did not find evidence of DJ-1 immunoreactivity in Lewy bodies with the available antibodies, we did find that the DJ-1 immunoreactivity co-localizes within a subset of pathological tau inclusions in a diverse group of neurodegenerative disorders, supporting the view that different neurodegenerative diseases may have similar pathogenetic mechanisms, which likely include DJ-1.[43] This finding raises the question of whether tau pathology is also present in the brain of patients with DJ-1 mutations. Interestingly, tau pathology has been found in patients with parkin disease. Therefore, if this would also be the case in DJ-1-related disease, it would suggest the existence of a common pathological signature in DJ-1- and parkin-related forms. Investigating the expression of the DJ-1 protein in the brain of patients with parkin disease and vice versa is also warranted.

The exact role of the DJ-1 protein remains unknown, but the available information about DJ-1 interactions provides clues to a possible function, and to possible ways of linking DJ-1 to neurodegeneration (chapter 2.9). An involvement of DJ-1 in processes as different as oncogenesis, male fertility, gene transcription, mRNA stability, and response to cell stress is suggested by the current evidence. Although much work remains to be done to clarify the biology of this protein, the possible involvement of DJ-1 as molecular chaperone and/or an antioxidant appears particularly intriguing in the light of our current understanding of the pathogenesis of PD (chapter 1.2).
However, the importance of identifying a novel gene causing PD is even greater if this leads to innovative ideas about pathogenesis. In the case of *DJ-1*, potential novel insights are the focus on the nuclear mechanisms and the control of gene expression in PD pathogenesis. Moreover, by linking sumoylation to the pathogenesis of PD, DJ-1 might extend the involvement of this ubiquitin-like protein-conjugation system in the neurodegenerative diseases characterized by protein aggregation in the cytoplasm (chapter 2.9).

Our finding that mutant DJ-1^{L166P} is localised to mitochondria once again suggests a possible link to oxidative stress (chapter 2.8). This localisation has recently been confirmed using untagged *DJ-1* constructs, therefore excluding tagging effects on the conformation of the mutant protein.[44] This localization is also intriguing in the light of the mitochondrial abnormalities described in classical PD,[45] and assessing the mitochondria functions in the patients with *DJ-1* mutations is therefore warranted.
Due to the high levels of expression in cell systems analysed,[46] we could not exclude that a fraction of wild type DJ-1 also localises to mitochondria, as recently suggested by others.[47] This possibility warrants further investigations. Furthermore, we observed that the mutant DJ-1^{L166P} protein is unstable and rapidly degraded, resulting in much lower steady state levels in both transfected cells and patient lymphoblasts.[44] The mutant DJ-1 could therefore be targeted to mitochondria as a result of abnormal structure, or, the reduced levels of expression could lead DJ-1 to occupy only its sites of highest affinity binding, which could include mitochondria and the nucleus (as the mutant retains the nuclear localization). The instability of DJ-1^{L166P} suggests that the mutant is misfolded and, as such, underlies rapid degradation, as observed in many other genetic diseases.[48] The instability of mutant DJ-1^{L166P} has been recently confirmed in an independent study.[47]

Important insights about the function of DJ-1 are coming from the structural biology studies. The crystal structure of human DJ-1, at resolutions ranging from 1.95 to 1.1 Å,

has been recently reported by four independent groups.[49-52] These structures are very similar to each other, with DJ-1 assuming an α/β sandwich fold similar to the so-called flavodoxin-like or Rossmann fold, that is conserved in the DJ-1-ThiJ-PfpI superfamily.
An important finding in these studies is that DJ-1 exists as dimer, a finding that we and others independently observed in gel filtration experiments.[44, 47, 50, 51] Most of the residues involved in the dimerization are highly conserved, but DJ-1 and its closest homologues share a peculiar dimerization pattern in the superfamily, which is partly determined by the presence of an additional C-terminal helix. A putative active site has been identified close to the dimer interface, with some similarities to the active site of cysteine proteases, the residues Cys106, His126, and perhaps Glu18 being likely involved.[49-51] However, these residues do not show the orientation required for the proton transfer which is typical of the cysteine protease catalysis.[51] In keeping with this, biochemical attempts to detect protease[49, 51] or kinase[51] activity of DJ-1 have been unsuccessful so far.
Moreover, the crystal studies confirm our prediction that the residue mutated in the PD patients (L166) is located in a C-terminal α-helix,[49-52] and show that this helix is part of a hydrophobic core formed by three helices (two contributed by the C-terminal and one by the N-terminal part of the monomer), which is involved in the dimerization.[50, 51] The L166P mutation appears to disrupt the C-terminal domain and the dimerization capability, suggesting that the dimerization is functionally important. A novel disease-linked missense mutation (M26I) has been recently reported.[40] Intriguingly, the residue Met26 is located in the N-terminal helix which contributes to the same hydrophobic core, and is spatially close to Leu166; furthermore, this N-terminal helix contributes to the putative active site of DJ-1.[50]
Our findings in gel filtration studies also suggest that the mutant does not form dimers but adopts a different higher order structure or complexes with other proteins.[44] Interestingly, a deletion mutant lacking residues 173 to 189 is also reported to form higher aggregates.[50] Taken as a whole, these evidences suggest that the dimeric structure is important for the function of DJ-1.
Despite the rapid progress in elucidating the structure of DJ-1, the biochemical function of this protein remains elusive. Whether it possesses one of more of the proposed enzymatic properties (protease, hydrolase, kinase, amidotrasferase, catalase), or whether it functions as a chaperone, or a transcription factor, or it has more than one function, is unknown. Initial biochemical studies suggest that protease, kinase, and amidotransferase activities are unlikely, whereas catalase or other catalytic activities remain unexplored and still intriguing possibilities. Moreover, it remains to be seen whether the dimer represents the active form, or whether in response to specific signals, DJ-1 undergoes other structural changes that make it functionally active.
Interestingly, it has been reported that replacement of the Cys53 residue, located in the dimer interface, abolished the pI shift of DJ-1 in response to oxidative stimuli, suggesting that this shift is mediated by the oxidative conversion of the sulphydrylic group of cysteine to cysteine sulfinic acid.[49] It has been proposed that this modification leads to functional activation of the protein in response to oxidative stress.[49] However, the observation that another conserved cysteine (Cys106) displays extreme sensitivity to radiation damage, suggests that Cys106 also mediates the oxidative conversion of DJ-1.[51] The presence of one or more cysteines, which react to oxidative conditions, supports the idea that DJ-1 is an oxidation-response protein.
The *E.coli* stress-inducible chaperone EcHsp31belongs to the DJ-1-ThiJ-PfpI superfamily, and possesses a putative hydrolytic site.[53] It has been suggested that it might switch from chaperone to protease function on the basis of the temperature shift,[53] and

this has previously been observed for another bacterial protein.[54] Similarly, one could speculate that DJ-1 has also dual function of chaperone and enzyme depending on the cell stress level. Despite the fact that many questions remain unanswered, the resolution of the structure of DJ-1 has been an important step forward to clarify the exact function of the protein.

The effect of the loss of DJ-1 function, either alone or in combination with mutations in other PD-related genes, is now being investigated in cell culture and animal models, including mice and flies, and models of PD induced by dopaminergic neurotoxins, like MPTP and rotenone. In transgenic flies, it will be possible to rapidly screen for genetic modifiers, either enhancers or suppressors of any resulting *DJ-1*-related phenotype.
Transgenic mice overexpressing the human *DJ-1* gene have been generated and show no influence on fertility in comparison to the wild type animals.[55] Expression of the transgene was high in testis but also in the brain, and these mice might also be useful to investigate putative brain functions of DJ-1, including resistance to dopaminergic toxins (MPTP, rotenone), oxidative stress and protein misfolding.
Expression profiling in cell cultures in which the *DJ-1* gene is manipulated, or in patient-derived cells, might facilitate the characterization of *DJ-1*-related pathways. Biochemical and cell biology studies have also been initiated in order to understand further the function of DJ-1, identify its interacting partners, and explore possible relationships between DJ-1 and the proteins encoded by genes, which are firmly implicated in PD and other common neurodegenerative diseases. Initial studies in our lab suggest that DJ-1 is not directly interacting with α-synuclein and tau.[44] These complementary approaches will help clarifying the pathogenesis of *DJ-1*-related disease, but, more importantly, they will provide new insights for understanding of the molecular mechanisms of classical PD.

Reflections and perspectives for future studies
During the last few years molecular genetics has provided tremendous contributions to our understanding of the mechanisms of neurodegeneration in general and PD in particular. Most of this progress has come from the analysis of rare inherited forms of the disease. We know that other monogenic forms of PD exist (chapter 1.1), and the identification of the genes involved holds the promise of yielding further significant steps-forward. The importance of the classical linkage mapping and positional cloning strategies in Mendelian forms of PD should not be underscored, and further potentially critical contributions are expected from this approach in the near future.
Yet, we are just beginning to disentangle the complexity of the common forms of the disease. In this regard, the genome-wide linkage screens have only recently produced the first statistically significant results (chapter 1.1). It remains to be seen whether these findings will be confirmed, and whether they will lead to the identification of biologically relevant genetic variants determining susceptibility to the disease. Nevertheless, more and more studies in the coming years will be based on small families with affected relative pairs, and on patient cohorts from genetically isolated populations.[56]
Modelling neurodegenerative diseases in transgenic animals has also revealed to be a very powerful strategy. These studies contributed significantly to support the contention that critical proteins such as tau, β-amyloid precursor, polyglutamine repeats, or α-synuclein play primary roles in the pathogenesis.[57] Moreover, these studies demonstrated that it is possible to modify the neurodegenerative process by manipulating components of the molecular chaperones, or of the ubiquitin-proteasome systems, thus suggesting novel avenues to design future therapeutic approaches.[57] As

novel genes for PD are identified, experiments in transgenic models will continue to explore their role and their relationship with the other genes, which were previously implicated in the pathogenesis. Animal and cell culture models will also be used to test novel ways of therapeutic intervention, for instance, silencing of dominant mutations by small interfering RNAs,[58] or overexpressing genes for neuroprotective proteins (such as parkin or DJ-1).[59]

There is no evidence of a spontaneous PD-like neurodegenerative disease in other animals.[60] Moreover, the full pathological picture of AD (the most frequent human neurodegenerative disease) has never been reported in non-human primates.[61, 62] The apparent absence of these neurodegenerative diseases in our closest evolutionary relatives, leads to the idea that the biological bases of these common disorders of the human brain are somehow related to what makes us unique among primates. The genomes of humans and chimpanzees (the closest evolutionary relatives) are almost 99% identical,[63] and it appears unlikely that this little genotypic divergence alone explains the wide phenotypic differences, such as those in cognitive performances and susceptibility to neurodegeneration. An alternative hypothesis is that inter-species differences might be due to quantitative changes in the level of gene or protein expression, and that this mechanism might have been particularly important for the evolution of the brain differences.[64] It is possible that our susceptibility to common age-related neurodegeneration is also related to the peculiar levels of brain gene and protein expressions, which make us humans.

Perhaps the next few years will see the shift from the current "anatomical" screening of the genome in PD, to a more functional analysis exploring quantitative changes in gene and protein expression. Profiling the transcriptome and the proteome in human tissue, animal and cell-based models might provide functional signatures of the disease process, allowing recognition of pathways and development of integrative views.

It is important that these studies are made in the different stages, including ideally the pre-symptomatic phase of the disease, in order to distinguish the primary from secondary events, and the factors determining the initiation from those influencing the progression of the disease.

REFERENCES

1. Hughes AJ, Daniel SE, Kilford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J Neurol Neurosurg Psychiatry 1992; 55:181-4.
2. Rajput AH, Rozdilsky B, Rajput A. Accuracy of clinical diagnosis in parkinsonism--a prospective study. Can J Neurol Sci 1991; 18:275-8.
3. Lang AE, Lozano AM. Parkinson's disease. First of two parts. N Engl J Med 1998; 339:1044-53.
4. Gelb DJ, Oliver E, Gilman S. Diagnostic criteria for Parkinson disease. Arch Neurol 1999; 56:33-9.
5. Schmidt ML, Martin JA, Lee VM, Trojanowski JQ. Convergence of Lewy bodies and neurofibrillary tangles in amygdala neurons of Alzheimer's disease and Lewy body disorders. Acta Neuropathol (Berl) 1996; 91:475-81.
6. Trojanowski JQ, Goedert M, Iwatsubo T, Lee VM. Fatal attractions: abnormal protein aggregation and neuron death in Parkinson's disease and Lewy body dementia. Cell Death Differ 1998; 5:832-7.
7. Mori H, Oda M, Komori T, et al. Lewy bodies in progressive supranuclear palsy. Acta Neuropathol (Berl) 2002; 104:273-8.
8. Arawaka S, Saito Y, Murayama S, Mori H. Lewy body in neurodegeneration with brain

iron accumulation type 1 is immunoreactive for alpha-synuclein. Neurology 1998; 51: 887-9.
9. Gibb WR, Lees AJ. The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson's disease. J Neurol Neurosurg Psychiatry 1988; 51:745-52.
10. Perl DP, Olanow CW, Calne D. Alzheimer's disease and Parkinson's disease: distinct entities or extremes of a spectrum of neurodegeneration? Ann Neurol 1998; 44:S19-31.
11. Li YJ, Scott WK, Hedges DJ, et al. Age at onset in two common neurodegenerative diseases is genetically controlled. Am J Hum Genet 2002; 70:985-93.
12. Spira PJ, Sharpe DM, Halliday G, Cavanagh J, Nicholson GA. Clinical and pathological features of a Parkinsonian syndrome in a family with an Ala53Thr alpha-synuclein mutation. Ann Neurol 2001; 49:313-9.
13. Gasser T, Muller-Myhsok B, Wszolek ZK, et al. A susceptibility locus for Parkinson's disease maps to chromosome 2p13. Nat Genet 1998; 18:262-5.
14. Mori H, Kondo T, Yokochi M, et al. Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology 1998; 51:890-2.
15. Funayama M, Hasegawa K, Kowa H, Saito M, Tsuji S, Obata F. A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. Ann Neurol 2002; 51:296-301.
16. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392:605-8.
17. Lucking CB, Abbas N, Durr A, et al. Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism. The European Consortium on Genetic Susceptibility in Parkinson's Disease and the French Parkinson's Disease Genetics Study Group. Lancet 1998; 352:1355-6.
18. Leroy E, Anastasopoulos D, Konitsiotis S, Lavedan C, Polymeropoulos MH. Deletions in the Parkin gene and genetic heterogeneity in a Greek family with early onset Parkinson's disease. Hum Genet 1998; 103:424-7.
19. Abbas N, Lucking CB, Ricard S, et al. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Hum Mol Genet 1999; 8:567-74.
20. Hedrich K, Marder K, Harris J, et al. Evaluation of 50 probands with early-onset Parkinson's disease for Parkin mutations. Neurology 2002; 58:1239-46.
21. Periquet M, Latouche M, Lohmann E, et al. Parkin mutations are frequent in patients with isolated early-onset parkinsonism. Brain 2003; 126:1271-8.
22. Foroud T, Uniacke SK, Liu L, et al. Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. Neurology 2003; 60:796-801.
23. Oliveira SA, Scott WK, Martin ER, et al. Parkin mutations and susceptibility alleles in late-onset Parkinson's disease. Ann Neurol 2003; 53:624-9.
24. Kann M, Jacobs H, Mohrmann K, et al. Role of parkin mutations in 111 community-based patients with early-onset parkinsonism. Ann Neurol 2002; 51:621-5.
25. Golbe LI. Young-onset Parkinson's disease: a clinical review. Neurology 1991; 41:168-73.
26. Klein C, Pramstaller PP, Kis B, et al. Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: expanding the phenotype. Ann Neurol 2000; 48:65-71.
27. Farrer M, Chan P, Chen R, et al. Lewy bodies and parkinsonism in families with parkin mutations. Ann Neurol 2001; 50:293-300.
28. Khan NL, Graham E, Critchley P, et al. Parkin disease: a phenotypic study of a large case series. Brain 2003; 126:1279-92.
29. Maruyama M, Ikeuchi T, Saito M, et al. Novel mutations, pseudo-dominant inheritance, and possible familial affects in patients with autosomal recessive juvenile parkinsonism. Ann Neurol 2000; 48:245-50.
30. Kobayashi T, Matsumine H, Zhang J, Imamichi Y, Mizuno Y, Hattori N. Pseudo-autosomal dominant inheritance of PARK2: two families with parkin gene mutations. J Neurol Sci 2003; 207:11-7.
31. Hilker R, Klein C, Ghaemi M, et al. Positron emission tomographic analysis of the nigrostriatal dopaminergic system in familial parkinsonism associated with mutations in the parkin gene. Ann Neurol 2001; 49:367-76.
32. Khan NL, Brooks DJ, Pavese N, et al. Progression of nigrostriatal dysfunction in a parkin

kindred: an [18F]dopa PET and clinical study. Brain 2002; 125:2248-56.
33. Lohmann E, Periquet M, Bonifati V, et al. How much phenotypic variation can be attributed to parkin genotype? Ann Neurol 2003; 54:176-85.
34. West A, Periquet M, Lincoln S, et al. Complex relationship between Parkin mutations and Parkinson disease. Am J Med Genet 2002; 114:584-91.
35. West AB, Maraganore D, Crook J, et al. Functional association of the parkin gene promoter with idiopathic Parkinson's disease. Hum Mol Genet 2002; 11:2787-92.
36. Oliveira SA, Scott WK, Nance MA, et al. Association study of Parkin gene polymorphisms with idiopathic Parkinson disease. Arch Neurol 2003; 60:975-80.
37. Hernan MA, Takkouche B, Caamano-Isorna F, Gestal-Otero JJ. A meta-analysis of coffee drinking, cigarette smoking, and the risk of Parkinson's disease. Ann Neurol 2002; 52:276-84.
38. Pankratz N, Nichols WC, Uniacke SK, et al. Genome screen to identify susceptibility genes for Parkinson disease in a sample without parkin mutations. Am J Hum Genet 2002; 71: 124-35.
39. Hague S, Rogaeva E, Hernandez D, et al. Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation. Ann Neurol 2003; 54:271-4.
40. Abou-Sleiman PM, Healy DG, Quinn N, Lees AJ, Wood NW. The role of pathogenic DJ-1 mutations in Parkinson's disease. Ann Neurol 2003; 54:283-286.
41. Hedrich K, Djarmati A, Schafer N, et al. DJ-1 mutations are less frequent than Parkin mutations in early-onset Parkinson's disease. Submitted for publication 2003.
42. Periquet M, Lucking C, Vaughan J, et al. Origin of the mutations in the parkin gene in Europe: exon rearrangements are independent recurrent events, whereas point mutations may result from Founder effects. Am J Hum Genet 2001; 68:617-26.
43. Rizzu P, Hinkle DA, Zhucareva V, et al. DJ-1 co-localizes with pathological tau inclusions: a possible link between parkinsonism and dementia. Ann Neurol in press.
44. Macedo MG, Anar B, Bronner IF, et al. The DJ-1 L166P mutant protein associated with early onset Parkinson's disease is unstable and forms higher order protein complexes. Hum Mol Genet in press.
45. Orth M, Schapira AH. Mitochondrial involvement in Parkinson's disease. Neurochem Int 2002; 40:533-41.
46. Bonifati V, Rizzu P, van Baren MJ, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 2003; 299:256-9.
47. Miller DW, Ahmad R, Hague S, et al. L166P mutant DJ-1, causative for recessive Parkinson's disease, is degraded through the ubiquitin-proteasome system. J Biol Chem 2003; 278:36588-95.
48. Bross P, Corydon TJ, Andresen BS, Jorgensen MM, Bolund L, Gregersen N. Protein misfolding and degradation in genetic diseases. Hum Mutat 1999; 14:186-98.
49. Honbou K, Suzuki NN, Horiuchi M, et al. The crystal structure of DJ-1, a protein related to male fertility and Parkinson's disease. J Biol Chem 2003; 278:31380-4.
50. Tao X, Tong L. Crystal structure of human DJ-1, a protein associated with early-onset Parkinson's disease. J Biol Chem 2003; 278:31372-9.
51. Wilson MA, Collins JL, Hod Y, Ringe D, Petsko GA. The 1.1-A resolution crystal structure of DJ-1, the protein mutated in autosomal recessive early onset Parkinson's disease. Proc Natl Acad Sci U S A 2003; 100:9256-61.
52. Huai Q, Sun Y, Wang H, et al. Crystal structure of DJ-1/RS and implication on familial Parkinson's disease. FEBS Lett 2003; 549:171-5.
53. Quigley PM, Korotkov K, Baneyx F, Hol WG. The 1.6-A crystal structure of the class of chaperones represented by Escherichia coli Hsp31 reveals a putative catalytic triad. Proc Natl Acad Sci U S A 2003; 100:3137-42.
54. Spiess C, Beil A, Ehrmann M. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell 1999; 97:339-47.
55. Okada M, Matsumoto K, Niki T, Taira T, Iguchi-Ariga SM, Ariga H. DJ-1, a target protein for an endocrine disrupter, participates in the fertilization in mice. Biol Pharm Bull 2002; 25: 853-6.
56. Heutink P, Oostra BA. Gene finding in genetically isolated populations. Hum Mol Genet 2002; 11:2507-15.

57. Zoghbi HY, Botas J. Mouse and fly models of neurodegeneration. Trends Genet 2002; 18: 463-71.
58. Gonzalez-Alegre P, Miller VM, Davidson BL, Paulson HL. Toward therapy for DYT1 dystonia: allele-specific silencing of mutant TorsinA. Ann Neurol 2003; 53:781-7.
59. Yang Y, Nishimura I, Imai Y, Takahashi R, Lu B. Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in Drosophila. Neuron 2003; 37:911-24.
60. Gerlach M, Riederer P. Animal models of Parkinson's disease: an empirical comparison with the phenomenology of the disease in man. J Neural Transm 1996; 103:987-1041.
61. Gearing M, Rebeck GW, Hyman BT, Tigges J, Mirra SS. Neuropathology and apolipoprotein E profile of aged chimpanzees: implications for Alzheimer disease. Proc Natl Acad Sci U S A 1994; 91:9382-6.
62. Kiatipattanasakul W, Nakayama H, Yongsiri S, et al. Abnormal neuronal and glial argyrophilic fibrillary structures in the brain of an aged albino cynomolgus monkey (Macaca fascicularis). Acta Neuropathol (Berl) 2000; 100:580-6.
63. Varki A. A chimpanzee genome project is a biomedical imperative. Genome Res 2000; 10:1065-70.
64. Enard W, Khaitovich P, Klose J, et al. Intra- and interspecific variation in primate gene expression patterns. Science 2002; 296:340-3.

SUMMARY

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, with a prevalence of 1-2% in the population aged ≥ 65. The disease is clinically defined by the presence of parkinsonism (the combination of akinesia, resting tremor, and muscular rigidity), and a good response to dopaminergic therapy. These features are associated at the pathological level with neuronal loss and gliosis, mainly in the *substantia nigra pars compacta* but also in other brain areas, and formation of cytoplasmic inclusions called Lewy bodies in the surviving neurons.
PD is mostly a sporadic disorder, but in 10-15% of cases it runs in families without a clear-cut Mendelian pattern. More rarely, it segregates as a Mendelian trait with either autosomal dominant or recessive inheritance. The common form of PD is therefore likely to be a complex trait, determined by several genetic as well as non-genetic factors.
In the last few years, family-based linkage analysis and positional cloning have led to the identification of several loci and genes for the rare monogenic forms and more recently, of two loci harboring susceptibility genes for the common, non-Mendelian forms. Although the monogenic forms explain a very small fraction of PD cases at the population level, they are promoting the understanding of the molecular events and the metabolic pathways involved in the common forms of PD as well.

The first section of this thesis (Introduction) reviews the genetics of PD, and the pathogenesis of two monogenic forms previously identified. In **chapter 1.1**, the clinical and molecular genetic aspects of the Mendelian forms of PD are reviewed, and the results of genome-wide screens to localise genes involved in the common, non-Mendelian forms of the disease are also discussed. In **chapter 1.2**, the pathogenesis of PD forms caused by mutations in *α-synuclein* and *parkin*, the two genes which have been firmly associated with PD in the past, are reviewed, highlighting the implications of these knowledge for the understanding of the mechanisms underlying the common forms of PD.

The research work on which this thesis is based is presented in the second section, and it deals with molecular genetic studies and phenotypical characterization of autosomal recessive, early onset PD forms associated with mutations in the *parkin* (**chapters 2.1-2.4**) and the *DJ-1* (**chapters 2.5-2.8**) gene.
Soon after the cloning of the *parkin* gene in 1998, other groups confirmed the presence of mutations in PD families. However, the frequency of involvement of *parkin* in early onset PD and the mutational spectrum were not fully appreciated until the study described in **chapter 2.1**, which explored for the first time this gene in a large dataset of families and isolated early-onset cases, including genomic sequencing and gene copy dosage. The results revealed mutations in this gene in about half of the families compatible with recessive inheritance and PD onset before age of 45, and in about 20% of the isolated cases with early onset. Another important finding of the study is that half of the mutations consists of large genomic rearrangements, and therefore, for a sensitive screening gene copy analysis is needed in addition to gene sequencing.
If the frequency of recessive mutations in a given gene is high in the population, the mating of a homozygous (or a compound heterozygous) and a heterozygous carrier might lead to appearance of the recessive disease in subsequent generations, mimicking a dominant pattern of transmission (pseudo-dominance). These matings might also result in allele heterogeneity within the same sibship. These phenomena were demonstrated in different studies in Japanese and Italian families (**chapters 2.2,**

2.3), suggesting that *parkin* gene mutations might be frequent in some populations. Genotype phenotype correlations for parkin-related disease are still poorly understood. However, the analysis of a large number of cases is providing indications that a specific genotype might influence the phenotype (**chapter 2.4**).

A novel locus for autosomal recessive, early-onset PD (PARK7) was localised to the chromosome 1p36 region by a genome-wide scan and homozygosity mapping in a large consanguineous family with four affected individuals, belonging to a genetically isolated population in the south-west of the Netherlands (**chapter 2.5**). Linkage to the PARK7 region was later confirmed in a second, consanguineous family with three siblings affected by early-onset PD and coming from central Italy (**chapter 2.6**). The phenotype in both families is characterized by parkinsonism of early onset (ranging from age 27 to 40), good levodopa response and slow progression. Behavioral and psychic disturbances, and dystonic features (including blepharospasm) are also present, and a positron emission tomography study in the Dutch family showed dopaminergic presynaptic dysfunctions (**chapter 2.7**).
Fine mapping studies and a positional cloning strategy led to the identification of pathogenic mutations in the *DJ-1* gene in the two families definitely linked to PARK7 (**chapter 2.8**). The *DJ-1* gene has been highly conserved in evolution and is abundantly and ubiquitously expressed in the brain and other body tissues. The exact role of the DJ-1 protein remains mostly unknown but a review of the literature provides clues to a possible function and allows hypotheses linking DJ-1 to neurodegeneration to be formulated (**chapter 2.9**). The discovery that mutations in *DJ-1* cause autosomal recessive early-onset forms of PD establishes that the loss of the DJ-1 function leads to human neurodegeneration. Clarifying the mechanisms of *DJ-1*-related disease might also potentially shed light on novel mechanisms of brain neuronal maintenance and promote the understanding of pathogenesis of common forms of PD.

The **chapter 3** contains the general discussion of the results of our experimental work. First, it deals with the implications of the knowledge resulting from the genetic studies for the nosology of PD. Moreover, it discusses the place of parkin-related disease in the context of PD, highlighting the importance of this form for the diagnostic work-up of early-onset patients, and the issue of the genotype phenotype correlation. Last, the contribution resulting from the discovery of *DJ-1* mutations in PARK7, and the most recent findings from biochemical and structural biology studies on the DJ-1 protein are discussed. The thesis ends with some reflections and perspectives for the future studies aiming at elucidating further the causes and molecular mechanisms of PD.

SAMENVATTING

De ziekte van Parkinson, na de ziekte van Alzheimer de meest voorkomende neurodegeneratieve aandoening, komt voor bij 1-2% van de bevolking ≥ 65 jaar. De klinische diagnose van de ziekte kan worden gesteld indien parkinsonisme wordt aangetoond (de combinatie van akinesie, tremor en musculaire rigiditeit) en indien er een positieve reactie is op behandeling met dopamine. Pathologisch wordt de ziekte gekenmerkt door selectief neuronaal verlies en gliose, welke voornamelijk voorkomt in de *substantia nigra pars compacta,* maar ook in andere gebieden van de hersenen, en door de vorming van cytoplasmatische insluitsels, genaamd Lewy-lichaampjes, in de overlevende neuronen.
De ziekte van Parkinson is een sporadisch voorkomende aandoening. Echter, in 10-15% van de gevallen is de aandoening familiair, maar vaak zonder een helder en duidelijk Mendeliaans overervingspatroon. In zeldzame gevallen ziet men overerving volgens de wetten van Mendel met ofwel een autosomaal dominante of recessieve vorm van overerving. Aangenomen wordt dat bij het ontstaan van de meest gangbare vorm van de ziekte van Parkinson zowel genetische als niet-genetische factoren een rol spelen. Gedurende de laatste jaren heeft linkage-analyse binnen families, gevolgd door positionele klonering, geleid tot identificatie van verschillende loci en genen voor zeldzame monogenetische vormen. Meer recent zijn twee loci geïdentificeerd waar zich waarschijnlijk genen bevinden voor de meer algemene, niet Mendeliaanse vormen van overerving.
Alhoewel de monogenetische vormen van overerving maar een klein deel van de gevallen van de ziekte van Parkinson binnen de populatie verklaren, zorgen zij er toch voor dat er meer inzicht ontstaat in de moleculaire en metabole processen welke betrokken zijn bij de ziekte van Parkinson.

Het eerste deel van dit proefschrift (Introduction) geeft een overzicht van de genetica van de ziekte van Parkinson en de pathogenese van twee monogenetische vormen van de ziekte waarvan de genen zijn geïdentificeerd. In **Hoofdstuk 1.1** worden de klinische en moleculair genetische aspecten nader beschreven, en worden de resultaten besproken van genoom-analyse voor het lokaliseren van genen betrokken bij de meer algemene, niet-Mendeliaanse vormen van overerving van de ziekte. In **Hoofdstuk 1.2** wordt de pathogenese besproken van de ziekte van Parkinson, veroorzaakt door mutaties in het *alpha-synuclein* en *parkin* gen, de twee genen die in het verleden al werden geassocieerd met de ziekte van Parkinson. De nadruk wordt gelegd op de implicaties van deze kennis voor het begrijpelijk maken van de mechanismen welke betrokken zijn bij de meer algemene vormen van de ziekte van Parkinson.

Het onderzoek waarop dit proefschrift is gebaseerd, wordt gepresenteerd in de tweede deel, en deze sectie heeft betrekking op de moleculair genetische studies en phenotypische karakterisering van autosomaal recessieve, vroeg beginnende vormen van de ziekte van Parkinson, welke worden geassocieerd met mutaties in het *parkin* (**hoofdstuk 2.1-2.4**) gen en het *DJ-1* **(hoofdstuk 2.5-2.8**) gen.

Al snel na de klonering van het *parkin* gen in 1998 bevestigden andere onderzoeksgroepen de aanwezigheid van mutaties in genen van families waarin de ziekte van Parkinson voorkomt. Echter, de frequentie waarin het *parkin* gen betrokken is bij de vroegbeginnende vorm van de ziekte van Parkinson en het spectrum van

mutaties in dit gen kwamen pas duidelijk aan het licht na de studie beschreven in **Hoofdstuk 2.1**. Voor het eerst werd in families en geïsoleerde gevallen de betrokkenheid van dit gen onderzocht bij de vroegbeginnende vormen van de ziekte van Parkinson, waarbij DNA analyse werd uitgevoerd en het aantal aanwezige kopieën van het *parkin* gen werden bepaald. In ongeveer de helft van de families met recessieve overerving, waarin de ziekte van Parkinson aanvangt voor het 45ste levensjaar, en in 20% van de geïsoleerde gevallen met een vroegbeginnende vorm van de ziekte van Parkinson werden mutaties in dit gen gezien. Een andere belangrijke ontdekking gedurende deze studie is dat de helft van de mutaties bestaat uit grote veranderingen op DNA-niveau. Daarom is een gevoelige screening van het aantal genkopieën nodig ter aanvulling van de reguliere mutatie-analyse. Als binnen de algemene populatie de frequentie van recessieve mutaties in een gegeven gen hoog is, dan bestaat de kans dat een homozygoot (of een samengesteld heterozygoot) individu en een heterozygote drager voor nageslacht zorgen. Dit kan leiden tot het voorkomen van de recessieve vorm van de ziekte in opeenvolgende generaties, waarbij een dominant patroon van transmissie wordt nagebootst (pseudo-dominantie). Dit kan resulteren in heterogeniteit van allelen binnen een familie. Dit fenomeen werd aangetoond in verschillende studies binnen Japanse en Italiaanse families (**hoofdstukken 2.2, 2.3**), hetgeen suggereert dat *Parkin* gen mutaties frequent voorkomen in sommige populaties.
Genotype fenotype correlaties voor de ziekte gerelateerd aan het *parkin* gen zijn nog onderwerp van nadere studie. De analyse van een groot aantal gevallen geeft een indicatie dat een specifiek genotype invloed zou kunnen hebben op het fenotype (**hoofdstuk 2.4**).

Een nieuwe locus voor een autosomaal recessieve, vroegbeginnende vorm van de ziekte van Parkinson (PARK7) werd gelokaliseerd binnen de chromosomale 1p36 regio. Met behulp van een genetische scan van het hele genoom werd een homozygote regio gevonden binnen een grote consanguine familie met vier aangedane personen, die afkomstig is uit een genetisch geïsoleerde populatie in het Zuid-Westen van Nederland (**hoofdstuk 2.5**).
Koppeling van de ziekte met de PARK7 regio is later bevestigd in een tweede, consanguine familie met drie bloedverwanten uit midden Italië met de vroegbeginnende vorm van de ziekte van Parkinson (**hoofdstuk 2.6**). Het fenotype binnen beide families wordt gekenmerkt door vroegbeginnende Parkinsonisme (variërend van 27 tot 40 jaar), goede levodopa respons en langzame progressie. Gedrags- en psychische stoornissen, en dystonische gelaatstrekken (inclusief blepharospasmus) worden ook gezien en een positron emissie tomografie studie in de Nederlandse familie liet ook dopaminerge presynaptische dysfuncties zien (**hoofdstuk 2.7**).

Karakterisering van de PARK7 regio en positionele klonering leidde tot de identificatie van mutaties in het *DJ-1* gen in de twee families waar PARK7 zeker voorkomt (**hoofdstuk 2.8**). Het *DJ-1* gen is zeer goed geconserveerd gebleven gedurende de evolutie en komt hoog tot expressie in de hersenen en andere lichaamsweefsels. De preciese rol van het *DJ-1* eiwit is vooralsnog onbekend maar in de literatuur worden aanwijzingen gevonden over de mogelijke functie van het eiwit. Dit geeft de mogelijkheid tot het poneren van hypotheses waarin *DJ-1* wordt gerelateerd aan neurodegeneratie (**hoofdstuk 2.9**). De ontdekking dat mutaties in *DJ-1* de oorzaak zijn van autosomaal recessieve, vroegbeginnende vormen van de ziekte van Parkinson bewijst dat verlies van de *DJ-1* functie leidt tot humane neurodegeneratie. Verklaring van de mechanismen van de *DJ-1* gerelateerde ziekte kan eventueel ook licht werpen op

nieuwe mechanismen van onderhoud van de neuronale hersencellen en leiden tot meer begrip van de pathogenese van de algemene vormen van de ziekte van Parkinson.

Hoofdstuk 3 bevat de discussie van de resultaten van ons experimentele werk. Ten eerste worden hier de implicaties behandeld van de kennis, welke resulteerde uit de genetische studies naar de ziekte van Parkinson. Bovendien wordt de bijdrage van de Parkin gerelateerde ziekte binnen de context van de ziekte van Parkinson besproken, waarbij de nadruk wordt gelegd op het belang voor de diagnostiek bij patiënten met de vroegbeginnende vorm van de ziekte van Parkinson, en de mogelijke genotype phenotype correlatie. Tenslotte wordt de ontdekking van *DJ-1* mutaties in PARK7 besproken, en wordt aandacht besteed aan de meest recente bevindingen uit biochemische en structurele biologische studies naar het *DJ-1* eiwit.
Dit proefschrift eindigt met enkele reflecties, en mogelijkheden voor onderzoek in de toekomst, gericht op het nader verklaren van de oorzaken en de moleculair mechanismen van de ziekte van Parkinson.

RIASSUNTO

La malattia di Parkinson (MP) e' la forma piu' frequente di malattia neurodegenerativa dopo la malattia di Alzheimer, con una prevalenza intorno all'1-2% nella popolazione con piu' di 65 anni. La malattia e' definita sul piano clinico dalla presenza di parkinsonismo (la combinazione di acinesia, tremore a riposo, e rigidita' muscolare), e da una buona risposta alla terapia dopaminergica. Sul piano neuropatologico, queste caratteristiche sono associate a perdita neuronale e gliosi, principalmente a carico della *substantia nigra pars compacta*, ma anche in altre aree cerebrali, con formazione di inclusioni citoplasmatiche, chiamate corpi di Lewy, nei neuroni superstiti.
La MP e' per lo piu' una malattia sporadica, ma nel 10-15% dei casi ricorre nella famiglia in assenza di un chiaro pattern di tipo Mendeliano. Piu' raramente, la malattia segrega come un tratto Mendeliano con trasmissione di tipo autosomico dominante o recessivo. Pertanto, la forma comune di MP e' probabilmente un tratto complesso, determinato da molti fattori di tipo genetico e non-genetico. Negli ultimi anni, gli studi di linkage in forme familiari, e gli studi di "positional cloning" hanno portato alla identificazione di diversi loci e geni per forme monogeniche rare, e piu' recentemente, di due loci per geni di suscettibilita' per le forme comuni, non-Mendeliane della malattia. Sebbene le forme monogeniche spieghino una piccolissima parte dei casi di MP a livello di popolazione, esse stanno facilitando la comprensione degli eventi molecolari e delle vie metaboliche implicate anche nelle forme comuni della malattia.

La prima sezione di questa tesi (Introduzione) passa in rassegna la genetica della MP, e la patogenesi delle due forme monogeniche identificate in precedenza. Il **capitolo 1.1** contiene una rassegna degli aspetti genetici clinici e molecolari delle forme Mendeliane di MP, e dei risultati degli studi genomici volti alla localizzazione di geni implicati nelle forme comuni, non-Mendeliane della malattia. Nel **capitolo 1.2** viene trattata la patogenesi delle forme di MP causate dalle mutazioni di *α-synuclein* e *parkin*, i due geni sicuramente associati alla MP noti in precedenza, evidenziando le implicazioni di queste conoscenze per la comprensione dei meccanismi alla base delle forme comuni della malattia.

Le ricerche su cui si basa questa tesi sono presentate nella seconda sezione, e riguardano studi genetici molecolari e fenotipici in forme autosomiche recessive di MP ad esordio precoce, associate alle mutazioni dei geni *parkin* (**capitoli 2.1-2.4**) e *DJ-1* (**capitoli 2.5-2.8**).
Poco dopo il clonaggio del gene *parkin* nel 1998, altri gruppi hanno confermato la presenza di mutazioni in famiglie con MP. Tuttavia, la frequenza del coinvolgimento del *parkin* nella MP ad esordio precoce e lo spettro mutazionale sono stati compresi pienamente grazie allo studio descritto nel **capitolo 2.1**, che ha esplorato per la prima volta questo gene in una ampia casistica di famiglie e di casi isolati con esordio precoce, mediante sequenziamento a livello genomico e dosaggio delle copie del gene. Lo studio ha rivelato mutazioni in questo gene in circa meta' delle famiglie compatibili con ereditarieta' di tipo recessivo ed esordio della MP prima dei 45 anni, ed in circa il 20% dei casi isolati con esordio precoce. Un ulteriore importante risultato dello studio e' che meta' delle mutazioni consistono in vasti riarrangiamenti genomici, e pertanto, per effettuare uno screening di sensibilita' accettabile, e' necessario il dosaggio delle copie geniche oltre al sequenziamento.
Se nella popolazione la frequenza di mutazioni di tipo recessivo in un certo gene e'

elevata, l'unione di un omozigote (o un eterozigote composto) con un portatore eterozigote puo' condurre alla comparsa della malattia recessiva in generazioni successive, simulando cosi' un pattern di trasmissione dominante (pseudo-dominanza). Queste unioni possono anche dare luogo ad eterogeneita' allelica all'interno di una stessa fratria. Questi fenomeni sono stati dimostrati in diversi studi in famiglie Giapponesi ed Italiane (**capitoli 2.2, 2.3**), suggerendo che le mutazioni nel gene *parkin* siano frequenti in certe popolazioni. La comprensione delle correlazioni genotipo-fenotipo nella malattia della parkina e' ancora limitata. Tuttavia, dalla analisi di una ampia serie di casi proviene l'indicazione che genotipi particolari possono influenzare il fenotipo (**capitolo 2.4**).

Un nuovo locus per una forma di MP autosomica recessiva ad esordio precoce (PARK7) e' stato da noi localizzato sul cromosoma 1p36 mediante una scansione genomica con mappatura di omozigosi in una estesa famiglia consanguinea contenente quattro individui affetti, ed appartenente ad una popolazione geneticamente isolata della parte sud-occidentale dei Paesi Bassi (**capitolo 2.5**). Il linkage per la regione PARK7 e' stato successivamente confermato in una seconda famiglia consanguinea contenente tre germani affetti da MP ad esordio precoce, e proveniente dalla Italia centrale (**capitolo 2.6**). In entrambe le famiglie il fenotipo e' caratterizzato da parkinsonismo ad esordio precoce (dai 27 ai 40 anni), buona risposta alla levodopa e lenta progressione. Sono inoltre presenti disturbi psichici e comportamentali e distonie (tra cui blefarospasmo), ed uno studio con tomografia ad emissione di positroni nella famiglia Olandese ha mostrato disfunzioni dopaminergiche di tipo presinaptico (**capitolo 2.7**).
Studi di mappaggio fine ed una strategia di "positional cloning" hanno condotto alla identificazione di mutazioni patogene nel gene *DJ-1* nelle due famiglie definitivamente legate al PARK7 (**capitolo 2.8**). Il gene DJ-1 e' stato strettamente conservato nel corso dell'evoluzione ed e' espresso in maniera abbondante ed ubiquitaria sia nel cervello che in altri tessuti. Il ruolo esatto della proteina DJ-1 rimane in gran parte sconosciuto, ma una revisione della letteratura ci fornisce indizi circa una possibile funzione e ci permette di formulare ipotesi per mettere in collegamento il *DJ-1* e la neurodegenerazione (**capitolo 2.9**). La scoperta che mutazioni nel *DJ-1* causano forme autosomiche recessive di MP ad esordio precoce permette di stabilire che nell'uomo, la perdita della funzione del DJ-1 conduce a neurodegenerazione. Il chiarimento dei meccanismi alla base della malattia correlata alla proteina DJ-1 puo' fare luce su nuovi meccanismi che regolano il mantenimento dei neuroni nel cervello, e puo' favorire la comprensione della patogenesi delle forme comuni di MP.

Il **capitolo 3** contiene la discussione generale dei risultati del nostro lavoro sperimentale. Dapprima, sono trattate le implicazioni delle conoscenze derivanti dagli studi genetici per la nosologia della MP. La malattia correlata alla parkina viene quindi discussa nel contesto generale della MP, evidenziando l'importanza di questa forma nell'*iter* diagnostico dei pazienti con esordio precoce, e le correlazioni genotipo-fenotipo. Infine, viene discusso il contributo apportato dalla scoperta delle mutazioni del *DJ-1* nel PARK7, ed i piu' recenti risultati degli studi biochimici e di biologia strutturale sulla proteina DJ-1. La tesi si conclude con alcune riflessioni e prospettive per gli studi futuri volti a chiarire ulteriormente le cause ed i meccanismi della MP.

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SELECTED PUBLICATIONS

1. Meco M, *Bonifati V*, Collier WL, Ramacci MT, Amenta F. Enzyme histochemistry of monoamine oxidase in the heart of aged rats. *Mech Ageing Dev* 1987;38(2):145-55.

2. Meco G, *Bonifati V*, Cusimano G, Fabrizio E, Vanacore N. Hallucinations in Parkinson disease: neuropsychological study. *Ital J Neurol Sci* 1990;11(4):373-9.

3. Cusimano G, Capriani C, *Bonifati V*, Meco G. Hypothalamo-pituitary function and dopamine dependence in untreated parkinsonian patients. *Acta Neurol Scand* 1991;83(3):145-50.

4. Meco G, Pratesi L, *Bonifati V*. Cardiovascular reflexes and autonomic dysfunction in Parkinson's disease. *J Neurol* 1991;238(4):195-9.

5. Meco G, *Bonifati V*, Bedini L, Bellatreccia A, Vanacore N, Franzese A. Relations between on-off phenomena and cognitive functions in Parkinson disease. *Ital J Neurol Sci* 1991;12(1):57-62.

6. Formisano R, Pratesi L, Modarelli FT, *Bonifati V*, Meco G. Rehabilitation and Parkinson's disease. *Scand J Rehabil Med* 1992;24(3):157-60.

7. Lestingi L, *Bonifati V*, Stocchi F, Antonozzi I, Meco G. TRH test and the continuous dopaminergic stimulation in complicated Parkinson's disease. *Eur Neurol* 1992;32(2):65-9.

8. Vanacore N, *Bonifati V*, Bellatreccia A, Fabrizio E, Meco G. Mortality rates for Parkinson's disease and parkinsonism in Italy (1969-1987). *Neuroepidemiology* 1992;11(2):65-73.

9. *Bonifati V*, Vanacore N, Bellatreccia A, Meco G. Mortality rates for parkinsonism in Italy (1969 to 1987). *Acta Neurol Scand* 1993;87(1):9-13.

10. *Bonifati V*, Vanacore N, Meco G. Anticipation of onset age in familial Parkinson's disease. *Neurology* 1994;44(10):1978-9.

11. Meco G, *Bonifati V*, Vanacore N, Fabrizio E. Parkinsonism after chronic exposure to the fungicide maneb (manganese ethylene-bis-dithiocarbamate). *Scand J Work Environ Health* 1994;20(4):301-5.

12. *Bonifati V*, Fabrizio E, Cipriani R, Vanacore N, Meco G. Buspirone in levodopa-induced dyskinesias. *Clin Neuropharmacol* 1994;17(1):73-82.

13. Meco G, Alessandri A, *Bonifati V*, Giustini P. Risperidone for hallucinations in levodopa-treated Parkinson's disease patients. *Lancet* 1994;343(8909):1370-1.

14. Meco G, Vanacore N, *Bonifati V*. The mortality rates for multiple sclerosis in Italy (1969-1987). *Neuroepidemiology* 1994;13(4):162-74.

15. *Bonifati V*, Fabrizio E, Vanacore N, De Mari M, Meco G. Familial Parkinson's disease: a clinical genetic analysis. *Can J Neurol Sci* 1995;22(4):272-9.

16. Menniti-Ippolito F, Spila-Alegiani S, Vanacore N, *Bonifati V*, Diana G, Meco G, et al. Estimate of parkinsonism prevalence through drug prescription histories in the Province of Rome, Italy. *Acta Neurol Scand* 1995;92(1):49-54.

17. Vanacore N, Corsi L, Fabrizio E, *Bonifati V*, Meco G. [Relationship between exposure to environmental toxins and motor neuron disease: a case report]. *Med Lav* 1995;86(6):522-33.

18. Gasser T, Muller-Myhsok B, Supala A, Zimmer E, Wieditz G, Wszolek ZK, Vieregge P, *Bonifati V*, Oertel WH. The CYP2D6B allele is not overrepresented in a population of German patients with idiopathic Parkinson's disease. *J Neurol Neurosurg Psychiatry* 1996;61(5):518-20.

19. Gasparini M, Fabrizio E, *Bonifati V*, Meco G. Cognitive improvement during Tolcapone treatment in Parkinson's disease. *J Neural Transm* 1997;104(8-9):887-94.

20. Gasser T, Muller-Myhsok B, Wszolek ZK, Durr A, Vaughan JR, *Bonifati V*, et al. Genetic complexity and Parkinson's disease. *Science* 1997;277(5324):388-9.

21. Meco G, Alessandri A, Giustini P, *Bonifati V*. Risperidone in levodopa-induced psychosis in advanced Parkinson's disease: an open-label, long-term study. *Mov Disord* 1997;12(4): 610-2.

22. Bennett P, *Bonifati V*, Bonuccelli U, Colosimo C, De Mari M, Fabbrini G, et al. Direct genetic evidence for involvement of tau in progressive supranuclear palsy. *Neurology* 1998;51(4):982-5.

23. Fabbrini G, Vanacore N, *Bonifati V*, Colosimo C, Meco G. Presymptomatic hypertension in progressive supranuclear palsy. *Arch Neurol* 1998;55(8):1153-5.

24. Gasser T, Muller-Myhsok B, Wszolek ZK, Oehlmann R, Calne DB, *Bonifati V*, et al. A susceptibility locus for Parkinson's disease maps to chromosome 2p13. *Nat Genet* 1998;18(3):262-5.

25. Lucking CB, Abbas N, Durr A, *Bonifati V*, Bonnet AM, de Broucker T, et al. Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism. *Lancet* 1998;352(9137):1355-6.

26. Meco G, Fabrizio E, Alessandri A, Vanacore N, *Bonifati V*. Risperidone in levodopa induced dyskinesiae. *J Neurol Neurosurg Psychiatry* 1998;64(1):135.

27. Tassin J, Durr A, de Broucker T, Abbas N, *Bonifati V*, De Michele G, et al. Chromosome 6-linked autosomal recessive early-onset Parkinsonism: linkage in European and Algerian families, extension of the clinical spectrum, and evidence of a small homozygous deletion in one family. *Am J Hum Genet* 1998;63(1): 88-94.

28. Vaughan J, Durr A, Tassin J, Bereznai B, Gasser T, *Bonifati V*, et al. The alpha-synuclein Ala53Thr mutation is not a common cause of familial Parkinson's disease: a study of 230 European cases. *Ann Neurol* 1998;44(2):270-3.

29. Vaughan JR, Farrer MJ, Wszolek ZK, Gasser T, Durr A, Agid Y, *Bonifati V*, et al. Sequencing of the alpha-synuclein gene in a large series of cases of familial Parkinson's disease fails to reveal any further mutations. *Hum Mol Genet* 1998;7(4):751-3.

30. *Bonifati V*, Joosse M, Nicholl DJ, Vanacore N, Bennett P, Rizzu P, et al. The tau gene in progressive supranuclear palsy: exclusion of mutations in coding exons and exon 10 splice sites, and identification of a new intronic variant of the disease-associated H1 haplotype in Italian cases. *Neurosci Lett* 1999;274(1):61-5.

31. Abbas N, Lucking CB, Ricard S, Durr A, *Bonifati V*, De Michele G, et al. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. *Hum Mol Genet* 1999;8(4):567-74.

32. *Bonifati V*, Meco G. New, selective catechol-O-methyltransferase inhibitors as therapeutic agents in Parkinson's disease. *Pharmacol Ther* 1999;81(1):1-36.

33. Harhangi BS, Farrer MJ, Lincoln S, *Bonifati V*, Meco G, De Michele G, et al. The Ile93Met mutation in the ubiquitin carboxy-terminal-hydrolase-L1 gene is not observed in European cases with familial Parkinson's disease. *Neurosci Lett* 1999;270(1):1-4.

34. Nicholl DJ, Bennett P, Hiller L, *Bonifati V*, Vanacore N, Fabbrini G, et al. A study of five candidate genes in Parkinson's disease and related neurodegenerative disorders. *Neurology* 1999;53(7):1415-21.

35. Lucking CB, Durr A, *Bonifati V*, Vaughan J, De Michele G, Gasser T, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene. *N Engl J Med* 2000;342(21):1560-7.

36. Meco G, Vanacore N, Locuratolo N, *Bonifati V*, Vella C, Giovani A, et al. Heart rate variability in Parkinson's disease patients treated with tolcapone. *Parkinsonism Relat Disord* 2000;6(4):223-227.

37. Vanacore N, *Bonifati V*, Fabbrini G, Colosimo C, Marconi R, Nicholl D, et al. Smoking habits in multiple system atrophy and progressive supranuclear palsy. *Neurology* 2000;54(1):114-9.

38. *Bonifati V*, Lucking CB, Fabrizio E, Periquet M, Meco G, Brice A. Three parkin gene mutations in a sibship with autosomal recessive early onset parkinsonism. *J Neurol Neurosurg Psychiatry* 2001;71(4):531-4.

39. van Duijn CM, Dekker MC, *Bonifati V*, Galjaard RJ, Houwing-Duistermaat JJ, Snijders PJ, et al. Park7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36. *Am J Hum Genet* 2001;69(3):629-34.

40. *Bonifati V*. Monogenic Parkinsonisms and the genetics of Parkinson's disease. *Funct Neurol* 2001;16(1):35-44.

41. Periquet M, Lucking C, Vaughan J, *Bonifati V*, Durr A, De Michele G, et al. Origin of the mutations in the parkin gene in Europe: exon rearrangements are independent recurrent events, whereas point mutations may result from Founder effects. *Am J Hum Genet* 2001;68(3):617-26.

42. *Bonifati V*, De Michele G, Lucking CB, Durr A, Fabrizio E, Ambrosio G, et al. The parkin gene and its phenotype. *Neurol Sci* 2001;22(1):51-2.

43. Lucking CB, *Bonifati V*, Periquet M, Vanacore N, Brice A, Meco G. Pseudo-dominant inheritance and exon 2 triplication in a family with parkin gene mutations. *Neurology* 2001;57(5):924-7.

44. Casali C, *Bonifati V*, Santorelli FM, Casari G, Fortini D, Patrignani A, et al. Mitochondrial myopathy, parkinsonism, and multiple mtDNA deletions in a Sephardic Jewish family. *Neurology* 2001;56(6):802-5.

45. Colosimo C, Vanacore N, *Bonifati V*, Fabbrini G, Rum A, De Michele G, et al. Clinical diagnosis of multiple system atrophy: level of agreement between Quinn's criteria and the consensus conference guidelines. *Acta Neurol Scand* 2001;103(4):261-4.

46. Fabbrini G, Barbanti P, *Bonifati V*, Colosimo C, Gasparini M, Vanacore N, et al. Donepezil in the treatment of progressive supranuclear palsy. *Acta Neurol Scand* 2001;103(2):123-5.

47. Gasparini M, *Bonifati V*, Fabrizio E, Fabbrini G, Brusa L, Lenzi GL, et al. Frontal lobe dysfunction in essential tremor: a preliminary study. *J Neurol* 2001;248(5):399-402.

48. *Bonifati V*, Breedveld GJ, Squitieri F, Vanacore N, Brustenghi P, Harhangi BS, et al. Localization of autosomal recessive early-onset parkinsonism to chromosome 1p36 (PARK7) in an independent dataset. *Ann Neurol* 2002;51(2):253-6.

49. Valente EM, Brancati F, Ferraris A, Graham EA, Davis MB, Breteler MM, Gasser T, *Bonifati V*, et al. PARK6-linked parkinsonism occurs in several European families. *Ann Neurol* 2002;51(1):14-8.

50. West A, Periquet M, Lincoln S, Lucking CB, Nicholl D, *Bonifati V*, et al. Complex relationship between Parkin mutations and Parkinson disease. *Am J Med Genet* 2002;114(5):584-91.

51. Rawal N, Periquet M, Durr A, de Michele G, *Bonifati V*, Teive HA, et al. Exclusion of the Nurr1 gene in autosomal recessive Parkinson's disease. *J Neurol* 2002; 249(8): 1127-9.

52. *Bonifati V*, Dekker MC, Vanacore N, Fabbrini G, Squitieri F, Marconi R, et al. Autosomal recessive early onset parkinsonism is linked to three loci: PARK2, PARK6, and PARK7. *Neurol Sci* 2002;23(Suppl 2):S59-60.

53. *Bonifati V*. Deciphering Parkinson's disease--PARK8. *Lancet Neurol* 2002;1(2):83.

54. *Bonifati V*, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* 2003;299(5604): 256-9.

55. Dekker M, *Bonifati V*, van Swieten J, Leenders N, Galjaard RJ, Snijders P, et al. Clinical features and neuroimaging of PARK7-linked parkinsonism. *Mov Disord* 2003;18(7):751-7.

56. Dekker MC, *Bonifati V*, van Duijn CM. Parkinson's disease: piecing together a genetic jigsaw. *Brain* 2003;126(Pt 8):1722-33.

57. Duzcan F, Zencir M, Ozdemir F, Cetin GO, Bagci H, Heutink P, *Bonifati V*, Sahiner T. Familial influence on parkinsonism in a rural area of Turkey (Kizilcaboluk-Denizli): a community-based case-control study. *Mov Disord* 2003;18(7):799-804.

58. Hague S, Rogaeva E, Hernandez D, Gulick C, Singleton A, Hanson M, Johnson J, Weiser R, Gallardo M, Ravina B, Gwinn-Hardy K, Crawley A, St George-Hyslop PH, Lang AE, Heutink P, *Bonifati V*, Hardy J, Singleton A. Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation. *Ann Neurol* 2003;54(2):271-4.

59. Lohmann E, Periquet M, *Bonifati V*, Wood NW, De Michele G, Bonnet AM, et al. How much phenotypic variation can be attributed to parkin genotype? *Ann Neurol* 2003;54(2):176-85.

60. *Bonifati V*, Heutink P. Chromosome 1 and other hotspots for Parkinson disease genes. In: Kahle P and Haass C, (Editors), *Molecular Mechanisms of Parkinson's Disease*. Landes Bioscience Publishers, in press.

61. Macedo MG, Anar B, Bronner IF, Cannella M, Squitieri F, *Bonifati V*, et al. The DJ-1^{L166P} mutant protein associated with early onset Parkinson's disease is unstable and forms higher order protein complexes. *Hum Mol Genet*, in press.

62. Rizzu P, Hinkle DA, Zhucareva V, *Bonifati V*, Severijnen L-A, Martinez D, et al. DJ-1 co-localizes with pathological tau inclusions: a possible link between Parkinsonism and dementia. *Ann Neurol*, in press.

63. Bertoli-Avella AM, Giroud-Benitez JL, *Bonifati V*, Alvarez-Gonzalez E, Heredero-Baute L, van Duijn C, Heutink P. Suggestive linkage to chromosome 19 in a large Cuban family with late-onset Parkinson's disease. *Mov Disord*, in press.

64. *Bonifati V*, Oostra BA, Heutink P. Linking DJ-1 to neurodegeneration offers novel insights for understanding the pathogenesis of Parkinson's disease. *J Mol Med*, in press.

Dankwoord

In my view, this thesis is the result of ten years dedicated to the clinical characterisation of familial forms of Parkinson's disease, to the collection of biological samples, and to extensive molecular genetic research. I'm therefore indebted to many colleagues and friends, who helped me during this long period.
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